Current Issues in Medicine Vol. 3

ADVANCES IN SURGICAL AND MEDICAL SPECIALTIES

edited by

Raj Bawa

ADVANCES IN SURGICAL AND MEDICAL SPECIALTIES

Current Issues in Medicine

Series Editor
Raj Bawa

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Current Issues in Medicine Vol. 3

Advances in Surgical and Medical Specialties

edited by

Raj Bawa, PhD, MD

Patent Agent, Bawa Biotech LLC, Ashburn, Virginia, USA
Vice President and Chief IP Officer, Guanine Inc., Rensselaer, New York, USA
Scientific Advisor, Teva Pharmaceutical Industries, Ltd., Israel

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Note from the Series Editor and the Publisher

Extensive efforts have been made to make the information provided herein as accurate and as up-to-date as possible. It is important to note that knowledge and best practices in the various fields represented in this book (pathology, clinical microbiology, vaccines, nutrition, surgical tools and procedures, stem cell research, biochemistry, drug delivery, nanomedicine, precision medicine, genomics, tissue engineering, pharmaceutical sciences, etc.) are constantly evolving. This book is no substitute for individual patient assessment based on health care professionals' examination of each patient and consideration of specific factors unique to that patient. These include, but are not limited to, the following: age, weight, height, gender, current and past medical history, family medical data, laboratory data, etc. Therefore, it is imperative that the reader not rely solely on the information presented herein. The reader should always consult appropriate medical professionals (physicians, pharmacists, licensed healthcare professionals, etc.), product manufacturer, federal agencies (FDA, CDC, NIH, etc.), drug and device product labels (regarding use, warnings, directions, etc.) before consuming any drug product, using any medical device, selecting any diagnostic procedure, or undergoing any surgery. To the fullest extent of the law, neither the publisher nor the editor or authors make any representations or warranties, express or implied, with respect to the information presented in this book, for its use or misuse, or interpretation thereof. In this regard, they assume no liability for any injury and/or damage to persons or property as a matter of product liability, negligence, or otherwise.

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Advances in Surgical and Medical Specialties

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Dedication

Integrity is doing the right thing, even when no one is watching.

—C. S. Lewis

The world is changed by your example, not by your opinion.

—Paulo Coelho

In order to discover the character of people we have only to observe what they love.

—Saint Augustine

The book is dedicated with love to my wonderful family: my wife and parents.

My late mother, Mrs. Sudesh Bawa (1935–2020), blessed me with immeasurable love and joy. She guided me to pursue my goals with integrity, inspired me with her perseverance, and instilled in me the desire to aid the helpless. Although her medical care in Upstate New York was not perfect, she courageously endured her pain and suffering without bitterness. Most incredulously, she tried to lessen our emotional pain even while enduring her physical discomforts. Her loss has left me saddened beyond comprehension and I miss her dearly.

My 93-year-old father, Dr. S. R. Bawa, a retired anatomy professor/chair, who now lives with us in Virginia and whose caregiving I relish, has always been an inspirational role model for me. His remarkable life and professional accolades are subject of a chapter in this volume. These are complimented by his kindness, generosity of spirit, and loving nature – all accompanied by a remarkably strong character. He is the best teacher I have ever had.

I thank my loving wife, Sangita, for providing me stability, support, and guidance. Her gentle nature and sweet demeanor remind me every day of my mother. Thank you for walking beside me and for wanting me at your side.

The Editor

Raj Bawa, PhD, MD, is president of Bawa Biotech LLC (founded in 2002), a biotech/pharma consultancy and patent law firm based in Ashburn, Virginia, USA. Trained as a microbiologist and biochemist, he is an inventor, author, entrepreneur, professor, and registered patent agent (since 2002) licensed to practice before the US Patent & Trademark Office. He is currently a scientific advisor to Teva Pharmaceutical Industries, Israel (since 2010), a visiting research scholar at the Pharmaceutical Research Institute of Albany College of Pharmacy, Albany, New York, and full professor (adjunct) at Northern Virginia Community College, Annandale, Virginia (since 2004). He is vice president and chief IP officer at Guanine, Inc., Rensselaer, New York (since 2017), a company focused on rapid, accurate detection of infective pathogens. He has served as a principal investigator of various National Cancer Institute (NCI) research grants, most recently as a principal investigator of a Centers for Disease Control and Prevention (CDC) grant to develop an assay for carbapenemase resistant bacteria. He was an adjunct professor at Rensselaer Polytechnic Institute, Troy, New York, from 1998 to 2018. After earning a BSc (Honors School) in microbiology, he earned a MS in cancer biology, a PhD in biophysics/biochemistry and an MD. In the 1990s, Dr. Bawa held various positions at the US Patent & Trademark Office, including primary examiner from 1996–2002. Currently, he is a life member of Sigma Xi, cochair of the nanotech and precision medicine committees of the American Bar Association, and founding director of the American Society for Nanomedicine (established in 2008). He has authored over 100 publications, edited 10 texts, and serves on the editorial boards of numerous peer-reviewed journals, including serving as an associate editor of *Nanomedicine* (Elsevier).

Contents

Corresponding Authors

Fatma Khinaifis Al-thoubaity, MD
Department of Surgery
Faculty of Medicine
King Abdulaziz University
Jeddah, Saudi Arabia
fatmalthoubaity@yahoo.com
fthoubaity@kau.edu.sa

Yong-Sun Bahn, PhD
Department of Biotechnology
College of Life Science and Biotechnology
Yonsei University
Seoul, Republic of Korea
ysbahn@yonsei.ac.kr

Raj Bawa, PhD, MD
Bawa Biotech LLC, Ashburn, Virginia, USA
Guanine Inc., Rensselaer, New York, USA
Teva Pharmaceutical Industries Ltd., Israel
bawa@bawabiotech.com

Eric Bruckert, MD
Hopital Pitié Salpêtrière et Université Paris Sorbonne
Paris, France
eric.bruckert@aphp.fr

Javier Cañueto, MD, PhD
Instituto de Biología Molecular y Celular del Cáncer
Centro de Investigación del Cáncer
Salamanca, Spain
jcanueto@usal.es

Linda Clare, PhD, ScD
Centre for Research in Ageing and Cognitive Health
College of Medicine and Health
University of Exeter
Exeter, UK
l.clare@exeter.ac.uk

Raymund B. Dantes, MD
Division of Hospital Medicine
Department of Medicine
Emory University School of Medicine
Atlanta, Georgia, USA
Raymund.dantes@emoryhealthcare.org

Paul R. De Lay, MD
Independent Consultant
Washington, DC, USA
pdelay1@gmail.com

Mariacruz L. Díaz, DVM, MSc, PhD
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
Departamento de Reproducción Animal
Madrid, Spain
lopez.maria@inia.es

Sabrina Donzelli, MD
ISICO – Italian Scientific Spine Institute
Milan, Italy
sabrina.donzelli@isico.it

Margarida Fardilha, PhD
Laboratory of Signal Transduction
Department of Medical Sciences
Institute of Biomedicine
University of Aveiro
Aveiro, Portugal
mfardilha@ua.pt

Luca Fiorillo, DDS, MSc, PhD
Department of Biomedical and Dental Sciences
Morphological and Functional Images
University of Messina
Policlinico G. Martino
Messina, Italy
lfiorillo@unime.it

JoAnne L. Flynn, PhD
Department of Microbiology and Molecular Genetics
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania, USA
joanne@pitt.edu

Luigi Fontana, MD, PhD
Charles Perkins Centre
Faculty of Medicine and Health
University of Sydney
Sydney, New South Wales, Australia
luigi.fontana@sydney.edu.au

Mario Ganau, MD, PhD, MBA
Department of Neurosciences
John Radcliffe Hospital
Oxford University, UK
mario.ganau@alumni.harvard.edu

Richard P. Grose, PhD
Centre for Tumour Biology
Barts Cancer Institute
Queen Mary University of London
London, UK
r.p.grose@qmul.ac.uk

Glaiza S. de Guzman, MD
Department of Obstetrics and Gynecology
University of the Philippines
Philippine General Hospital
Manila, Philippines
gsdeguzman1@up.edu.ph

Kurt A. Jellinger, MD, PhD
Institute of Clinical Neurobiology
Vienna, Austria
kurt.jellinger@univie.ac.at

Jasmine N. Khouja, PhD
MRC Integrative Epidemiology Unit at the University of Bristol
Bristol, UK
jasmine.khouja@bristol.ac.uk

Hiroshi Kitoh, MD, PhD
Department of Orthopaedic Surgery
Aichi Children's Health and Medical Center
Obu, Aichi, Japan
Department of Comprehensive Pediatric Medicine
Nagoya University Graduate School of Medicine
Nagoya, Aichi, Japan
hkitoh420@gmail.com
hiroshi_kitou@sk00106.achmc.pref.aichi.jp

Gregory Y. H. Lip, MD
Liverpool Centre for Cardiovascular Science
University of Liverpool and Liverpool Heart & Chest Hospital
Liverpool, UK
Gregory.lip@liverpool.ac.uk

Daniel López-López, DP, PhD
Research, Health and Podiatry Group
Department of Health Sciences
Faculty of Nursing and Podiatry
Universidade da Coruña
Ferrol, Spain
daniel.lopez.lopez@udc.es

Alexander M. Markin, PhD
Laboratory of Infection Pathology and Molecular Microecology
Institute of Human Morphology
Moscow, Russia
alexander.markin.34@gmail.com

Ray Marks, EdD
Department of Health and Behavior Studies
Columbia University
Teachers College
New York, New York, USA
rm226@columbia.edu

Eran Meshorer, PhD
Department of Genetics and the Edmond and Lily Center for Brain Sciences
Hebrew University of Jerusalem
Jerusalem, Israel
meshorer@cc.huji.ac.il

Alexander N. Orekhov, PhD
Laboratory of Infection Pathology and Molecular Microecology
Institute of Human Morphology
Moscow, Russia
a.h.opexob@gmail.com

Nicolas Papon, PhD
Univ Angers, Univ Brest, IRF, SFR ICAT
Angers, France
nicolas.papon@univ-angers.fr

Adjanie Patabendige, PhD
Brain Barriers Group
School of Biomedical Sciences and Pharmacy
The University of Newcastle
Callaghan, New South Wales, Australia
adjanie.patabendige@newcastle.edu.au

Pedro Póvoa, PhD
Polyvalent Intensive Care Unit
Hospital de São Francisco Xavier
Centro Hospitalar Lisboa Ocidental
Estrada do Forte do Alto do Duque
Lisbon, Portugal
pedrorpovoa@gmail.com

Shubhadeep Roychoudhury, PhD
Department of Life Science and Bioinformatics
Assam University
Silchar, India
shubhadeep1@gmail.com

M. Zahid Saeed, MD
Trauma and Orthopaedic Department
University College London Medical School
Royal Free Hospital
London, UK
z.saeed@nhs.net
mzahidsaeed@hotmail.com

Beata Sarecka-Hujar, PhD, DSc
Department of Basic Biomedical Science
Faculty of Pharmaceutical Sciences in Sosnowiec
Medical University of Silesia in Katowice
Sosnowiec, Poland
beatasarecka@poczta.onet.pl
bsarecka-hujar@sum.edu.pl

Gabriele Savioli, MD, PhD
Emergency Department
IRCCS Policlinico San Matteo
Pavia, Italy
gabrielesavioli@gmail.com

Angelos K. Sikalidis, PhD
Department of Food Science and Nutrition
California Polytechnic State University
San Luis Obispo, California, USA
asikalid@calpoly.edu

Camilo G. Sotomayor, MD
Department of Internal Medicine
University Medical Center Groningen
University of Groningen
Groningen, The Netherlands
c.g.sotomayor.campos@umcg.nl

Jacqueline M. Stephens, PhD
Adipocyte Biology Laboratory
Pennington Biomedical Research Center
Baton Rouge, Louisiana, USA
jsteph1@lsu.edu

Karin Sundström, MD, PhD
Department of Laboratory Medicine
Division of Pathology
Karolinska Institute
Stockholm, Sweden
karin.sundstrom@ki.se

Jacob I. Sznajder, MD
Division of Pulmonary and Critical Care Medicine
Northwestern University Feinberg School of Medicine
Chicago, Illinois, USA
j-sznajder@northwestern.edu

Eleonora Tamilia, PhD
Fetal Neonatal Neuroimaging and Developmental Science Center
Department of Pediatrics
Boston Children's Hospital
Boston, Massachusetts, USA
Eleonora.Tamilia@childrens.harvard.edu

Giuseppe Tisone
Hepatobiliary Surgery and Transplant Unit
Department of Surgical Sciences
University of Rome Tor Vergata
Rome, Italy
tisone@uniroma2.it

Lale Tokgözoğlu, MD
Department of Cardiology
Hacettepe University
Ankara, Turkey
laletok@gmail.com

Doug M. Turnbull, PhD, MD
Wellcome Centre for Mitochondrial Research
Clinical and Translational Research Institute
Faculty of Medical Sciences
Newcastle University
Newcastle, UK
doug.turnbull@ncl.ac.uk
amy.vincent@ncl.ac.uk

Daria Van Tyne, PhD
Department of Medicine
Division of Infectious Diseases
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania, USA
vantyne@pitt.edu

Alice Wang, PhD
Division of Global HIV & Tuberculosis
Center for Global Health
Centers for Disease Control and Prevention
Atlanta, Georgia, USA
ilm1@cdc.gov

Holly N. Wilkinson, PhD
Centre for Atherothrombosis and Metabolic Disease
Hull York Medical School
The University of Hull
Hull, UK
h.n.wilkinson@hull.ac.uk

Ezequiel Jose Zaidel, MD
Cardiology Department
Sanatorio Güemes and School of Medicine
University of Buenos Aires
Buenos Aires, Argentina
ezaidel@fsg.edu.ar

Note from the Series Editor

A hallmark of medicine is that it is continuously evolving, its knowledge base continuously expanding. Clearly, the pace and sophistication of advances in medicine in the past two decades have been truly breathtaking. This has necessitated a growing need for a comprehensive reference that highlights the current issues in specific sectors of medicine. Keeping this in mind, each volume in the *Current Issues in Medicine* series is a stand-alone text that provides a broad survey of various critical topics in a focused area of medicine—all accomplished in a user-friendly yet interconnected format. The series not only highlights current issues and advances but also explores related topics such as translational medicine, precision medicine, nanomedicine, regulatory science, neglected global diseases, emerging pandemics (COVID-19, RSV, Ebola, etc.), FDA and patent law, immunotoxicology, theranostics, big data, artificial intelligence, novel imaging tools and techniques, combination drug products, and novel drug delivery therapies. While bridging the gap between basic research and clinical medicine, this series provides a thorough understanding of medicine's potential to address health problems from both the patient's and the provider's perspectives in a healthcare setting. Each volume is an excellent resource for medical practitioners, medical students, nurses, fellows, residents, undergraduate and graduate students, educators, venture capitalists, policymakers, and biomedical researchers. The multidisciplinary approach of the series makes it a valuable reference for health care systems, the pharmaceutical industry, academia, and governments. However, unlike other series on medicine or medical texts, this series focuses on current trends, perspectives, and critical issues in medicine that are central to healthcare delivery in the 21st century.

The first two volumes in this series focus on the current issues in basic medical sciences, subjects that are fundamental to the practice of medicine. These subjects, traditionally taught in the first two years of medical school that precede clinical instruction, provide a core of basic knowledge crucial for later success in clinical medicine during rotations, training, and medical practice. Subsequent volumes are dedicated to clinical topics or specialties in medicine. In addition, a separate volume on medical history and another on medical perspectives/editorials are in preparation.

Surgical and medical specialties have all aided in treatment and prevention of diseases throughout human history. They have reduced suffering and saved lives. We as humans would certainly not exist in the capacity that we do in 2023 without advances in surgery and medical interventions. While recognizing how expansive and multifaceted these areas are, the current volume is not a textbook that provides comprehensive information. Instead, it addresses crucial recent advances and current issues in surgical and medical specialties. Specifically,

knowledge and experience of experts from academia and practicing surgeons is integrated. Medical practitioners today continue to improve upon techniques and technologies to provide procedures for patients that are safer, faster, less invasive, and more accurate—a direct consequence of advances in technological breakthroughs from a variety of medical and engineering fields. In order to render modern patient care, it is imperative that surgeons and medical practitioners stay current with these latest advances in their respective specialties. Given this backdrop, the specific topics covered in this volume and the expertise of the contributing authors accurately reflect the rapidly evolving areas within surgical and medical specialties.

Raj Bawa, PhD, MD
Series Editor

Chapter 1

Surgical and Medical Specialties: *A Journey in Pictures*

Raj Bawa, PhD, MD

Patent Law Department, Bawa Biotech LLC, Ashburn, Virginia, USA
Guanine Inc., Rensselaer, New York, USA
Teva Pharmaceutical Industries Ltd., Israel

bawa@bawabiotech.com

Medicine is a science of uncertainty and an art of probability.

— William Osler, 1849–1919

The science of medicine is as incomprehensible as the ocean. It cannot be fully described even in hundreds and thousands of verses. Dull people who are incapable of catching the real import of the science of reasoning would fail to acquire a proper insight into the science of medicine if dealt with elaborately in thousands of verses.

— Sushruta, c. 700 BC

Wherever the art of Medicine is loved, there is also a love of Humanity.

— Hippocrates, c. 400 BC

Healthcare is the most critical issue of our time and will be so for the foreseeable future. Surgical and medical specialties[1] all have aided in the treatment and prevention of diseases throughout human history. They have reduced suffering and saved lives. We as humans would certainly not exist in the capacity that we do in 2023 without advances in surgery and medical interventions. My intention with this chapter is to provide a glimpse into the richness of medical history and the superb progress mankind has made on the path of medical miracles. The focus of this chapter is rather broad: surgery and medical specialities. Even at the time of Galen (p. 10), specialization was common among Roman physicians. The system of modern medical specialties evolved gradually during the 19th century

[1]A medical specialty is a branch of medicine that is focused on a defined group of patients, diseases, skills, or philosophy. Examples include those branches of medicine that deal exclusively with children (pediatrics), cancer (oncology), microbial diseases (medical microbiology), primary care (family medicine), etc. Following medical school or other basic training, physicians and other clinicians generally further their education in a specific medical specialty to become a specialist.

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as we moved away from superstitions, magic and the placebo effect.[2] It continues with a greater impact of cutting-edge technologies and novel techniques. The pace of medical understanding, and technological advances that impact it, will continue to accelerate.

This chapter celebrates the wonders of surgery and medicine: it looks at the best and brightest minds of medicine, as well as the surgical instruments and techniques that have revolutionized it. Detailed are some of the greatest medical inventions, discoveries, milestones and miracles in the context of surgery and medical specialization viz-a-viz interventions, diagnosis, tools, techniques, prophylaxis, and therapies. This vivid story is presented here via photographs, paintings, etches, and images. Historical images have been sprinkled throughout the chapter to provide context and perspective while highlighting the long span of medical advances and patient care. Each entry is short – at most only a few paragraphs. My intention is that the reader jump in and ponder a subject without having to sort through verbiage.

Obviously, it is impossible to include all the significant surgical and medical milestones in a single chapter. It is impossible to be fluent in all aspects of medicine, even more so for medical history. I plan on creating a separate, more comprehensive volume in this series where more entries can be compiled. Since, this is an ongoing project and a labor of love, I welcome suggestions and criticisms. Many of the topics included here appeal to me personally. My bias and intellectual shortcomings will be apparent. I have been forced to omit many important medical marvels.

This chapter should appeal to a wide audience: medical historians and laypersons, medical students and their parents, practicing physicians and their patients, professor in academia and their medical fellows, and so on. In essence, it is a brief guide to significant medical milestones, ideas, tools and techniques, thinkers and influencers in surgery and medicine. Let's discover the origins of some of the greatest medical innovations in history, as we embark on this fascinating journey in pictures.

I dedicate this chapter to my sunshine, my late mother, Mrs. Sudesh Bawa (1935–2020), who blessed me with immeasurable love and joy. She guided me to pursue my goals with integrity, inspired me with her perseverance, taught me to appreciate people for their inherent beauty, and instilled in me the desire to aid the helpless. It is these remarkable values that serve as the basis of the newly formed *Sudesh Bawa Medical Foundation*.

[2]In the modern era, the start of specialization can be traced to the major hospitals of Paris in the 1830s from where it rapidly expanded to Vienna and other European cites in the 1850s. In the US, specialization slowly grew after the Civil War.

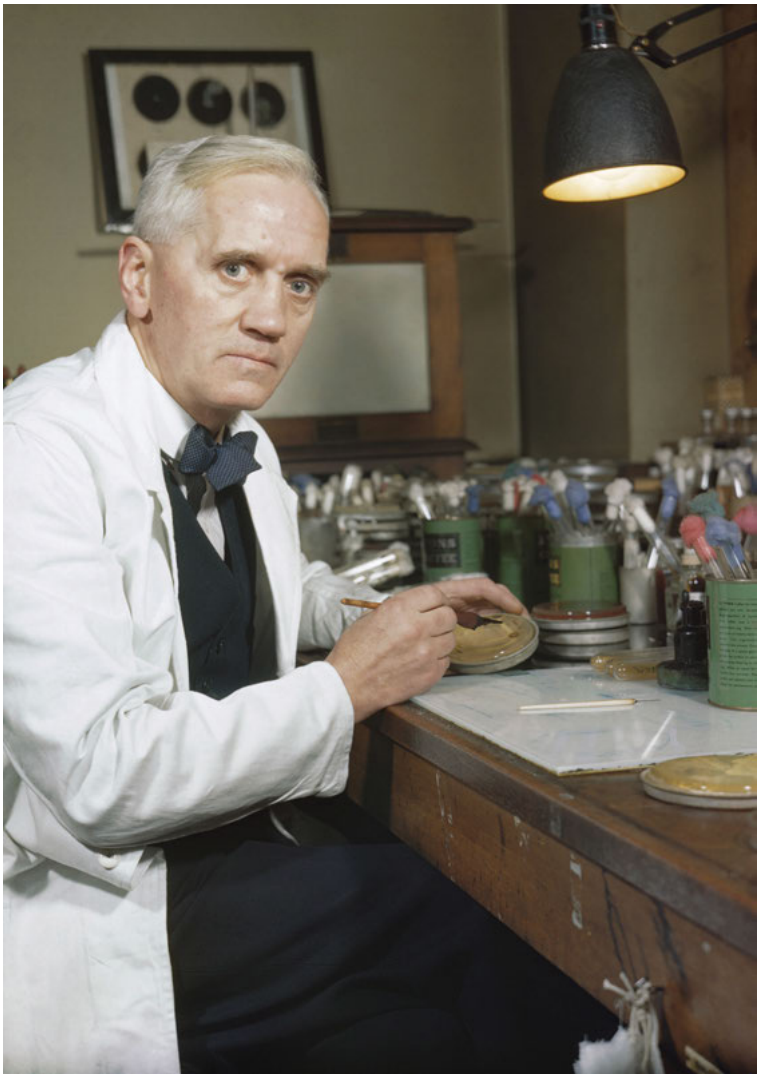
Alexander III of Macedon (Ancient Greek: Ἀλέξανδρος, Romanized: Alexandros; 356 BC–323 BC), commonly known as Alexander the Great, was a king of the ancient Greek kingdom of Macedon. Alexander the Great's confidence in his physician Philip of Acarnania is reflected in this classic oil painting by Benjamin West. In the scene from 333 BC, Alexander is about to drink the medicine for fever prepared for him by Philip. Alexander made a quick and full recovery. It is uncertain whether Philip was present some years later when Alexander claimed, "I am dying with the help of too many physicians." Although communications has become an integral part of modern medical education curricula, misunderstanding between physician and patient persists. It is imperative that medical students learn to integrate communication and clinical skills in their future medical practice. However, the communication skills of Philip in this picture are questionable, as he appears to be reading a scroll and avoids eye contact with his patient Alexander.

William Cheselden (1688–1752), an English anatomist and surgeon, giving an anatomical demonstration to six spectators (possibly students) in the anatomy theatre of the Barber-Surgeons' Company, London. Oil painting, c. 1730–1740. Cheselden was influential in establishing surgery as a scientific medical profession. Via the medical missionary Benjamin Hobson, his work also helped revolutionize medical practices in China and Japan in the 19th century. Cheselden published two seminal texts in medical education – *Anatomy of the Human Body* and *The Anatomy of Bones*. Most striking about this image is that the various activities the learners are undertaking – observing, listening, and discussing – are all standard in medical education today.

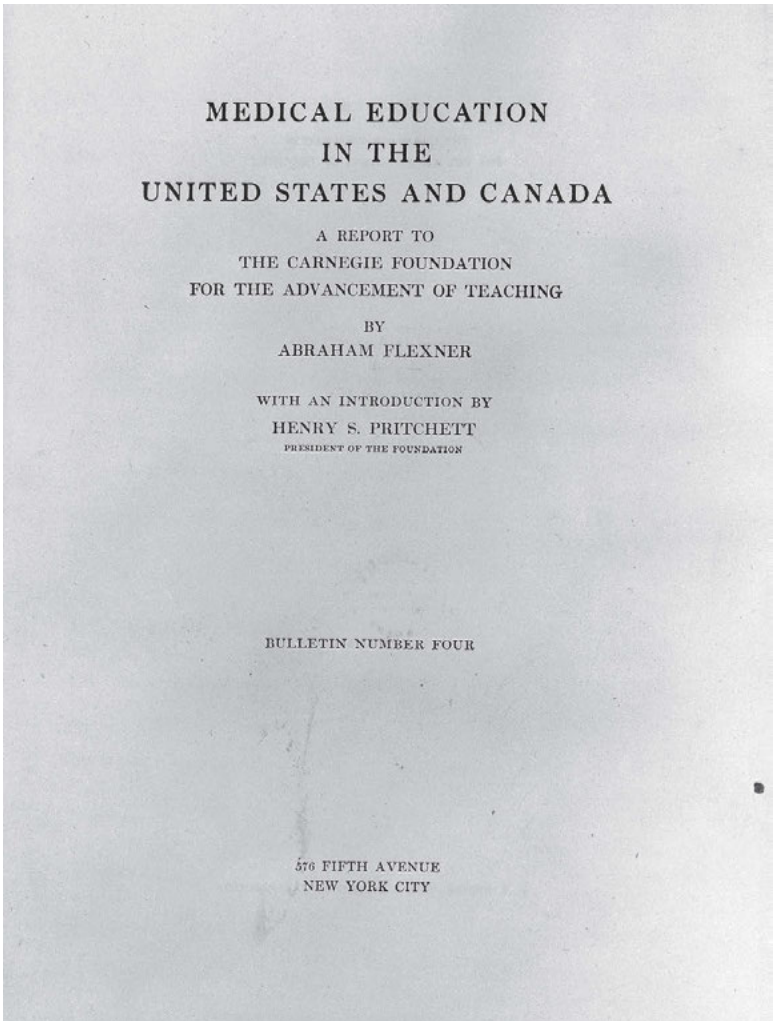
Paul Georges Dieulafoy (1839–1911), a French surgeon is shown in this photograph, dated 1900, in his pomp, surrounded by assistants and students. He perfected a pump-like device for use in thoracentesis, and extensively studied pleurisy and liver conditions including hydatid disease and epidemic hepatitis. However, he is perhaps best known for his study of appendicitis. Dieulafoy described the constellation of signs for acute appendicitis, which is eponymously named "Dieulafoy's triad:" hyperesthesia of the skin, exquisite tenderness and guarding over McBurney's point, considered a classic sign of acute appendicitis. He also discovered Dieulafoy's lesion (a rare cause of upper gastrointestinal bleeding) and invented Dieulafoy's apparatus (a pump to evacuate pleural effusions). This photograph shows Dieulafoy at the Hôtel-Dieu (founded in 651), the oldest and still working hospital in Paris, France.

Edward Jenner (1749–1823) was a British physician and scientist who pioneered the concept of vaccines including creating the smallpox vaccine, the world's first vaccine. The terms vaccine and vaccination are derived from *Variolae vaccinae* ("pustules of the cow"), the term devised by Jenner to denote cowpox. He used it in 1798 in the title of his *Inquiry into the Variolae vaccinae* known as the Cow Pox, in which he described the protective effect of cowpox against smallpox. In the West, Jenner is often called "the father of immunology," and his work is said to have saved more lives than the work of any other human. In 1980, the WHO declared smallpox an eradicated disease, the result of coordinated public health efforts, but vaccination was an essential component. Although the disease was declared eradicated, some pus samples still remain in laboratories at the CDC in Atlanta in the US, and in State Research Center of Virology and Biotechnology VECTOR in Koltsovo, Novosibirsk Oblast, Russia. Courtesy of Wikipedia.

In this painting from 1910, Edward Jenner is performing his first vaccination on James Phipps, a boy of age 8, on May 14, 1796.



Alexander Fleming in his laboratory at St Mary's, Paddington, London, c. 1943. Alexander Fleming (1881–1955) was a Scottish physician and microbiologist, best known for discovering the world's first broadly effective antibiotic substance, which he named penicillin. His discovery in 1928 of what was later named benzylpenicillin (or penicillin G) from the mould *Penicillium rubens* is described as the "single greatest victory ever achieved over disease." Fleming's discovery of penicillin in 1928 marks the start of the modern era of antibiotics. He remarked: "One sometimes finds, what one is not looking for. When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer. But I suppose that was exactly what I did." For this discovery, he shared the Nobel Prize in Physiology or Medicine in 1945 with Howard Florey and Ernst Boris Chain. He also discovered the enzyme lysozyme from his nasal discharge in 1922, and along with it a bacterium he named *Micrococcus Lysodeikticus*, later renamed *Micrococcus luteus.*



MEDICAL EDUCATION
IN THE
UNITED STATES AND CANADA

A REPORT TO
THE CARNEGIE FOUNDATION
FOR THE ADVANCEMENT OF TEACHING

BY
ABRAHAM FLEXNER

WITH AN INTRODUCTION BY
HENRY S. PRITCHETT
PRESIDENT OF THE FOUNDATION

BULLETIN NUMBER FOUR

576 FIFTH AVENUE
NEW YORK CITY

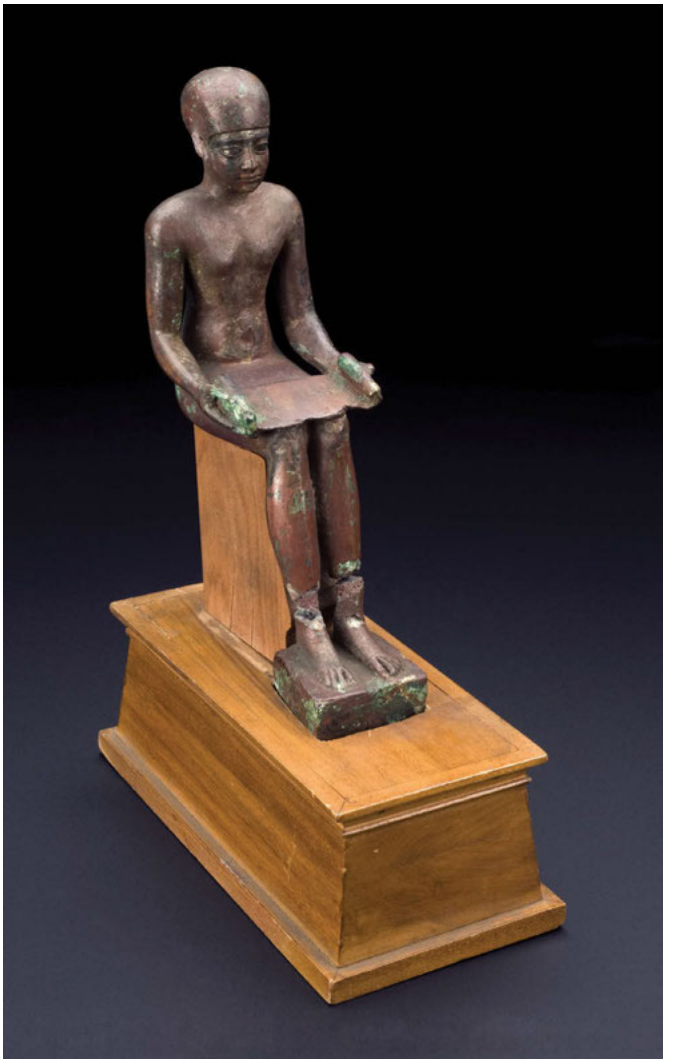
The image above shows the cover page of the simply and aptly titled *Medical Education in the United States and Canada. A Report to the Carnegie Foundation for the Advancement of Teaching.* It is attributed to Abraham Flexner (1866–1959), an American educator, best known for his role in the 20th century reform of medical and higher education in the US and Canada. He was an authority in innovative methods of medical education and was commissioned to evaluate medical education in North America. Flexner questioned many traditional aspects of medical education and challenged physicians to justify their practice in teaching: "what sound reason can be given for requiring the able and the less able, the industrious and the less industrious, to complete practically the same course of instruction in the same period of time?" (A. Flexner. (1924). Medical education, 1909–1924. *JAMA* 82(11):833–838.). In 1910, Flexner published the *Flexner Report*, which examined the state of American medical education and led to far-reaching reform in the training of doctors not only in North America but also worldwide. Indeed, it was to set the landscape for medical education for the new century. Image courtesy of the Wellcome Library.

Line engraving showing Galen in action – sallying forth holding a book and an ointment jar. Source: A. J. Brock. (1963). *On the Natural Faculties*. Heinemann, London, UK, p. 57.

Aelius Galenus or Claudius Galenus (Greek: Κλαύδιος Γαληνός; 129 – c. 216), often Anglicized as Galen or Galen of Pergamon, was a Greek physician, anatomist, surgeon and philosopher in the Roman Empire. Although his knowledge of anatomy was based not on human dissection but on the dissection of monkeys and pigs, he contributed enormously to medical wound management. Galen's ideas were to influence Western medical science for more than a thousand years. Many of his ideas were correct (voice comes from the larynx) while others were incorrect (venous blood was created in the liver and arterial blood in the heart). Galen correctly advocated ongoing medical education and keeping an open mind: "The fact is that those who are enslaved to their sects are not merely devoid of all sound knowledge, but they will not even stop to learn!"

William Harvey demonstrating his theory of circulation of the blood before King Charles I. Oil painting by Ernest Board. In this oil painting, Harvey is standing and the learner is relaxing in his chair; this role-reversal is due to the fact that the learner is King Charles I.

William Harvey (1578–1657) was an English physician, who was among the first to describe the circulation of the blood, and published his findings in his treatise *On the Motion of the Heart and Blood.* Before Harvey, it was thought that the arterial and venous systems were largely separate and only came in contact through pores in the ventricles. It is interesting to note that he was never able to show how blood passed from arteries to veins, but suspected that small blood vessels must enable this passage. Harvey lectured extensively on anatomy and highlighted the use of dissection and pathological demonstration in education: "I profess both to learn and to teach anatomy, not from books but from dissections; not from positions of philosophers but from the fabric of nature."

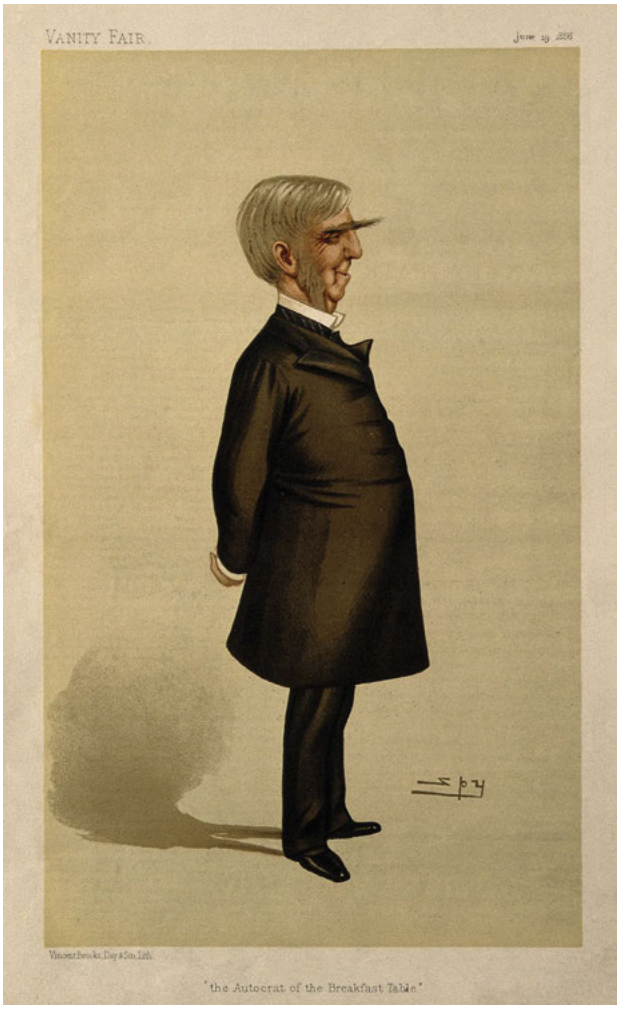


A statuette of Imhotep showing his typical pose – carefully studying a sheet of papyrus. It is from Egypt and made around 600–630 BC. Courtesy of the Science Museum, London.

Imhotep (/ɪm'hoʊtɛp/; Ancient Egyptian: ỉí-m-ḥtp "(the one who) comes in peace"; 2650–2600 BC) was an Egyptian physician and priest, who is often suggested as the author of the *Edwin Smith Papyrus,* which outlined detailed anatomical and pathological observations. "Case-based learning – a pedagogical technique in which the student applies knowledge to real-world scenarios – is ubiquitous in medical education today, and Imhotep is considered the earliest advocate of this teaching. Case-based learning unquestionably promotes self-directed learning and rational clinical problem-solving in medicine. However, it wasn't all seriousness in Imhotep's teachings. Possibly, the earliest attribution of the famous phrase, "eat, drink and be merry for tomorrow we shall die," is to Imhotep.

Shown above is a popular colored lithograph reproduction of a painting of Florence Nightingale by Henrietta Rae, 1891. She is seen with her lamp at a patient's bedside during the Crimean War.

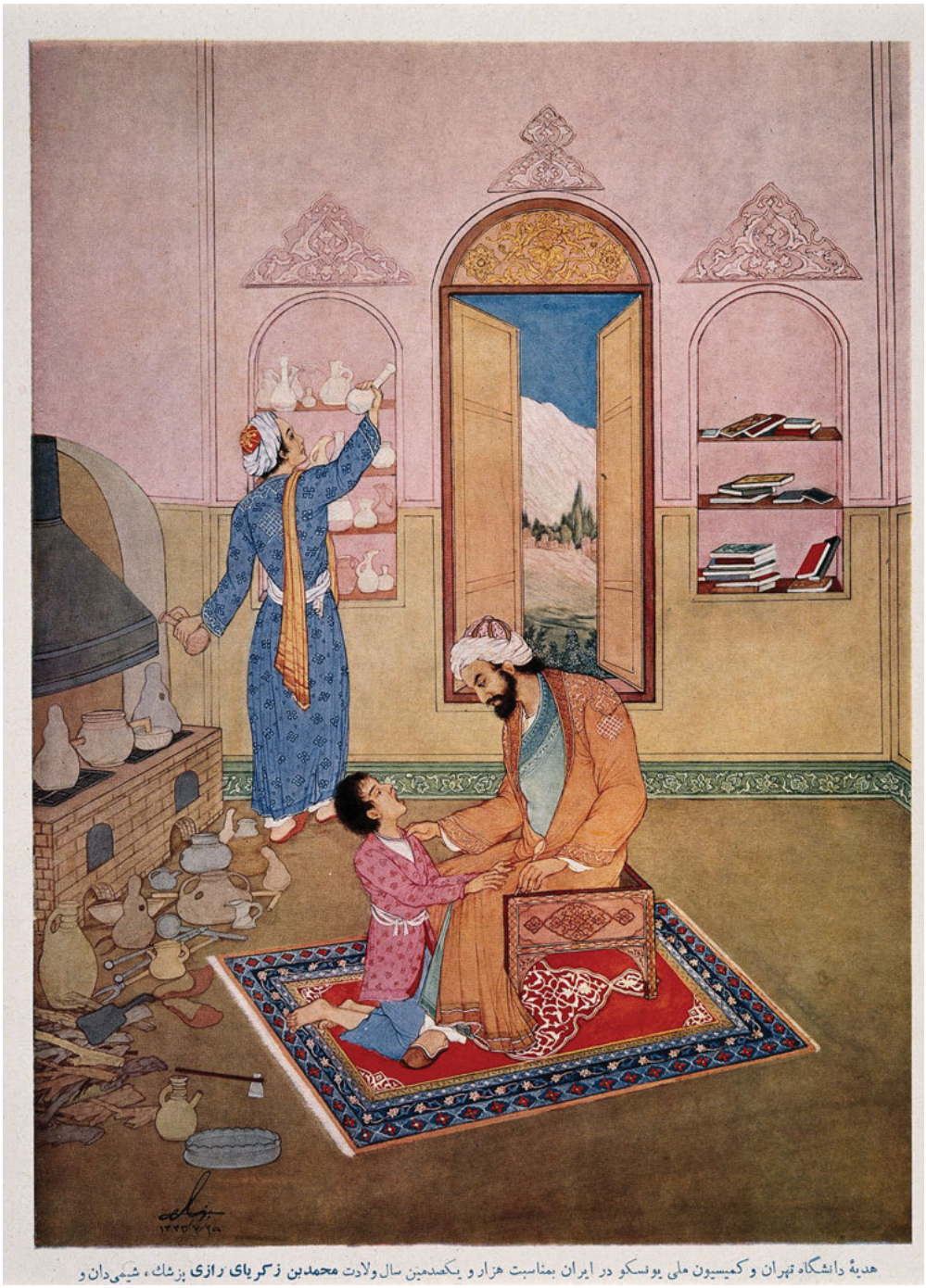
Florence Nightingale (1820–1910) was an English social reformer, statistician and the founder of modern nursing. Nightingale came to prominence while serving as a manager and trainer of nurses during the Crimean War, in which she organized care for wounded soldiers at Constantinople. She significantly reduced death rates by improving hygiene and living standards. Nightingale gave nursing a favorable reputation and became an icon of Victorian culture, especially in the persona of "The Lady with the Lamp" making rounds of wounded soldiers at night. In recognition for her pioneering work in nursing is the Nightingale Pledge, which is the oath taken by new nurses; the Florence Nightingale Medal, which is the highest international distinction a nurse can achieve; and International Nurses Day, which is celebrated on her birthday. Her social reforms included improving healthcare for all sections of British society, advocating better hunger relief in India, helping to abolish prostitution laws that were harsh for women, and expanding the acceptable forms of female participation in the workforce. Nightingale was fluent in four languages: English, French, German and Italian. Courtesy of Wikipedia.



Oliver Wendell Holmes, Sr. (1809–1894) was an American polymath**,** physician, poet, educator and author. After graduating from Harvard in 1829, he briefly studied law before turning to the medical profession. I have had a similar path to medicine. He coined the term "Boston Brahmin" to describe those of the upper castes of Boston society – from which he himself had originated. A dedicated reformer, he sometimes became frustrated by the slow pace of change in medicine – once famously saying, "it is so hard to get anything out of the dead hand of medical tradition!" This colored lithograph by Leslie Matthew Ward shows Holmes as *The Autocrat of the Breakfast Table*; this also was the title of a collection of his essays. Holmes' students called him "Uncle Oliver." He became an advocate for various medical reforms, and notably posited the controversial idea that doctors were capable of carrying puerperal fever from patient to patient. Holmes was well respected by his peers, and garnered a large, international following throughout his long life. Particularly noted for his intelligence, he was named by American theologian Henry James Sr. as "intellectually the most alive man I ever knew."

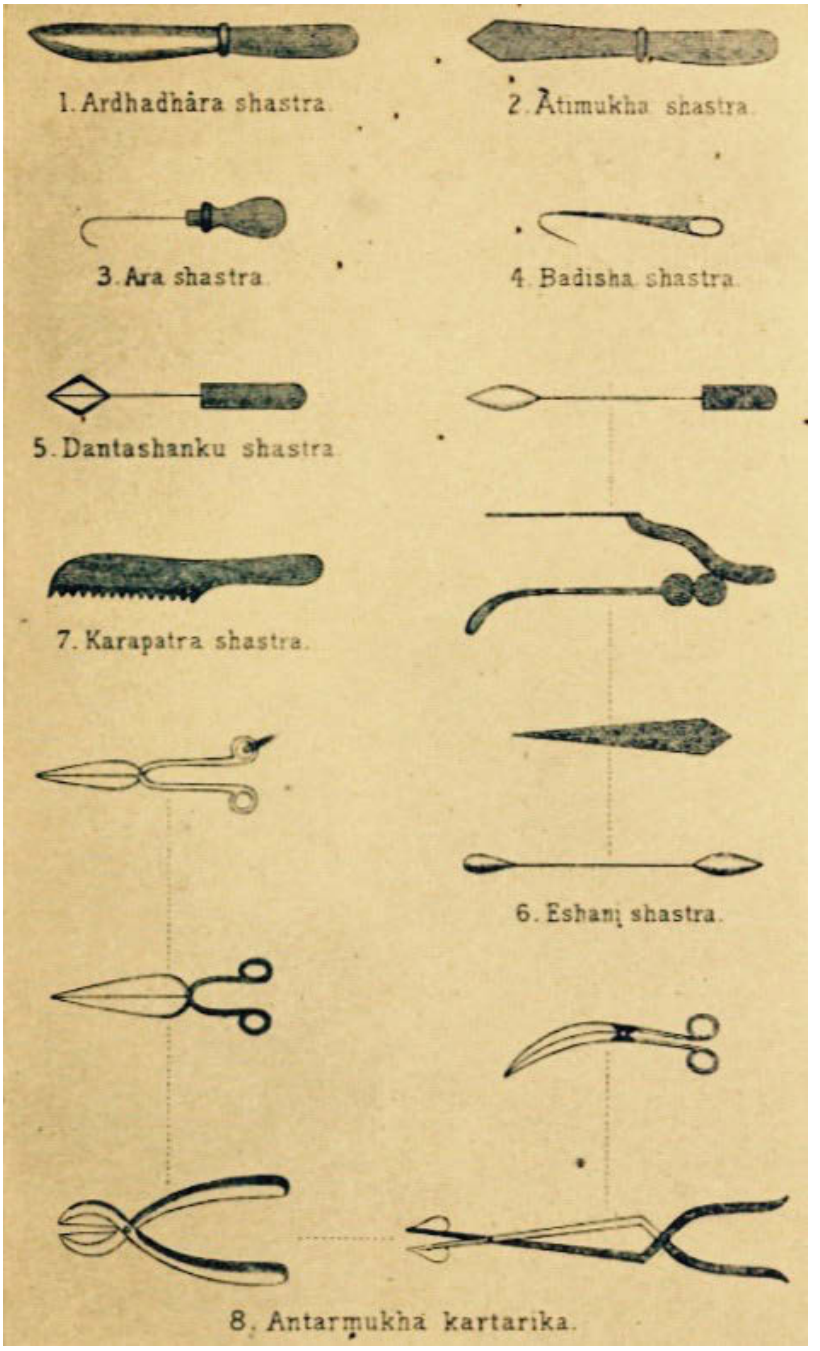
A plate from *Osteographia* (or "the Anatomy of Bones"), published in 1733 by William Cheselden, an English surgeon and teacher of anatomy and surgery. Cheselden was influential in establishing surgery as a scientific medical profession. *Osteographia* was the first full and accurate description of the anatomy of the human skeletal system. Image kindly provided by the National Library of Medicine.

Portrait of Rhazes (al-Razi) (AD 865–925), physician and alchemist who lived in Baghdad (in present-day Iraq). Abū Bakr al-Rāzī (full name: محمد بن زكرياء الرازي أبو بكر, Abū Bakr Muḥammad bin Zakariyyāʾ al-Rāzī, often known as (al-)Razi or by his Latin name Rhazes, also rendered Rhasis) was a Persian physician, philosopher and alchemist who lived during the Islamic Golden Age. Rhazes was born in Rey, Persia. He was a physician, philosopher, and chemist. He was also a prolific author of more than 200 manuscripts. He was the first to clinically distinguish between smallpox and measles, and suggest sound treatment for the former. His medical teaching often was based on the questions of his students. However, he always allowed other students to attempt to answer questions before he intervened. This method of peer education persists to this day. He also wrote about the ethics of medicine: "the doctor's aim is to do good, even to our enemies, so much more to our friends, and my profession forbids us to do harm to our kindred, as it is instituted for the benefit and welfare of the human race, and God imposed on physicians the oath not to compose mortiferus remedies." Image courtesy of the Wellcome Library.



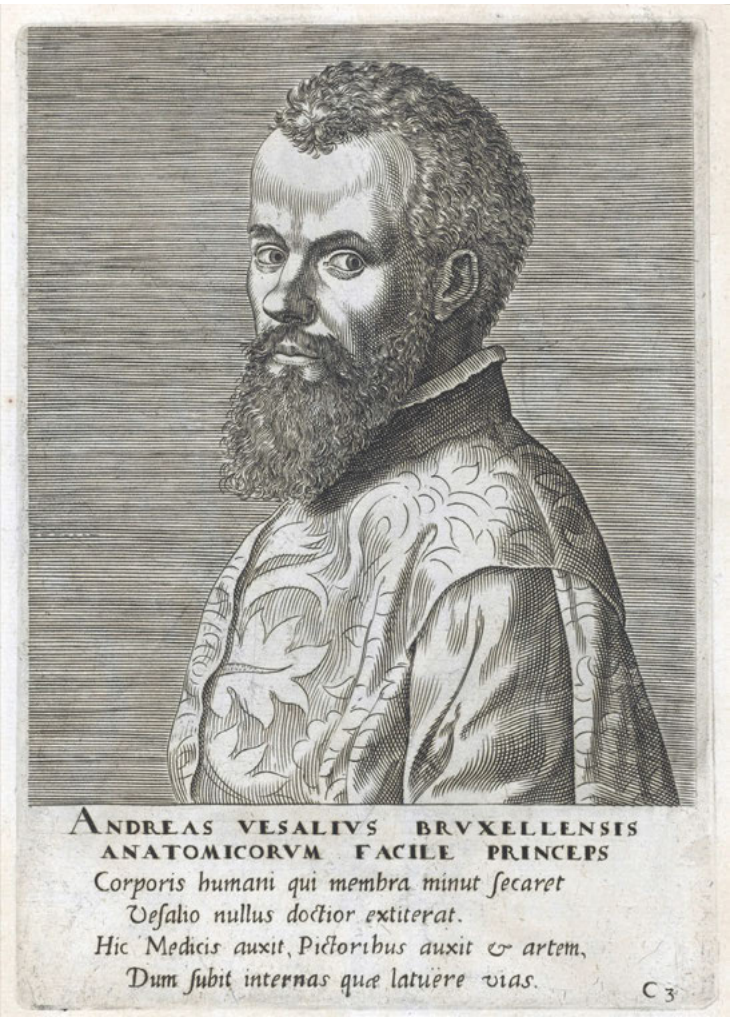
This miniature painting by Hossein Behzad shows Rhazes (al-Razi) examining a patient. Rhazes examines a kneeling boy, who has his mouth wide open. They are in a surgery full of equipment.

Sushruta, or *Suśruta* (Sanskrit: सुश्रुत, IAST: *Suśruta*, lit. "well heard" c. 700 BC) was a remarkable ancient Indian physician, surgeon and author who probably lived in the sixth century BC. The *Sushruta Samhita* (*Sushruta's Compendium*), a treatise ascribed to him, is one of the most important surviving ancient treatises on medicine and is considered a foundational text of Ayurveda. The treatise addresses all aspects of general medicine, but the impressive chapters on surgery have led to the false impression that this is its main topic. He is often dubbed, "the father of plastic surgery" because of these detailed accounts of surgery. Perhaps most extraordinary is a section on plastic surgery, which mentions the use of flaps for a number of different purposes; it also gives a basic outline on how to reconstruct the nose after an injury. The text, furthermore, touches on how best to study medicine and emphasizes the importance of good study habits: "A pupil who is pure, obedient to his preceptor, applies himself steadily to his work, and abandons laziness and excessive sleep, will arrive at the end of the science he has been studying." This watercolor depicts Susruta clearly, but what is he demonstrating?



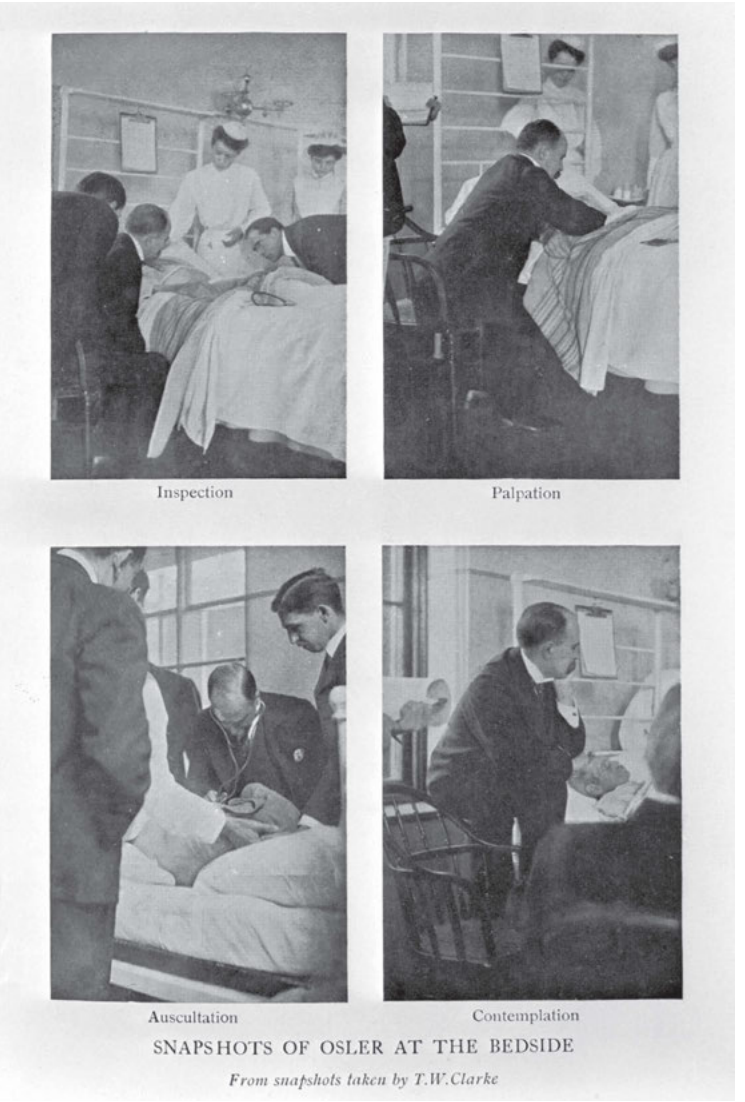
This is Plate No. 3 of four plates published in the 1907 book, *An English Translation of the Sushruta Samhita in Three Volumes* (Volume 1), on page LXIX of the Introduction section. It represents the following surgical instruments: 1 Ardhadhara shastra, 2 Atimukha, 3 Ara, 4 Badisha, 5 Dantashanku, 6 Eshani shastra, 7 Karapatra, and 8 Antarmukha kartarika.

Palm leaves of the *Sushruta Samhita* or *Sahottara-Tantra* from Nepal, stored at Los Angeles County Museum of Art. The text is dated 12th-13th century, while the art is dated 18th-19th century.



Portrait of Andreas Vesalius Bruxellensis Anatomicorum Facile Princeps. 1572. Source: P. Galle (1572). *Virorum doctorum de disciplinis bene merentium effigies XLIIII.* Antuerpiae.

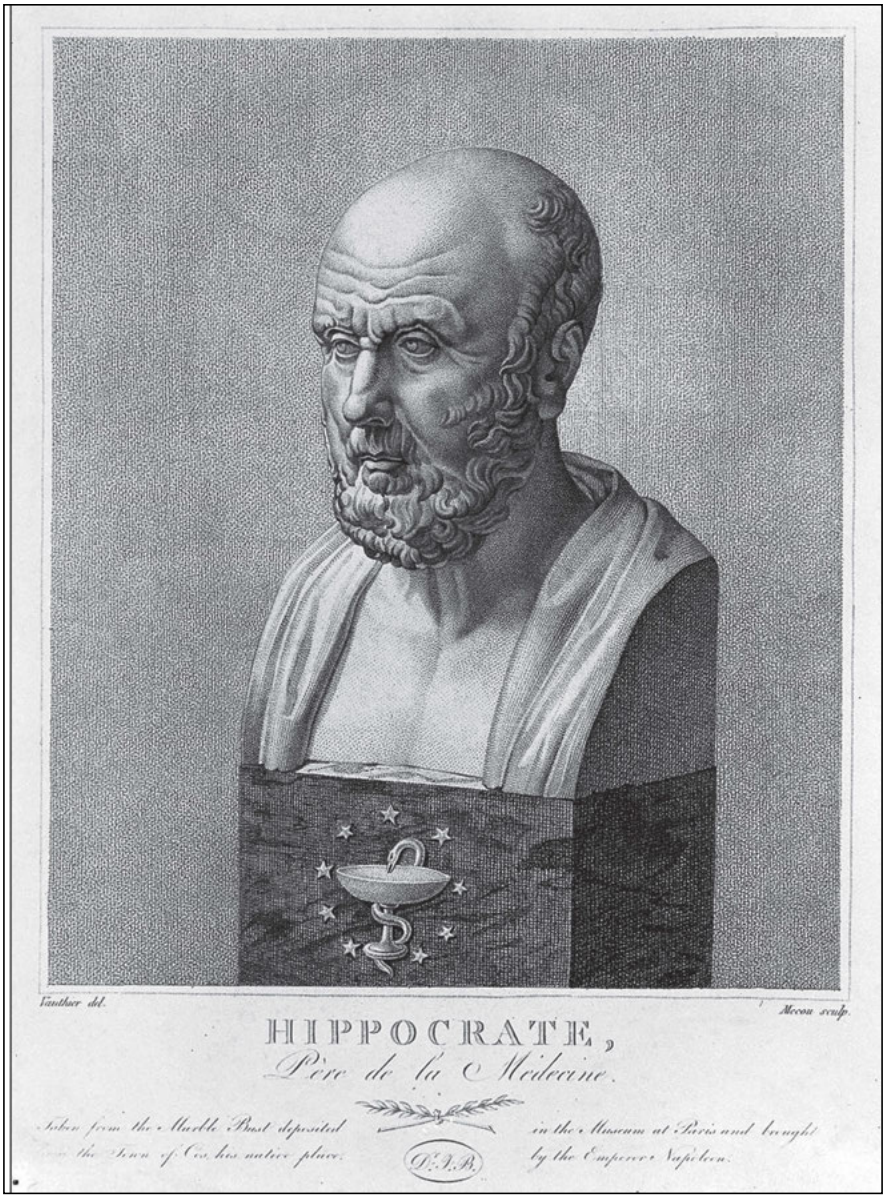
Andreas Vesalius (1514–1564) was a Belgian physician and anatomist who wrote *De Humani Corporis Fabrica*, an iconic textbook of anatomy. At the start of the sixteenth century, Vesalius was one of the first to test the findings of medical giants like Galen and other ancient physicians. In what seemed like blasphemy at the time, he was to prove Galen wrong on a number of counts. For example, he demonstrated that the mandible was made of only one bone and not two as Galen had thought. Vesalius emphasized the active role of the learner in medical education: "I strive that in public dissection the students do as much as possible so that if even the least trained of them must dissect a cadaver before a group of spectators, he will be able to perform it accurately with his own hands; and by comparing their studies one with another they will properly understand this part of medicine."



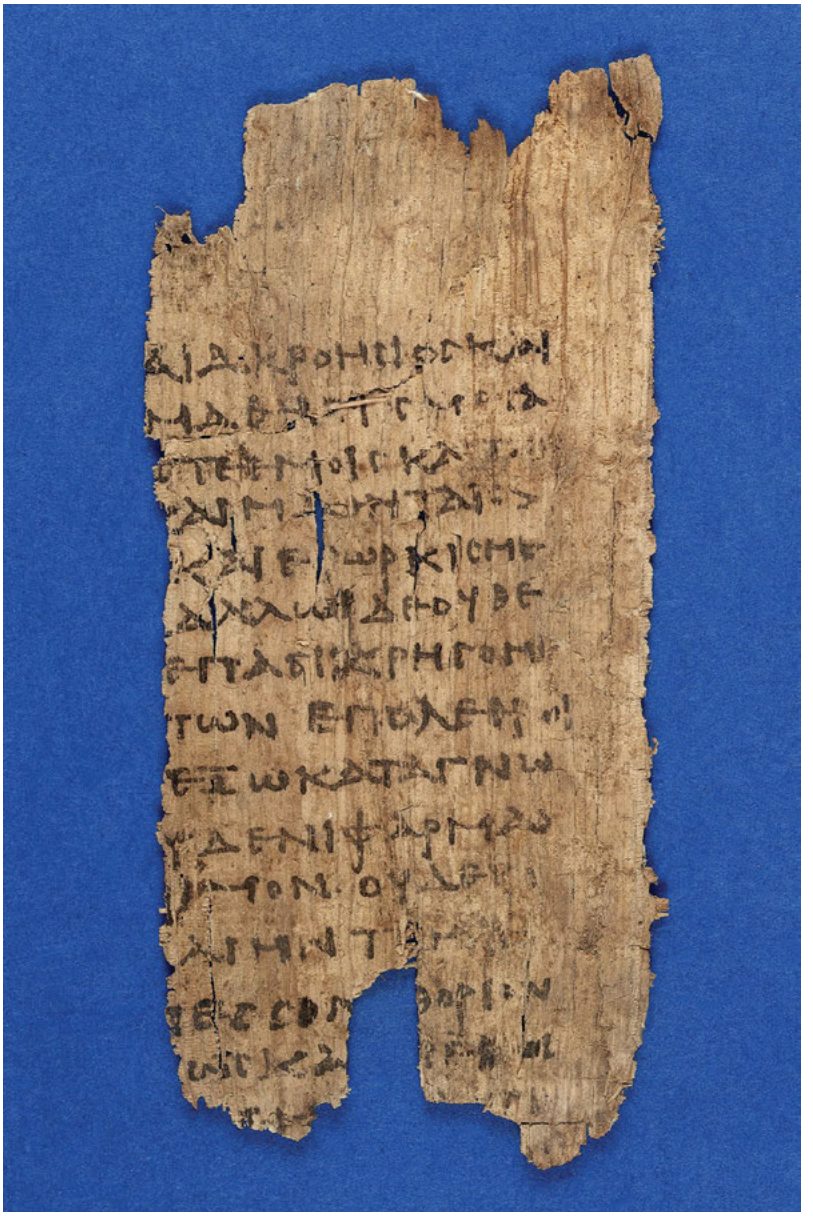
Shown above is a page from *The Life of Sir William Osler* by Harvey Cushing, published by Clarendon Press, Oxford, 1925, Volume 1, Facing page 552. Courtesy of the Wellcome Library, London.

Physician and educator, Sir William Osler (1849–1919), a founding father of Johns Hopkins Hospital, was a pioneer of experiential education. He introduced clinical training at the bedside for medical students and trainee doctors and set up residencies for doctors in training. The pictures above highlight his approach to training doctors through bedside demonstration of four critical skills – inspection, palpation, auscultation, and contemplation. He accomplished this through his hands-on "grand rounds" with medical residents. These relatively simple skills would help him discover the following eponymous signs, syndromes and diseases: Osler's nodes, Osler's sign, Osler–Rendu–Weber disease, and Osler–Libman–Sacks syndrome.

The above photograph was obtained from *Description of the Johns Hopkins Hospital*, John Shaw Billings, Press of J. Friedenwald, Baltimore, 1890, p. 19. It shows the octagonal shape of the Octagon Ward at John Hopkins Hospital that facilitated the training of medical residence through "rounds," where residents and their physician educators, such as William Osler, moved from patient to patient. This environment led to the experiential education of medical residents in facilities that provide high-quality patient care. Residents also learn critical skills in communication, team building, and mentorship, in addition to their clinical skills development.



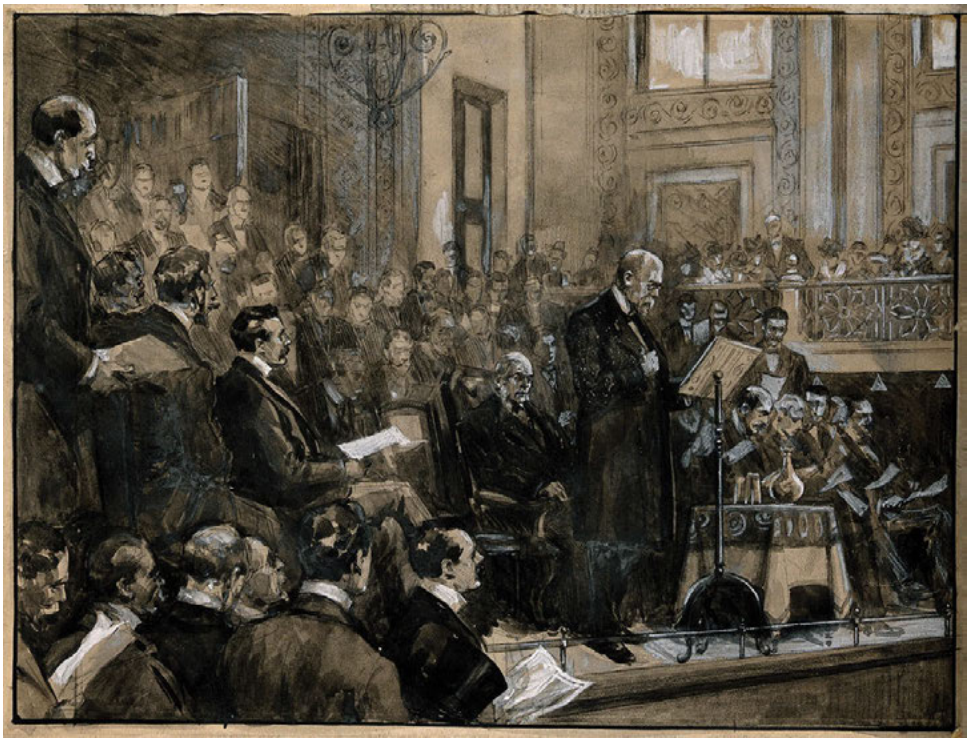
Engraving depicting the marble bust of Hippocrates by A. Mecou after Vauthier after a statue in the Louvre. Hippocrates of Kos (Greek: Ἱπποκράτης ὁ Κῷος, translit. *Hippokrátēs ho Kóos*), also known as Hippocrates II (460–370 BC), was a Greek physician, who is considered one of the most outstanding figures in the history of medicine. Hippocrates is traditionally referred to as the "Father of Medicine" in recognition of his lasting contributions to the field, such as the use of prognosis, clinical observation, and the systematic categorization of diseases. He was the first to describe many diseases and, also, to set out standards of ethical behavior to which physicians were expected to adhere: "Cure sometimes, treat often, comfort always." Image courtesy of the Wellcome Library, London.



This torn papyrus text shows a fragment of the Hippocratic Oath. The Hippocratic Oath is attributed to the Greek physician Hippocrates (460–370 BC). Ironically, it is not certain that Hippocrates actually wrote it. Most modern scholars believe that it originated after his death. The Hippocratic Oath is taken by physicians who promise to practice medicine to high ethical and professional standards. These include the principles of medical confidentiality and non-maleficence. It often is said that the exact phrase, "*First, do no harm* (Latin: *Primum non nocere*)," is a part of the original Hippocratic oath. Image kindly provided by the Wellcome Library.

This image shows a reconstruction of the facade of the temple of Asclepius at Epidaurus, Greece. Today, only the foundation of the temple is preserved. However, the original temple has had a long-lasting influence on the architecture of buildings associated with medicine. Watercolor. Source: H. Lechat. (1895). *Epidaure, restauration & description des principaux monuments du sanctuaire d'Asclépios*, Librairies-Imprimeries Reunies, Paris, Plate III.

Asclepius (Greek: Ἀσκληπιός *Asklēpiós* [asklɛːpiós]; Latin: *Aesculapius*), or Hepius, is a hero and god of medicine in ancient Greek religion and mythology. The temple of Asclepius at Epidaurus was built in the 4th century BC. Medicine and medical education have been intertwined with religion for centuries. For example, the witch-doctor (with his psychosomatic associations), the priests of ancient times (with access to the gods of health), practitioners treating diseases (with their religious prayers, dances or ceremonies), and religious shamans (with their magical cures) tended to underline the importance of medicine as an art or mystique, rather than a science with application of technique and – now – technology. Over the millennia, medicine slowly has shed its religious origins. However, a few remnants persist: Latin continues to have a strong influence over the language of both religion and medicine.



Robert Koch addressing a conference at St James's Hall, Piccadilly, London. Gouache by F. C. Dickinson, 1901. Robert Koch (1843–1910) was a German physician and medical microbiologist. He discovered the anthrax disease cycle (1876), and the bacteria responsible for tuberculosis (1882) and cholera (1883). He also worked extensively on tropical diseases, including African trypanosomiasis and malaria. He is considered to be one of the founders of medical bacteriology and the father of microbiology (with Louis Pasteur). In my view, his discovery of *Bacillus anthracis* in 1876 marks the birth of modern bacteriology. He was the first to grow bacteria in laboratory using agar and glass plates (later developed as Petri dishes by his assistant Julius Petri). In 1905, he was awarded the Nobel Prize in Physiology or Medicine for his work on tuberculosis. Koch also claimed to have found a cure for tuberculosis. Unfortunately, this was not the case as the drug, Tuberculine, turned out to be useful for diagnosing tuberculosis rather than treating it. In fact, Tuberculine, which is a purified protein derivative (PPD) from cultures of tubercle bacillus, still is used today in a skin test by hypodermic injection for infection with or immunity to tuberculosis. Koch established guidelines for determining whether a particular microorganism is the causative agent of a specific infectious disease. He remained a modest man until the end of his life: "If my efforts have led to greater success than usual, this is due, I believe, to the fact that during my wanderings in the field of medicine, I have strayed onto paths where the gold was still lying by the wayside. It takes a little luck to be able to distinguish gold from dross, but that is all."

Ignaz Philipp Semmelweis (1818–1865) (German: ['ɪgnaːts 'zɛmḭvaɪs]; Hungarian: *Semmelweis Ignác Fülöp* ['sɛmmɛlvɛjs 'ignaːts 'fyløp]) was a Hungarian physician who discovered that simple antiseptic procedures such as handwashing could markedly reduce the occurrence of puerperal (or childbed) fever and save the lives of mothers in maternity wards. He was popularly known as "the saviour of mothers." To avoid spreading microbes like SARS-CoV-2, the least controversial and most effective tactic today is to "properly" wash hands with soap and water. However, in the 19th century, this simple practice was considered scandalous. His doctrine was eventually accepted by medical science and control of infection was hailed by Joseph Lister: "I think with the greatest admiration of him and his achievement and it fills me with joy that at last he is given the respect due to him." In my humble opinion, Semmelweis deserves to be portrayed as one of the greatest physicians and public health advocates of all time. Semmelweis University is a research-led medical school in the stunningly beautiful city of Budapest in Hungary. Founded in 1769, it was renamed in 1969 in honor of Semmelweis. I have regularly visited this outstanding institution for conferences and research activities for the past 15 years.

Joseph Lister with his house surgeons and dressers. Photograph by Barrauds, between 1890 and 1899.

Joseph Lister (1827–1912) was an English surgeon who introduced antiseptics during surgery. Based on the concepts of Pasteur, he employed carbolic acid to clean surgical wounds and insisted that his surgical team use aseptic techniques during surgery. Before Lister's studies of surgery, it was believed that chemical damage from exposure to "bad air" (*miasma*) was responsible for infections in wounds. Lister's studies showed that using antiseptics, washing hands, and wearing gloves drastically reduced the incidence of surgical site infections—basic hygiene principles that are critical in this era of COVID-19. He is remembered and honored for his remarkable accomplishments that earned him the title "Father of Modern Surgery." Lister summarized the conclusions of his research: "But since the antiseptic treatment has been brought into full operation, and wounds and abscesses no longer poison the atmosphere with putrid exhalations, my wards, though in other respects under precisely the same circumstances as before, have completely changed their character; so that during the last nine months not a single instance of pyemia, hospital gangrene, or erysipelas has occurred in them." Although asepsis and sterile techniques have replaced antisepsis as the primary principle in combating infection, Lister's application of germ theory laid the foundation for surgery.

This colored etching by J. B. Wunder (c. 1832) shows a man who has barricaded himself with a panoply of protections against the cholera epidemic. The latter is represented as a hag, implying an overabundance of useless advice concerning protection against cholera.

Shown above is a colored etching by R. I. Cruikshank (c. 1832) that depicts a cholera patient experimenting with remedies.

Cholera is an infection of the small intestine by strains of the bacteria *Vibrio cholerae*. The classic symptom is large amounts of watery diarrhea that lasts a few days; vomiting and muscle cramps may also occur. It is spread mostly by unsafe water and unsafe food that has been contaminated with human feces containing the bacteria. Humans are the only known host for the bacteria. Prevention methods against cholera include improved sanitation and access to clean water. Cholera vaccines, oral rehydration salts (ORS), Zinc supplementation, intravenous fluids, etc., are all used used for therapy and prophylaxis (with varying effectiveness).

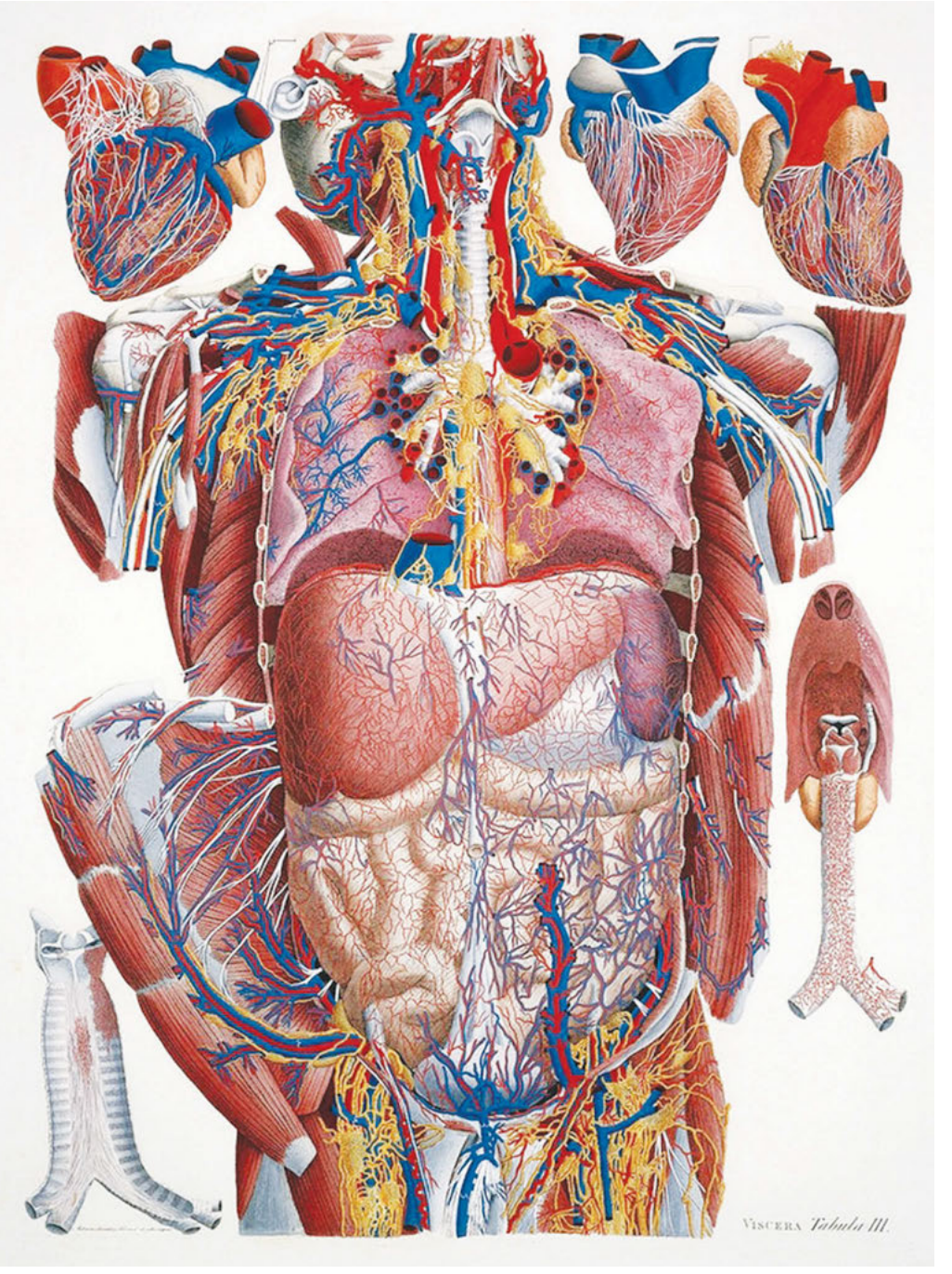
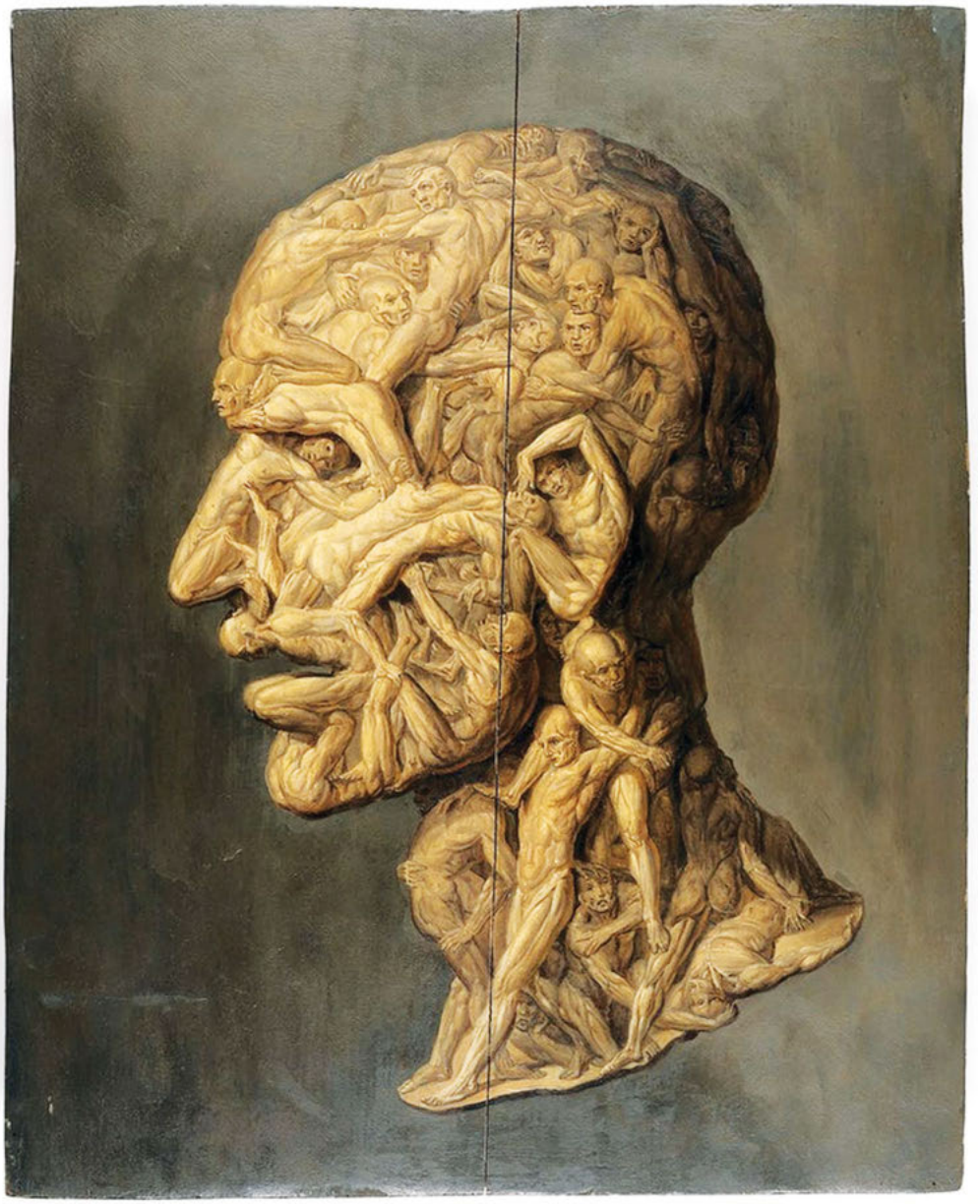


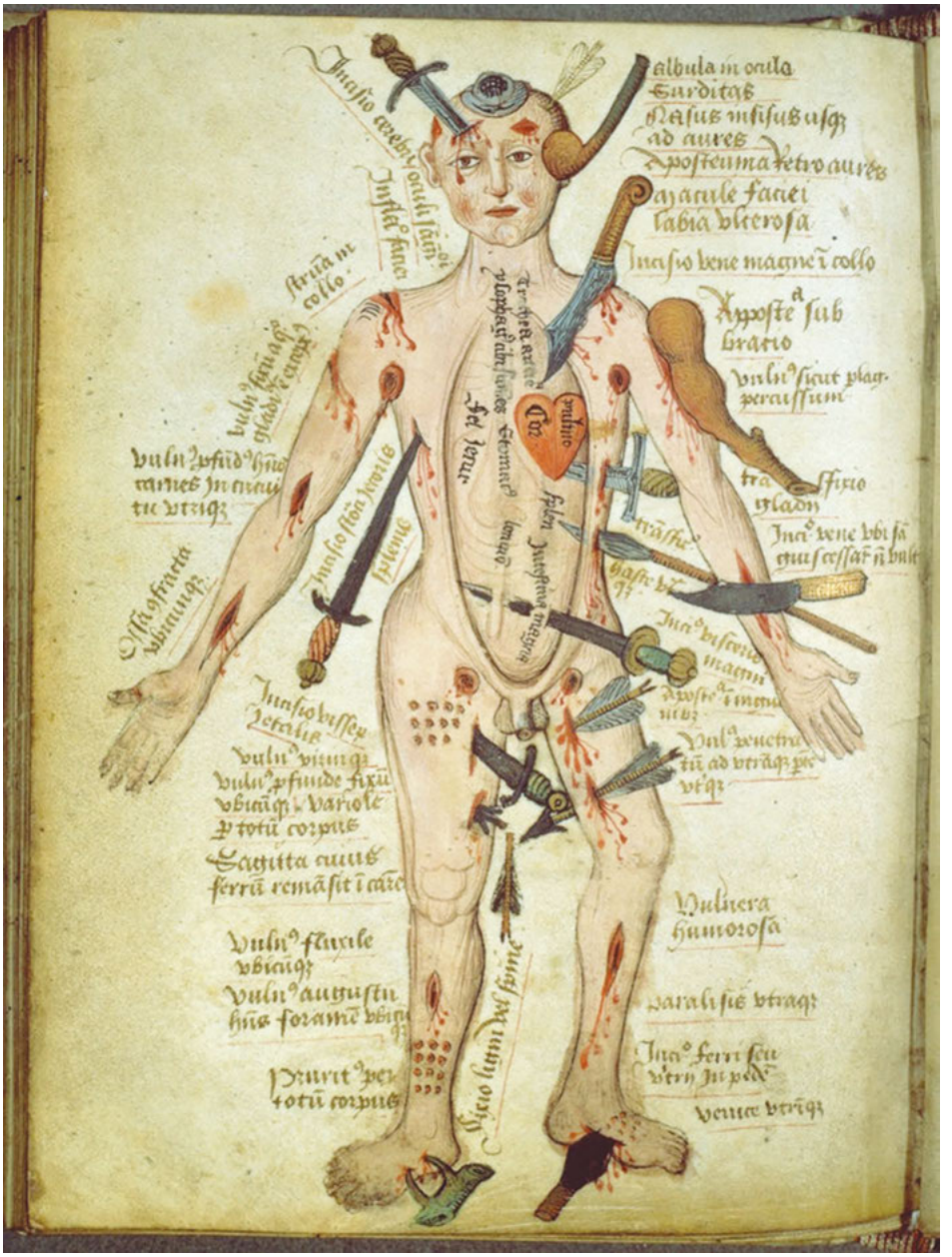
Illustration of human viscera by the Italian anatomist, Paulo Mascagni (1752 or 1755–1815), from his *Anatomia Universa* (1823–31). Image courtesy of the Wellcome Library, London.



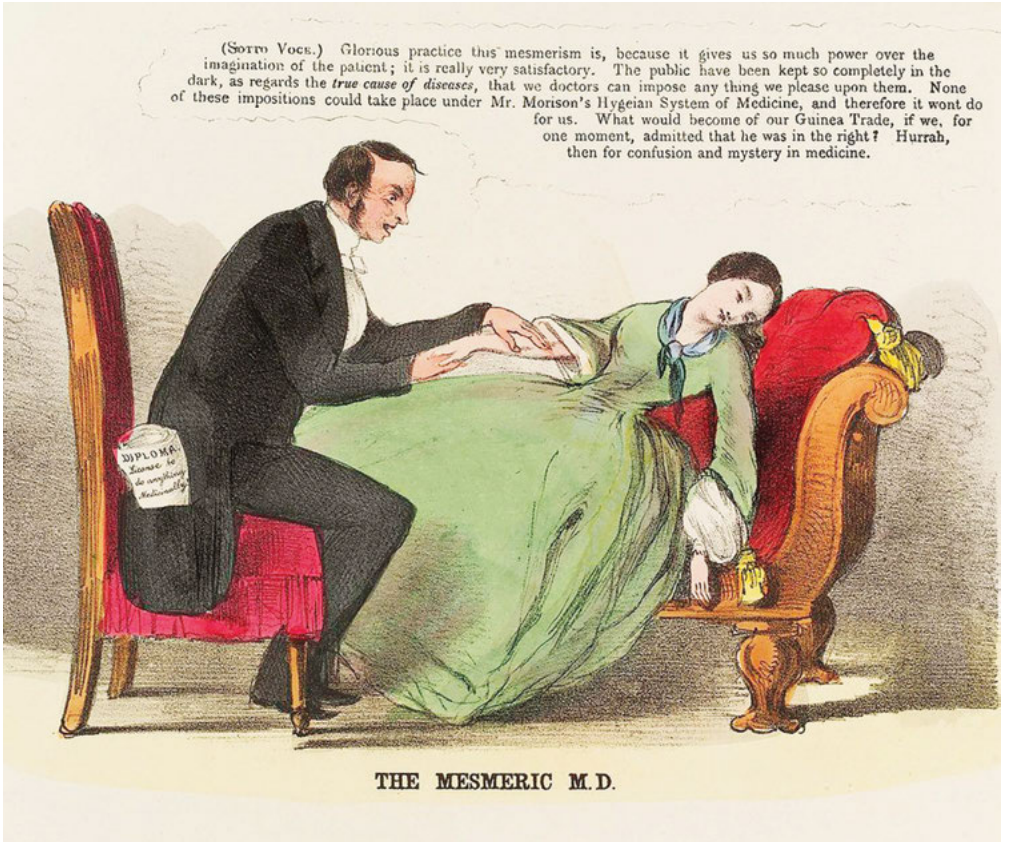
Testa Anatomica (1854); profile view of a male human head composed of writhing, apparently tormented naked men, by Filippo Balbi. Courtesy of the Wellcome Library, London.

An image from an Indian manuscript in Sanskrit, c. 1469. This manuscript is from the genre of *karmavipāka*, meaning "the ripening of karma." It begins with a salutation to the sage Dhanvantari, the traditional author of the original works on Ayurveda, one of the world's oldest medical systems. Image courtesy of the Wellcome Library, London.

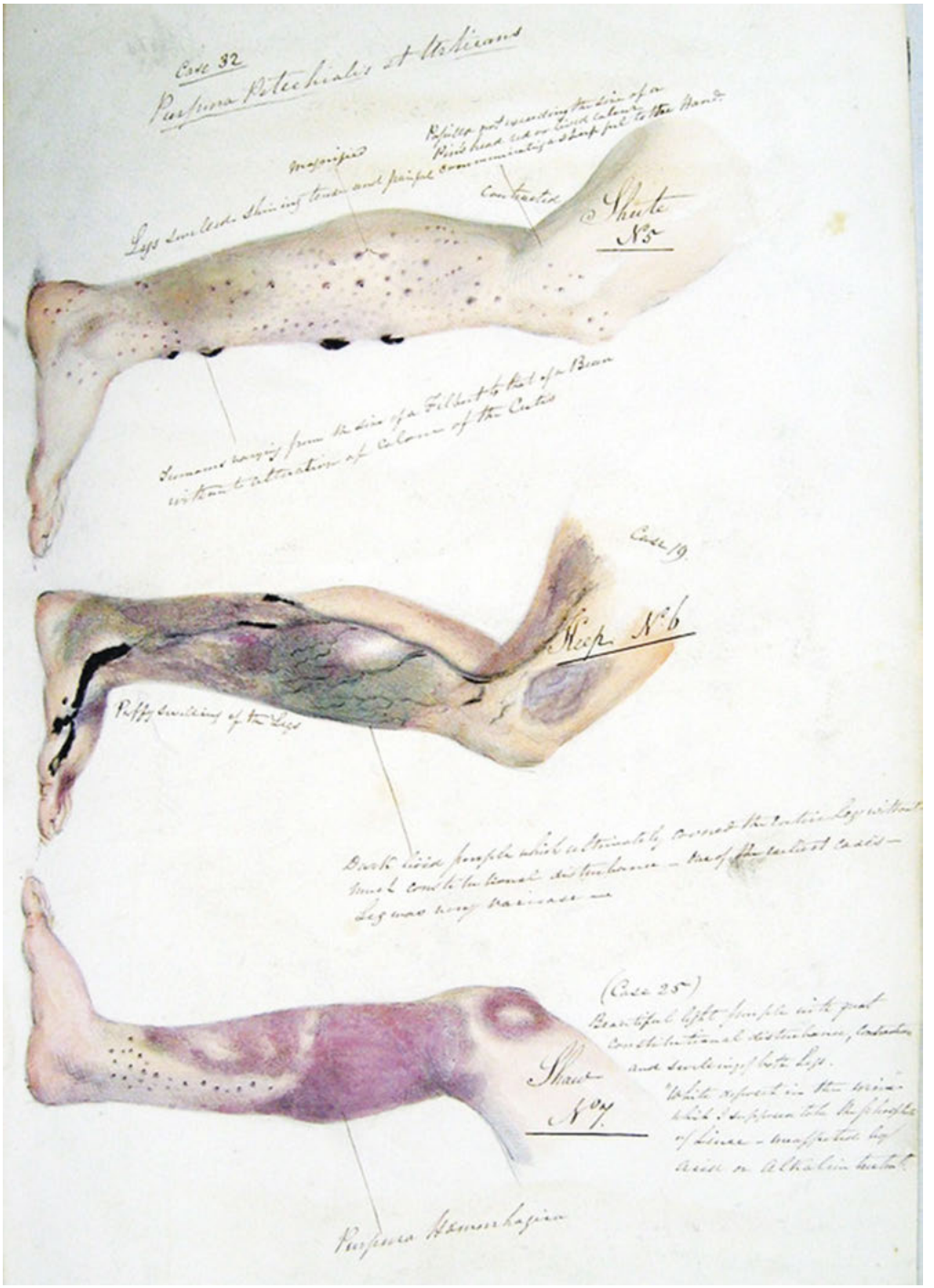
Illustrations of Tibetan *materia medica* from the *Sman bla'i dgongs rgyan rgud bzhi'i nang gi 'khrungs dpe re zhig,* meaning, "A Selection of Substances used for the Production of Medicine based on the Teaching of the four medical Tantras." Specifically, the image shows two leaves detailing traditional medicines. A manuscript such as this would often have been carried by a Tibetan doctor to refer to while treating patients. Image courtesy of the Wellcome Library, London.



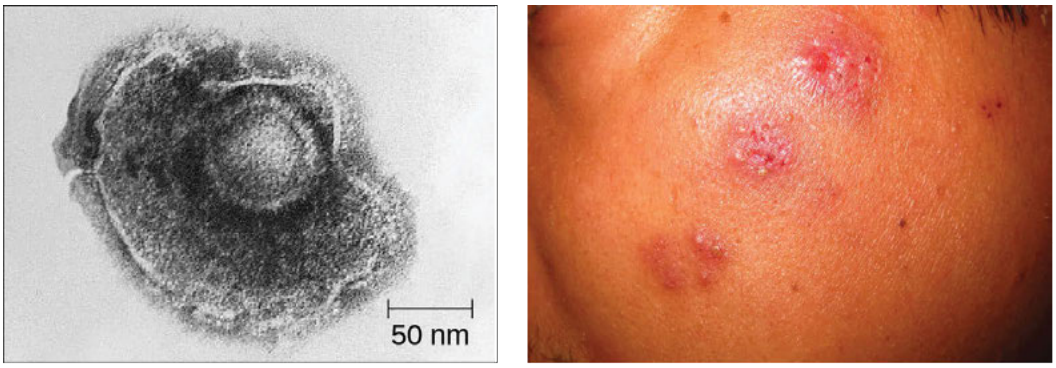
Wound Man image, folio 53 verso, from Claudius (Pseudo) Galen's *Anathomia,* a 15th century English anatomical manuscript. Note that the words in Latin do not provide any treatment regimen but merely describe the injury ("penetration by a sword" or "an arrow whose point has remained in the thigh"). The weapons are shown as they pierce the body, and the positions of internal organs are indicated. Figures like these can be found in several manuscripts and printed books produced in the 15th and 16th centuries. They might have served as a reminder of the injuries to which the human body is prone.



A color lithograph from 1852 depicting a mesmeric physician taking advantage of his female patient. In his pocket can be seen a diploma reading "License to do anything medicinally." Courtesy of the Wellcome Library.

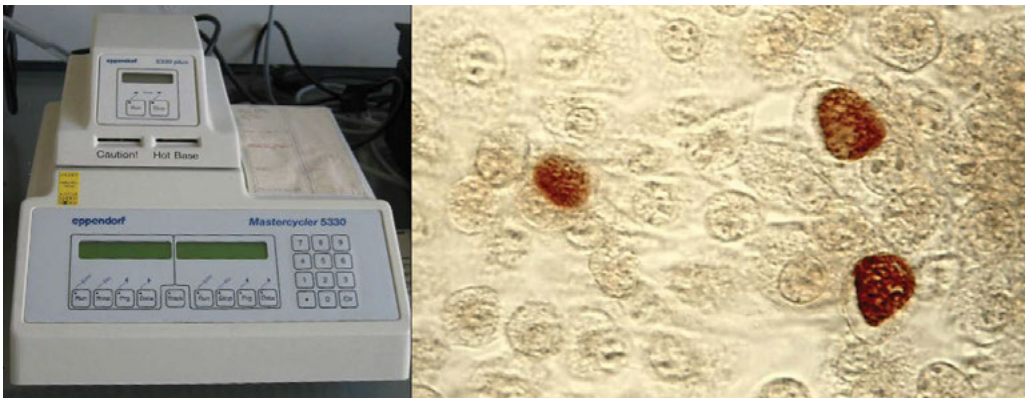


A page from the journal of Henry Walsh Mahon showing the effects of scurvy from his time aboard the HM convict ship *Barrosa* (1841–42). Courtesy of the Wellcome Library.

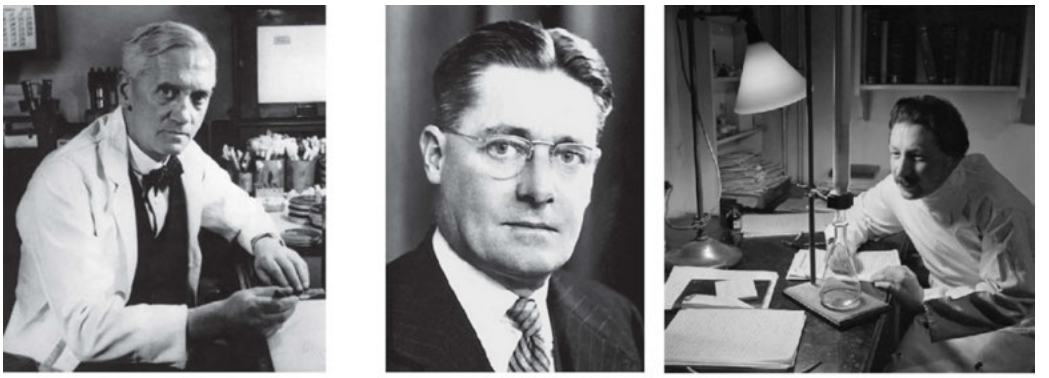


(a) (b)

Images depicting: (a) Varicella-zoster, the virus that causes chickenpox, has an enveloped icosahedral capsid visible in this transmission electron micrograph. Its double-stranded DNA genome becomes incorporated in the host DNA. (b) After a period of latency, the virus can reactivate in the form of shingles, usually manifesting as a painful, localized rash on one side of the body. Image (a) courtesy of Erskine Palmer and B.G. Partin—scale-bar data from Matt Russell. Image (b) courtesy of Paulo O/Flickr (CC-BY). Text courtesy of OpenStax (https://openstax.org).

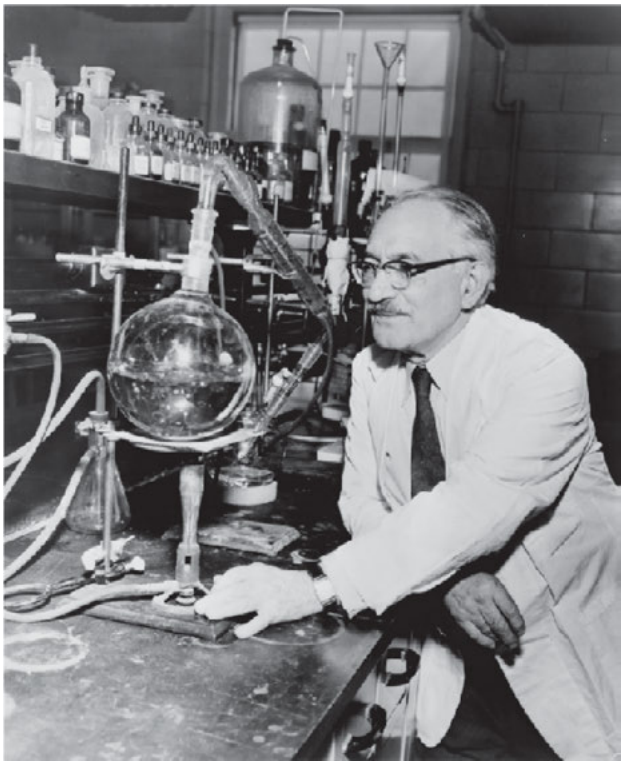


A thermal cycler (left) is used during a polymerase chain reaction (PCR). PCR amplifies the number of copies of DNA and can assist in diagnosis of infections caused by microbes that are difficult to culture, such as *Chlamydia trachomatis* (right). *C. trachomatis* causes chlamydia, one of the most common sexually transmitted diseases in the US, and trachoma, the world's leading cause of preventable blindness. Courtesy of the Centers for Disease Control and Prevention and OpenStax (https://openstax.org).



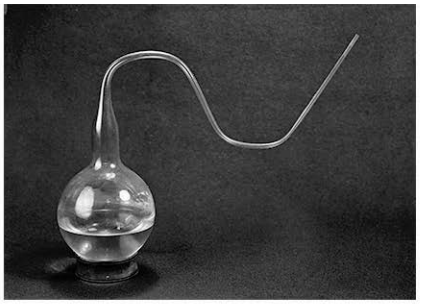
(a) (b)

(a) Alexander Fleming was the first to discover a naturally produced antimicrobial, penicillin in 1928. (b) Howard Florey and Ernst Chain discovered how to scale up penicillin production. Then, they figured out how to purify it and showed its efficacy as an antimicrobial in animal and human trials in the early 1940s. Courtesy of OpenStax (https://openstax.org).

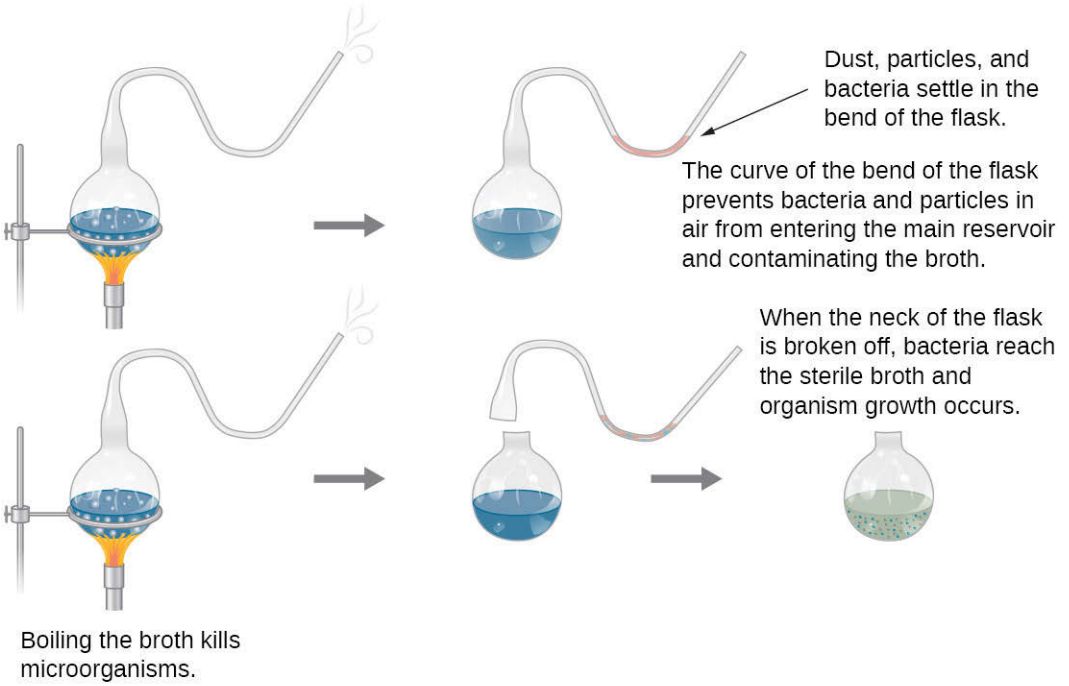


Selman Waksman was the first to show the vast antimicrobial production capabilities of a group of soil bacteria, the actinomycetes. Courtesy of OpenStax (https://openstax.org).

(a)

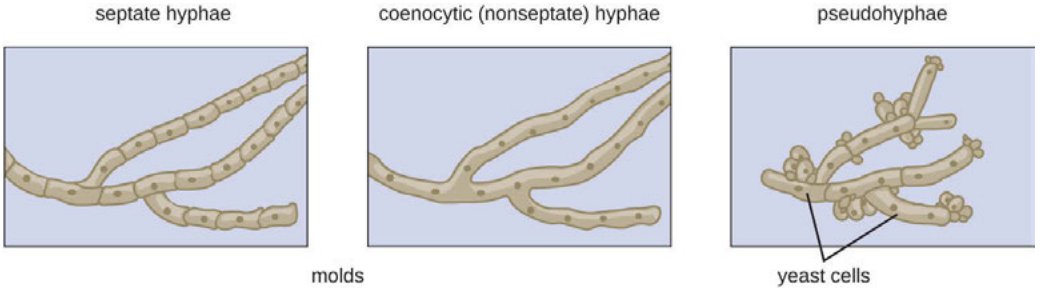


(b)

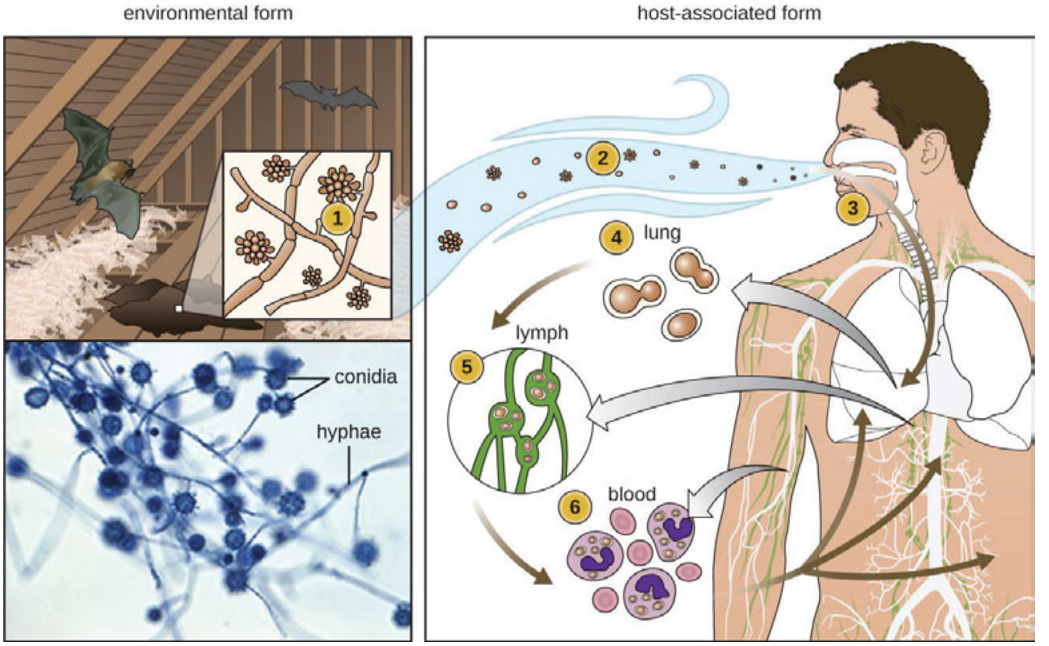


(c)

(a) French microbiologist Louis Pasteur, who definitively refuted the long-disputed theory of spontaneous generation. (b) The unique swan-neck feature of the flasks used in Pasteur's experiment allowed air to enter the flask but prevented the entry of bacterial and fungal spores. (c) Pasteur's experiment consisted of two parts. In the first part, the broth in the flask was boiled to sterilize it. When this broth was cooled, it remained free of contamination. In the second part of the experiment, the flask was boiled and then the neck was broken off. The broth in this flask became contaminated. Courtesy of Wellcome Images/Wikimedia Commons and OpenStax (https://openstax.org).

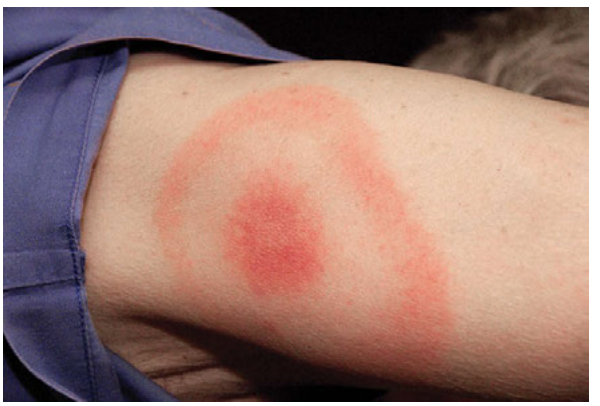


Multicellular fungi (molds) form hyphae, which may be septate or nonseptate. Unicellular fungi (yeasts) cells form pseudohyphae from individual yeast cells. Courtesy of OpenStax (https://openstax.org).

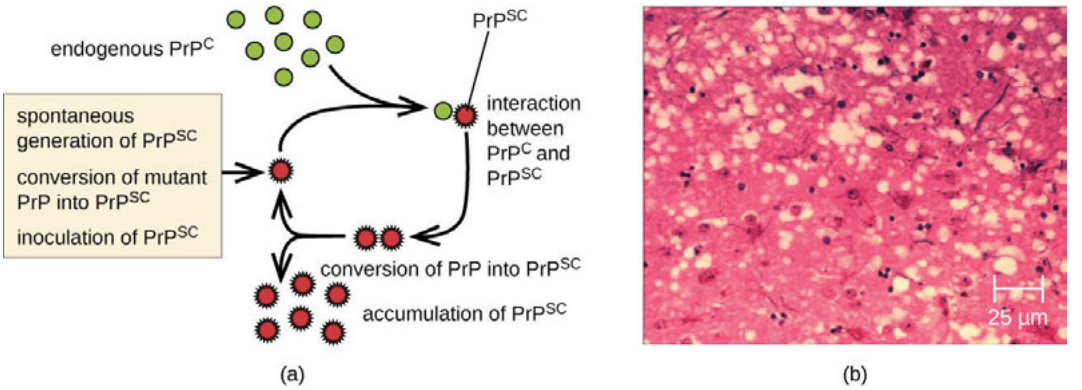


Histoplasma capsulatum is a dimorphic fungus that grows in soil exposed to bird feces or bat feces (guano) (top left). Its sexual form is called *Ajellomyces capsulatus*. It can change forms to survive at different temperatures. In the outdoors, it typically grows as a mycelium (as shown in the micrograph, bottom left), but when the spores are inhaled (right), it responds to the high internal temperature of the body (37°C) by turning into a yeast that can multiply in the lungs, causing the chronic lung disease histoplasmosis. In 1905, Samuel Taylor Darling serendipitously identified this protozoan-like microorganism in an autopsy specimen while trying to understand malaria, which was prevalent during the construction of the Panama Canal. He named it such, because it invaded the cytoplasm (plasma) of histiocyte-like cells (Histo) and had a refractive halo mimicking a capsule (capsulatum), a misnomer. Courtesy of the Centers for Disease Control and Prevention.

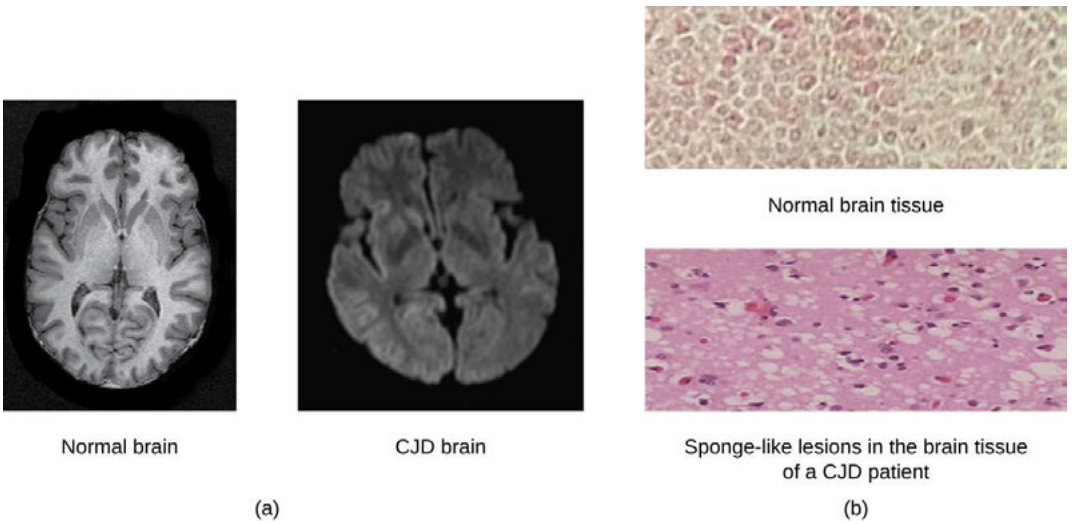
Photograph of Willy Burgdorfer inoculating Ornithodoros ticks. Burgdorfer (1925–2014) was an American scientist and an international leader in medical entomology. He discovered the bacterial pathogen that causes Lyme disease, a spirochete *Borrelia burgdorferi*, named in his honor. Burgdorfer's research explored the interactions between animal and human disease agents and their transmitting arthropod vectors, particularly ticks, fleas and mosquitoes. His research contributions are published in more than 225 papers and books. They cover a wide field of investigations, including those on relapsing fevers, plague, tularemia, Colorado tick fever, Rocky Mountain spotted fever, as well as other bacterial and viral diseases. Photograph courtesy of the Rocky Mountain Laboratories, NIH.



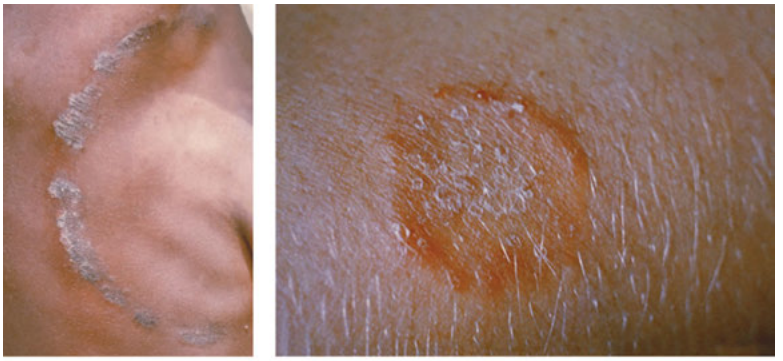
A bulls-eye rash is one of the common symptoms of Lyme diseases, but up to 30% of infected individuals never develop a rash. Erythema migrans or erythema chronicum migrans is an expanding rash often seen in the early stage of Lyme disease, and also – but less commonly – can be caused by southern tick-associated rash illness (STARI). It can appear anywhere from one day to one month after a tick bite. This rash does not represent an allergic reaction to the bite, but rather an actual skin infection of one of the Lyme bacteria species from the genus *Borrelia*. The rash's name comes from New Latin for "migrating redness." Text courtesy of Wikipedia. Image courtesy of the Centers for Disease Control and Prevention.



Endogenous normal prion protein (PrPc) is converted into the disease-causing form (PrPsc) when it encounters this variant form of the protein. PrPsc may arise spontaneously in brain tissue, especially if a mutant form of the protein is present, or it may originate from misfolded prions consumed in food that eventually find their way into brain tissue. Courtesy of the US Department of Agriculture and OpenStax (https://openstax.org).

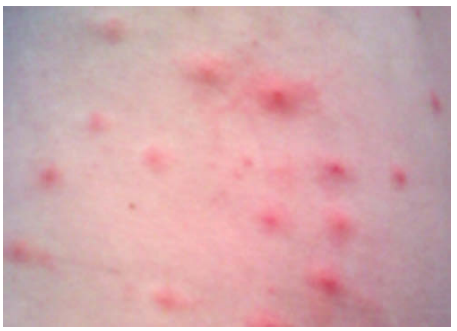


Creutzfeldt-Jakob disease (CJD) is a fatal disease that causes degeneration of neural tissue. (a) These brain scans compare a normal brain to one with CJD. (b) Compared to a normal brain, the brain tissue of a CJD patient is full of sponge-like lesions, which result from abnormal formations of prion protein. (credit (a) (right): Dr. Laughlin Dawes; credit (b) (top): Suzanne Wakim; credit (b) (bottom): the Centers for Disease Control and Prevention). Courtesy of and OpenStax (https://openstax.org).

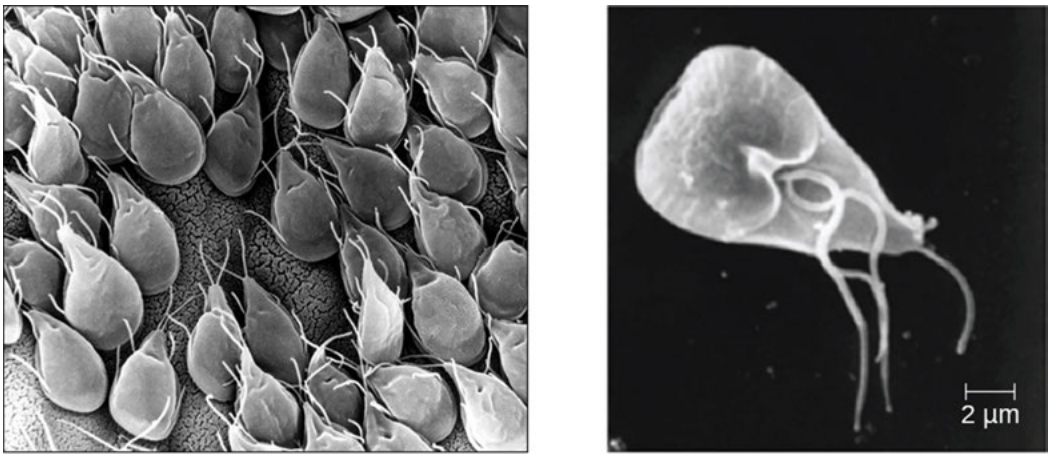


(a) (b)

Dermatophytosis, also known as ringworm, is a fungal infection of the skin. It presents as a raised ring, which is gray or brown on brown or black skin (a), and red on lighter skin (b). Typically it results the red, itchy, scaly, circular rash shown in (b) above. Hair loss may occur in the area affected. Symptoms usually start 4-14 days following exposure. Multiple areas can be affected at a given time. The most common term for the infection, "ringworm," is a misnomer, since the condition is caused by fungi of several different species and not by parasitic worms. Risk factors include using public showers, contact sports such as wrestling, excessive sweating, contact with animals, obesity, and poor immune function. Ringworm can spread from other animals or between people. Diagnosis is often based on the appearance and symptoms. It may be confirmed by either culturing or looking at a skin scraping under a microscope. Prevention is accomplished by keeping the skin dry, not walking barefoot in public, and not sharing personal items. Treatment is typically with antifungal creams such as clotrimazole or miconazole. If the scalp is involved, antifungals by mouth such as fluconazole may be needed. Globally, up to 20% of the population may be infected by ringworm at any given time. Infections of the groin are more common in males, while infections of the scalp and body occur equally in both sexes. Infections of the scalp are most common in children while infections of the groin are most common in the elderly. Descriptions of ringworm date back to ancient history. Text courtesy of Wikipedia. Images courtesy of the Centers for Disease Control and Prevention.

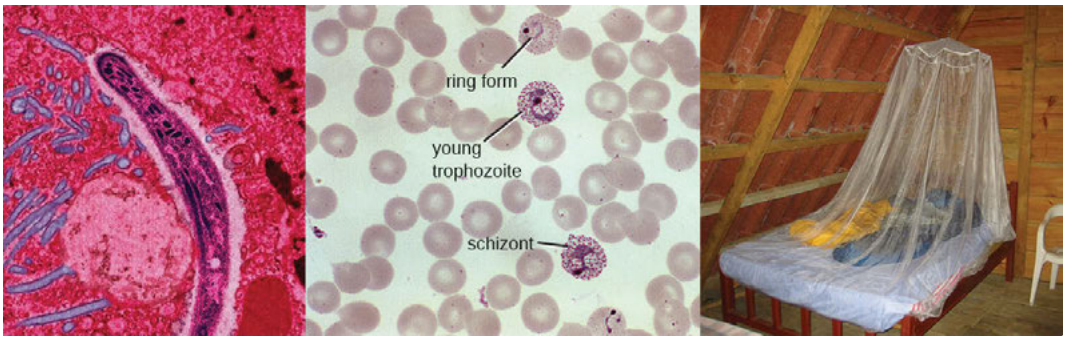


Exposure to *Pseudomonas aeruginosa* in the water of a pool or hot tub can sometimes cause a skin infection that manifests as "hot tub rash." Courtesy of "Lsupellmel"/Wikimedia Commons.

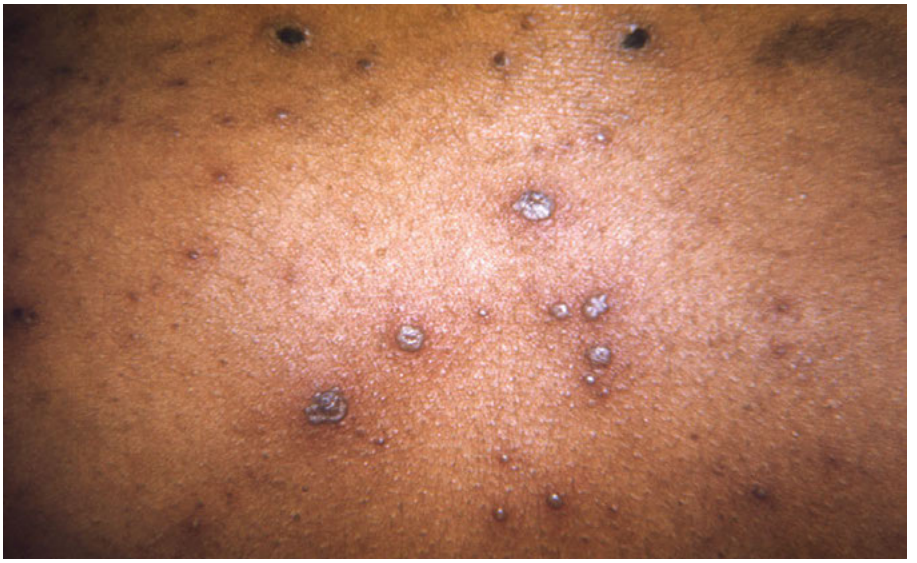


(a) (b)

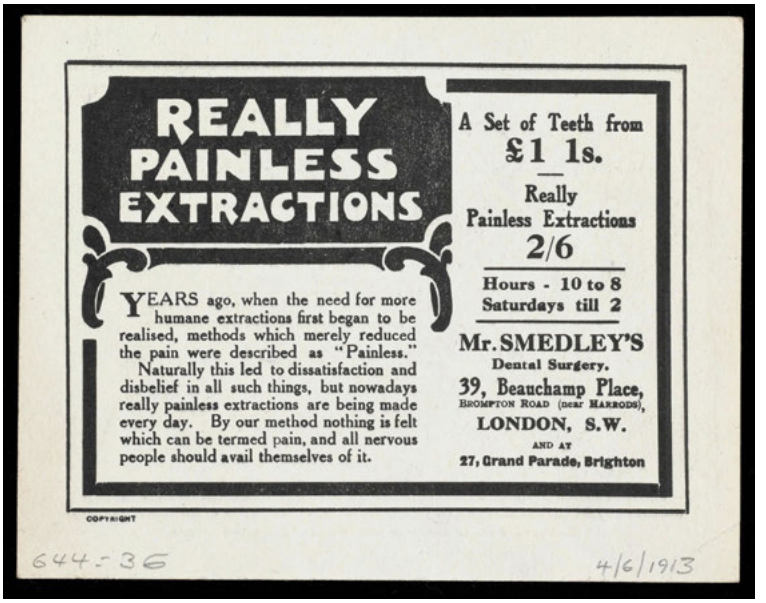
(a) A scanning electron micrograph shows many *Giardia* parasites in the trophozoite, or feeding stage, in a gerbil intestine. The mucosa surface is almost entirely obscured by attached trophozoites. (b) An individual trophozoite of *G. lamblia* is visualized here in a scanning electron micrograph. This waterborne protist causes severe diarrhea when ingested. *Giardia* is a genus of anaerobic flagellated protozoan parasites that colonize and reproduce in the small intestines of several vertebrates, causing the disease giardiasis. Their life cycle alternates between a swimming trophozoite and an infective, resistant cyst. Giardia were first described by Leeuwenhoek in 1681 while the genus is named after the French zoologist Alfred Mathieu Giard. Micrographs courtesy of the Centers for Disease Control and Prevention.



Malaria is a disease caused by a eukaryotic parasite transmitted to humans by mosquitos. Micrographs (left and center) show a sporozoite life stage, trophozoites, and a schizont in a blood smear. On the right, a primary defense against mosquito-borne illnesses like malaria is depicted – mosquito netting. (Credit left: Ute Frevert; credit middle: the Centers for Disease Control and Prevention; credit right: Tjeerd Wiersma). Courtesy of OpenStax (https://openstax.org).



Skin rash, shown above due to varicella or chickenpox, is caused by the highly contagious varicella-zoster virus (VZV). The characteristic rash seen here is partly a result of inflammation associated with the body's immune response to the virus. Inflammation is a response mechanism of innate immunity that helps the body fight off a wide range of infections. The disease results in a characteristic skin rash that forms small, itchy blisters, which eventually scab over. It usually starts on the chest, back, and face. It then spreads to the rest of the body. The rash and other symptoms, such as fever, tiredness, and headaches, usually last five to seven days. Complications may occasionally include pneumonia, inflammation of the brain, and bacterial skin infections. The disease is usually more severe in adults than in children. Chickenpox is an airborne disease which spreads easily from one person to the next through the coughs and sneezes of an infected person. The incubation period is 10–21 days, after which the characteristic rash appears. It may be spread from one to two days before the rash appears until all lesions have crusted over. It may also spread through contact with the blisters. Those with shingles may spread chickenpox to those who are not immune through contact with the blisters. The disease can usually be diagnosed based on the presenting symptom. However, in unusual cases it may be confirmed by polymerase chain reaction (PCR) testing of the blister fluid or scabs. Testing for antibodies may be done to determine if a person is immune. People usually only get chickenpox once. Although reinfections by the virus occur, these reinfections usually do not cause any symptoms. Since its introduction in 1995, the varicella vaccine has resulted in a decrease in the number of cases and complications from the disease. It protects about 70–90 percent of people from disease with a greater benefit for severe disease. Routine immunization of children is recommended in many countries. Immunization within three days of exposure may improve outcomes in children. Treatment of those infected may include calamine lotion to help with itching, keeping the fingernails short to decrease injury from scratching, and the use of paracetamol (acetaminophen) to help with fevers. For those at increased risk of complications, antiviral medication such as acyclovir are recommended. Text courtesy of Wikipedia. Image courtesy of John Noble, Centers for Disease Control and Prevention.



A 1913 advertisement from London titled "Really Painless Extractions." But, were such extractions possible over a century ago?

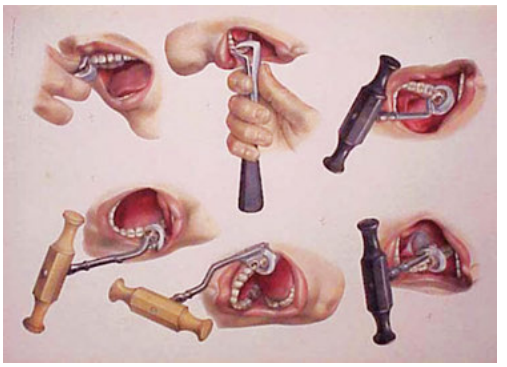


Illustration demonstrating the use of the dental key for extracting teeth. Drawing by Christophe Francois Delabarre, in "Odontologie, ou, observation sur les dents humaines," 1815, France.

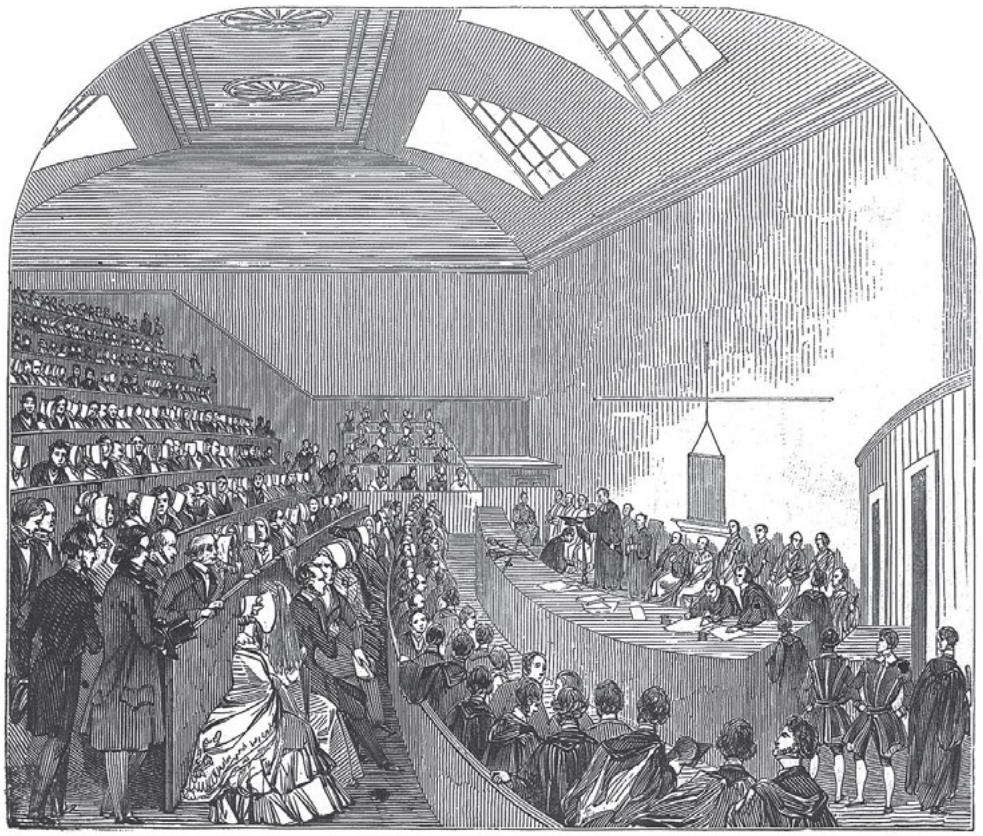
A dental extraction (also referred to as tooth extraction, exodontia, exodontics, or informally, tooth pulling) is the removal of teeth from the dental alveolus (socket) in the alveolar bone. Historically, dental extractions have been used to treat a variety of illnesses. Before the discovery of antibiotics, chronic tooth infections were often linked to a variety of health problems, and therefore removal of a diseased tooth was a common treatment for various medical conditions. Instruments used for dental extractions date back several centuries. In the 14th century, Guy de Chauliac invented the dental pelican, which was used through the late 18th century. The pelican was replaced by the dental key which, in turn, was replaced by modern forceps in the 19th century. As dental extractions can vary tremendously in difficulty, depending on the patient and the tooth, a wide variety of instruments exist to address specific situations. Rarely, tooth extraction was used as a method of torture, e.g., to obtain forced confessions. Courtesy of Wikipedia.

A surgeon, sitting on the floor, attends to a patient's leg, as he lies on a bed. Gouache painting by an Indian artist, c. 1825.

A crowd of medical staff standing around a woman patient in bed in a hospital ward. The "ward round" has been a source of medical learning since the time of Osler, but what is it like for the patient? I find it outrageous that around 20 people are crowing this patient. I am not sure if they all appeared for the photograph or if they all were part of the ward round. In any case, in my view, the patient in this photograph seems uncomfortable and worried. Is this why I note murals of foliage and allegories painted on the walls? But, they are likely to provide little comfort to the patient. However, over the years, much has been done to improve the experience of the patient during teaching ward rounds: (i) patient consent is now essential before taking medical students to see them; and (ii) now-a-days teaching rounds are often conducted separate from the clinical rounds so that patient care can be prioritized. I feel that medicine needs to be even more patient-centric. Image source: Seeberger Freres, c. 1910, France. (CC BY 2.0).

This wood engraving by G. Morrow from London, 1914, depicts a medical student being examined for his bedside manner by a group of senior doctors. Teaching and assessing communication skills is a vital duty for the medical educator. However, even today, patients only play a peripheral role in the assessment of physicians and medical care they are receiving. Often, their role is little more than tokenism. In my view, patient involvement in both curriculum design and assessment is essential. Things are gradually changing, though, and efforts are being made at some institutions to empower the patient.

The WHO defines empowerment as: "A process in which patients understand their role, are given the knowledge and skills by their health-care provider to perform a task in an environment that recognizes community and cultural differences and encourages patient participation."



"CAPPING" OF DOCTORS OF MEDICINE, AT EDINBURGH.

Doctors receiving their degrees in a degree ceremony in Edinburgh. Source: *Illustrated London News*, London, 1845. Here we can see the traditional "capping" ceremony taking place with the new physician at the front bowing to receive his cap. In my view, it is important that formal ceremonies like this graduation ceremony, white coat ceremony, etc. take place. Despite emphasis on such events, continuous professional development must be emphasized and be a pivotal part of medicine. In this context, William Henry Welch (1850–1934), known as the '"Dean of American Medicine," was an early advocate of lifelong learning and in 1892 remarked: "medical education is not completed at the medical school: it is only begun." Here, it is worth providing an excerpt from an address given on October 1, 1910 by Dr. Sprigge at the opening of the Medical Session at St. George's Hospital, London: "But education does not happen to end with registration, or qualification, with the obtaining of a commission, a diploma, or a degree, and there is no professional life in which this truth can be seen more clearly than in the medical life, in relation to which it is stark staleness to say that education never ceases and that the longer we practise our calling the more we have the opportunity of learning, of testing that learning, and of obtaining its rewards."

A wood engraving from 1854 depicting *A meeting of the college for the Harveian lecture*, The Royal College of Physicians, Trafalgar Square, London. According to Jules Older (*BMJ* 1985; 290:930), medical meetings "should be about learning and change (never mind job seeking, flirtation, tax breaks, drinking, and the rest of the conference's hidden curriculum)." And, how often should physicians attend them? According to Francis Martin Rouse Walshe (*Perspect Biol Med* 1959; 2:197), "symposia, like hard liquor, should be taken in reasonable measure, at appropriate intervals." Image courtesy of the Wellcome Library.

A MEDICAL OPINION.

Eminent Physician. "I FEEL VERY QUEER. I WONDER WHAT CAN BE THE MATTER?"

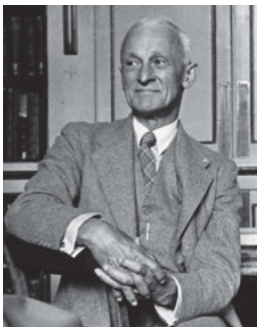
Anxious Wife. "SHALL I SEND FOR DOCTOR PILCOX OR DOCTOR SQUILLS?"

E. P. "NO, NO." *A. W.* "OR ANY OTHER DOCTOR?"

E. P. "NO; WE ALL GO IN FOR THINKING EACH OTHER SUCH HUMBUGS!"

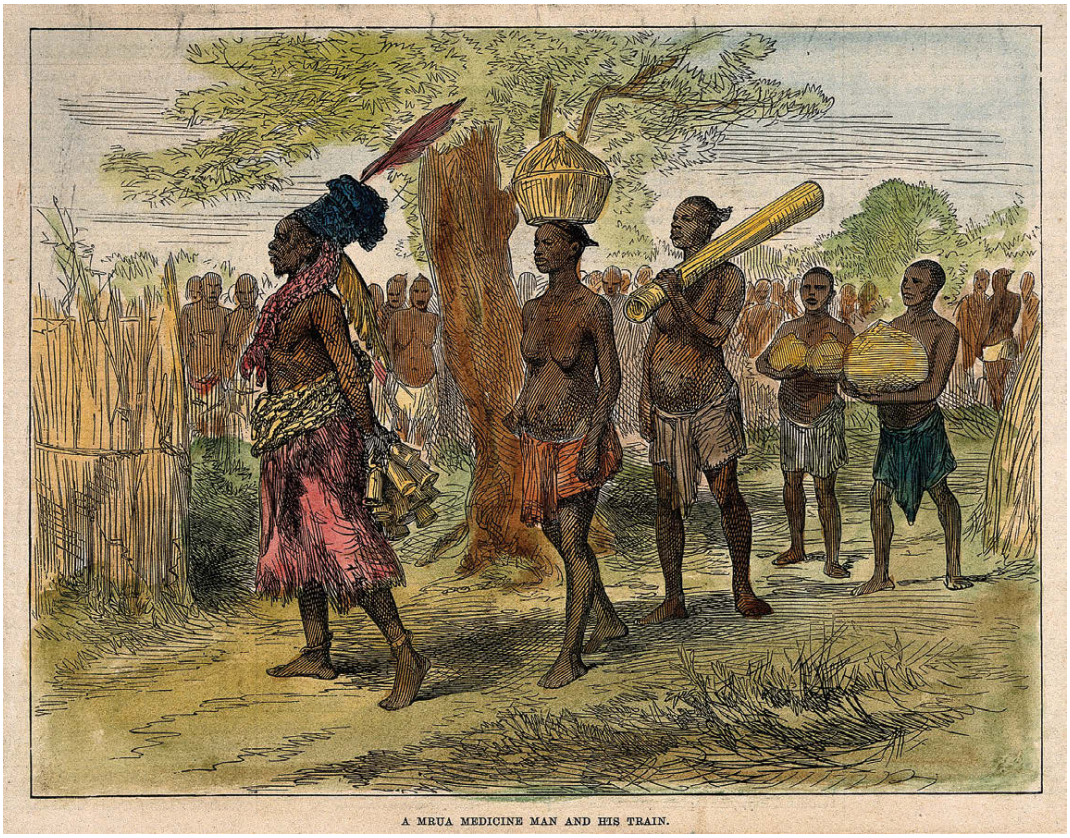
A wood engraving after G. Du Maurier, London showing an ill physician refusing to let his wife call another doctor. Often, good doctors make bad patients. In my view, a physician should be able to gauge how unwell he or she is and know when to stop rendering medical care rather than continue on and possibly harm patients in the process. Society places a lot of pressure on physicians and views them as superheroes lacking any weakness. I have observed this in every culture and every country that I have visited. We doctors are human and suffer from the same ailments as others. Image courtesy of the Wellcome Library.

Charles Horace Mayo (1865–1939) was an American surgeon, who, along with his brother William James Mayo, founded the Mayo Clinic. He was among the first to recognize the relationship between teaching, learning and patient care: "the safest thing for a patient is to be in the hands of a man engaged in teaching medicine. In order to be a teacher of medicine the doctor must always be a student" (*Proceedings of the Staff Meetings of the Mayo Clinic* 1927; 2:223).



Harvey Williams Cushing (1869–1939) was an America neurosurgeon and educator who described Cushing's syndrome and the Cushing reflex. Cushing's syndrome is a collection of signs and symptoms due to prolonged exposure to glucocorticoids such as cortisol. Cushing's syndrome is caused by either excessive cortisol-like medication (e.g., prednisone), or a tumor that either produces or results in the production of excessive cortisol by the adrenal glands. Cases due to a pituitary adenoma are known as Cushing's disease. A number of ectopic tumors may also cause Cushing's.

Like him, I firmly believe that medical learning and teaching go hand-in-hand and is a lifelong endeavor. Cushing was a dedicated teacher and an enthusiastic proponent of self-directed learning: "It is someone's business in every medical school to teach laboratory methods to the students but it is no one's particular business to teach them how to use medical literature …Short talks on the use of the library might well be made an obligatory sectional exercise for students" (H. Cushing. Bookshelf browsing: The doctor and his books. *American Journal of Surgery* 1928; 4(1):100–110). It is often said that a good surgeon knows how to operate while a great surgeon knows when to operate. In this regard, Cushing was an early advocate of these "non-surgical aspects of surgical education": "I would like to see the day when somebody would be appointed surgeon somewhere who had no hands, for the operative part is the least part of the work" (Letter to Dr. Henry Christian, November 20, 1911). Photo dated 1938 is kindly provided by the Wellcome Trust, UK.



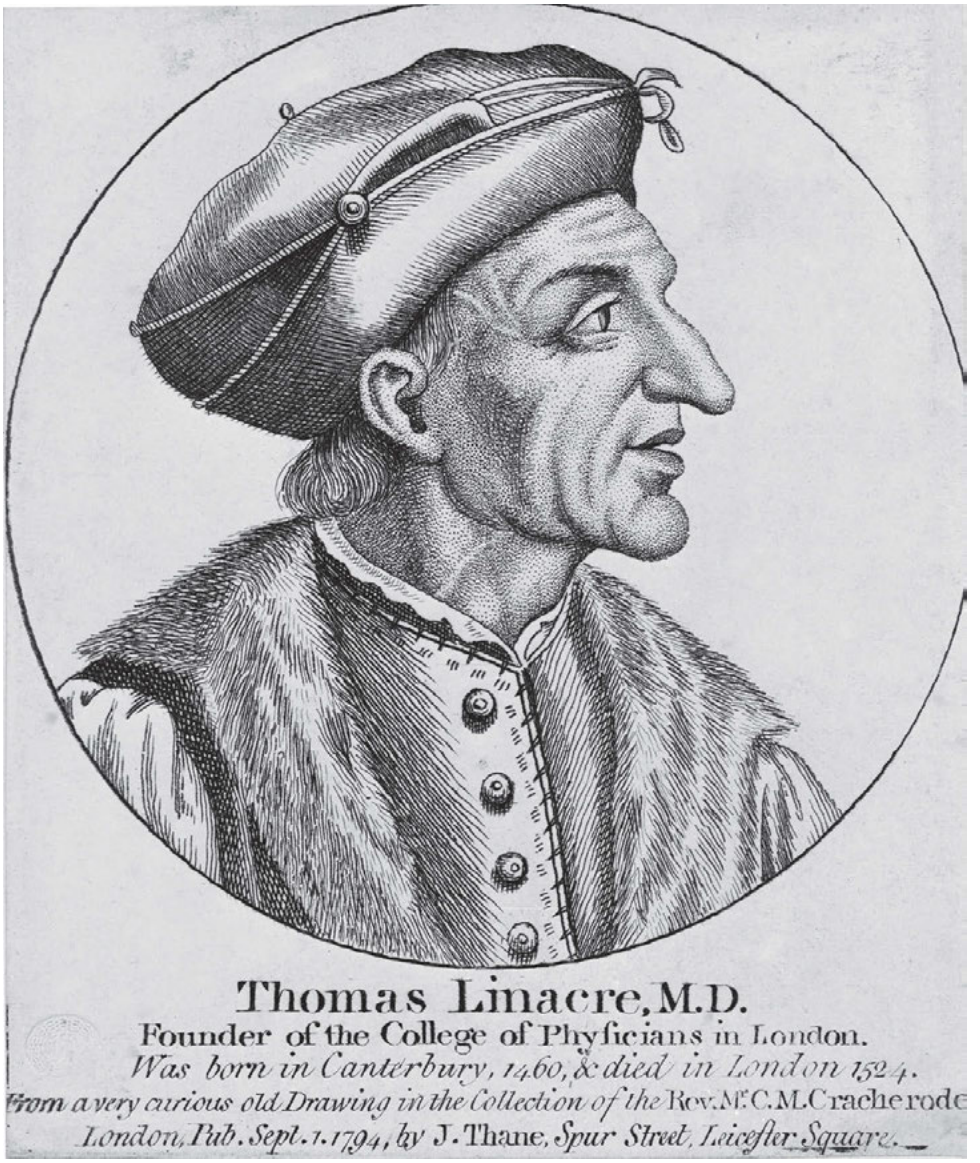
Colored wood engraving after a sketch by Lieutenant Cameron depicts a Mrua medicine man or shaman with his assistants, Central Africa. Courtesy of the Wellcome Library.

A physician reading a recipe instructs his assistant who is mixing with a pestle and mortar. Engraving after a twelfth century manuscript. Courtesy of the Wellcome Library.

Moses ben Maimon (1138–1204), commonly known as Maimonides (/maɪˈmɒnɪdiːz/) and also referred to by the acronym Rambam (Hebrew: רמב"ם), was a medieval Sephardic Jewish philosopher who became one of the most prolific and influential Torah scholars of the Middle Ages. Photogravure. Courtesy of the Wellcome Trust, UK.

Mondino de Luzzi, or de Liuzzi or de Lucci, (c. 1270–1326), also known as Mundinus, was an Italian physician, anatomist and professor of surgery, who worked in Bologna. In this oil painting he is making his first dissection in the anatomy theatre in Bologna, 1318. He is often credited as the restorer of anatomy because he reintroduced the practice of public dissection of human cadavers and writing the first modern anatomical text. Oil painting by Ernest Board, c. 1910.

Early fourteenth century pen and wash drawing showing a standing female healer, perhaps of Trotula, clothed in red and green with a white headdress, holding up a urine flask to which she points with her right hand. Source: Miscellanea medica XVIII. Folio 65 recto (=33 recto).

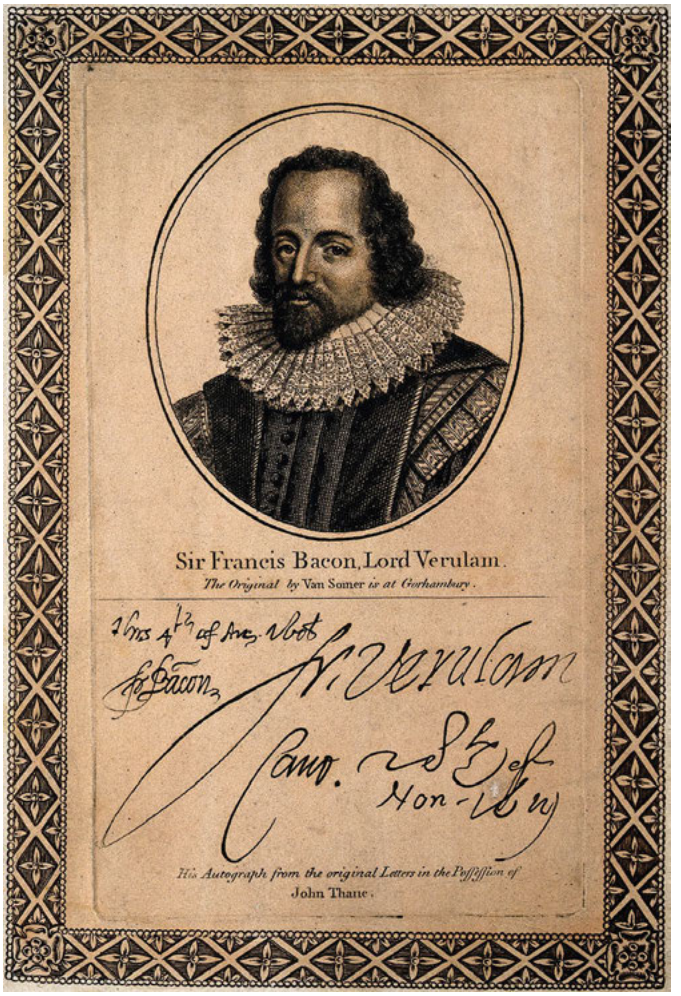


An engraving dated 1794 of Thomas Linacre or Lynaker (c. 1460–1524), an English humanist scholar and physician.

Ambroise Paré, as an apprentice barber surgeon in a busy shop in Paris. Wood engraving by E. Morin, after J. Ansseau.

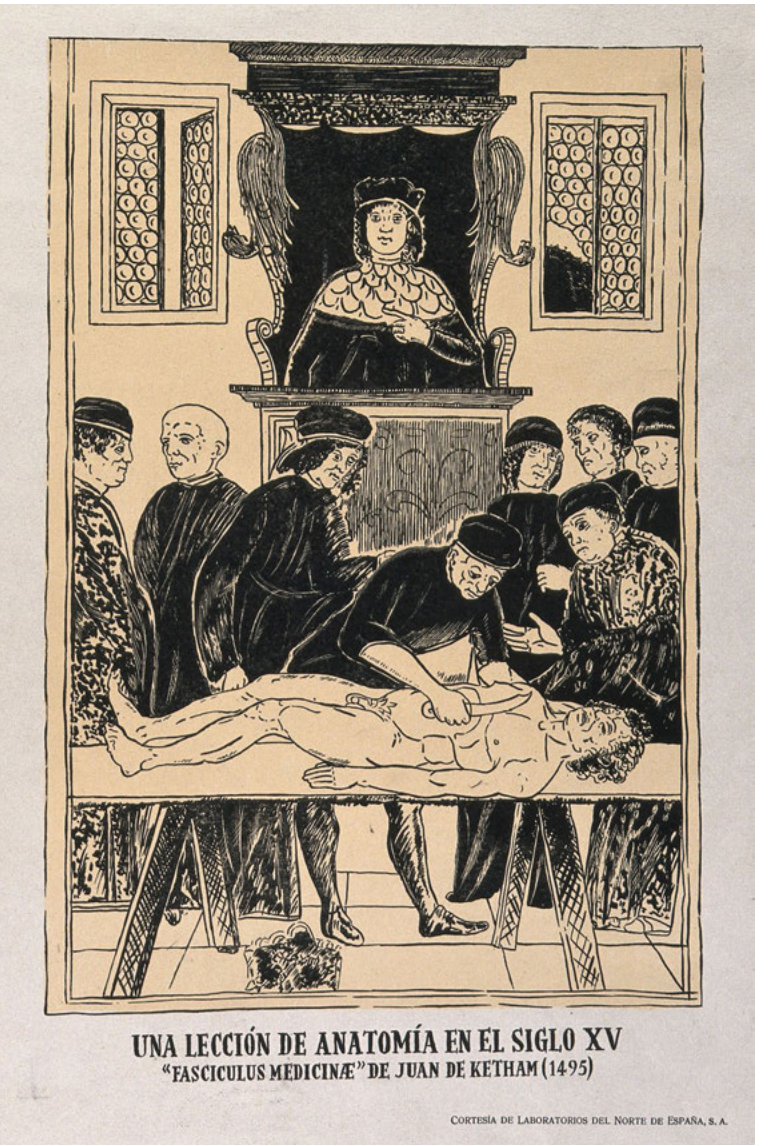
Bloodletting set of a barber surgeon, beginning of 19th century, Märkisches Museum Berlin. Photograph kindly provided by Wikipedia.

John Banister (1533–1610), an English anatomist, surgeon and teacher, giving the "visceral lecture" to surgeons at the Barber-Surgeons' Hall, London, in 1581. He published *The Historie of Man, from the most approved Authorities in this Present Age* in 1578. Oil painting by Jack Orr.

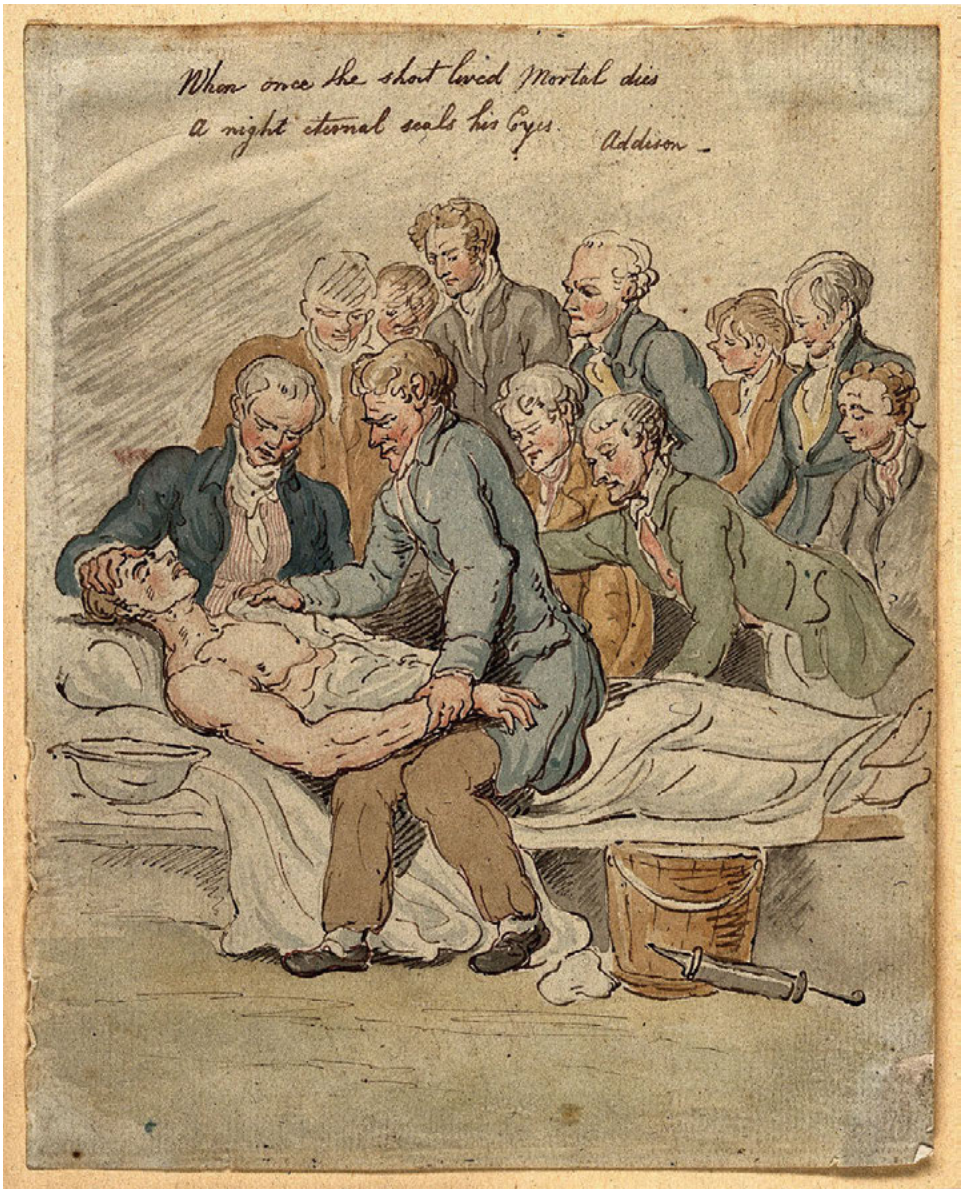


Francis Bacon (1561–1626), also known as Lord Verulam, was an English philosopher and statesman who served as Attorney General and Lord Chancellor of England. Bacon led the advancement of both natural philosophy and the scientific method and his works remained influential even in the late stages of the Scientific Revolution. Bacon has been called the father of empiricism. He argued for the possibility of scientific knowledge based only upon inductive reasoning and careful observation of events in nature. He believed that science could be achieved by the use of a sceptical and methodical approach whereby scientists aim to avoid misleading themselves. Although his most specific proposals about such a method, the Bacoonian method, did not have long-lasting influence, the general idea of the importance and possibility of a sceptical methodology makes Bacon one of the later founders of the scientific method. His portion of the method based in scepticism was a new rhetorical and theoretical framework for science, whose practical details are still central to debates on science and methodology. Image kindly provided by the Wellcome Library. Text courtesy of Wikipedia.

A physician in traditional costume holding an ointment jar is supervising an apprentice who is mixing a concoction in a pot over a fire. Woodcut, Early Sixteenth Century.



A dissection is in progress with the anatomy professor at his lectern. Line block, after a drawing after a woodcut, 1493. After: Mondino dei Luzzi. Published: Laboratorios del Norte de España (Spain, 1950?). This image shows the classic way of teaching/learning gross anatomy via dissection of donated cadavers. The anatomy professor is professing at his pulpit, the demonstrator is performing the actual dissection, and the students are mere observers. Reforms would lead to professors descending from their pulpits and doing the dissection, and finally to the students themselves doing the dissections under supervision. I learnt gross anatomy via a variety of means: human cadaver dissection, dissected material, life models, radiological images, and the digitally-interactive 3D Anatomage table. Image courtesy of the Wellcome Library.

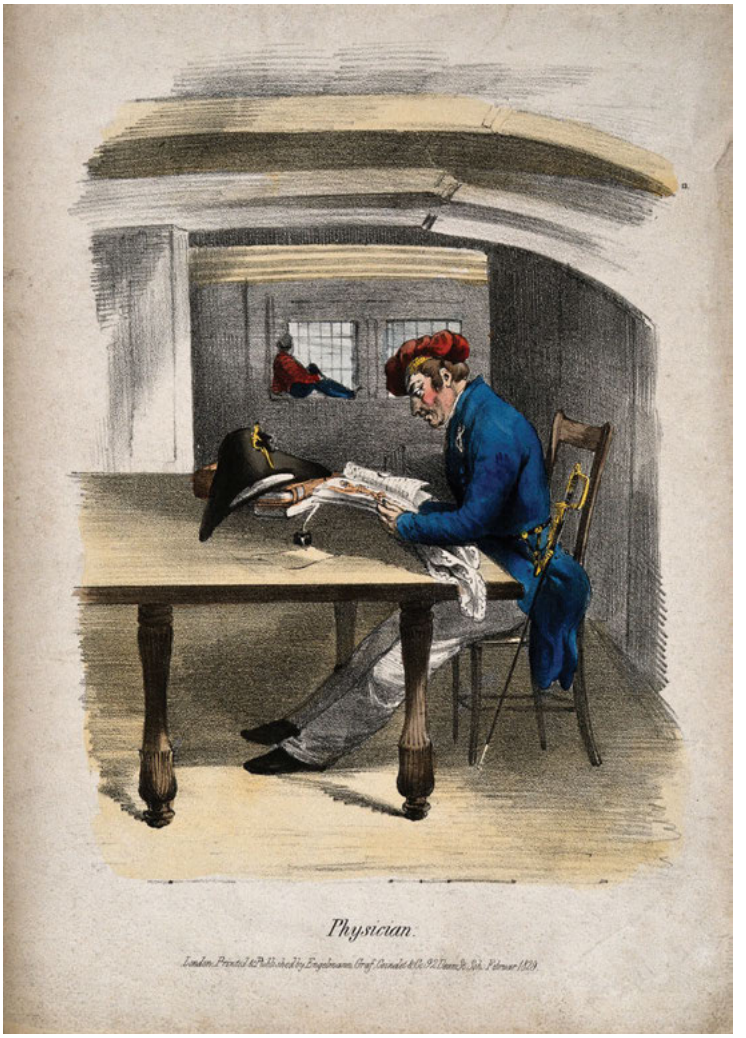


Watercolor painting showing a dying patient surrounded by a group of doctors and medical students. This painting is as notable for what is missing as for what is present: there are no nurses, patient's family, intravenous catheters, intubation equipment, cardiorespiratory monitors or other modern sacramental instruments of death and dying. I note a possible enema delivery device on the floor. The inscription at the top of the image reads: "When once the short lived mortal dies, a night eternal seals his eyes." Courtesy of the Wellcome Library.

Memorial, erected by the inhabitants of Dundalk in Ireland, to Geo Gillichan, MD, for his work during the Irish fever epidemic. He died in 1817. The memorial depicts a traveler helping an ill man by the roadside – possibly an allusion to the Good Samaritan. The image was taken from the book F. Barker and J. Cheyne. (1821). *An Account of the Rise, Progress, and Decline of the Fever Lately Epidemical in Ireland, Together with Communications from Physicians in the Provinces.* Baldwin, Cradock and Joy, Dublin.

Thomas Henry Huxley (1825–1895), an English biologist and anthropologist specializing in comparative anatomy, was nicknamed "Darwin's bulldog" for his aggressive defense of Darwin's ideas. This cartoon shows the "bulldog" in an aggressive posture. The caricaturist was Carlo Pellegrini, nicknamed Ape, who worked for *Vanity Fair.*

Huxley had a strong influence on the modernization of medical curricula in the nineteenth century and was one of the first medical teachers to highlight the learning approach of medical students: "…and I am quite sure a very considerable number of young men spend a large portion of their first session simply learning how to learn in a fashion that is entirely new to them" T. Huxley. (1883). Introductory address on the intervention of the state in the affairs of the medical profession. *BMJ* 2:709). His forthright assessment of the primary purpose of education is clear here: "Perhaps the most valuable result of all education is the ability to make yourself do the thing you have to do, when it ought to be done, whether you like it or not."



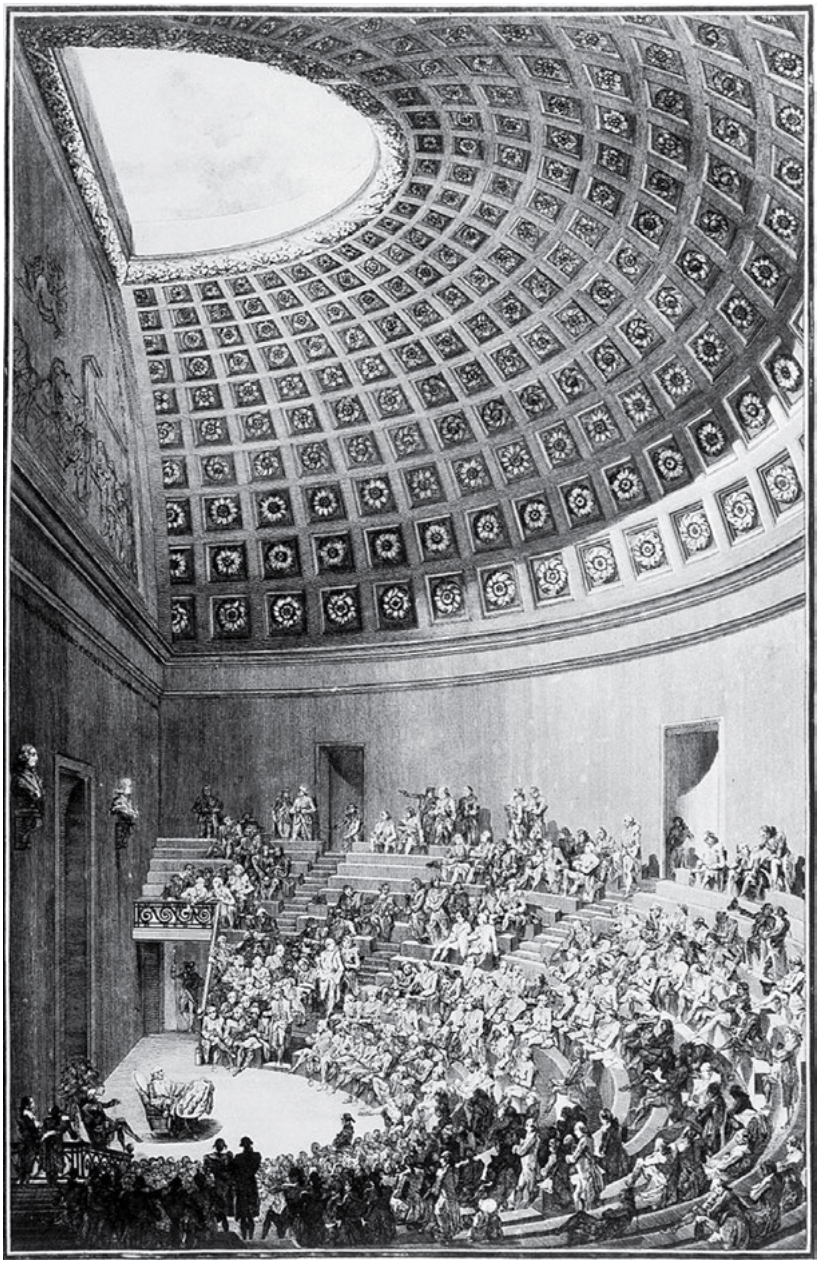
This color sketch titled "Physician" was published by Engelmann, Graf, Coindet & Co, London in 1829. In today's medical education, reading is central to learning and also to keeping up with advances in various specialities: "If we take into account the pivotal role that reading has in a doctor's continuous learning then reading should be generously honored, allowing doctors to meet at least half of any set annual standard of credit points by reporting their reading and its perceived influence on their practice" (H. A. Holm. (2000). Should doctors get CME points for reading? Yes: Relaxing documentation doesn't imply relaxing accountability. *BMJ* 320:394). However, the massive explosion of new medical knowledge published every year is impossible for a physician to keep abreast of. Therefore, the modern emphasis in continuous professional development has turned to critical reading skills, whereby physicians consider the validity and relevance of the content that they read and reflect on how they might apply it to their specific practice.

Portrait by Cecilia Beaux dated 1895 of John Shaw Billings (1838–1913), an American librarian, building designer, and surgeon who worked at the National Library of Medicine. In fact, he developed the National Library of Medicine (formerly the Library of the Surgeon General's Office in the US). He noted the importance of high-quality medical informatics in the delivery of both medical education and clinical care. He is credited with setting up *Index Medicus*, a comprehensive index of articles in medical journals. Today, the National Library of Medicine is the biggest medical library in the world. Like others, Billings was also a strong believer in the importance of ongoing medical education following qualification: "The education of the doctor which goes on after he has his degree is, after all, the most important part of his education" (*Boston Medical and Surgical Journal*, 1894; 131:140).

Jean Baptiste Pierre Antoine de Monet Lamarck (1744–1829). Colored etching by J. M. Frémy, after C. Thévenin, 1801.

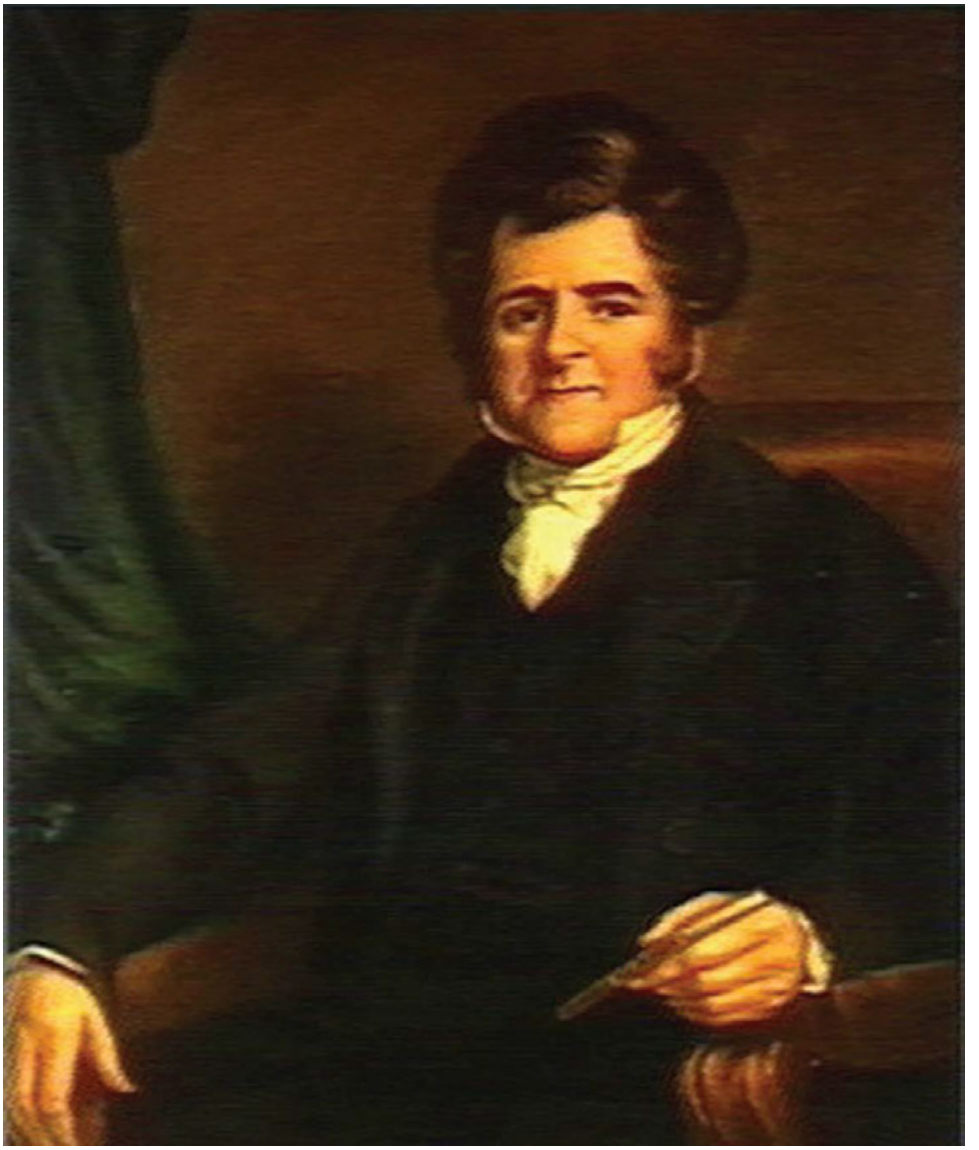
A foppish medical student smoking a cigarette – denoting a cavalier attitude. Sketch, dated 1840, by Joseph Kenny Meadows after William Wordsworth and John Orrin Smith. Image courtesy of the Wellcome Library.

A physician shouting at his assistant. In this print, the assistant is scared, confused and unlikely to learn or to carry out the instructions asked of him. Bullying in medicine remains a significant problem to this day, with medical students, nurses, hospital staff, and physicians in training the typical victims of bullying. Hierarchical medical structures are to blame, in part to bullying. It is a vicious cycle where the bullied eventually turns into the bully. Bullying is clearly harmful to the entire medical enterprise: "...bullying, intimidation, and humiliation are essential in maintaining all hierarchies, but I still can't find the evidence that they are any good for producing doctors" (*Fraser's Magazine* 1862; 66:574). It also negatively impacts productivity: "The negative impact that bullying and harassment have on the wellbeing of students and doctors, overall morale in the medical workforce, and recruitment and retention in the profession demand our continuing efforts to resolve these problems" (K. Walsh, P. H. Gompertz, and A. G. Rudd. (2002). Stroke care: How do we measure quality? *Postgrad Med J* 78(920): 322–326).



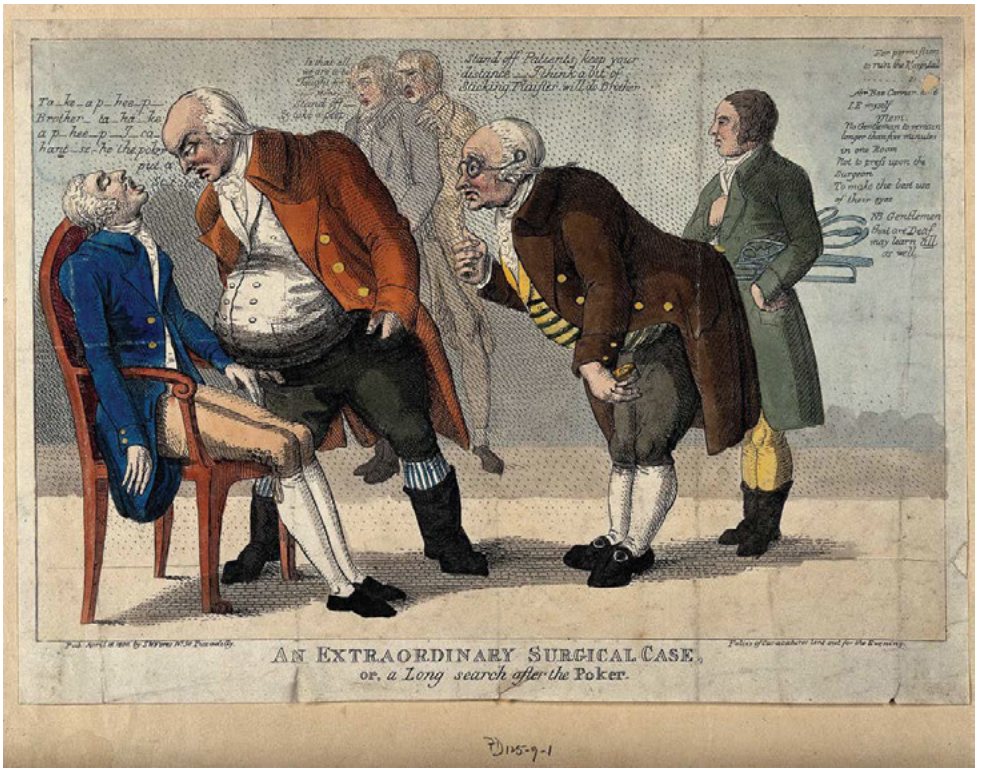
Ecole de chirurgie, Paris, scene in theatre showing anatomical demonstration. Source: J. Gondoin. (1780). *Description des ecoles de chirurgie*. P. D. Pierres, Paris. Plate XXIX.

Etching from 1781 by D. N. Chodowiecki, Leipzig, depicting doctors disputing while the patient is unattended and ignored. Have I seen this before? Disputing doctors who ignore their patients should become museum pieces – both in medical education and in clinical care. The pivotal role of patients today cannot be underestimated, their role is certain to only expand in the future: "Let the young know they will never find a more interesting, more instructive book than the patient himself" (Giorgio Baglivi, 1668–1707).



Oil painting of Richard Bright (1789–1858), an English physician, educator and researcher. This rather formal and posed painting is by Frederick Richard Say. Source: Thomas Joseph Pettigrew, *Medical Portrait Gallery,* vol. 2 (1838).

Bright was an early pioneer in the research of kidney disease. He conducted research into kidney disease at Guy's Hospital, London and discovered nephritis (originally called Bright's disease). It is interesting to note that his contemporaries at Guy's included Thomas Addison and Thomas Hodgkin.

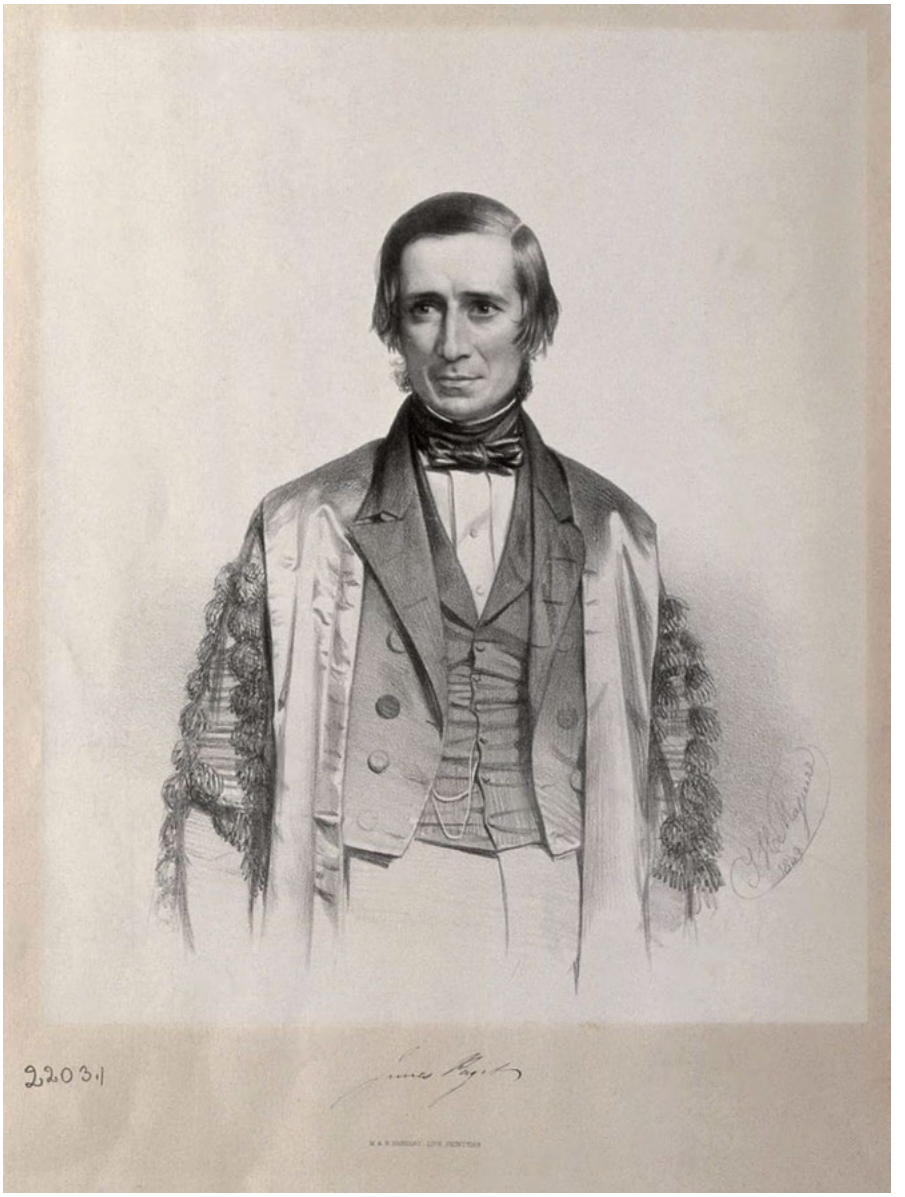


A colored etching of a gagging man surrounded by confused consultants and medical students. It was published by S.W. Fores, London on April 18, 1800. Medical education has evolved since 1800; it has become more patient-centric. However, as I study this image, it is hard for me to wonder if these 19th century teachers and learners truly cared about the patient they were examining and learning from. It appears to me that they are horrified, not by the plight of the patient, but whether the patient is contributing in the process of their education or merely serving as a silent prop. Why do I think so? Well, read the caption beside them that reads: "Is that all we are to be taught for our money?" (I had to use a magnifying glass to read the caption due to the print size).

An inexperienced student doctor taking the pulse of a patient in his bed. Colored etching by A. M. Mills, 1806, Bowles & Carver, London. Image courtesy of the Wellcome Library.

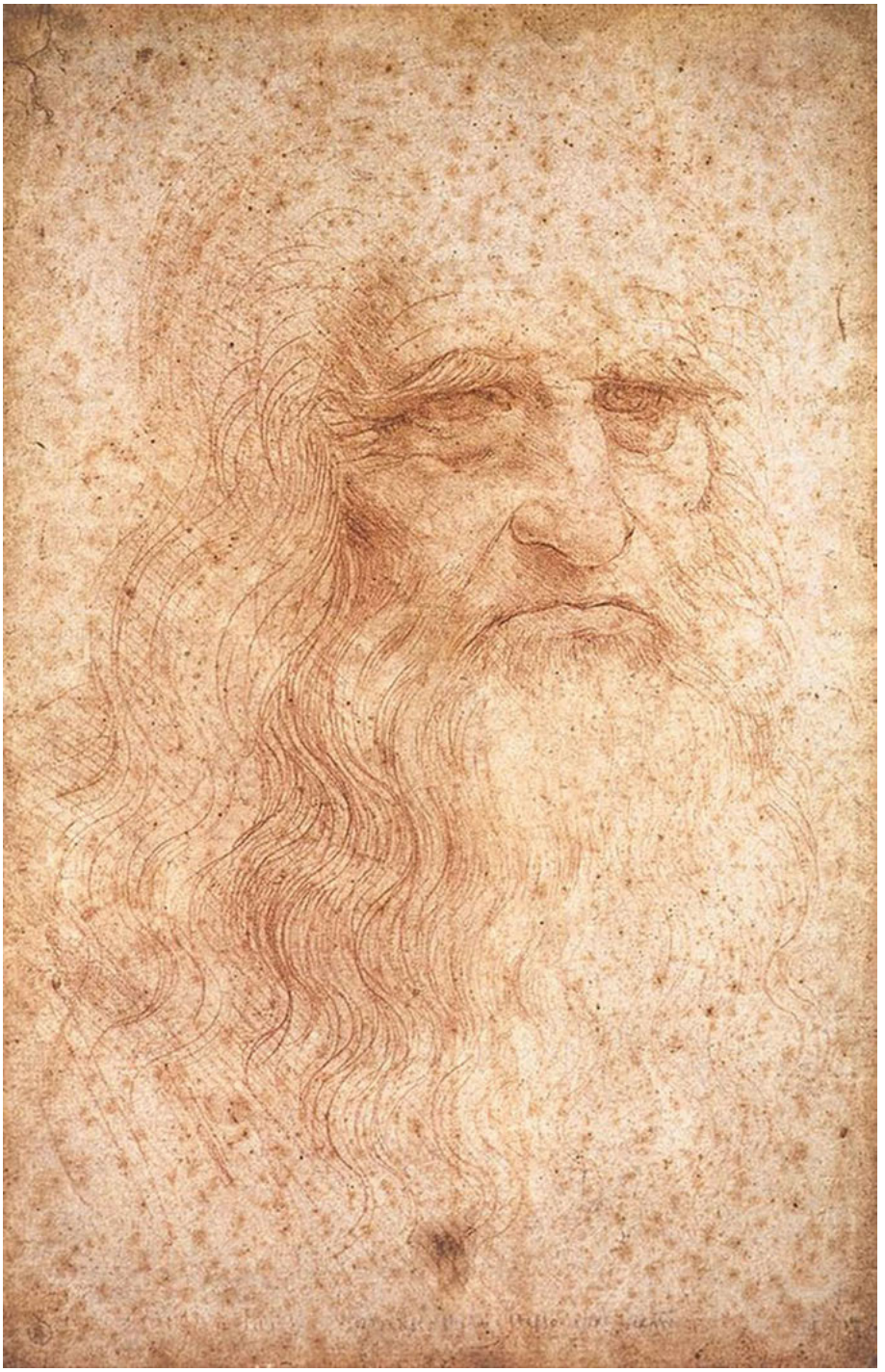
A doctor reading out a letter from a dissatisfied patient to his wife over breakfast. Bears dialogue: 'Doctor. "Um! Most insolent!" (To his wife.) "Listen to this, my dear." (Reads letter aloud.) "Sir, – I enclose a P.O. order for thirteen shillings and sixpence, hoping it will do you as little good as your two very small bottles of "Physic" did me.'" Wood engraving by C. Keene, London, 1878.

A 19th century lithograph of a medical student, smoking, with a tankard and *Quain's Anatomy* on the table. The lithograph was published by Brewer and Co./Leoni Lee, London. *Quain's Anatomy* was one of the principal anatomical textbooks of its day. However, it was not intended to serve as a beer mat for students' tankards – as it is in this lithograph. Recent data from literature establishes that physicians have a higher rate of alcohol and substance dependency than that found in the general population. With this background, it may be wise to revisit a sage remark by a literary giant from the past: "a parcel of lazy, idle fellars, that are always smoking and drinking and lounging …a parcel of young cutters and carvers of live people's bodies, that disgraces the lodgings" (C. Dickens. (1837). *The Pickwick Papers*. Chapman and Hall, London).

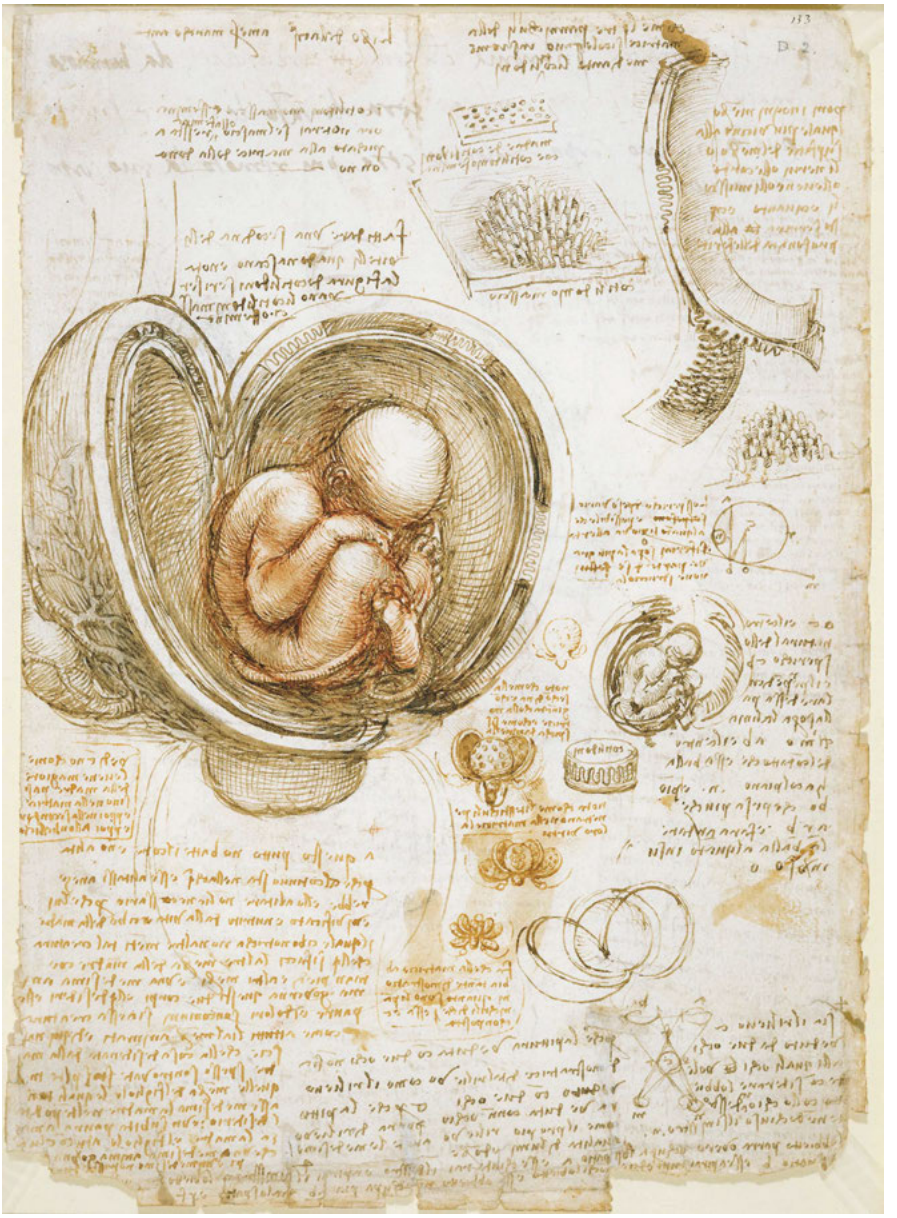


James Paget (1814–1899) was an English surgeon and pathologist who described osteitis deformans (Paget's disease of the bone) and intraductal breast cancer (Paget's disease of the nipple; mammary and extramammary). He is considered, together with Rudolf Virchow, as one of the founders of scientific medical pathology. His famous works included *Lectures on Tumours* (1851) and *Lectures on Surgical Pathology* (1853). This half-length portrait of the robed Paget is by Thomas Herbert Maguire. Academics wearing robes dates back to the Middle Ages when universities were places of religion as well as scholarship.

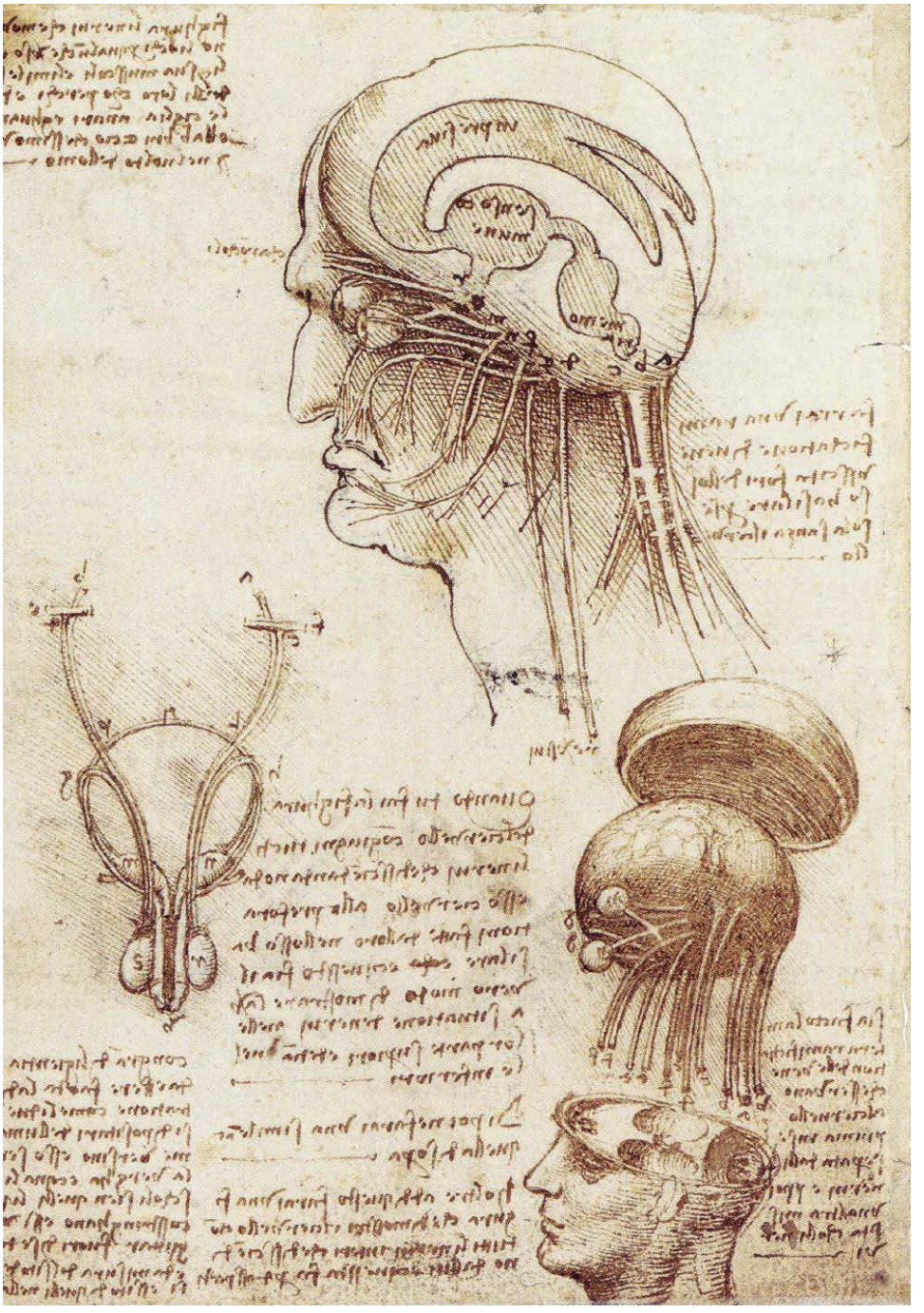
A doctor telling his apprentice how to use language correctly. Wood engraving after J. Leech, 1817–1864. Courtesy of the Wellcome Library.



Presumed self-portrait (c. 1510) of the genius, Leonardo da Vinci (1452–1590) at the Royal Library of Turin, Italy. A reproduction of this self-portrait appears in *Del Cenacolo di Leonardo da Vinci* by Giuseppe Bossi, 1810.



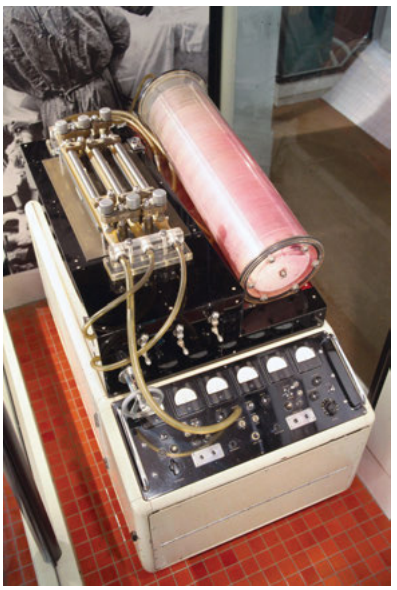
The fetus in the womb drawing by Leonardo da Vinci. *Studies of the Fetus in the Womb* are two colored annotated sketches by Leonardo done around 1511. Here one is shown. The studies correctly depict the human fetus in its proper position inside a dissected uterus. Leonardo depicted the uterus with one chamber, in contrast to theories that the uterus had multiple chambers which many believed divided fetuses into separate compartments in the case of twins. He also correctly drew the uterine artery and the vascular system of the cervix and vagina. Leonardo studied human embryology with the help of anatomist Marcantonio della Torre and saw the fetus within a cadaver. Text and drawing kindly provided by Wikipedia.



Leonardo's physiological sketch of the human brain and skull (c. 1510).

Blood transfusion apparatus, UK, 1914–1918**.** It's 1917, and you are a wounded soldier at a casualty clearing station on the Western Front. You are bleeding badly and going into shock. You are in danger of dying and urgently need blood – where's the nearest blood bank? It is right next to you. Your blood transfusion will come directly from another patient. Is it safe? It's the best method available at the time, particularly if your surgeon is Lieutenant Geoffrey Keynes of the Royal Army Medical Corps. Keynes designed and pioneered this portable blood transfusion kit, with a special device in the flask for regulating flow. Why didn't Keynes use stored blood? It didn't keep very well. It needed to be refrigerated – a difficult task in field hospitals, and it clotted into lumps unless an anticoagulant was added. While it became technically possible to do this during the First World War, the patient-to-patient method was still more widely used. Matching of blood groups was recommended, but there was not always time. The first blood banks stored O type blood – suitable for all recipients. But in the meantime, you were lucky to have reached a casualty clearing station, to take your chances with an emergency transfusion. And who better than Lieutenant Geoffrey Keynes? In 1921 he co-founded London's Blood Transfusion Service, and a year later published Britain's first textbook on the subject. Courtesy of the Science Museum, London and the Wellcome Collection.

Simpson type obstetrical forceps, London, 1871–1900. These long obstetrical forceps are made of steel and ebony. They follow a forceps design by James Young Simpson (1811–1870), a Scottish physician and obstetrician. The forceps are longer and heavier than his short-type forceps. They delivered babies from higher up the birth canal. Simpson's long and short forceps were adapted in many later designs. They were manufactured by Mayer and Melzer, London. Courtesy of the Science Museum, London and the Wellcome Collection.



Heart-lung machine, London, 1955–1960. The heart-lung machine performs the functions of the heart and lungs during surgery. A pump takes over the action of the heart, supplying the body with blood. The heart can then be stopped, making it easier to operate on. The patient's blood flows over the rotating discs where oxygen is blown across it, effectively taking over the action of the lungs. This machine with a pump oxygenator was conceived by Denis Melrose (1921–2007) at the Postgraduate Medical School in Hammersmith in London, in the early 1950s. Melrose also developed a way to stop the heart beating during heart surgery using potassium citrate. The technique is still used today and is called cold cardioplegia. The machine shown here was the first to be used in open heart surgery operations at the Postgraduate Medical School. It was manufactured by New Electronic Products, London. Courtesy of the Science Museum, London and the Wellcome Collection.

Surgical instrument set, Germany, 1939. This extensive steel surgical instrument set contains a wide range of the equipment an army surgeon may have needed at a German field hospital. Some of the instruments, such as the amputation knives and saw, are for specific tasks. Other instruments, such as the forceps for clamping bones, arteries, and intestines, have a more general use. The kit is labelled with the German word Hauptbesteck and the date. Hauptbesteck translates as "main cutlery," "cutlery" being another word for surgical instruments. The use of steel for the case as well as the instruments ensured that this equipment could be sterilized easily. It was manufactured by Jetter and Scheerer, Tuttlingen, Baden-Württemberg, Germany. Courtesy of the Science Museum, London and the Wellcome Collection.

Lithotomy set, Paris, 1820–1860. Lithotomy was a procedure where a stone in the bladder or urethra was removed by means of surgery. This thirty-piece instrument set contained all the necessary instruments to carry out the operation, including four gorgets, specialized knives used in lithotomy, and six lithotomy staffs, which are grooved to guide the gorget towards the stone. This set was made by Charrière, a surgical instrument maker based in Paris, France. Courtesy of the Science Museum, London and the Wellcome Collection.

Self-administering enema syringe, Europe, before 1935. Enema syringes vary in shape and material but they are all intended to introduce liquids such as medications or purgatives into the body via the rectum – a once very common medical procedure. After the ivory nozzle was inserted into the anus, liquid was pumped through the metal tubing from the metal reservoir. It allowed a person to give themselves the enema rather than relying on a physician or nurse and so was probably used in the home. It was produced somewhere in Europe but the manufacturer is unknown. Courtesy of the Science Museum, London and the Wellcome Collection.

Otosporin drops, 1968. One of a series of leaflets (traditional remedies) issued by Burroughs Wellcome & Co. advertising Otosporin, a remedy for otitis. This photo shows a man undergoing a traditional medical procedure to his ear by a woman at the dinner table. Courtesy of Burroughs Wellcome & Co. and the Wellcome Collection.

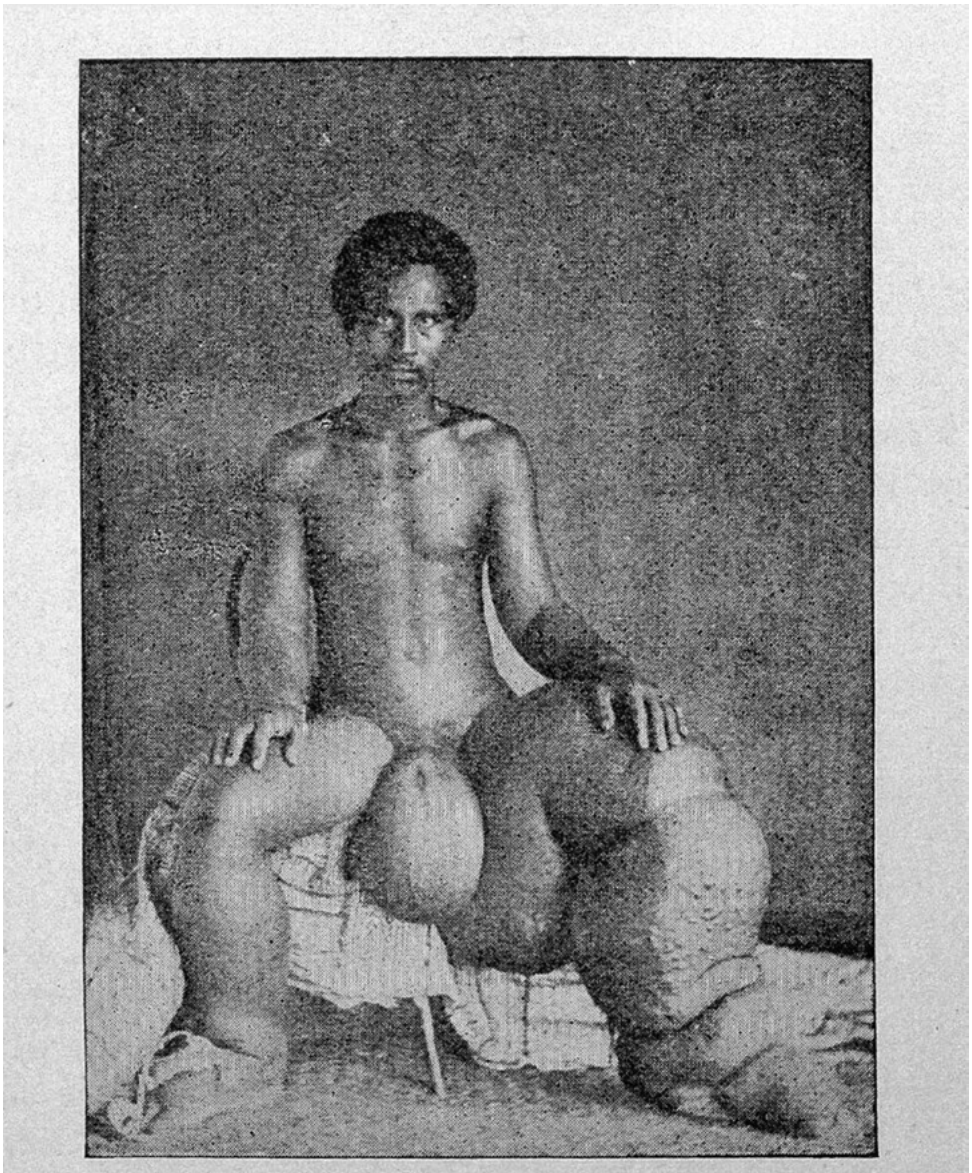
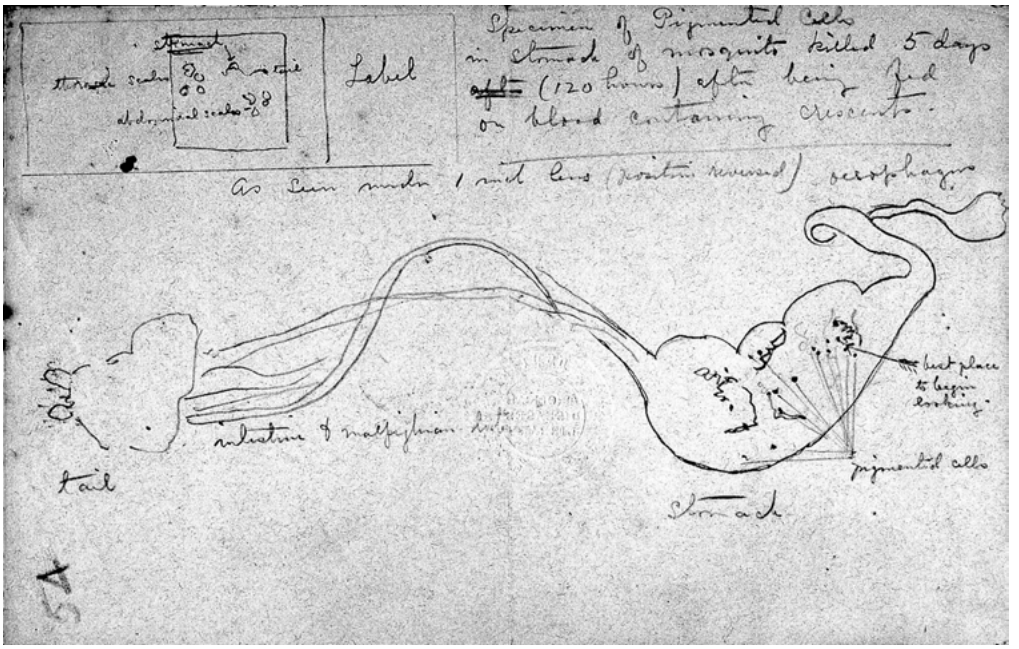


Fig. 47.—Elephantiasis of legs : scrotum and right arm slightly affected. (*From a photograph by Dr. Turner, Samoa.*)

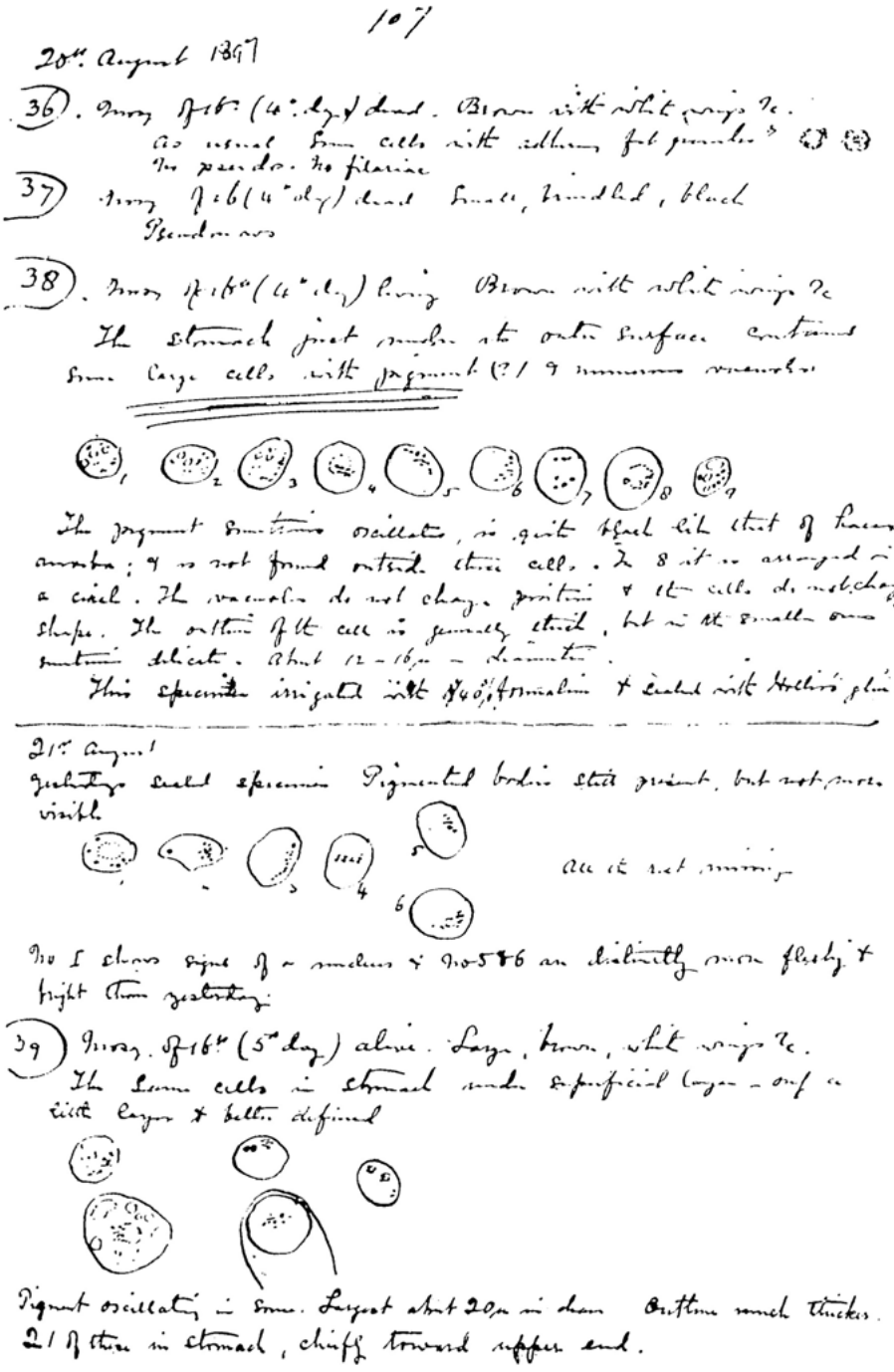
Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. It is a painful and profoundly disfiguring disease caused by infection with parasites classified as nematodes (roundworms) that are transmitted through the bites of infected mosquitoes. Note that the scrotum and right arm are slightly affected. Courtesy of Dr. Turner (Samoa) and the Wellcome Collection.

I. P. Pavlov and students, St. Petersburg, 1904. This photo is from the vivisection room, Physiology Department at the Imperial Institute of Experimental Medicine. Left to right: B.P. Babkin (pouring tea), S.V. Paraschuk (behind), V.V. Savich (foreground, drinking), V.N. Boldirev (in white coat), L.A. Orbeli (behind), E.A. Ghanike, P.A. Arbekov (seated at table), L.F. Piontkovskiy (?), V.P. Neelov, N.I. Heiman, G.B. Barlatskiy, I.S. Tsitovitch. Courtesy of the Wellcome Collection.

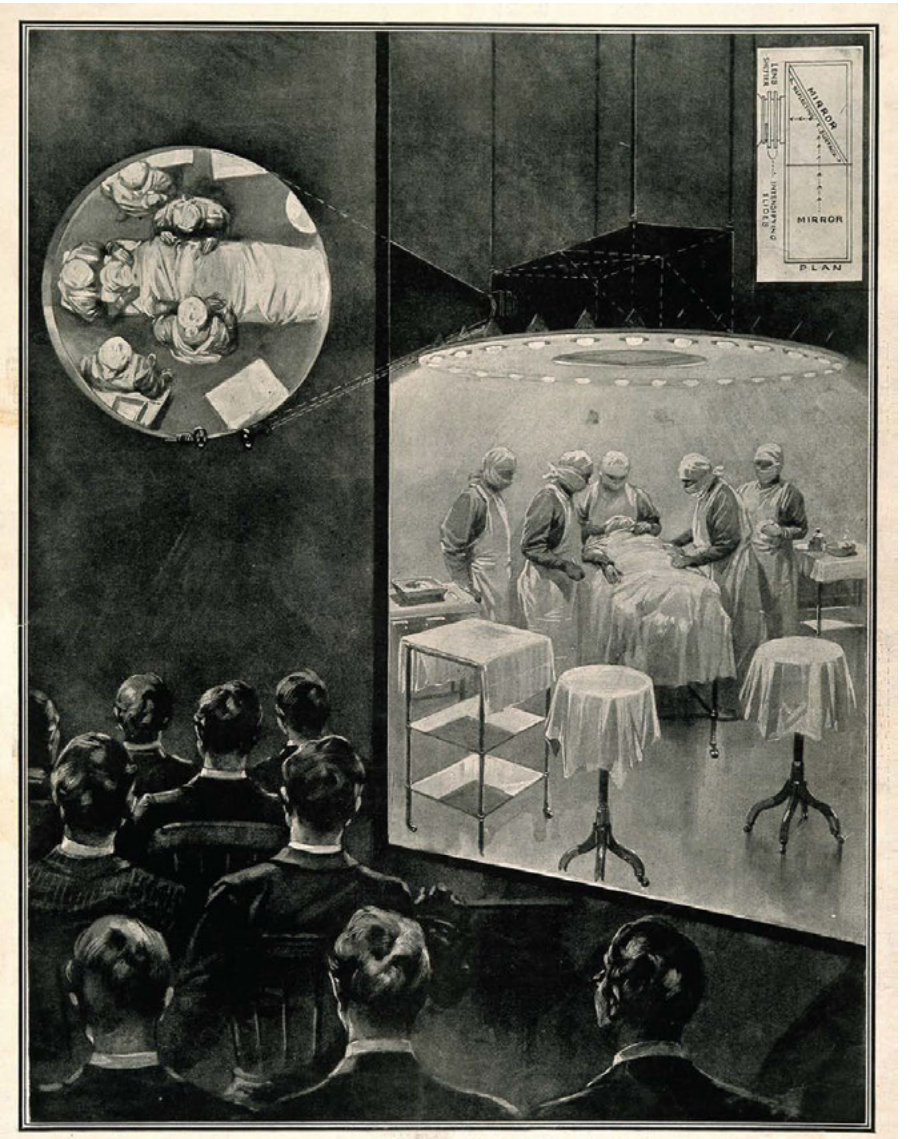
Ivan Petrovich Pavlov (Russian: Ива́н Петро́вич Па́влов, 1849–1936), was a Russian experimental neurologist, psychologist and physiologist known for his discovery of classical conditioning through his experiments with dogs. The concept for which Pavlov is famous is the "conditioned reflex" that he developed in 1901. Pavlov noticed that his dogs began to salivate in the presence of the technician who normally fed them, rather than simply salivating in the presence of the food. If a buzzer or metronome was sounded before the food was given, the dog would later come to associate the sound with the presentation of the food and salivate upon the presentation of the sound stimulus alone. He was awarded the 1904 Nobel Prize in Physiology or Medicine "in recognition of his work on the physiology of digestion, through which knowledge on vital aspects of the subject has been transformed and enlarged." Pavlov's dog, the Pavlovian session, and Pavlov's typology are named in his honor.



Dissection of malariated mosquito. Ronald Ross (1857–1932) was a British medical doctor who received the Nobel Prize for Physiology or Medicine in 1902 for his work on the transmission of malaria. His discovery of the malarial parasite in the gastrointestinal tract of a mosquito in 1897 proved that malaria was transmitted by mosquitoes, and laid the foundation for the method of combating the disease. Ross was a polymath, writing a number of poems, published several novels, and composed songs. He was also an amateur artist and mathematician. He worked in the Indian Medical Service for 25 years. It was during his service that he made the groundbreaking medical discovery. After resigning from his service in India, he joined the faculty of Liverpool School of Tropical Medicine, and continued as Professor and Chairman of Tropical Medicine of the institute for 10 years. In 1926, he became Director-in-Chief of the Ross Institute and Hospital for Tropical Diseases, which was established in honor of his works. He remained there until his death. Image courtesy of the Ross Archive at the London School of Hygiene and Tropical Medicine and the Wellcome Collection. Text courtesy of Wikipedia.



Malaria parasites. The page in Ross' notebook where he recorded on August 20, 1897 the "pigmented bodies" in mosquitoes that he later identified as malaria parasites. Source: R. Ross. (1923). *Memoirs with a Full Account of the Great Malaria Problem and Its Solution.* John Murray, London.

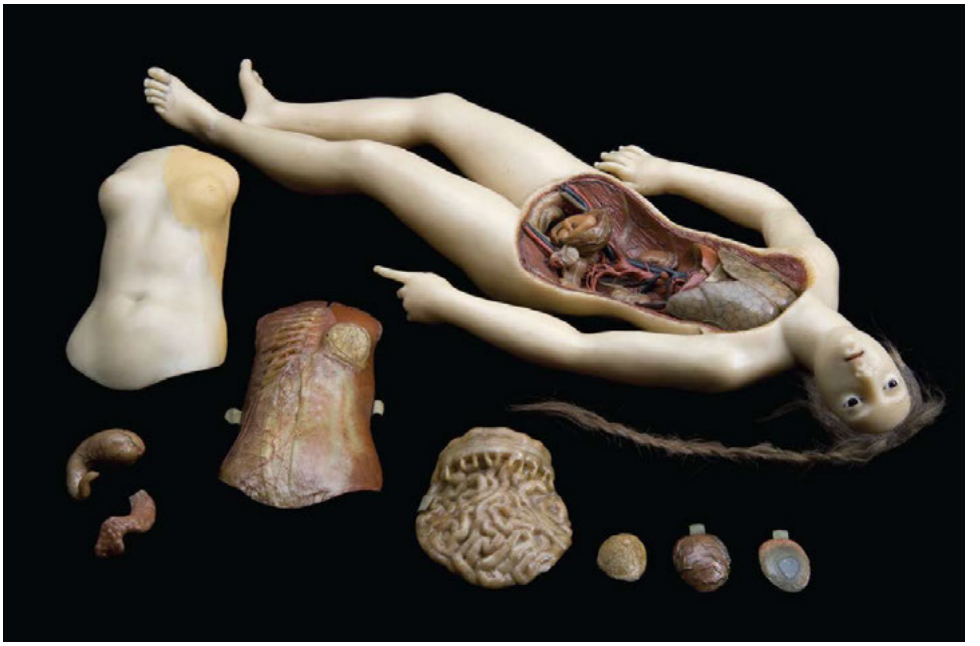


EVERY MOVEMENT OF THE SURGEON SHOWN ON A SCREEN IN THE ROOM NEXT TO THE OPERATING-ROOM.

Medical education is enhanced through the use of technology. This image, a halftone after a drawing by W. Woekkoek in 1909, depicts the use of a lantern screen. Lantern screens were used to allow medical students to view an operation from a viewpoint above an operating table through the use of a projecting periscope. The use of such technological advances have allowed students to more fully engage in medical procedures throughout their education. In this context, one is reminded of the aphorism: "Tell me and I'll forget. Show me and I may not remember. Involve me and I'll understand." Image source: W. Koekkoek. *The Illustrated London News*, 17 April 1909.

Oil on wood painting by Rembrandt van Rijn. Nicolaes Tulp (1593–1674), a Dutch surgeon from the Netherlands, teaching a group of students about human anatomy in The Hague. Tulp was a very well respected doctor and was the subject of this masterpiece by Rembrandt, titled *The Anatomy Lesson of Dr. Nicolaes Tulp*. He was also a college tutor and performed anatomy demonstrations on criminals who had been executed. The body is that of a male criminal and it is no coincidence that the cadaver's face is partly in shadow – this was known as the "shadow of death" and was a signature technique of Rembrandt. Image courtesy of the Wellcome Collection.

Painted by Franz Anton Maulbertsch (1724–1796) in the 18th century, *The Quack Doctor* shows a barber-surgeon pulling teeth at a temporary stall in town.

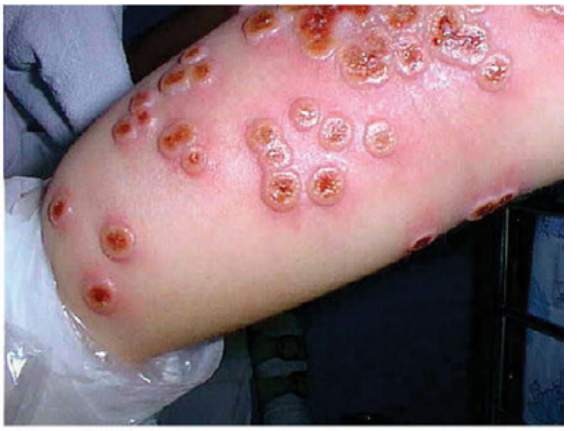


Wax anatomical figure of a reclining woman, Florence, Italy by Clemente Michelangelo Susini (1754–1814). The Italian sculptor became renowned for his wax anatomical models, that vividly and accurately depicted partly dissected corpses. Wax anatomical figures were used in medical education in the seventeenth and eighteenth centuries. They were typically male, and female figures emphasized how the female body was different. Image courtesy of the Science Museum, London and the Wellcome Collection.

Colored copper engraving (c. 1721) by Paul Fürst of a plague doctor of Marseilles (introduced as "Dr. Beaky of Rome"). The doctor's nose-case is filled with herbal material to keep off the plague. A plague doctor was a physician who treated victims of bubonic plague during epidemics. These physicians were hired by cities to treat infected patients regardless of income, especially the poor that could not afford to pay. Plague doctors had a mixed reputation, with some citizens seeing their presence as a warning to leave the area. In many cases these doctors were not experienced physicians or surgeons; instead, being volunteers, second-rate doctors, or young doctors just starting a career. They rarely cured patients, instead serving to record death tolls and the number of infected people for demographic purposes. Courtesy of Wikipedia.

A street scene (c. 1765) depicting an itinerant dentist of the seventeenth century. An orator is standing on a table. People are lined up behind another man who is sitting with two dental instruments, one in his hand and the other in his mouth. His patient is raising his right arm while resting his head in the dentist's lap. The rest of the town is going about their business as usual. Source: Leonaris Jacopo, US National Library of Medicine.

(a)



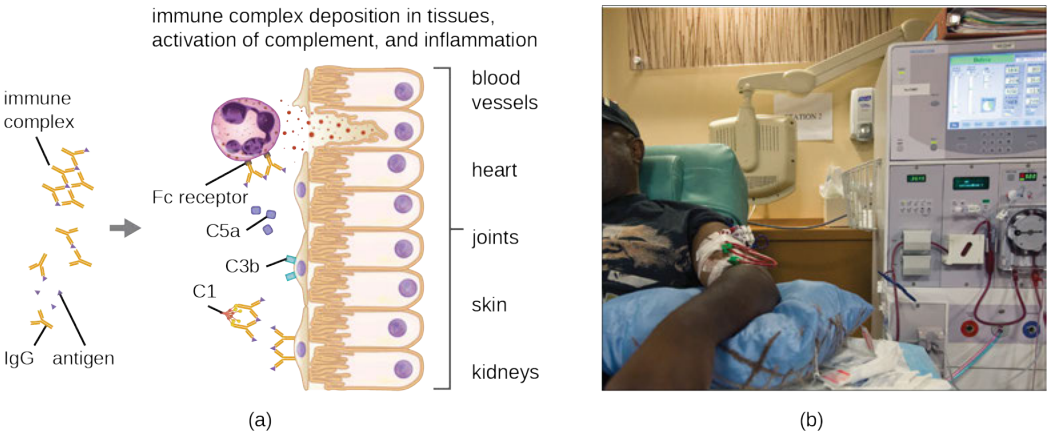
(b)

Left: A painting of Edward Jenner depicts a cow and a milkmaid in the background. *Right:* Lesions on a patient infected with cowpox, a zoonotic disease caused by a virus closely related to the one that causes smallpox.

Although variolation had been practiced for centuries, the English physician, Edward Jenner (1749–1823) is generally credited with developing the modern process of vaccination. Jenner observed that milkmaids who developed cowpox, a disease similar to smallpox but milder, were immune to the more serious smallpox. This led Jenner to hypothesize that exposure to a less virulent pathogen could provide immune protection against a more virulent pathogen, providing a safer alternative to variolation. In 1796, Jenner tested his hypothesis by obtaining infectious samples from a milkmaid's active cowpox lesion and injecting the materials into a young boy. The boy developed a mild infection that included a low-grade fever, discomfort in his axillae and loss of appetite. When the boy was later infected with infectious samples from smallpox lesions, he did not contract smallpox. This new approach was termed vaccination, a name deriving from the use of cowpox (Latin *vacca* meaning "cow") to protect against smallpox. Today, we know that Jenner's vaccine worked because the cowpox virus is genetically and antigenically related to the *Variola* viruses that caused smallpox. The success of Jenner's smallpox vaccination led other scientists to develop vaccines for other diseases. Perhaps the most notable was Louis Pasteur, who developed vaccines for rabies, cholera, and anthrax. During the 20th and 21st centuries, effective vaccines were developed to prevent a wide range of diseases caused by viruses (e.g., chickenpox and shingles, hepatitis, measles, mumps, polio, and yellow fever) and bacteria (e.g., diphtheria, pneumococcal pneumonia, tetanus, and whooping cough). Image (b) courtesy of the Centers for Disease Control and Prevention. Text courtesy of OpenStax (https://openstax.org).

David Vetter (1971–1984), popularly known as "The Bubble Boy," was born with Severe Combined Immunodeficiency (SCID) and lived most of his life isolated inside a plastic bubble. Here he is shown outside the bubble in a suit specially built for him by NASA. In his first years of life he lived mostly at Texas Children's Hospital in Houston, Texas. As he grew older, he lived increasingly at home with his parents and older sister Katherine in Dobbin, Texas.

Patients who suffer from SCID have B-cell and T-cell defects that impair T-cell dependent antibody responses as well as cell-mediated immune responses. Patients with SCID also cannot develop immunological memory, so vaccines provide them no protection, and live attenuated vaccines (e.g., for varicella-zoster, measles virus, rotavirus, poliovirus) can actually cause the infection they are intended to prevent. The most common form is X-linked SCID, which accounts for nearly 50% of all cases and occurs primarily in males. Patients with SCID are typically diagnosed within the first few months of life after developing severe, often life-threatening, opportunistic infection by *Candida* spp., *Pneumocystis jirovecii*, or pathogenic strains of *E. coli*. Without treatment, babies with SCID do not typically survive infancy. In some cases, a bone marrow transplant may successfully correct the defects in lymphocyte development that lead to the SCID phenotype, by replacing the defective component. However, this treatment approach is not without risks, as demonstrated by this famous case of David Vetter. Vetter, who lived in the protective plastic bubble to prevent exposure to opportunistic microbes, received a bone marrow transplant from his sister. Because of a latent Epstein-Barr virus infection in her bone marrow, however, he developed mononucleosis and died of Burkitt lymphoma at the age of 12 years. A Conroe ISD elementary school which opened in 1990 in The Woodlands in unincorporated Montgomery County, Texas, was named David Elementary after Vetter. The Paul Simon song "The Boy in the Bubble" was inspired by Vetter's story. Image kindly provided by NASA Johnson Space Center. Text courtesy of Sangita Bawa and OpenStax (https://openstax.org).



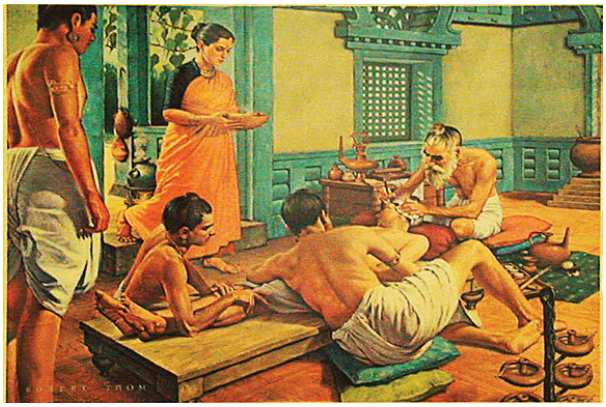
Type III hypersensitivities and the systems they affect. (a) Immune complexes form and deposit in tissue. Complement activation, stimulation of an inflammatory response, and recruitment and activation of neutrophils result in damage to blood vessels, heart tissue, joints, skin, and/or kidneys. (b) If the kidneys are damaged by a type III hypersensitivity reaction, dialysis may be required. Courtesy of OpenStax (https://openstax.org) and US Air Force.

Bee stings and other allergens can cause life-threatening, systemic allergic reactions. Sensitive individuals may need to carry an epinephrine auto-injector (e.g., EpiPen) in case of a sting. A bee-sting allergy is an example of an immune response that is harmful to the host rather than protective; epinephrine counteracts the severe drop in blood pressure that can result from the immune response. Courtesy of Carol Bleistine and OpenStax (https://openstax.org).

(a)

(b)

(a) The Aeromedical Biological Containment System (ABCS) is a module designed by the CDC and Department of Defense specifically for transporting highly contagious patients by air. (b) An isolation ward for Ebola patients in Lagos, Nigeria. Courtesy of the Centers for Disease Control and Prevention and OpenStax (https://openstax.org).

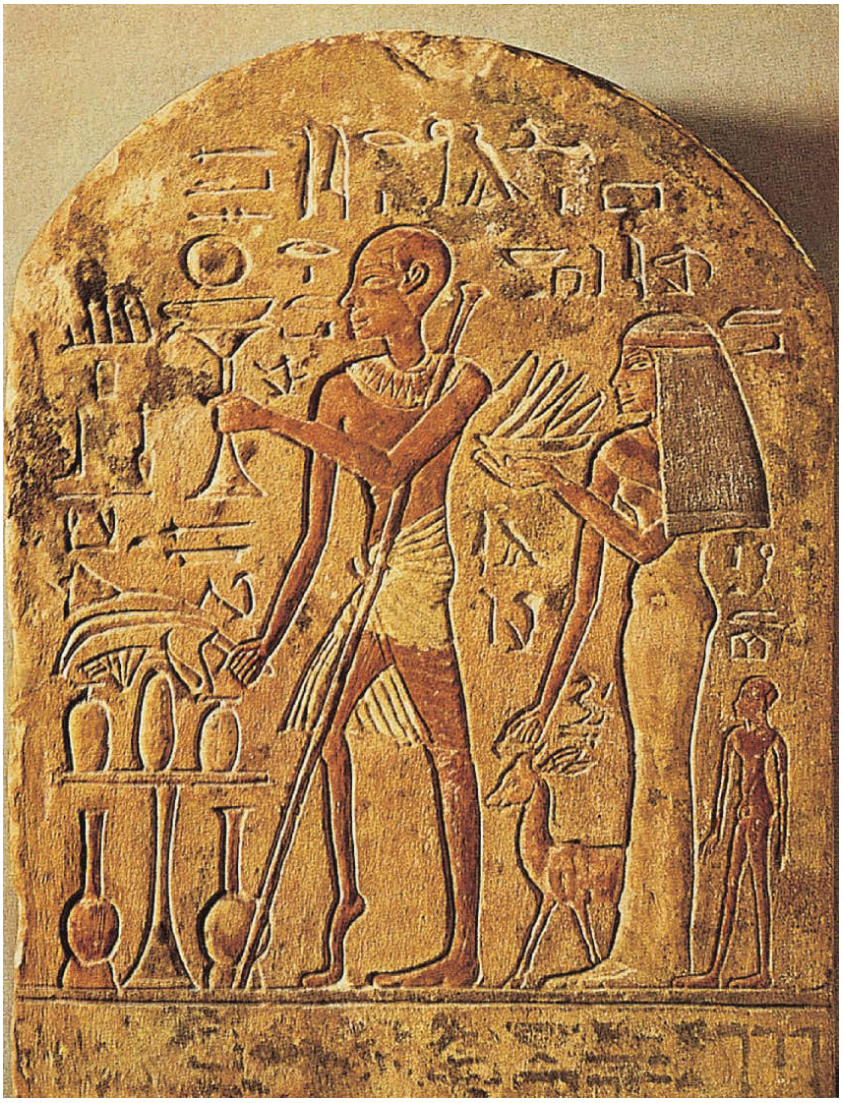


The painting above shows the great Indian surgeon, Sushruta, or Suśruta (Sanskrit: सुश्रुत, IAST: Suśruta, lit.'well heard'), generally considered as the "father of plastic surgery," performing an otoplastic operation (cosmetic ear surgery). Friends and relatives steady the patient, possibly drugging him with wine, as the great surgeon sets about fashioning an artificial earlobe. This following would most likely be the sequence of events in this surgery: a section of flesh will be cut from the patient's cheek; it will then be attached to the stump of the mutilated organ; then it will be treated with homeostatic powders; and finally it will be bandaged. The definition of an ideal surgeon according to Sushruta is: "A person who possesses courage and presence of mind, a hand free from perspiration, tremor less grip of sharp and good instruments and who carries his operations to the success and advantage of his patient who has entrusted his life to the surgeon. The surgeon should respect this absolute surrender and treat his patient as his own son."

Couching for cataract. This early 19th century drawing shows the procedure as described by Susruta.

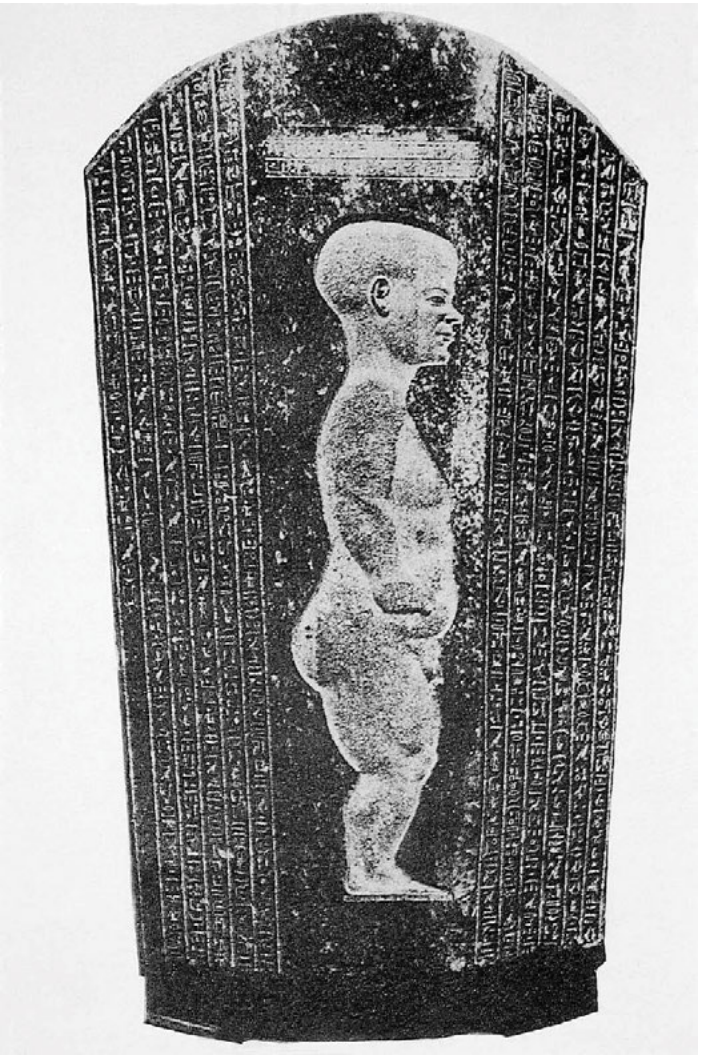
This photo shows a patient who has undergone rhinoplasty (surgery that changes the shape of the nose) based on Sushruta's technique. Although it is an old black-and-white photo, the scar on the forehead is clearly visible. This image is reproduced from the October 1794 issue of *The Gentleman's Magazine*, which ran uninterrupted in London for almost 200 years, from 1731 to 1922. It was the first to use the term magazine (French *magazine,* "storehouse") for a periodical.

The English surgeon, Joseph Constantine Carpue's (1764–1846) illustration of reconstruction of the nose using the Indian surgeon Susruta's technique. Carpue is known for performing the first rhinoplastic surgery in England, using a technique invented in India several centuries earlier by Susruta. In 1816, Carpue described the procedure in his publication of *Account of Two Successful Operations for Restoring a Lost Nose from the Integument of the Forehead.* The Indian rhinoplastic reconstruction involved using a flap of skin taken from the forehead, and was to become known in Europe as "Carpue's operation." It is hard for me not to question this as being classic plagiarism: the practice of taking someone else's work or ideas and passing them off as one's own.

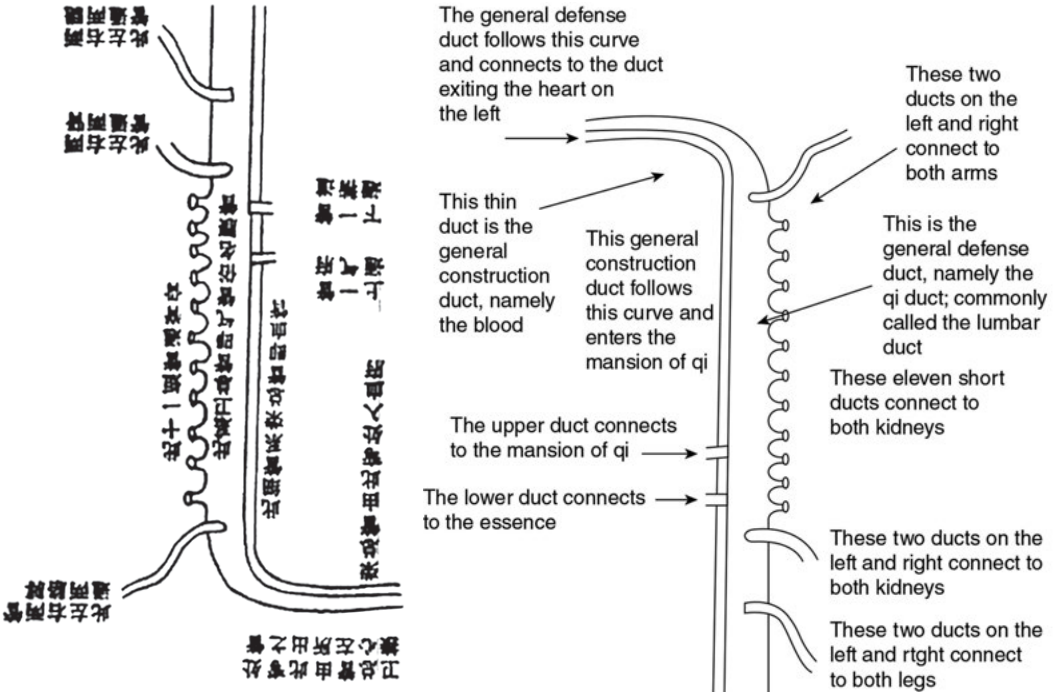


Poliomyelitis (polio) is a highly infectious disease caused by a virus belonging to the *Picornaviridae* family. It finds a mention even in ancient Egyptian paintings and carvings. The image above depicts an ancient Egyptian stone relief showing a patient with obvious poliomyelitis. Note the shortened right leg with muscle wasting and talipes equinovarus (TEV) together with the crutch. Poliomyelitis is a highly infectious viral disease that largely affects children under 5 years of age and is transmitted by person-to-person spread mainly through the fecal-oral route or, less frequently, by a common vehicle (e.g., contaminated water or food). It multiplies in the intestine, from where it can invade the nervous system and cause paralysis. The contribution of Salk and Sabin in the form of vaccines (oral polio vaccine (OPV) and the inactivated polio vaccine) has resulted in eradication of polio – it is a success story for medicine and public health and teaches us much about how to combat infectious diseases.

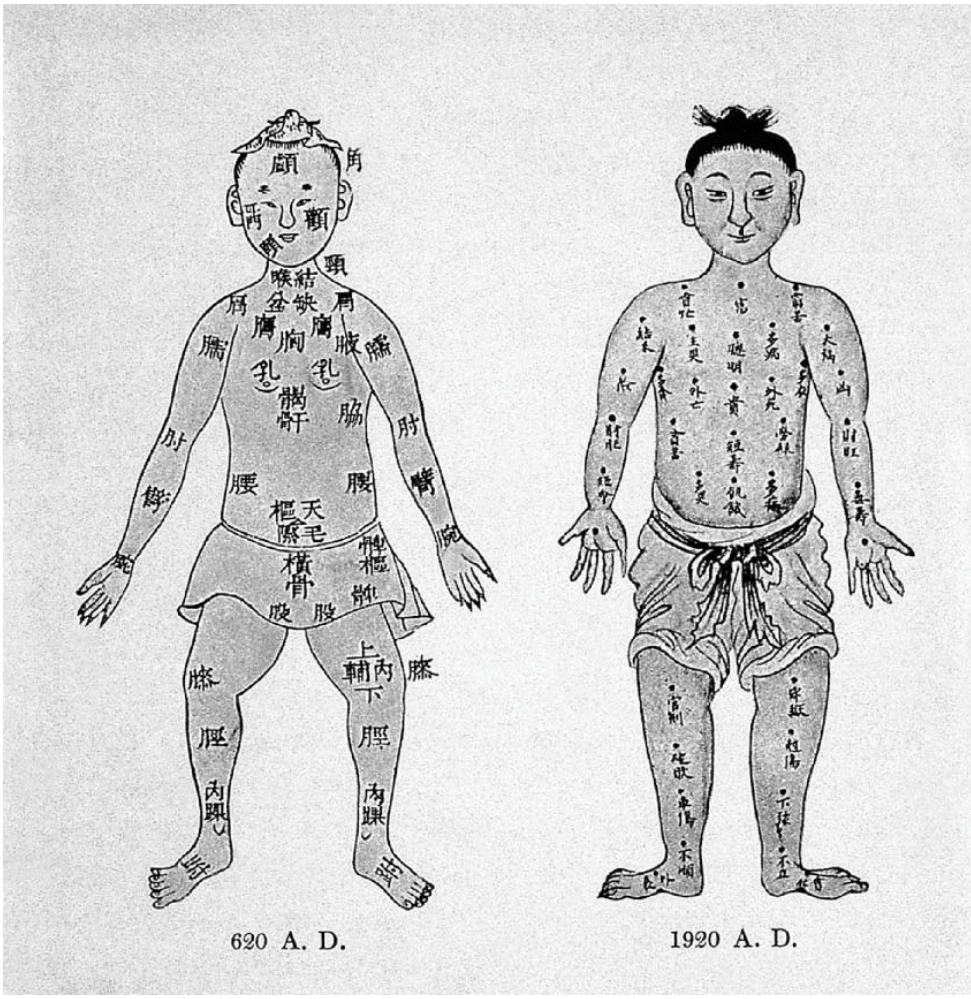
Model of a patient with a kyphosis (roundback) of the spine, probably tuberculosis. While a normal spine, when viewed from behind, appears straight, a spine affected by kyphosis shows evidence of a forward curvature of the vertebrae in the upper back area, giving an abnormally rounded or "humpback" appearance. Kyphosis is a spinal disorder, most common during adolescence, in which an excessive curve of the spine results in an abnormal rounding of the upper back or — in the case of a severe curve — as hunchback. Although the thoracic spine should have a natural kyphosis of 20 to 45 degrees, postural or structural abnormalities can result in a curve that is outside this normal range. Although the medical term for a curve that is greater than normal (greater than 50 degrees) is hyperkyphosis, the term kyphosis is generally used by physicians. Photo from the Cairo Museum, Egypt.



Stone relief of an achondroplastic. Achondroplasia is the most commonly occurring abnormality of bone growth (skeletal dysplasia) that causes disproportionate dwarfism. While dwarfism is defined as a condition of short stature as an adult, achondroplasia results in individuals who are short in stature with a normal sized torso and short limbs. This genetic disorder is caused by a mutation in the fibroblast growth factor receptor 3 *(FGFR3)* gene. It occurs as a result of a spontaneous genetic mutation in about 80% of patients and is inherited from a parent in the remaining 20%. It does not typically cause impairment or deficiencies in mental abilities. This genetic disorder is characterized by an unusually large head (macrocephaly), short upper arms (rhizomelic dwarfism), and short stature (adult height of ~4 feet). If the achondroplasia does not result in the bones that join the head and neck to compress the brainstem or upper spinal cord (craniocervical junction compression), then life expectancy is near normal. Photo from the Cairo Museum, Egypt.

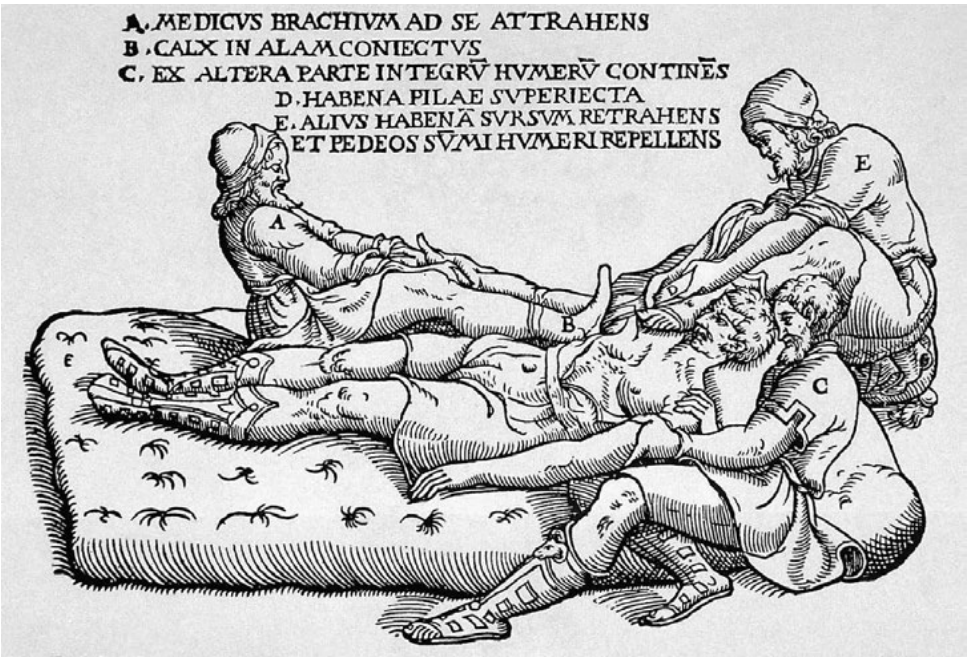


A depiction of the anatomy of the viscera and bowels from *Yi Lin Gai Cuo* ("Correcting the Errors in the Forest of Medicine") by Wang Qing-ren (1768–1831). Note that the original Chinese version is on the left and the translation is on the right. This "classic" of Chinese medicine was first published in 1830 and considered as a foundational work in modern Chinese medicine. The book has been republished many times in China, Japan, and Korea. Wang's insistence that doctors had been led astray by the descriptions of the internal organs in the classics was a provocative challenge to the physicians of his day. He wrote: "Among book-writing good doctors, there has not been a single perfect man. The reason is that earlier generations in their medical writings were mistaken about the viscera and bowels and later generations followed the established theories. The root of disease was lost early. [...]To write this book without understanding the internal organs, would not that be like an idiot talking nonsense? To treat illness without understanding the viscera and bowels is no different from a blind man walking in the dark!"

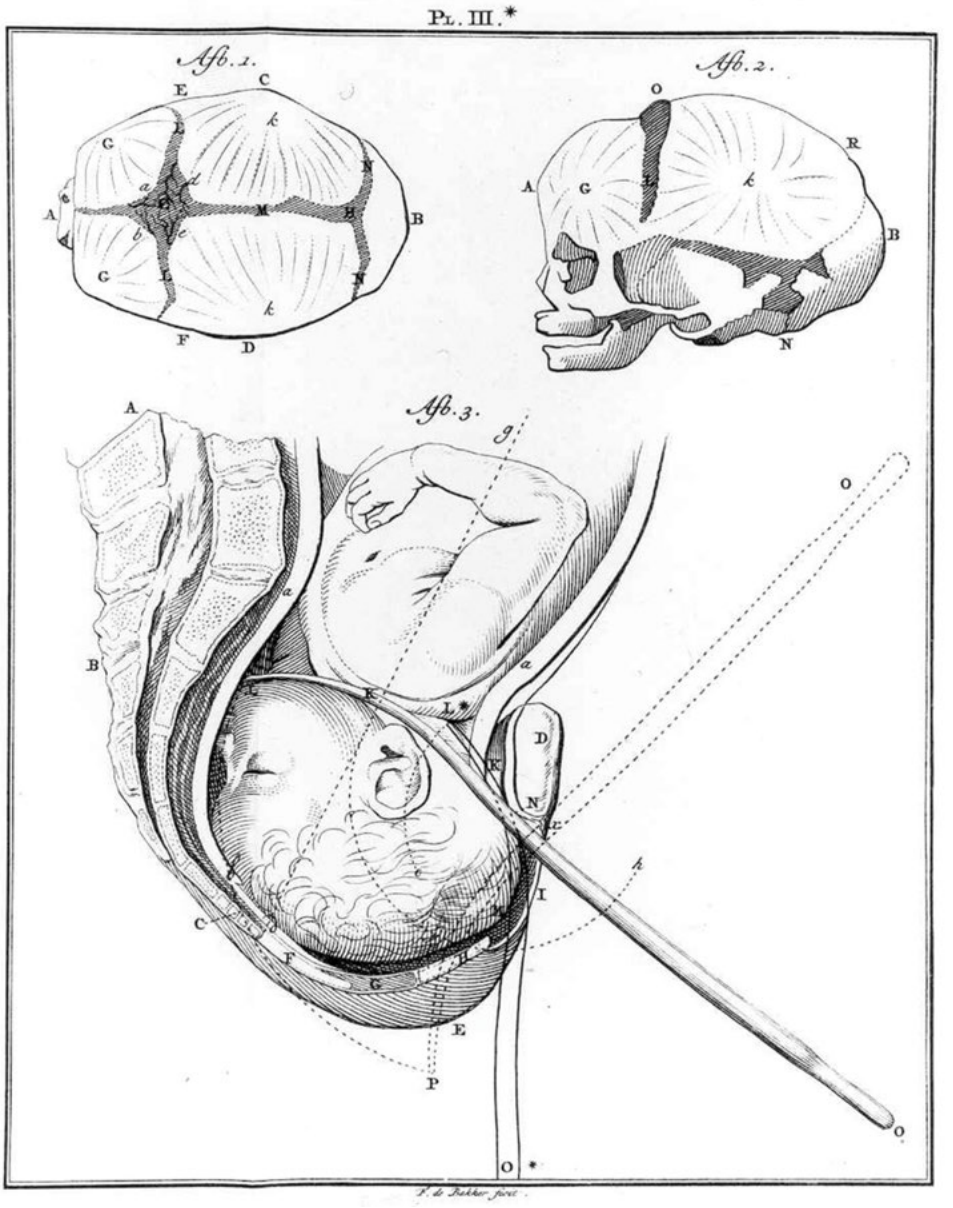


Chinese acupuncture diagrams from 620 AD and 1920. A key component of traditional Chinese medicine, acupuncture, is that it is most commonly used to treat pain. Acupuncture is the practice of penetrating the skin with thin metallic needles which are then activated through gentle and specific movements of the practitioner's hands or with electrical stimulation. Traditional Chinese medicine practitioners believe the human body has more than 2,000 acupuncture points connected by pathways or meridians. These pathways create an energy flow (Qi, pronounced "chee") through the body that is responsible for health; disruption of the energy flow can cause disease. Image source: W. Osler. (1921). *Evolution of Modern Medicine*. Yale University Press, New Haven.

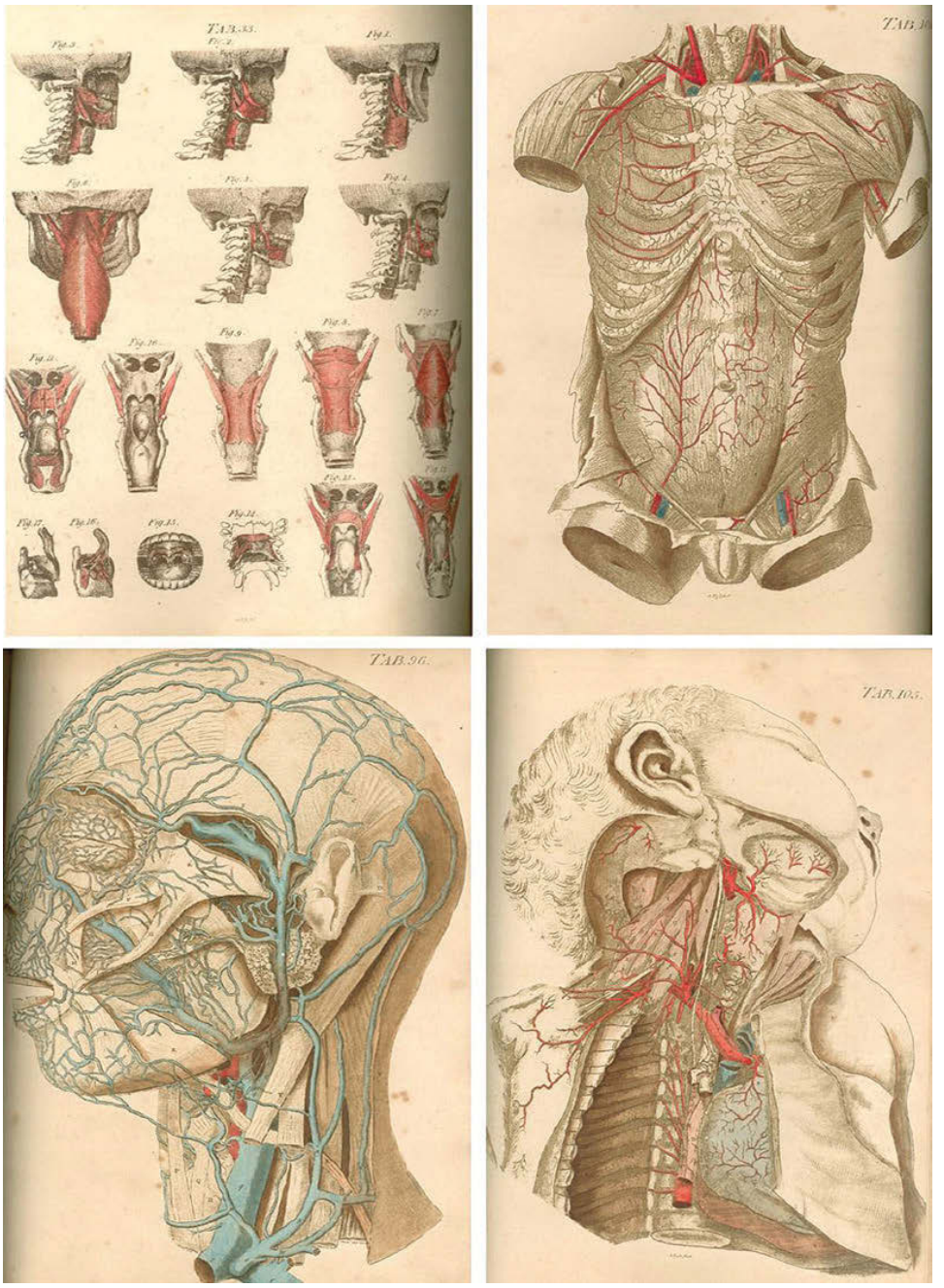
Two giants of medicine are shown in this fresco by the Italian Renaissance master, Raphael Sanzio. The elder Plato (left) walks alongside a younger Aristotle (right). Along with his teacher, Socrates, and his student, Aristotle, Plato is a central figure in the history of ancient Greek philosophy. This famous painting is referred to as *The School of Athens* (Italian: *Scuola di Atene*). The fresco was painted between 1509 and 1511 as a part of Raphael's commission to decorate the rooms now known as the *Stanze di Raffaello*, in the Apostolic Palace in the Vatican.



The Hippocratic method of reduction of a dislocated shoulder. This image has been taken from an edition of Galen's works published in Basel, Switzerland in 1562. Shoulder dislocations are the most common of all major joint dislocations and frequently present to clinics and the emergency rooms of hospitals. The shoulder joint is inherently unstable. The glenoid is shallow, allowing for a wide range of motion, with only a small portion of the humeral head articulating with the glenoid in any position. Reduction techniques can vary in terms of required force, time, equipment, and staff. Moreover, no single reduction method is successful in every instance. Hence, familiarity with several reduction techniques is critically important for a clinician. I found a study that give an overview of 23 different techniques for closed reduction for shoulder dislocations and 17 modifications of these techniques (*Turk J Emerg Med.* 2016; 16(4):155–168).

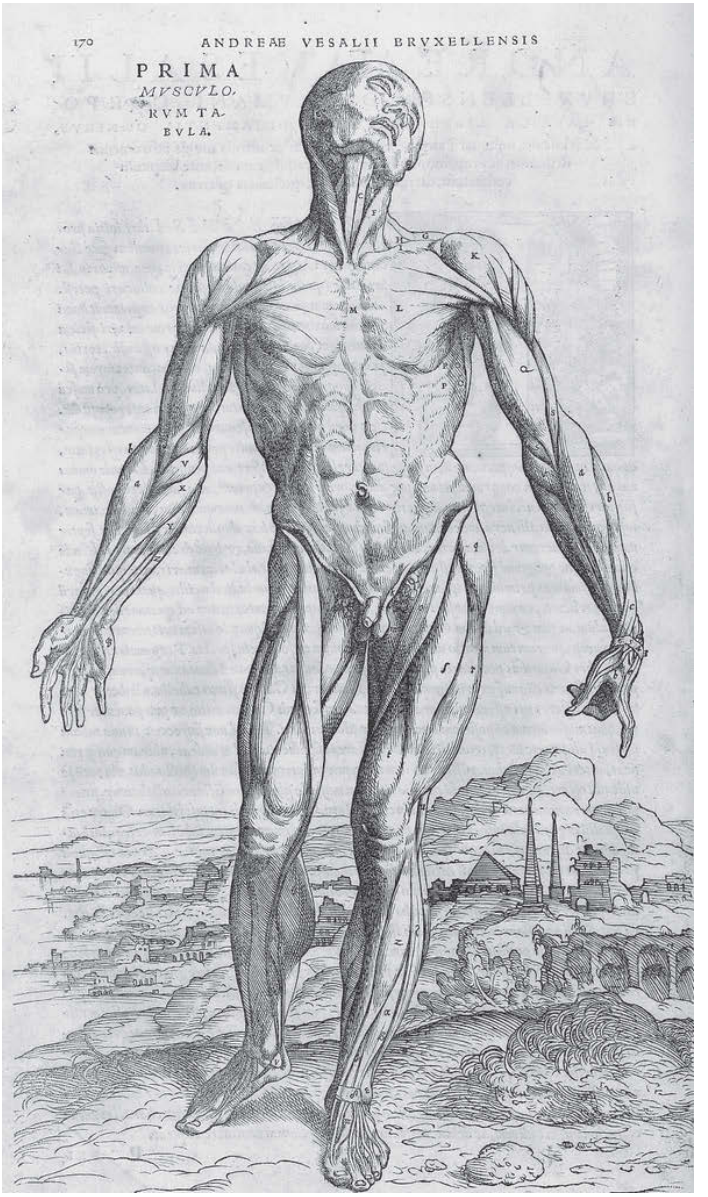


Original illustration of obstetric equipment created by Camper to assist in childbirth. Courtesy of the University of Groningen Library, the Netherlands.



Artwork drawn by Andrew Fyfe (1754–1824) used in his 1800 text, *Compendium of the Anatomy of the Human Body*.

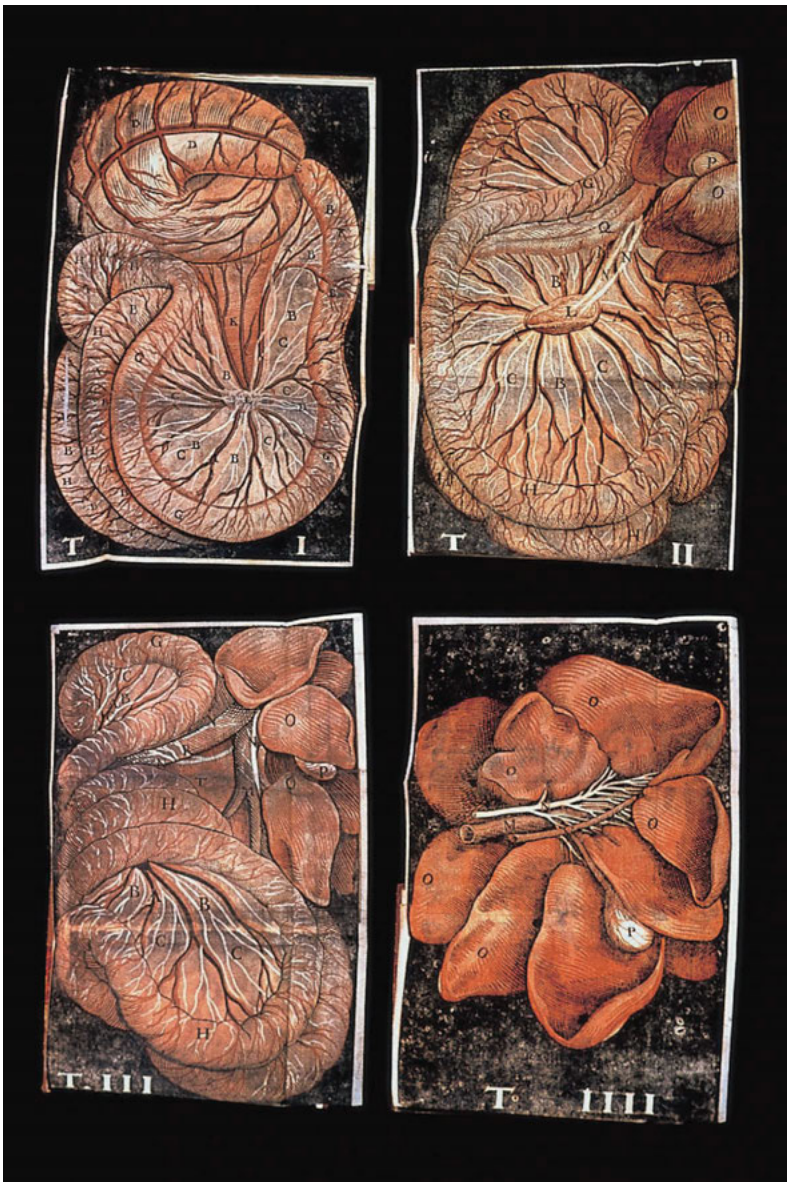
Andreas Vesalius (Latinized from Andries van Wezel) (/vɪˈseɪliəs/; 1514–1564) was a 16th century anatomist, physician, and author of one of the most influential books on human anatomy, *De Humani Corporis Fabrica Libri Septem*, published in 1543 (Latin, "On the fabric of the human body in seven books"). This image, which is the frontispiece of the *Fabrica*, shows Vesalius dissecting in front of his students at Padua. Vesalius was one of the first to accurately record and illustrate human anatomy based on his study of autopsies and dissections. Naturally, this led to improved understanding of the human body and enhanced surgery techniques. Vesalius is generally considered as the founder of modern human anatomy.



A woodcut from Vesalius' *Fabrica* shows muscles of the anterior aspect of the human body. Illustrations such as these are often better than those found in some many modern anatomy texts. Vesalius wrote in the *Fabrica:* "When I undertake the dissection of a human pelvis I pass a stout rope tied like a noose beneath the lower jaw and through the zygomas up to the top of the head... The lower end of the noose I run through a pulley fixed to a beam in the room so that I may raise or lower the cadaver as it hangs there or turn around in any direction to suit my purpose; ... You must take care not to put the noose around the neck, unless some of the muscles connected to the occipital bone have already been cut away."

Henry VIII presents the charter to the Company of Barber-surgeons, 1540. Thomas Vicary (c. 1490-1561), English physician, surgeon and anatomist, stands immediately to the right of the King.

John Bannister giving the visceral lecture at the Barber-surgeons Hall, 1581. From a print at Barber's Hall. The original is in the Glasgow University Library.



Gaspare Aselli's (or Asellio) (c. 1581–1625) demonstration of the lymphatics in the mesentery of the small intestine of the dog (the lacteals). This image represents the first demonstration of this system and the first-time color was used in a medical illustration. The Italian physician, noted for the discovery of the lacteal vessels of the lymphatic system, also discovered (or rediscovered) the chylous vessels, and studied systematically the significance of these vascular structures. This image is most likely based on the vivisection performed on July 23, 1622 on a dog where he saw these vessels, the white fluid they contained, and due to the milk-like character of this fluid the name *lacteal* was given. Image courtesy of the Royal College of Surgeons of England.

MEDICAL, CHIRURGICAL,
AND
ANATOMICAL CASES
AND
OBSERVATIONS.

By *LAURENCE HEISTER*, M. D.

Senior Profeſſor of Phyſic and Surgery in the Univerſity of HELMSTADT, firſt Phyſician and Aulic Counſellor to his ſerene Highneſs the Duke of BRUNSWICK, Member of the Imperial Academy of Sciences, and Fellow of the Royal Societies of LONDON and BERLIN.

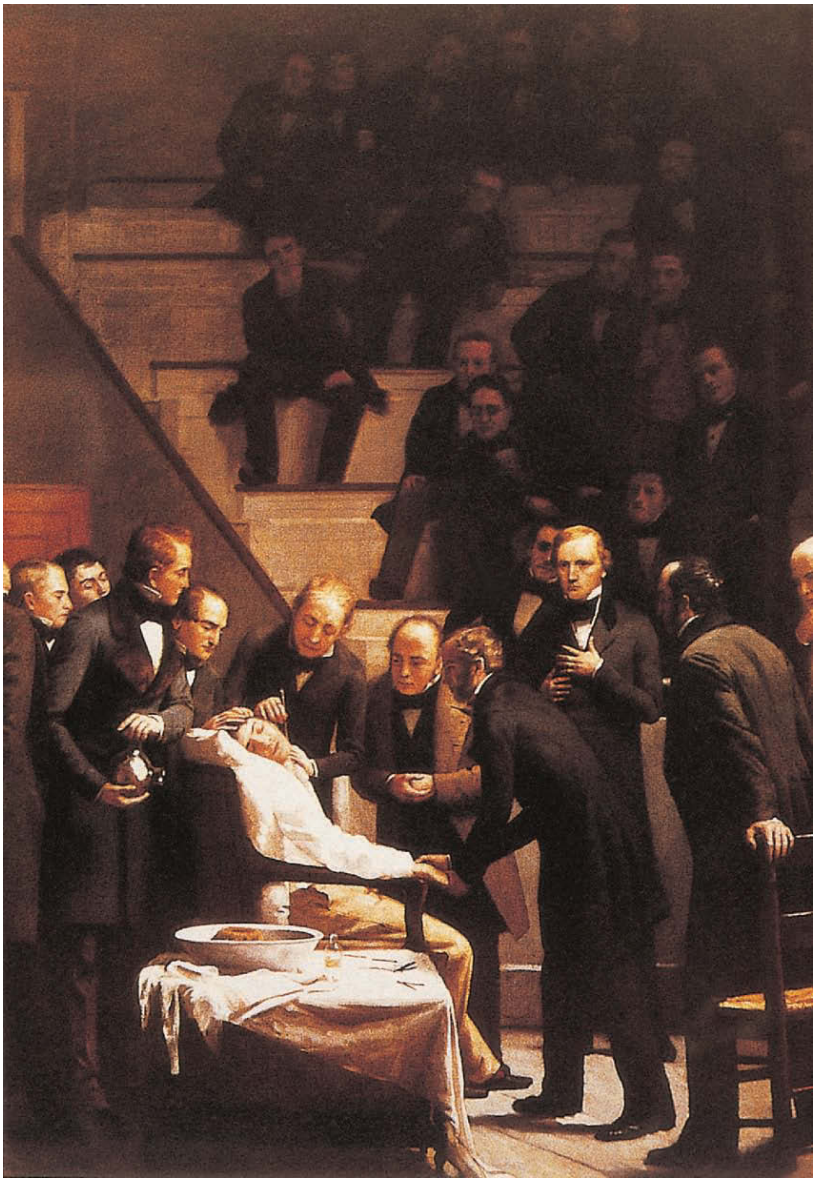
With COPPER-PLATES, illuſtrating the Deſcriptions in the reſpective Caſes.

Tranſlated from the German Original
By *GEORGE WIRGMAN.*

LONDON: Printed by *J. REEVES*,
For C. HITCH and L. HAWES, and J. BALDWIN in *Pater-noſter-Row*,
J. WHISTON and B. WHITE in *Fleet-ſtreet*, J. and J. RIVINGTON in
St. *Paul*'s *Church-yard*, and A. LINDE in *Catherine-ſtreet* in the *Strand*.

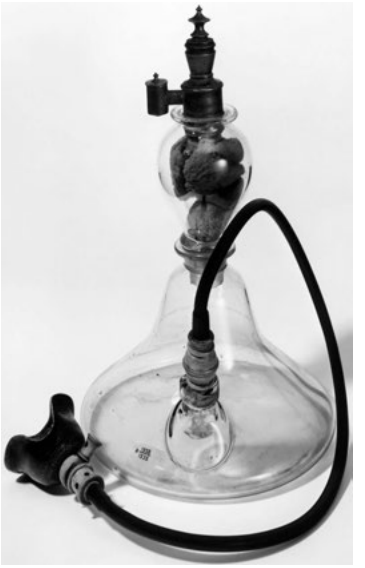
M DCC LV.

Frontispiece of Heister's *Medical, Chirurgical and Anatomical Cases and Observations*, English edition, 1755.



Painting depicting the first demonstration of surgical anesthesia. The anesthetist was William T. G. Morton, who holds his anaesthetic apparatus at the head of the table. On October 16, 1846, dentist William T. G. Morton (1819–1868) successfully demonstrated in front of the surgeons of Massachusetts General Hospital in Boston that inhalation of ether vapor could produce surgical anesthesia. This image is courtesy of the Wellcome Library.

"Now that anaesthetic agents are so much in vogue, it is seldom that we are obliged to tie our patients, or to roll them up in sheets and aprons, as was the custom prior to the discovery of these most useful remedies" (S. S. Gross. (1866). *A System of Surgery.* 4th Edition, Philadelphia, Henry C. Lea, page 479).



Copy of the Squire-type ether inhaler first used in 1846. The original Squire-type ether inhaler was used by Robert Liston (1794–1847) on December 21, 1846 to perform the first operation in England under anaesthetic, at University College Hospital London. He amputated a leg from Frederick Churchill, a chauffeur. After surgery was completed, the patient reported that he was unaware that the operation had even taken place. Vapors from ether-soaked sponges in the top of the inhaler collect in the chamber at the bottom. The vapors are inhaled by the patient through the metal face mask. The device is named after Peter Squire, a pharmacist commissioned by Liston to make the inhaler. Courtesy of the Wellcome Library, London.

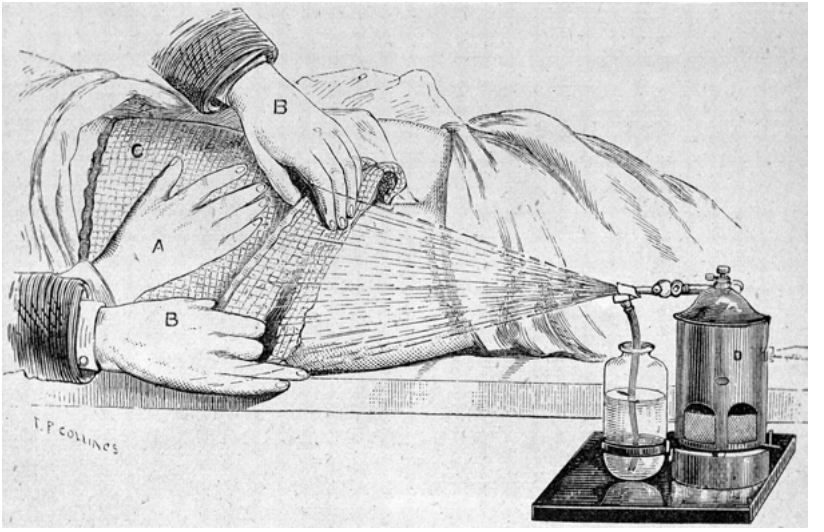
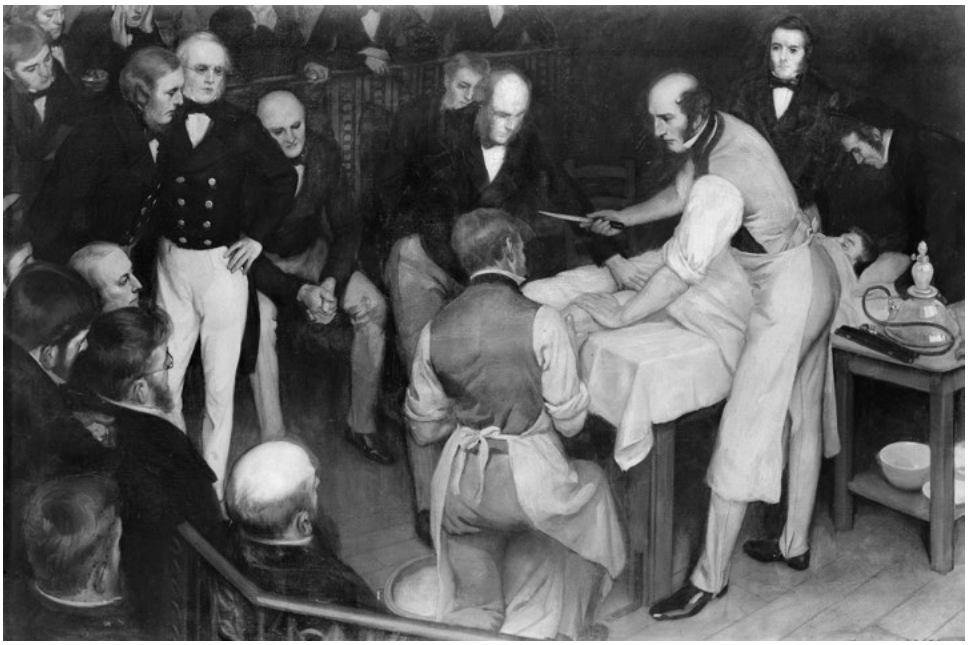


Image depicting antiseptic surgery. Source: W. W. Cheyne. (1882). *Antiseptic Surgery: Its Principles, Practice, History and Results*. Elder Smith, London.

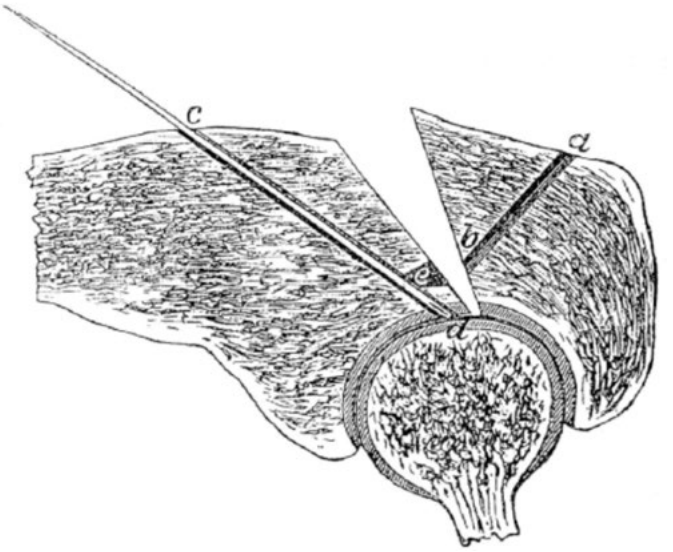


The first "capital" operation under ether at University College Hospital, London. Robert Liston (1794–1847) operates in his shirt sleeves. The British surgeon was noted for his speed and skill in an era prior to anesthetics, when speed made a difference in terms of pain and survival. The ether apparatus is placed on a small table.

Richard Gordon, a surgeon and medical historian, described Liston as "the fastest knife in the West End. He could amputate a leg in 2½ minutes." Indeed, Liston is reputed to have been able to complete operations in a matter of seconds, at a time (before anesthesia) when speed was essential to reduce pain and improve the odds of survival of a patient. In his most famous mishap, he was moving so fast that he took off a surgical assistant's fingers as he cut through a leg. Gordon described the scene thus: "He was six foot two, and operated in a bottle-green coat with wellington boots. He sprung across the blood-stained boards upon his swooning, sweating, strapped-down patient like a duelist, calling, "Time me gentlemen, time me!" to students craning with pocket watches from the iron-railinged galleries. Everyone swore that the first flash of his knife was followed so swiftly by the rasp of saw on bone that sight and sound seemed simultaneous. To free both hands, he would clasp the bloody knife between his teeth."

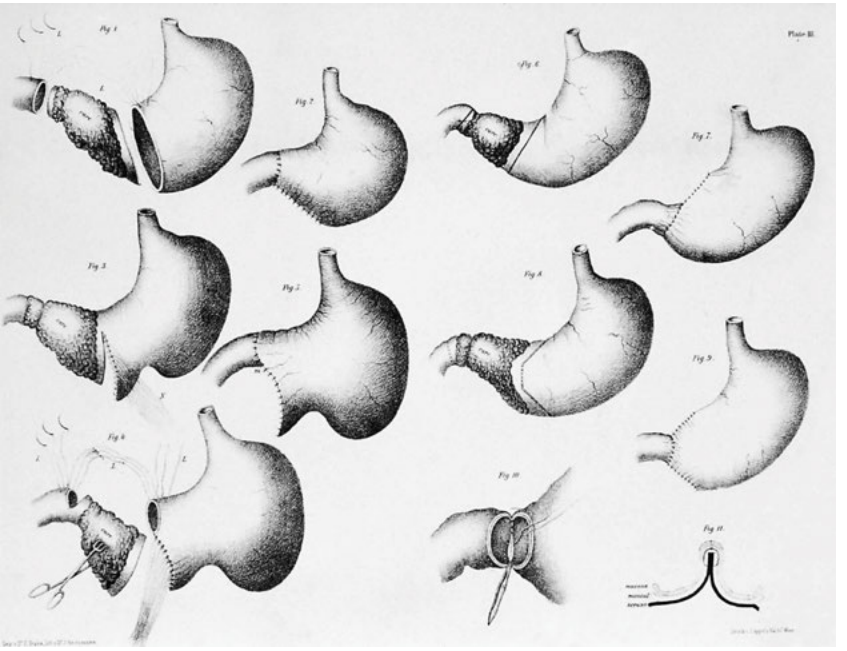
Side Note: In Florence Nightingale's *Notes on Nursing*, she states "there are many physical operations where *ceteris paribus* (all else being equal) the danger is in a direct ratio to the time the operation lasts; and *ceteris paribus* the operator's success will be in direct ratio to his quickness."

Text courtesy of Wikipedia. Photograph courtesy of the Wellcome Library, London.

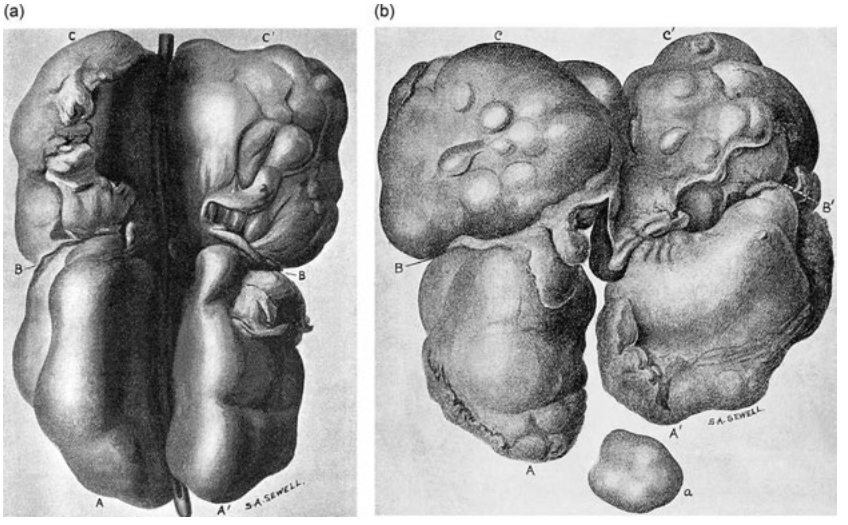


Joseph Lister's operation of wiring of a fractured olecranon. Source: J. Lister. (1883). An address on treatment of fractures of the patella. *British Medical Journal* 2, 855.

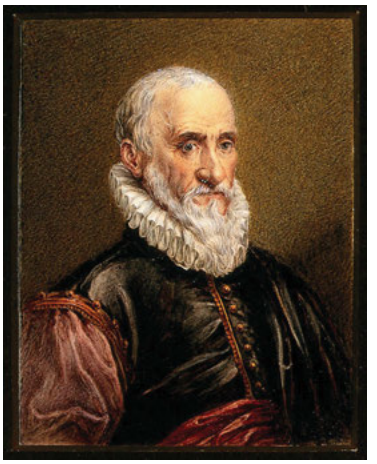
Lister's carbolic spray, nicknamed "the donkey," is shown above. It produced an aerosol of carbolic in the room, much to the discomfort of the surgeon and his assistants. This apparatus is preserved in the Hunterian Museum at the Royal College of Surgeons of England.



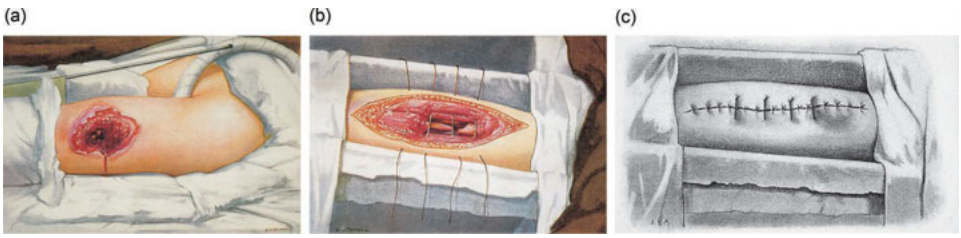
The gastrectomy on Frau Heller. Source: T. Billroth. (1891). *Clinical Surgery. Extracts from the Reports of Surgical Practice Between the Years 1860–1876*, The New Sydenham Society, London.



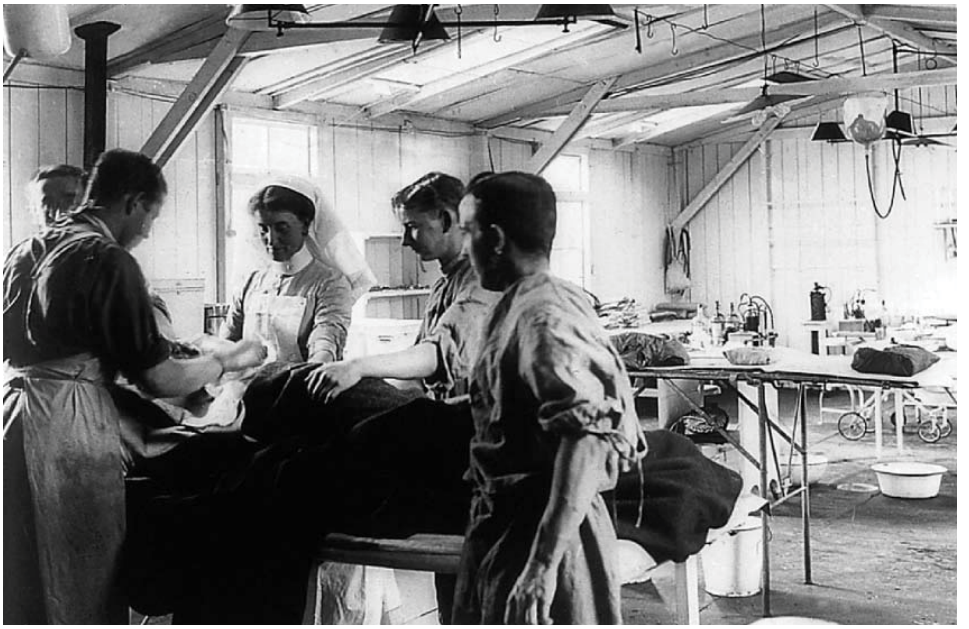
(a) Upper aspect of an enormous prostate, weighing 10½ ounces, removed from a patient aged 75. The catheter indicates the position occupied by the urethra. Portion A, A1, B, B1, lay in the bladder; B, B1, C, C1 outside the bladder between the pubic arch and the rectum. (b) Showing under aspect of the same prostate, with, below it, an adenoma detached from the prostate. Source: P. J. Freyer. (1902). *Clinical Lectures on Stricture of the Urethra and Enlargement of the Prostate*. Baillière, Tindall and Cox, London.



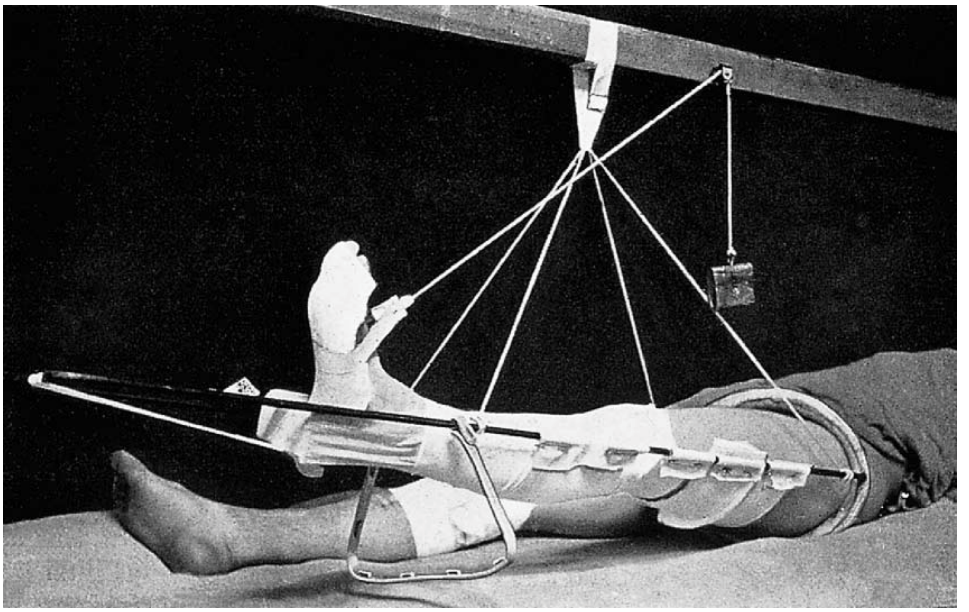
Ambroise Paré (c. 1510–1590) was a French barber surgeon who served in that role for kings Henry II, Francis II, Charles IX, and Henry III. He is considered one of the fathers of surgery and modern forensic pathology. A pioneer in surgical techniques and battlefield medicine, especially in the treatment of wounds, he invented several surgical instruments, and was a member of the Parisian barber surgeon guild. In his personal notes about the care he delivered to Captain Rat, in the Piémont campaign (1537–1538), Paré wrote: *Je le pansai, Dieu le guérit* ("I bandaged him and God healed him"). This epitomizes a philosophy that he used throughout his career. These words, inscribed on his statue in Laval, are reminiscent of the Latin adage *medicus curat, natura sanat*. Paré also reintroduced the ligature of arteries (first used by Galen) instead of cauterization during amputation. The usual method of sealing wounds by searing with a red-hot iron often failed to arrest the bleeding and caused patients to die of shock. For the ligature technique he designed the "Bec de Corbeau" ("crow's beak"), a predecessor to modern hemostats. Although ligatures often spread infection, it was still an important breakthrough in surgical practice. Paré detailed the technique of using ligatures to prevent hemorrhaging during amputation in his 1564 book *Treatise on Surgery*. Image courtesy of the Welcome Library. Text courtesy of Wikipedia.



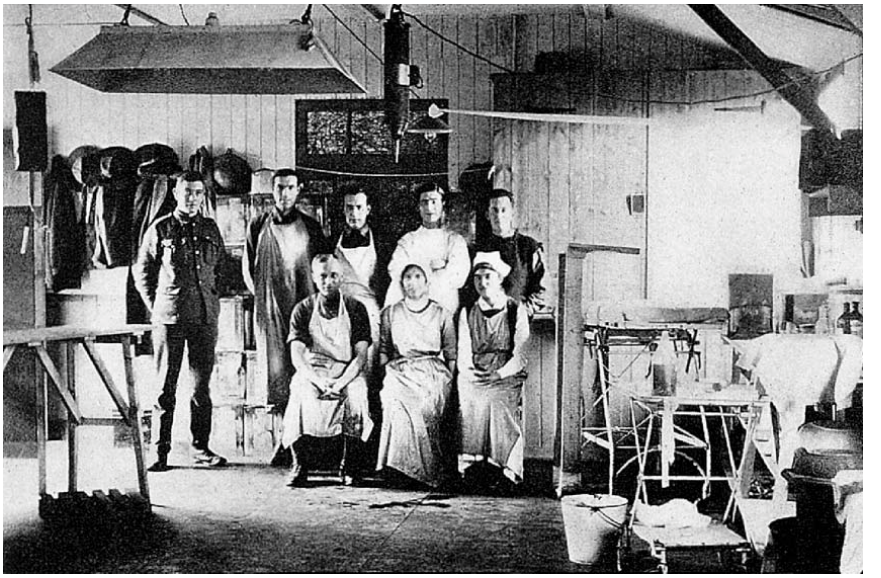
Stages of delayed primary suture. (a) Explosive exit wound in arm caused by rifle bullet 13 hours after infliction. Comminuted fracture of the humerus. (b) Wound after excision of damaged muscle and cleansing of the fracture. Deep sutures of silk in position. (c) Closure of the wound 7 days later. The wound healed by first intention. Source: F. Fraser et al. (1918). Primary and delayed primary suture of gunshot wounds. A report of research work at A C.C.S., December 27, 1917–March 1, 1918, with which is included a report on the bacteriology of wounds. *British Journal of Surgery* 6(21):92–124.



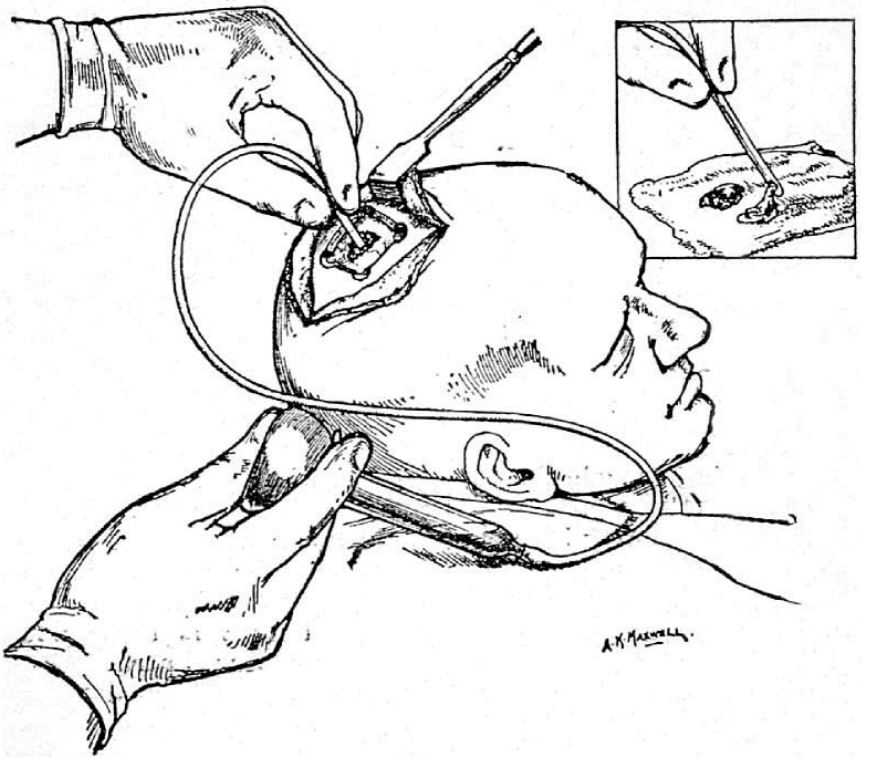
Operating theatre in a CCS, behind the line at the Battle of the Somme in 1916. Source: Imperial War Museum, London.



The Thomas splint used to treat a compound fracture of the femur. Source: C. Max Page and A. B. Le Mesurier. (1918). The early treatment of gunshot fractures of the thigh. *The British Journal of Surgery* 5, 66–99.



Harvey Cushing and his team at a CCS in 1917. Cushing sits in the front row on the left. Source: H. Cushing. (1936). *From a Surgeon's Journal 1915–1918*. Constable, London.



Cushing's technique of suction debridement of a cerebral wound track.

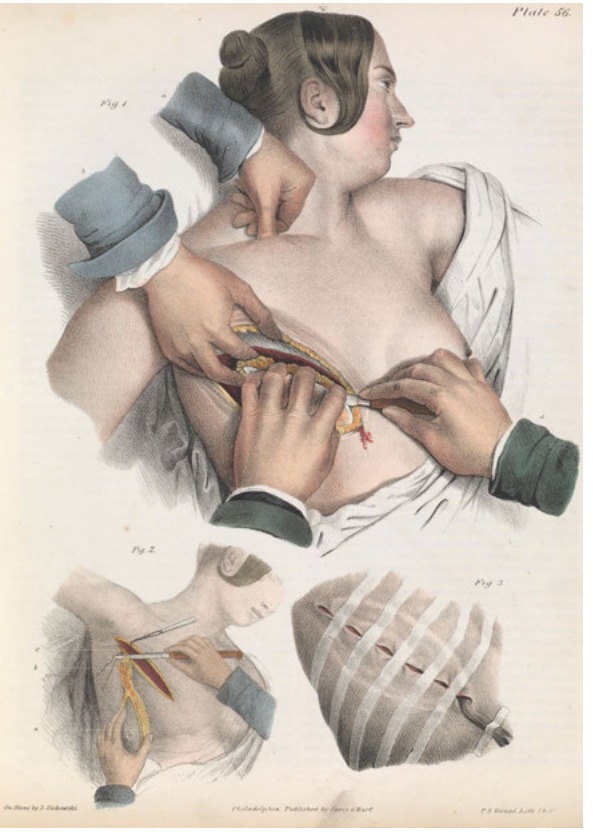
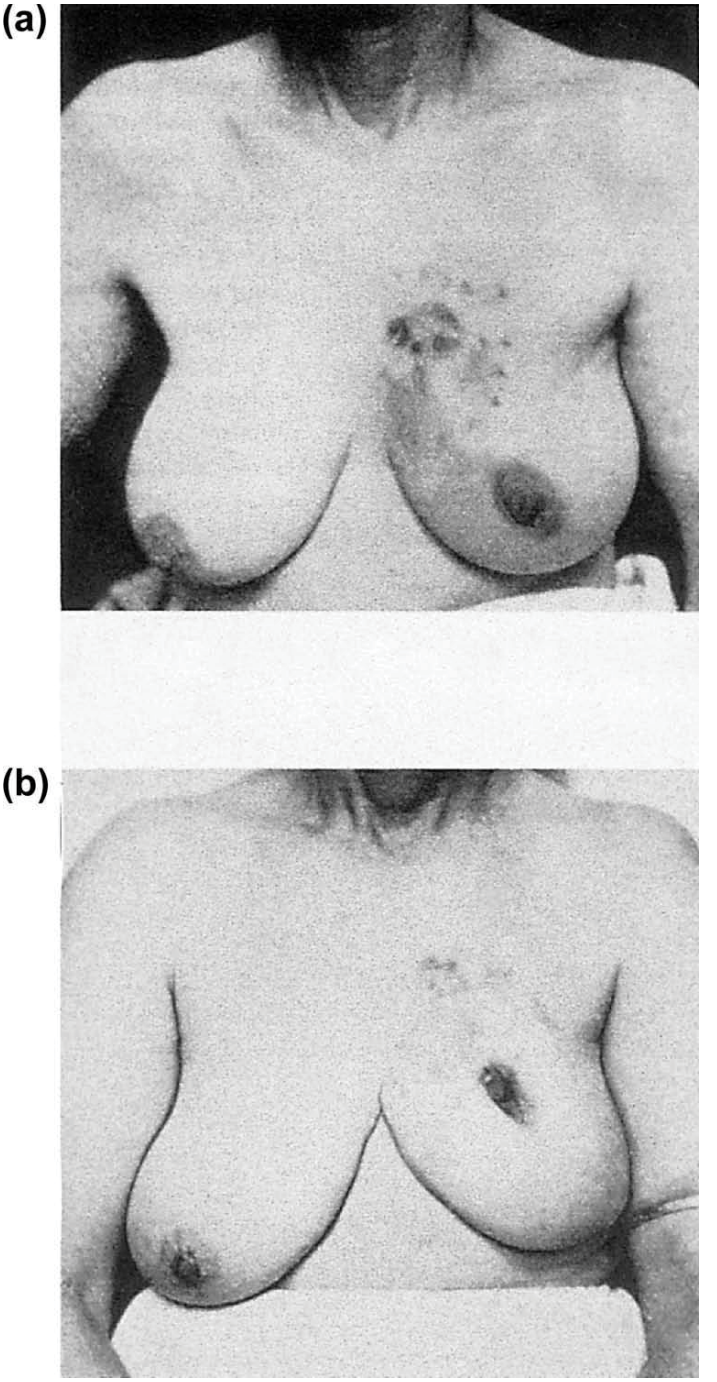


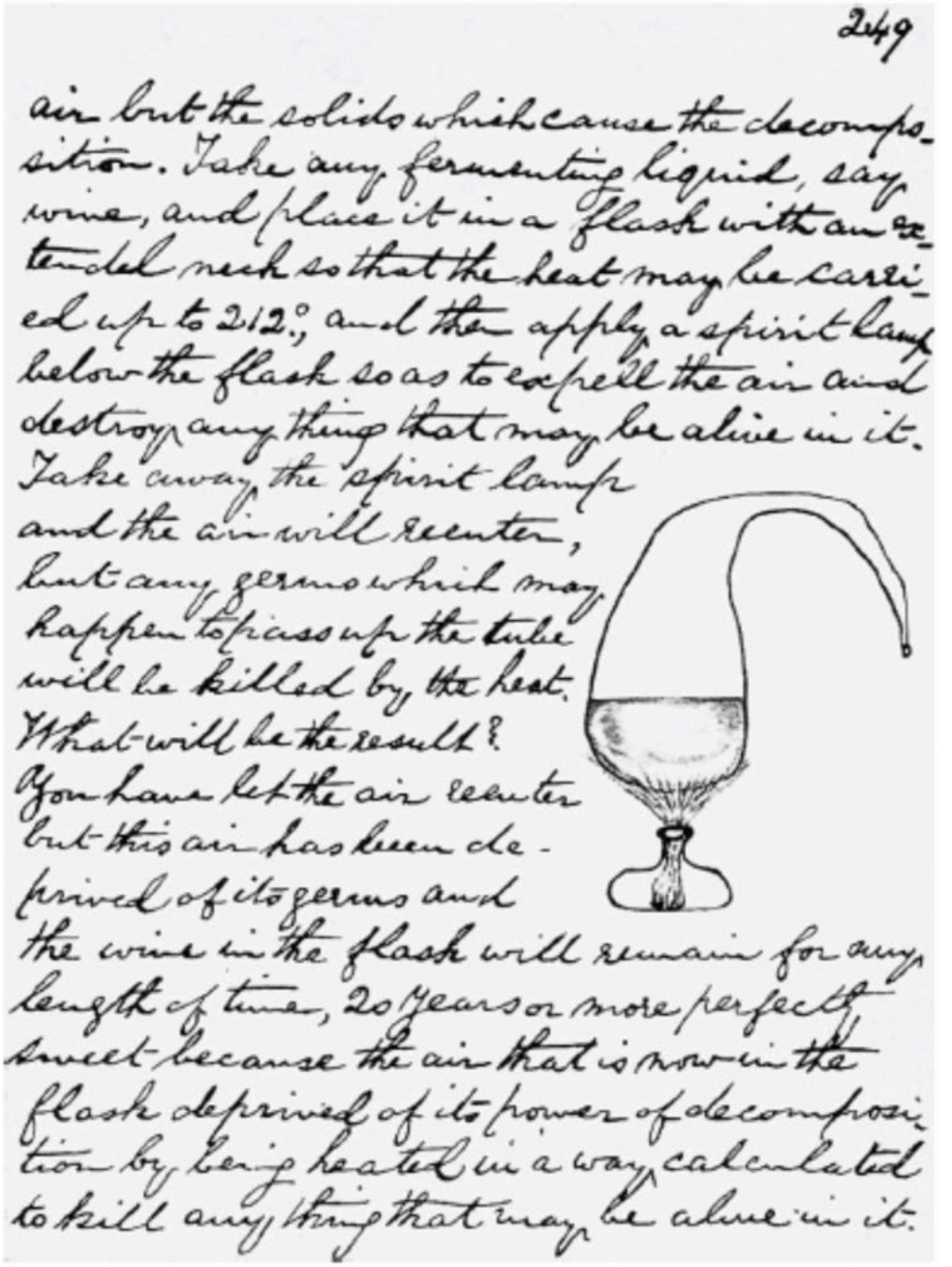
Plate LVI. Surgery for the removal of the mammary gland. Courtesy of the Wellcome Library, London.

An early example of treatment of breast cancer by irradiation, dated 1908. Note that the French radiotherapist wears no protection from the X-rays. Not surprising, many of these early pioneers, including Madame Marie Curie herself, developed serious complications that included skin cancer and aplastic anemia.



Patient treated by Keynes with radium implantation: (a) early result, (b) 7 years after treatment. Source: G. Keynes (1932). The radium treatment of carcinoma of the breast. *British Journal of Surgery* 19(75):415–480.

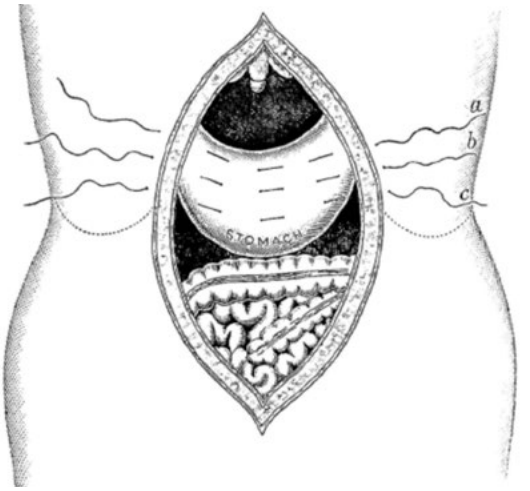
Pasteur's Portrait by Albert Edelfelt is the best-known portrait of the great French chemist Louis Pasteur (1822–1895). Painted in 1885, it shows Pasteur in his laboratory at the rue d'Ulm surrounded by his experimental apparatus, the innovative laboratory glassware used in bacteriology experimental methods, developed by him in the late 19th century. Pasteur is regarded as one of the main founders of bacteriology, and he is popularly known as the "father of microbiology." The painting had a great success at the Paris Salon of 1886, and became one of the most familiar images of the scientist. Courtesy of Wikipedia.



Pasteur's experiment with broth, illustrated in the lecture notes of Anderson, a medical student attending Lister's surgical lectures in Glasgow. Source: D. Guthrie. (1949). *Lord Lister, His life and Doctrine*. Livingstone, Edinburgh.

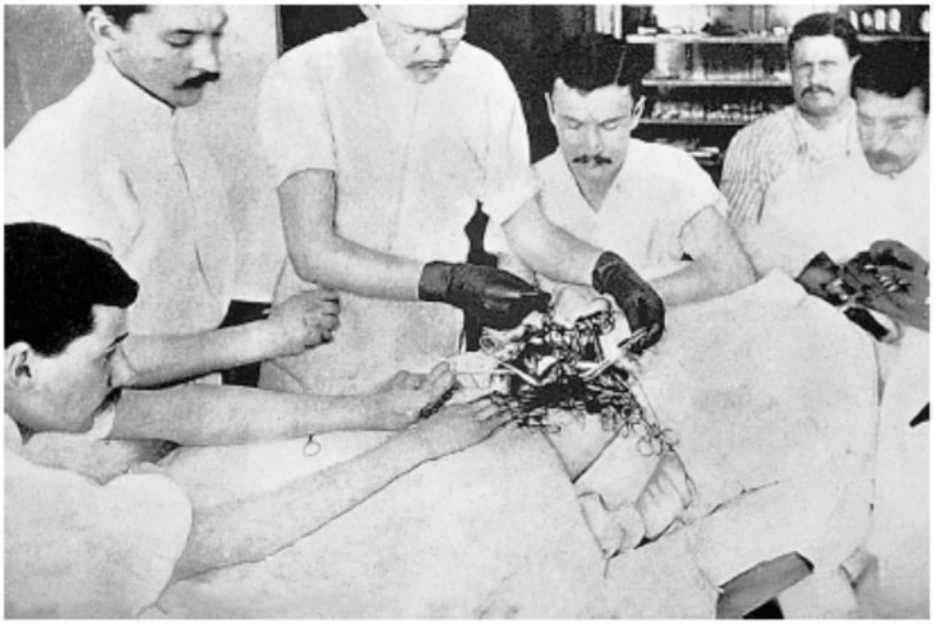
This image entitled "Narayana Thailum or The Gout, Rheumatism and Paralysis Destroyer." Taken from Ayurvedic medicines prepared by Ayurveda Marthanda Bhishangmani Pandit D. Gopalacharlu, A.V.S., at the Madras Ayurvedic Laboratory, Georgetown, Madras, 1909.

Ayurveda (/ˌɑːjʊərˈveɪdə, -ˈviː-/) is an alternative medicine system with historical roots in the Indian subcontinent. The theory and practice of Ayurveda is pseudoscientific. Ayurveda therapies have varied and evolved over more than two millennia. Therapies include herbal medicines, special diets, meditation, yoga, massage, laxatives, enemas, and medical oils. Ayurvedic preparations are typically based on complex herbal compounds, minerals, and metal substances (perhaps under the influence of early Indian alchemy or *rasashastra*). Ancient Ayurveda texts also taught surgical techniques, including rhinoplasty, kidney stone extractions, sutures, and the extraction of foreign objects. The main classical Ayurveda texts begin with accounts of the transmission of medical knowledge from the gods to sages, and then to human physicians. Printed editions of the *Sushruta Samhita* (Sushruta's Compendium), frame the work as the teachings of Dhanvantari, Hindu god of Ayurveda, incarnated as King Divodāsa of Varanasi, to a group of physicians, including Sushruta. The oldest manuscripts of the work, however, omit this frame, ascribing the work directly to King Divodāsa. Through well-understood processes of modernization and globalization, Ayurveda has been adapted for Western consumption, notably by Baba Hari Dass in the 1970s and Maharishi Ayurveda in the 1980s. Historical evidence for Ayurvedic texts, terminology and concepts appears from the middle of the first millennium BC onwards. Text courtesy of Wikipedia. Image courtesy of the Wellcome Library.

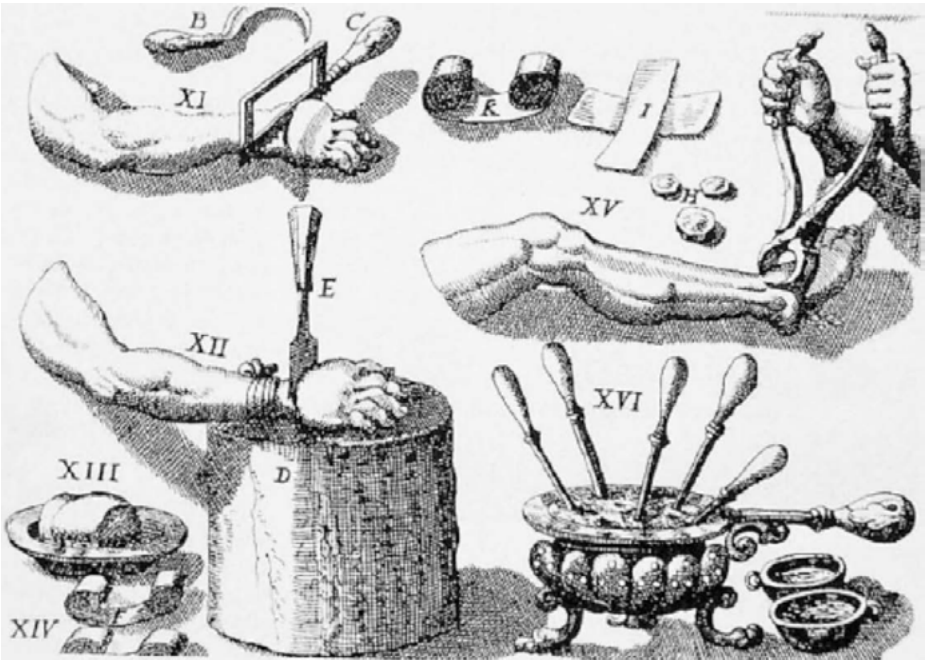


Rovsing's gastropexy for "ptosis of the stomach" is depicted in this image. Source: Thorkild Rovsing (1862–1927), professor of surgery at Copenhagen, Denmark.

A below-knee amputation in the 16th century. Note the patient in the background who has had his left hand amputated. Hans von Gersdorff. (1517). *Feldbuch der Wundartzney*. Strasburg.

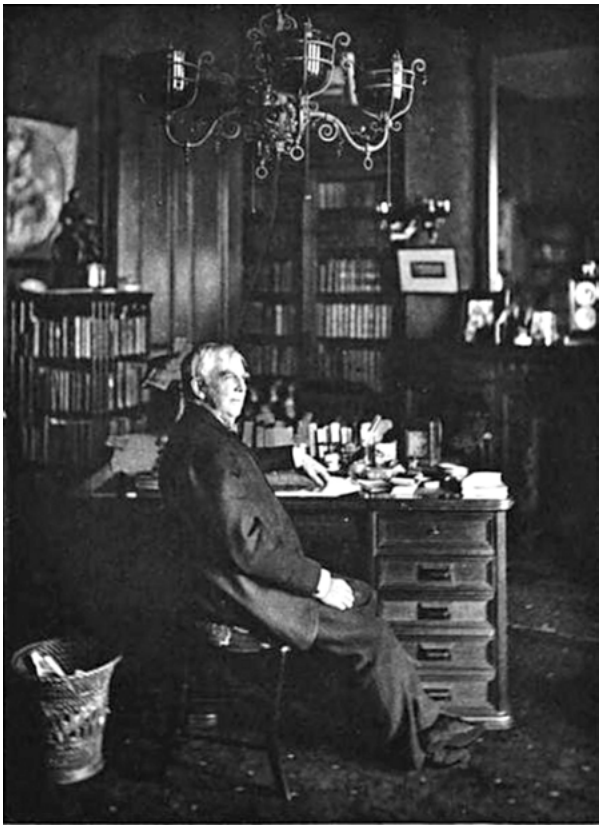


An operation in 1893. Only the surgeon is wearing rubber gloves while his assistants are barehanded.

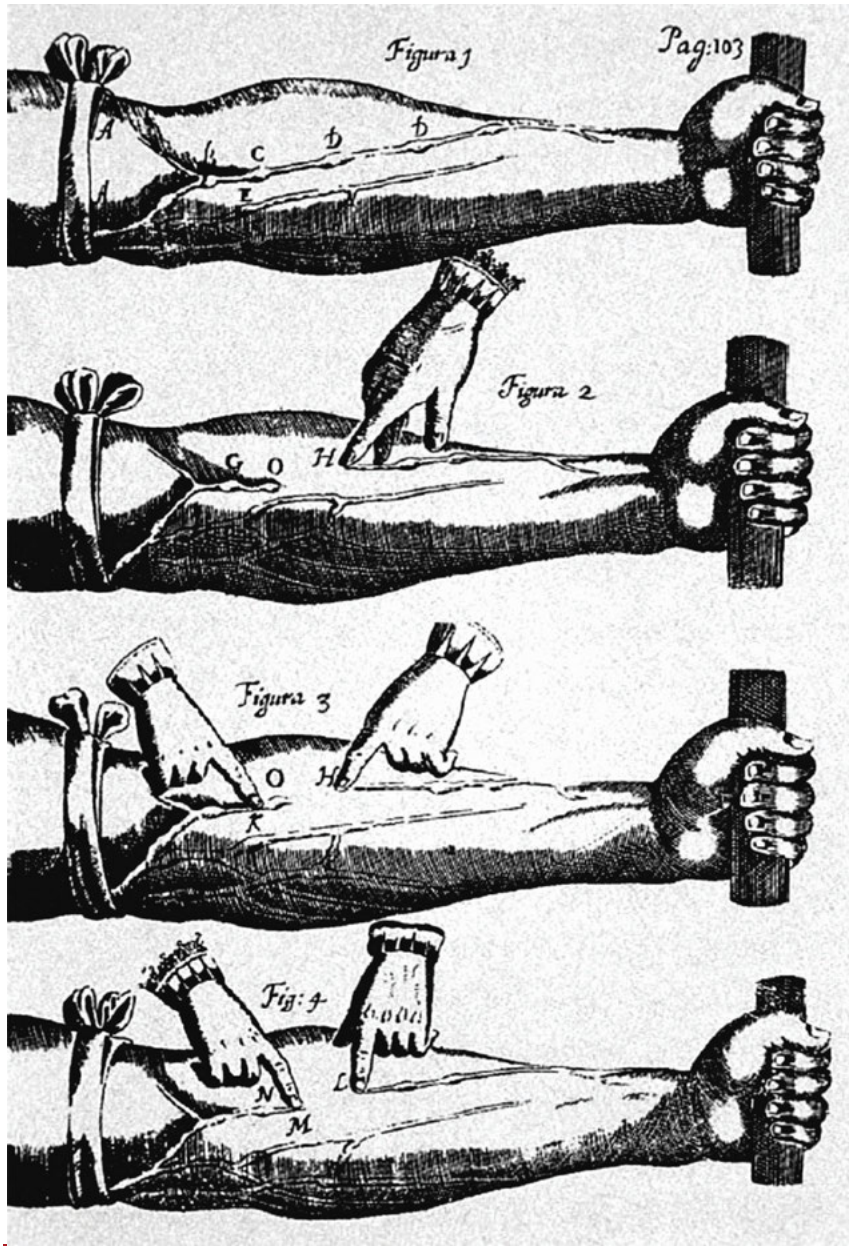


Amputations using a saw and a massive chisel, together with a selection of cauterizing irons. Source: *Armamentarium chirurgicum* by Johanni Scultetus, published in 1655.

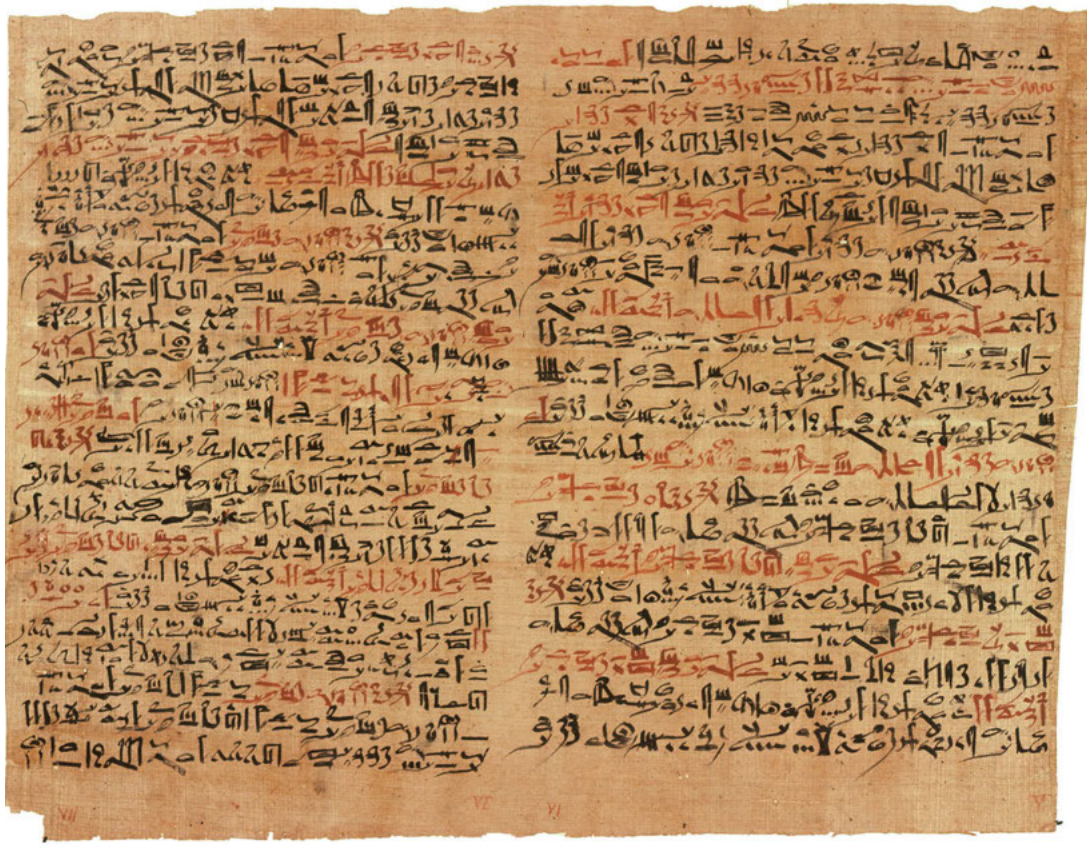
Photograph taken on the occasion of Oliver Wendell Holmes' retirement from Harvard in 1882, and just after the dedication of the new facility on Boylston Street. Holmes is seen to the far left of the photo.



Holmes in the study of his Boston home during his later life. Source: O. W. Holmes. (1904). *Ralph Waldo Emerson, John Lothrop Motley: Two Memoirs.* Houghton Mifflin, Boston and New York.



William Harvey's (1578–1657) ingenious demonstration of the function of the venous valves, using the superficial veins of the arm. He was the first to detail systemic circulation. When a vein was blocked with a tourniquet, it swelled up, the blood unable to escape back towards the heart. The classic image above is from his famous *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (Latin, "An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings"), commonly called *De Motu Cordis*. This landmark in the history of physiology was published in 1628.



The Edwin Smith Papyrus, the world's oldest surviving surgical document. Written in hieratic script in ancient Egypt around 1600 BC, the text describes anatomical observations and the examination, diagnosis, treatment, and prognosis of 48 types of medical problems in exquisite detail. Among the treatments described are closing wounds with sutures, preventing and curing infection with honey and moldy bread, stopping bleeding with raw meat, and immobilization of head and spinal cord injuries. Translated in 1930, the document reveals the sophistication and practicality of ancient Egyptian medicine. Recto Column 6 (right) and 7 (left) of the papyrus, pictured here, discuss facial trauma. (Cases 12–20)

Edward Jenner and two colleagues seeing off three anti-vaccination opponents, with dead smallpox victims littered at their feet. Color etching by Isaac Cruikshank, 1808.

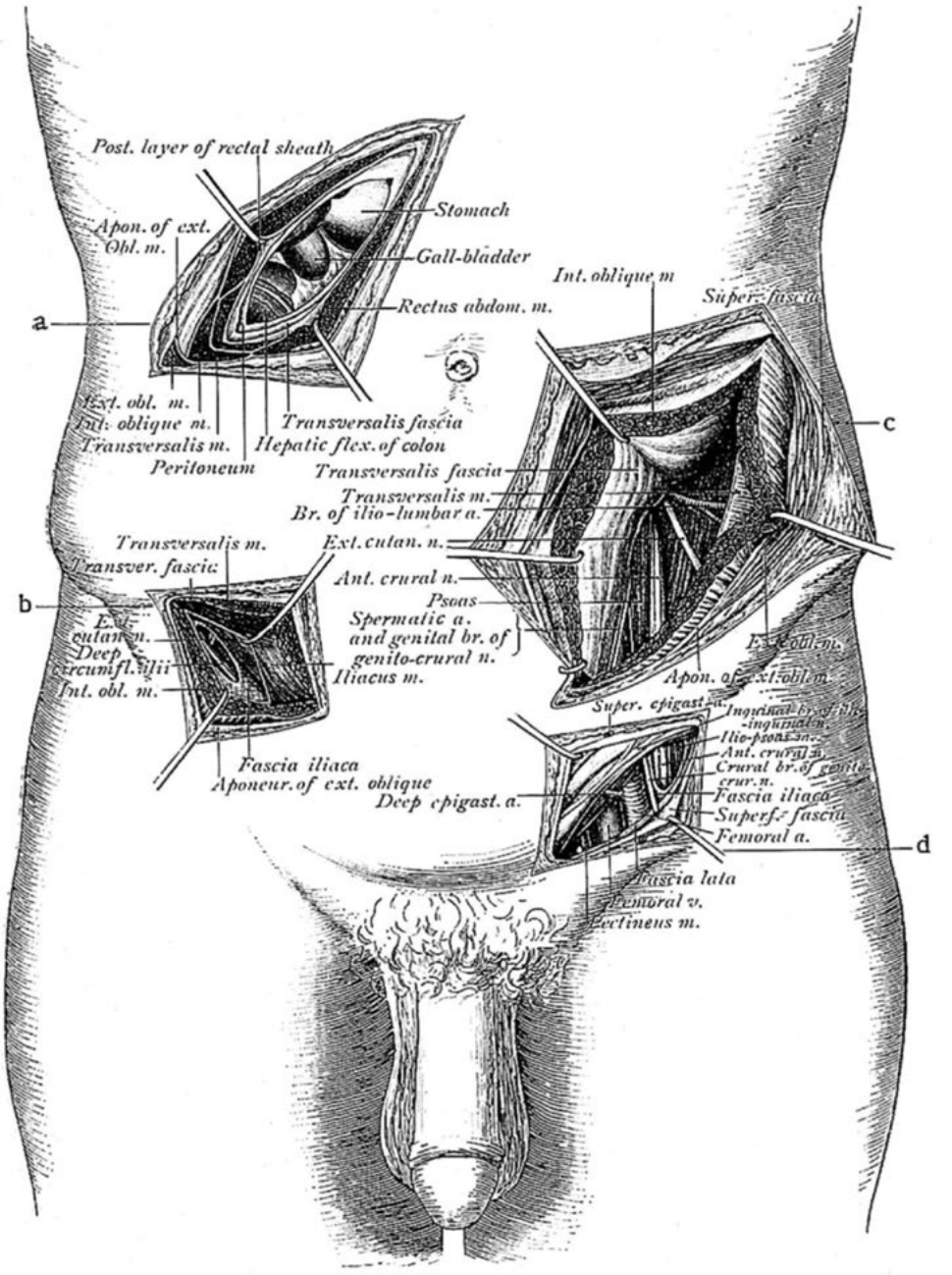
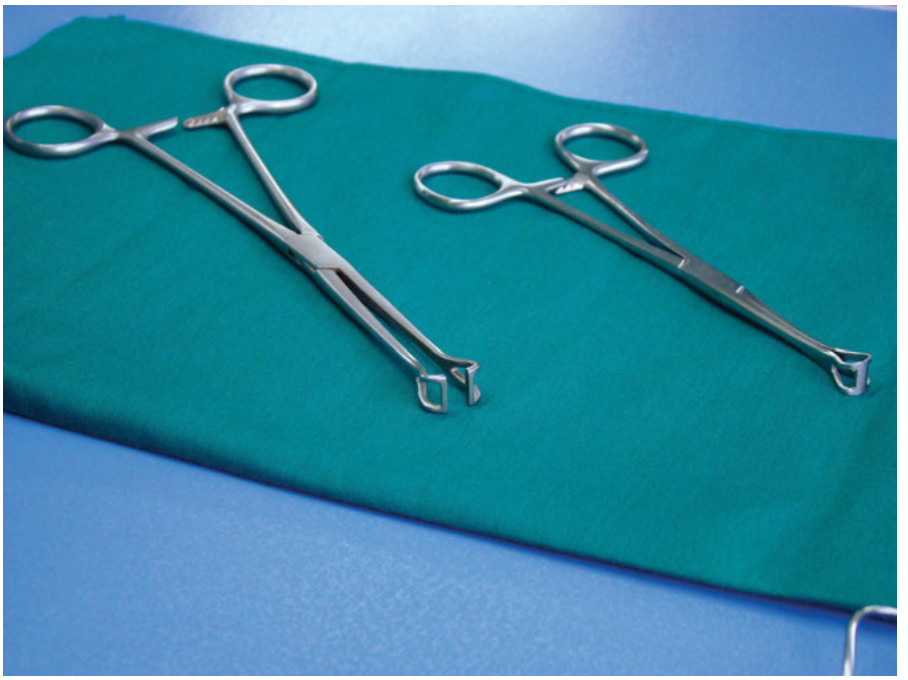
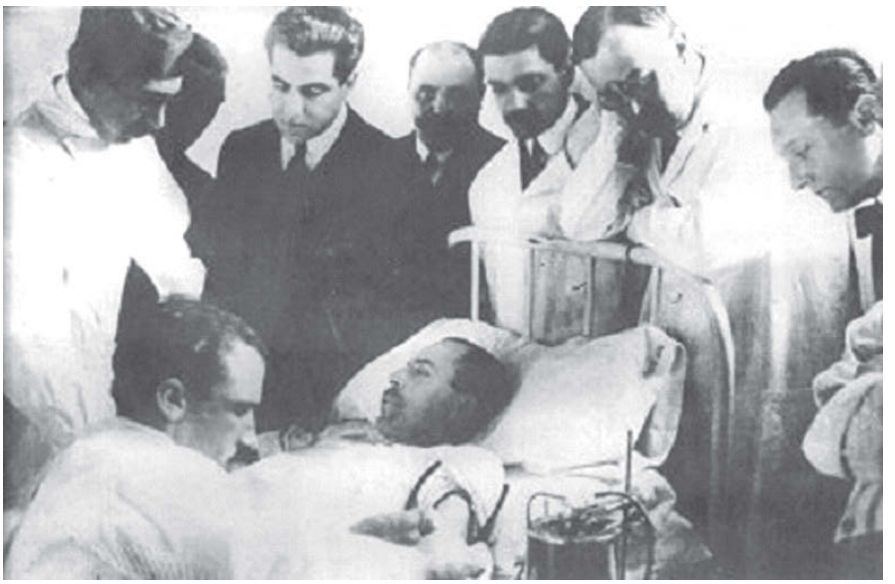


Illustration of various incisions from Kocher's 1903 *Textbook of Operative Surgery*, 2nd edition, Black, London. The uppermost incision is Kocher's incision for gall bladder surgery.



Babcock clamp is a medical tool that is used by doctors for surgery. The tool is more often used in tubal ligation which is the permanent form of birth control. The Babcock clamp holds the fallopian tube so it can be ligated to prevent any pregnancy.

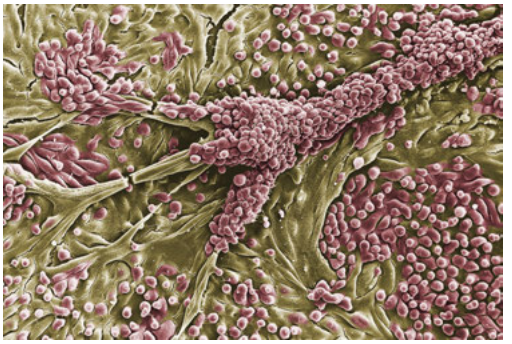


Luis Agote (1868–1954), second from right, overseeing one of the first safe and effective blood transfusions in 1914. He was the first to perform a non-direct blood transfusion using sodium citrate as an anticoagulant.

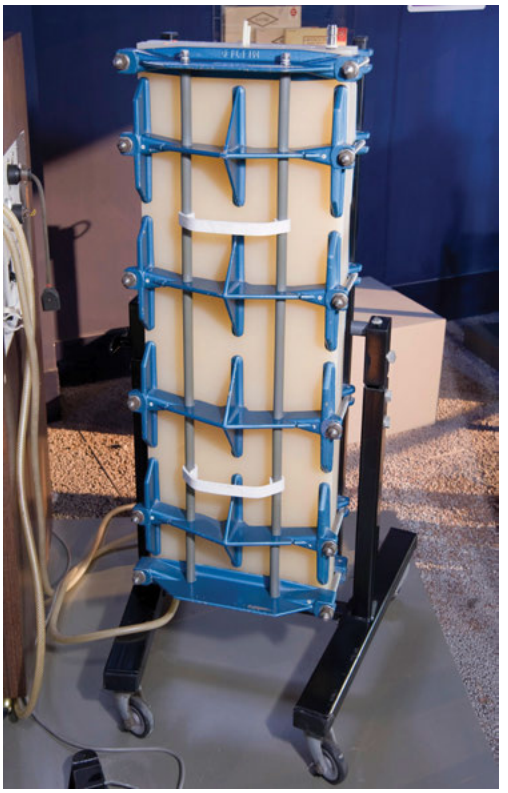
A watercolor, dated 1825, depicting a physician taking pulse in Delhi, India. Courtesy of the Wellcome Library.

Franz Anton Maulbertsch (1724–1796), an Austrian painter and engraver, painted *The Quack* (c. 1785) showing barber surgeons at work. In medieval Europe, surgery was practiced primarily by barbers (as opposed to surgeons) because surgery was viewed more as a craft than a profession at that time. Barbers carried out the "treatment" of bloodletting (bleeding people) as well as tooth extractions, amputations, enemas, selling medicines, and, of course, a shave and haircut, if desired. In fact, the red-and-white pole that still symbolizes a barbershop also symbolized the white napkins and blood-soaked bandages.

A barber-surgeon extracting stones from a woman's head. Note that images of a surgeon (often itinerant) making an incision in a patient's head in order to extract "stones" (implying madness in the individual) do not represent an actual operation, but are allegorical scenes referring to the subduction of "folly" (madness) from the body. Shown above is a watercolor done in 1787 by J. Cats, after B. Maton.



An artificial color SEM image of a human proximal tubule, showing the tubular structure and projections extending over the tissue surface. Kindly provided by David Gregory and Debbie Marshall.



Photograph of the Kiil kidney dialysis apparatus, England, 1980–1982. In 1961, Norwegian Frederik Kiil (1921–2015) introduced a kidney dialysis machine small enough to be used at home. Called the "sandwich," the machine had eight cellophane membranes to filter patients' blood. Larger areas of membranes meant better filtration of the blood. (The first kidney machine actually used sausage skins as membranes.) Patients had to dismantle and sterilize the machine at home. It was produced by Meltec Limited, Buckinghamshire, UK. Image courtesy of the Science Museum, London. Text courtesy of Wikipedia.

Chapter 2

Lymph Nodes—The Neglected Battlefield in Tuberculosis

Sharie Keanne C. Ganchua, PhD,[a] Alexander G. White, PhD,[a] Edwin C. Klein, PhD,[b] and JoAnne L. Flynn, PhD[a,c]

[a]*Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*
[b]*Division of Laboratory Animal Resources, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*
[c]*Center for Vaccine Research, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*

joanne@pitt.edu

Keywords: ^{18}F-fluorodeoxyglucose (FDG), adaptive immune system, alveolar macrophages, Bacille Calmette-Guerin (BCG), bronchoalveolar lavage (BAL), chromosomal equivalents (CEQs), colony forming unit (CFU), culture filtrate protein (CFP), cynomolgus macaques, dendritic cells (DC), DNA barcodes, extrapulmonary tuberculosis, Ghon complex, granulocyte-macrophage colony-stimulating factor (GMCSF), granuloma, hematoxylin–eosin, hilar lymphadenitis, interleukin-10 (IL-10), isoniazid (INH), latent tuberculosis, lymph node (LN), *Mycobacterium bovis, Mycobacterium tuberculosis* (Mtb), nonhuman primates (NHPs), phagocytic cells, positron emission tomography coupled with computed tomography (PET-CT), purified protein derivative (PPD), rifampicin (RIF), simian immunodeficiency virus (SIV), SIV/HIV infection, small granulomas, subclinical TB pathology, T helper type 1 (Th1), T helper type 17 (Th17), thoracic lymph nodes, toll-like receptor (TLR), tuberculin skin test (TST), tuberculosis (TB), tuberculosis lymphadenitis (TBLN), tumor necrosis factor (TNF), Ziehl-Neelsen staining

2.1 Introduction

Tuberculosis (TB) is an ancient disease that has plagued humans for thousands of years [1]. It has claimed millions of lives, killing 1.45 million people in 2018 alone, making it the leading cause of death by a single infectious agent. It is caused by bacteria, *Mycobacterium tuberculosis* (Mtb), which are spread in aerosolized droplets expelled from symptomatic individuals, i.e., those with active TB [2].

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Recent estimates suggest that approximately one-quarter of the world's human population is currently infected with this microbe without symptomatic and microbiological evidence of disease, which is clinically defined as latent TB [3]. Even though TB most commonly manifests as a pulmonary disease, extrapulmonary TB also occurs. In humans, Mtb infection usually results in a Ghon complex—a tuberculous lung lesion accompanied by a granuloma in a thoracic lymph node [4, 5]. Infected lymph nodes are considered to be extrapulmonary, even if they are within the thoracic cavity, and are the most common sites of extrapulmonary Mtb infection [6, 7]. Lymph nodes are niches for Mtb growth and persistence [8]. Early autopsy studies in humans found live Mtb in lymph nodes without signs of TB disease anywhere else in the body [9–11]. Even lymph nodes that appeared normal through gross inspection by a trained pathologist could harbor live Mtb [9]. In cattle, lymph nodes are the most common site of *M. bovis* infection [12]. In a small study of 15 cattle with evidence of bovine TB in lymph nodes, only 1 had identifiable pulmonary infection [13]. However, some authors as cited by Neill and colleagues [12] believe that a more comprehensive inspection of the bovine lungs should be performed since TB lesions can be small. It is widely accepted that, in bovine TB, lymph nodes get infected first while pulmonary lesions develop later during the infection [12, 14]. In our experience working with nonhuman primates (NHPs), lymph nodes are almost always infected with Mtb along with the lungs [8]. Occasionally, we find lymph nodes with no apparent granuloma also harboring live Mtb bacilli. Given these observations, it is understandable that Behr and Waters proposed TB as a lymphatic disease rather than strictly a pulmonary disease [15].

Reviews of human TB lymphadenitis (TBLN) focusing on epidemiology, clinical manifestations, pathology, diagnosis, and treatment have been published [16–20]. Here, we aim to review the pathogenesis of Mtb infection in lymph nodes, drawing on studies from animal models and humans.

2.1.1 From the Air to the Lymph Nodes

Infection begins when Mtb enters the airways in inhaled droplet nuclei expelled from individuals with active TB disease. Poulsen published 2 extensive studies in the 1950s detailing the early events in Mtb infection in 517 tuberculin skin test (TST) converters in the Faroe Islands [21, 22]. At the time that Poulsen conducted his study, this group of islands just north of the United Kingdom had a population of 30,000 living in isolated villages. A version of TST was done routinely on all inhabitants, and detailed medical histories were recorded. He determined that the incubation period—i.e., the time from Mtb exposure to the first clinical sign of infection (e.g., fever, erythema nodosum [reddish nodules of inflammation on the skin], TST conversion, X-ray showing hilar adenopathy or lung abnormalities)—is around 40 days. The first sign of infection was almost always onset of fever [21, 23]. The changes seen in chest radiographs were observed early, often coincident with the initial fever, and these changes consisted

mainly of enlarged and dense hilar shadows. The hila is composed of pulmonary arteries and veins, major bronchi, and lymph nodes. The common causes of enlarged hila are (1) lymphadenopathy and tumors, (2) arterial or venous hypertension, and (3) increase in pulmonary blood flow [24]. Often, these hilar changes remained for 1–2 years before receding. Pulmonary infiltrates were not as common, present only in a little more than one-third of children and less than one-third of adults [22], although the radiograph technology at the time was unlikely to be sufficient to detect small initial lung lesions. Of the 517 TST converters, 333 (64%) showed hilar lymphadenitis, which occurred more in children than in adults (78% of children versus 56% of adults). However, after prolonged observation, only approximately 10% of the TST converters developed clinically defined active TB, indicating that the early events involving lymph nodes and lungs occur in a large percentage of people following infection, even though only a fraction of these will go on to develop active disease.

The involvement of lymph nodes during the first month of Mtb infection is well established in mouse models of TB. After aerosol infection, Mtb is phagocytosed by alveolar macrophages, myeloid dendritic cells (DC) and neutrophils in the lungs [25]. While other respiratory viral and bacterial pathogens induce DC migration to the lymph nodes to activate the adaptive immune system by 1–3 days post infection [26–28], this important process is delayed in Mtb infection. Several studies have shown that Mtb-infected DCs do not migrate to the lymph node and prime T cells until 9–11 days post infection (Fig. 2.1) [29–31]. This delay in the dissemination of Mtb bacteria to the lymph nodes is thought to play a role in the increased susceptibility of C3H/HeJ mice to Mtb compared to C57BL/6 mice [29]. Wolf and colleagues also showed that the migration of DCs was transient, slowing down after peaking at 21 days post infection, an interesting observation given the chronic nature of TB. Not only are DC migratory functions dysregulated, but DCs and interstitial macrophages that transport Mtb to the lymph nodes are relatively poor at stimulating T-cell responses to Mtb antigens [30].

Lymph nodes are a major component of early Mtb infection in guinea pigs aerosolly infected with 20 colony forming units (CFU) [32–34]. One of the earliest (5–15 days post infection) observations in the lungs is inflammation of pulmonary lymphatic vessels [33]. Marked thoracic lymph node enlargement could be seen around 20 days post infection progressing to severe lymphadenopathy at 30 days post infection [32, 34]. Lymph node involvement has also been noted in rabbit models [35–37].

NHPs, particularly cynomolgus macaques, are excellent experimental TB models since they present with the full spectrum of human clinical TB (latent to active TB) when challenged with a low dose (≤25 CFU) and form granulomas identical to those formed in humans [38–46]. Although rhesus macaques are more susceptible and generally develop active TB, their TB pathology also recapitulates that of humans [47–49]. Macaques also have multiple thoracic lymph nodes that presumably drain different sections of the lungs, just like in humans,

as opposed to rodents having only a few lung-draining lymph nodes. NHPs provide an excellent model for studying the involvement of thoracic lymph nodes in Mtb infection.

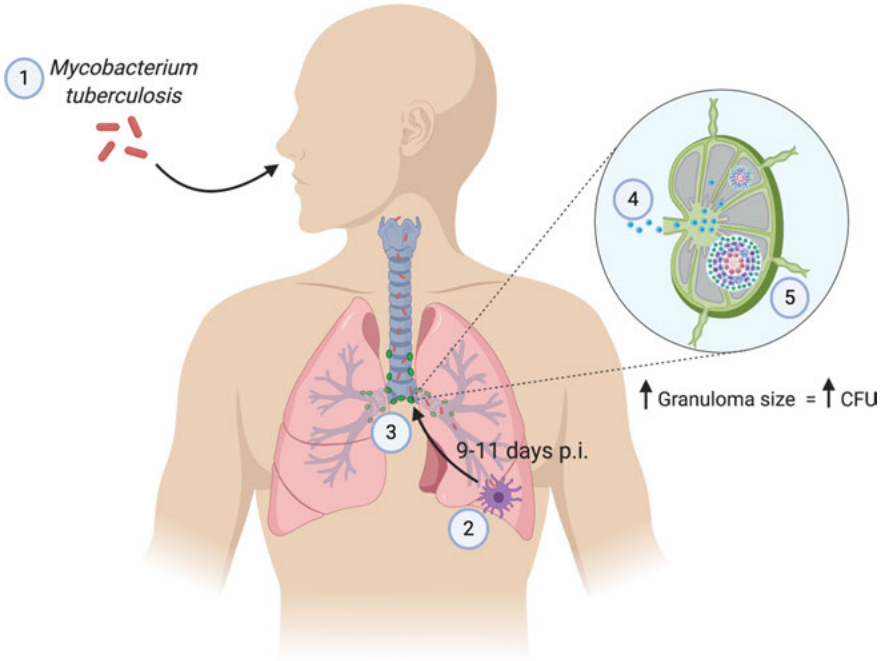

Figure 2.1 Mtb travels to the thoracic lymph nodes from the lungs. (1) Infection begins when a person inhales aerosolized droplets containing Mtb. (2) Mtb travels into the lungs and is taken up by phagocytic cells. (3) Mtb is then transported to a thoracic lymph node on the same side of the body. Steps 2 and 3 take 9–11 days in mice. (4) Mtb-containing phagocytic cells present antigen to naïve lymphocytes and generate an immune response. Activated lymphocytes travel back to the lungs to contain Mtb infection. (5) Live Mtb, either shuttled to the lymph nodes by phagocytic cells or carried by lymph fluid, begins to multiply and cause a granuloma to form. Mtb burden increases as the granuloma size increases. Lymph nodes are generally not able to eliminate the infection. *Abbreviations*: CFU, colony forming units; Mtb, *Mycobacterium tuberculosis*; p.i., post infection.

By performing serial positron emission tomography coupled with computed tomography (PET-CT) scans, we can track Mtb infection in the lungs and lymph nodes of NHPs over the course of infection [47, 50–55]. We used a radiolabeled glucose analog, ^{18}F-fluorodeoxyglucose (FDG), as our PET probe which is taken up and retained by metabolically active cells. FDG activity is a surrogate marker for inflammation in lung granulomas and lymph nodes [47, 50–55]. In contrast to lung granulomas, lymph nodes can be difficult to detect by PET-CT unless they are enlarged or FDG avid (metabolically active) [47, 53]. In macaques, one or more thoracic lymph nodes start to become FDG avid 2–4 weeks post infection, as do the lung granulomas [8, 52, 53]. These FDG-avid thoracic lymph nodes could

reflect immune cell activation or proliferation in response to priming, as well as an active site of Mtb infection. Combining PET-CT data with quantitative bacterial burden assessments in NHP, we reported that when thoracic lymph nodes were "hot" (SUVR, or maximum standard uptake ratio normalized to muscle ≥ 5), 96.3% contained culturable Mtb bacilli; however, only 50% of "warm" thoracic lymph nodes (SUVR ≥ 2.3 but < 5) had live Mtb. Interestingly, 40 of 240 lymph nodes that were not detectable by PET (SUVR < 2.3) also had culturable Mtb [8, 52]. In a previous study examining the early events of Mtb infection in cynomolgus macaques, granulomas assessed grossly were observed to form first in the thoracic lymph nodes before being detectable in the lungs [39]. Macaques euthanized at 3 weeks post infection had bilaterally enlarged hilar lymph nodes; however, no gross nor microscopic granuloma were seen in the thoracic lymph nodes or lungs of these animals. Macaques euthanized at 4 weeks post infection had bilaterally enlarged hilar lymph nodes, but only 1 of 2 macaques had a small granuloma grossly visible in the enlarged hilar lymph node. At this time point, multiple thoracic lymph nodes showed evidence of infection ranging from early (i.e., aggregates of epithelioid macrophages, sometimes with multinucleated giant cells) to more advanced (i.e., multifocal and coalescing areas of inflammation with central necrosis). One macaque had 1 granuloma in the lungs that was seen microscopically. By 5 to 6 weeks post infection, macaques exhibited greater pathology in both thoracic lymph nodes and lungs. Caseous granulomas were visible in both lungs and thoracic lymph nodes by gross assessment. T-cell responses in thoracic lymph nodes to mycobacterial proteins (culture filtrate protein [CFP]) can be detected by ELISpot at 3–4 weeks post infection, and this generally preceded responses in the blood and lungs. These observations suggest that pathology may progress from the thoracic lymph nodes to the lungs, and this coincides with the adaptive immune response being activated in the lymph nodes first before trafficking to the lungs [39]. However, it should be noted that this early study was done without the benefit of PET-CT imaging, and it is very possible that early small granulomas in lungs were missed during necropsy.

Once Mtb enters the airways and the lungs, only a fraction (9/98, 9.2%) gets transported to one or more thoracic lymph nodes and successfully infects them. By using DNA barcoded Mtb strains that allow discrimination of individual bacteria, Martin and colleagues [56] tracked infection dynamics in lungs and thoracic lymph nodes of cynomolgus macaques. While the majority of lung granulomas were formed by a single bacterium (only 1 barcode was found per granuloma), most lymph nodes were infected with multiple bacteria (≥2 barcodes). However, only a fraction (9/98, 9.2%) of the barcodes found in lung granulomas were also identified in culturable bacteria from lymph nodes. This suggests that after replicating in the lungs, either not all of Mtb that seeded granulomas were also able to disseminate to the lymph nodes and successfully replicate there, or they were transported to the lymph nodes but did not establish a productive infection.

2.1.2 Bacterial Dynamics in the Lymph Nodes

To the authors' knowledge, there is currently limited existing comprehensive analysis of Mtb bacterial dynamics in lymph nodes of small animal models. The reports indicate that Mtb gets shuttled into the lymph nodes mostly by DCs and interstitial macrophages around 9–11 days post infection [29–31], but since most of these studies were focused on the early events of T-cell priming, mice were euthanized at around day 28, and the fate of Mtb in these lymph nodes during long-term infection is unknown. In these T-cell priming studies, mice were infected at varying doses (<15 to 555 CFU), and the peak CFU was detected around days 14–21 post infection at approximately 10^5 to 10^6 numbers [25, 29–31, 57]. The degree and timing of peak CFU was correlated to the magnitude of the inoculation dose. A longer-term study on the effect of Bacille Calmette-Guerin (BCG) on Mtb burden in various tissues found that Mtb inoculated into the ear reaches peak CFU in ear-draining lymph nodes around 28 days post infection in unvaccinated C57BL/6 mice and remains relatively constant at 10^3 CFU until 120 days post infection [58]. Another study that followed Mtb infection in resistant (B10.MBR) and susceptible (B10.SM) strains of mice showed a rapid increase of Mtb to approximately 10^4 CFU in the thoracic lymph node 3 weeks post infection, which increased to 10^4–10^5 CFU by 10 weeks post infection [59]. In guinea pigs, Mtb can be cultured from lymph nodes in very low numbers (approximately 100 CFU) as early as 5 days post infection, reaching a peak at 20 days post infection (10^6 CFU) before decreasing and stabilizing by 60 days at 10^5 CFU [60]. These studies suggest that in mice and guinea pigs, once Mtb enters the lymph node, the host is unable to eliminate it, making the lymph node a bacterial reservoir.

Studying Mtb bacterial dynamics in human lymph nodes is extremely difficult since the timing of Mtb infection is usually unknown and thoracic lymph node biopsies are invasive. However, NHP models provide an opportunity to dissect the dynamics of lung and lymph node infections since infection timing and dose are known, and necropsies can be performed at various time points post infection. The 2 species of macaques most commonly used to model TB respond differently to Mtb infection. Following low dose infection, about half the cynomolgus macaques develop active TB disease, and the other half develop latent Mtb infection (defined as no clinical signs of disease and negative Mtb cultures from bronchoalveolar lavage (BAL) and gastric aspirate over 6 months) [40]. Rhesus macaques, on the other hand, are more susceptible to Mtb infection, always developing active TB disease when infected with a fully virulent strain of Mtb [47]. One difference between the 2 species is how their lymph nodes respond to Mtb infection. Rhesus macaque lymph nodes present with more extensive pathology and greater increases in size such that the lymph nodes can impinge on the airways, sometimes leading to lobe collapse. A recent publication from our group showed that live Mtb burden reaches its peak at 4–6 weeks post infection in both cynomolgus and rhesus macaques [8]. In cynomolgus macaques, this Mtb burden is reduced 100-fold by 11–14 weeks post infection and remains

constant until 16–29 weeks. In cynomolgus macaques that had latent Mtb infection (34–54 weeks post infection), one or a few lymph nodes had Mtb bacilli, and the bacterial numbers were significantly fewer compared to earlier time points post infection. In contrast, although rhesus macaque lymph nodes had approximately 10-fold-lower Mtb burden at 4 weeks post infection compared to cynomolgus macaques, this level of bacteria is maintained until 16–28 weeks post infection. We determined the chromosomal equivalents (CEQs; Mtb genomes quantified by quantitative PCR [qPCR]) in each lymph node as an approximation of the total number of bacteria (counting both live and dead Mtb) and found equivalent numbers in both macaque species at nearly all time points post infection. The ratio of live Mtb (CFU) to CEQ can be used to determine the killing capacity of each lymph node [61]. For both macaque species, there was minimal Mtb killing in the lymph nodes at 4–6 weeks post infection; however, cynomolgus macaque lymph nodes were able to kill at least a portion of Mtb at later time points post infection. The killing capacity of rhesus macaque lymph nodes never improved even at 16–28 weeks post infection. Thus, Mtb grew to the same level in the lymph nodes of both macaque species; however, rhesus macaque lymph nodes were not successful at killing Mtb, contributing to the more severe disease in the lymph nodes of these animals. We also found Mtb DNA in peripheral lymph nodes (axillary and inguinal) which do not drain the lungs. Since most of these lymph nodes were sterile and did not have granulomas apparent by histopathology, it seems that they have a high capacity for killing Mtb. However, trafficking of dead Mtb or Mtb genomes to these lymph nodes is also possible. In general, lymph nodes are poor killers of Mtb compared to lung granulomas [8, 61].

2.1.3 Immune Response of Lymph Nodes to Mtb

Once Mtb has reached the lymph nodes and an adaptive immune response is generated, the lymph node needs to contain or kill the growing number of Mtb bacteria inside it. Otherwise, the lymph node can be destroyed by necrosis. The primary mechanism to achieve bacterial killing is likely through the production of cytokines, chemokines, cytolytic and other effector molecules by cells in the lymph nodes [62, 63]. Although our study showed limited to no killing in the majority of thoracic lymph nodes in cynomolgus and rhesus macaques, we still found sterile lymph nodes with granulomas, albeit small in number (16 out of 200 [8%] lymph nodes with granulomas by microscopic histopathology were sterile). Thus, some lymph nodes are successful in killing Mtb; however, this is a rare occurrence. Comparing successful and unsuccessful immune response of lymph nodes may provide clues to immune control of TB.

Human studies investigating the immune response in Mtb-infected lymph nodes compared biopsied cervical lymph nodes of patients with TBLN with either healthy controls, patients with only pulmonary TB, and patients with other lymph node disease (e.g., cancer, non–TB-specific reactive lymphadenitis)

[64–67]. All studies obtained transcription profiles, but only one study examined protein levels. Moreover, only one study obtained data on bacterial burden in the lymph node samples from the TBLN patients. With these caveats in mind, we can only view these findings as the lymph node's response to Mtb infection without knowing whether that response was successful in killing the bacteria. In general, unstimulated cervical lymph nodes from patients with TBLN exhibited up-regulated transcripts related to viral defense, inflammatory response, toll-like receptor (TLR) signaling, tumor necrosis factor (TNF) signaling, and Th1-associated pathways compared to lymph nodes from healthy controls or patients with pulmonary TB only, TB meningitis, or lymph node cancer [64, 65]. Down-regulation of Th2 pathways was also observed [65]. When stimulated with Mtb-specific antigens, CFU+ lymph nodes had higher interleukin-10 (IL-10), T helper type 1 (Th1), T helper type 17 (Th17), and granulocyte-macrophage colony-stimulating factor (GMCSF) protein levels compared to CFU-lymph nodes from TBLN patients. No difference in levels of Th2 cytokines (IL-4, IL-5, and IL-13), IL-1β, or IL-18 was observed [66]. In contrast to other studies, Rahman and colleagues [67] showed that lymph nodes from children with TBLN had lower interferon alpha (IFNα), TNF, and IL-17 expression compared to non–TB-specific reactive lymph nodes and healthy tonsil controls. However, forkhead box protein 3 (Foxp3), transforming growth factor beta (TGFβ), and IL-13 mRNA were increased in lymph nodes from TBLN children. No changes in IL-4 or IL-10 were detected. Different experimental measures (mRNA versus protein), technique (microarray versus qPCR), samples and controls (TBLN patients versus variety of samples used as controls), and patients (adults versus children) could all contribute to variability in findings. The aggregate data support that lymph nodes respond to Mtb infection in a variety of ways, but whether these responses promote growth or killing of Mtb is unknown.

We examined Th1 (IFN-γ, TNF, IL-2), Th17 (IL-17), and IL-10 cytokine expression from T cells, B cells, and CD11b+ cells in thoracic lymph nodes of 24 cynomolgus macaques in response to Mtb antigen (6 kDa early secretory antigenic target [ESAT-6] and CFP-10) stimulation [8]. Uninfected lymph nodes (no granuloma by gross inspection or histopathology and no live Mtb) had higher proportions of CD3+ T cells than lymph nodes with granulomas. This is likely due to the destruction of lymph node architecture by granulomas. When compared to lymph nodes with live Mtb (CFU+), lymph nodes that were able to clear Mtb (with granulomas but CFU negative) had a significantly higher proportion of CD11b+ cells producing IL-10. On the other hand, CFU positive lymph nodes had a higher proportion of CD4+ T cells producing TNF. A significant negative correlation was found between IL-10-producing CD11b+ cells and bacterial burden, while a weak but significant positive correlation was found between CD4+ T cells producing TNF and bacterial burden. These data suggest that bacterial clearance is associated with CD11b+ macrophages producing IL-10 while TNF-producing CD4 T cells is associated with Mtb replication. The presence of IL-10

can be beneficial to lymph nodes as a balance of pro-inflammatory and anti-inflammatory signals and is associated with bacterial clearance in lung granulomas [68]. Neutralizing IL-10 in cynomolgus macaques also resulted in higher Mtb burden in lymph nodes at 4 weeks post infection [69].

2.1.4 Mtb Remodels Lymph Node Structure

Lymph nodes are organs whose function is tightly linked to their architecture. Different types of cells have predetermined spaces they call "home" (e.g., B cells in follicles, T cells in paracortex, macrophages distributed throughout the cortex, subcapsular sinus, and medullary region) (Fig. 2.2A). Antigen-presenting cells interact with T and B cells in set locations, and this facilitates initiation of the adaptive immune response [70–74]. Mtb infection of lymph nodes result in formation of granulomas, either in separate foci or coalescing, that destroy the lymph node's architecture (Fig. 2.2B–2.2D). We provided evidence that in lymph nodes where ≥50% of the area is occupied by a granuloma or coalescing granulomas, Mtb burden is higher compared to lymph nodes with granuloma(s) occupying <50%. This is true whether we examined live Mtb burden (CFU) or total Mtb burden (live and dead Mtb; CEQ). Minimal killing (CFU/CEQ) was observed irrespective of extent of granuloma involvement. Even a small granuloma composed of clusters of macrophages can push T cells out of their normal spatial arrangement, impinge on germinal centers, and disrupt the normal vasculature in these organs [8]. Similar disruption of lymph node architecture has been shown in humans with TBLN [67]. Granulomas that form in lymph nodes are structurally distinct from granulomas that form in the lungs. We showed that even though lymph node granulomas form in the T-cell and B cell regions of the lymph node, they lack B cell–rich tertiary lymphoid structures that form in the periphery of a lung granuloma. Distinct lymphocyte cuff regions found in lung granulomas are also negligible in lymph node granulomas. These observations suggest that the structural and compositional differences between lymph node and lung granulomas could be related to the poor Mtb killing potential of lymph nodes [8].

We compared the cytokine response (Th1, Th17, IL-10) and proliferation (Ki67) of CD4+ and CD8+ T cells to Mtb-specific antigens in lymph nodes without granulomas, with <50% granuloma involvement, and with >50% involvement. There was no difference in any of the cytokines or Ki67 measured among all groups, suggesting that the size of the granuloma inside a lymph node does not affect the overall function of the lymph node T cells [8]. Using immunohistochemistry, lymph nodes of human patients with TBLN also displayed extensive remodeling and enrichment of macrophages and DCs, with relatively stable T-cell proportions, while the number of B cells was reduced compared to patients with non–TB-specific reactive lymph nodes [67].

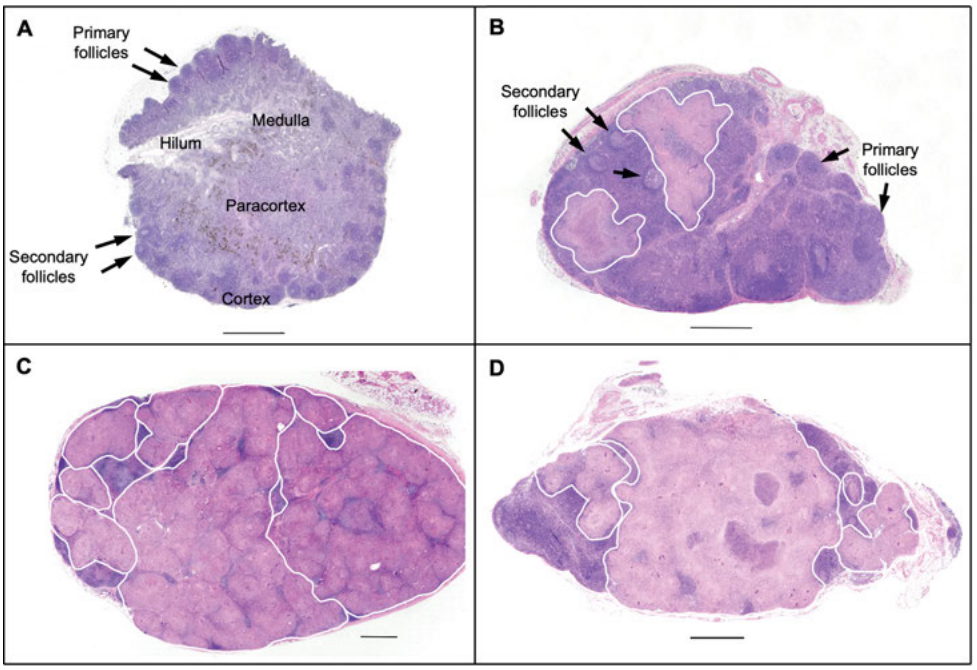

Figure 2.2 Mtb infection results in granuloma formation that disrupts the normal lymph node architecture. (A) Lymph node showing normal architecture without granuloma formation. (B) Lymph node with partially effacing granulomas. (C) Total nodal effacement by multiple coalescing non-necrotizing granulomas. (D) Near total nodal effacement by multiple coalescing caseous granulomas. Granulomas are outlined with a white line. Measuring bar = 1 mm. *Abbreviation*: Mtb, *Mycobacterium tuberculosis*.

2.1.5 Mtb Disseminates Ipsilaterally from Lungs to Lymph Nodes

To determine the pattern of Mtb dissemination from lungs to lymph nodes, we examined whether macaques that formed lung granulomas in the right lung lobes had live Mtb in thoracic lymph nodes on the right side (ipsilateral), left side (contralateral), or both sides (bilateral) of the airways. We assessed the presence of granulomas in each lung lobe and bacterial burden in each lymph node obtained during necropsy from 74 cynomolgus macaques 10–55 weeks post infection. The majority of macaques that formed granulomas on one side of the lungs (approximately 75%) had CFU+ lymph nodes on the same side of the airways (Fig. 2.3). Most macaques that had granulomas on both sides of the lungs also had CFU+ lymph nodes on both sides of the airways (Fig. 2.3). Only a small proportion (approximately 20%) of macaques that had granulomas in only one side of the lungs had bilateral lymph involvement. This suggests that Mtb primarily travels ipsilaterally (same side) from the lungs to the lymph nodes. Variability in draining of the lungs by lymph nodes, airway involvement, and lymph node to lymph node spread could explain the bilateral lymph node involvement in macaques with unilateral lung granulomas.

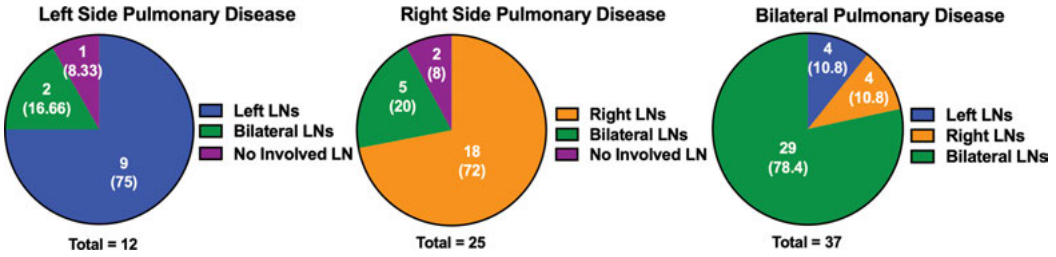

Figure 2.3 Mtb is spread ipsilaterally from the lungs to the thoracic lymph nodes in macaques. Cynomolgus macaques infected with low dose Mtb necropsied from 10 to 55 weeks post infection were assessed for lung disease by gross pathology during necropsy. Lymph node involvement was determined by quantitative culture for Mtb. The pie charts show the lymph node counts. The proportion is in parenthesis. *N* = 74 monkeys. *Abbreviations*: LN, lymph node; Mtb, *Mycobacterium tuberculosis*.

These NHP data are consistent with human data from an early study on 283 autopsies of children [4] and is consistent with the anatomy of the lymphatic system draining the lungs. In general, the right lung drains to the lymph nodes on the right side, and the left lung drains to the lymph nodes on the left, with the exception of the left lower lobe, which might cross over to the right via the lower tracheobronchial lymph nodes [75, 76].

2.1.6 Lymph Nodes Are Sites of TB Reactivation

Since true latency and reactivation models are either nonexistent or not well studied in small animal models, especially in relation to lymph nodes, we did not include them in this section. Based on human and macaque studies, lymph nodes can play a major role in reactivation of latent TB caused by immunosuppression. In NHPs, we define reactivation TB as a positive culture in BAL and/or gastric aspirate, increase in erythrocyte sedimentation rate, signs of disease such as coughing or weight loss, or the formation of a new granuloma by PET-CT after latent Mtb infection was established [51, 77–80]. In CD4 T-cell–depleted cynomolgus macaques, lower CD4+ T-cell levels in hilar lymph nodes was associated with reactivation [80]. In TNF-neutralized macaques, early signs of reactivation (i.e., non-necrotizing granuloma formation adjacent to established and often mineralized granulomas) were observed microscopically in the lymph nodes [78]. Latently Mtb-infected macaques with a high risk of reactivating after TNF neutralization had a smaller proportion of sterile thoracic lymph nodes, highly metabolically active (by PET-CT) lymph nodes, and increased live Mtb burden in lymph nodes compared to low-risk animals [51]. In a separate study [79], DNA barcoded Mtb bacteria, which allows for the discrimination of individual bacteria, was used to track Mtb dissemination during reactivation of latent TB (latent TB defined in our laboratory as animals with no clinical signs or culturable BAL or gastric aspirate and normal erythrocyte sedimentation rate up to 6 months post-Mtb infection [38, 39, 78]) in cynomolgus macaques induced by

simian immunodeficiency virus (SIV) co-infection. New lung granulomas that arose during reactivation were assessed for DNA barcodes and compared to the DNA barcoded bacilli found in old granulomas (those present prior to SIV infection) or in thoracic lymph nodes. Almost 50% of the DNA barcodes in new granulomas matched DNA barcodes from bacteria only found in lymph nodes and not in the old granulomas. Moreover, Mtb recovered from extrapulmonary sites (e.g., liver and spleen) had the same barcodes as Mtb from the lymph nodes. This suggests that Mtb dissemination during reactivation can originate from the lymph nodes dispersing to the lungs and other organs (Fig. 2.4). In antiretroviral-naïve humans with latent TB co-infected with HIV, abnormal FDG uptake in lymph nodes was associated with reactivation. Ten participants determined to have subclinical TB pathology were more likely to develop abnormal uptake of FDG in thoracic lymph nodes compared to participants without subclinical TB disease. Participants with subclinical TB pathology were also significantly more likely to develop active TB disease (4/10) during the 6-month follow-up period compared with the 25 participants with no subclinical pathology of which none developed active TB disease [81]. These data suggest that reactivation of latent TB—whether by SIV/HIV infection, CD4 T-cell depletion, or TNF neutralization—can start in the lymph nodes and can be predicted by visualizing the metabolic activity of lymph nodes by PET-CT.

2.1.7 Lymph Nodes Influence Effectiveness of BCG Vaccin

BCG is a live attenuated *M. bovis* strain and the only licensed vaccine for TB. It is effective at protecting infants and children against the more serious forms of the disease such as miliary disease or TB meningitis but variable in efficacy in protecting against pulmonary TB in adults [82]. For a vaccine to successfully elicit an immune response, it is required to reach secondary lymphoid organs such as the lymph nodes. A study in C57BL/6 mice compared the efficacy of 3 BCG vaccination routes (intradermal [ID], subcutaneous [s.c.], and intralymphatic injection) in eliciting a robust immune response and protection from Mtb challenge [83]. Direct injection of BCG to the inguinal lymph nodes resulted in tremendous transient swelling of not just the injected lymph nodes but all the other lymph nodes as well (e.g., mesenteric, axillary, brachial, thoracic, and cervical nodes). This is in contrast to s.c. and ID vaccination, which caused minimal swelling of any of the lymph nodes examined. Lymph nodes from intralymphatically vaccinated mice harbored greater numbers of BCG by Ziehl-Neelsen staining compared to s.c.-vaccinated animals. Intralymphatic vaccination also elicited a more robust immune response compared to s.c.-vaccinated animals. Significantly more proliferation and stronger TNF, IL-2, IL-17, and IFNγ responses up to 40 days post vaccination were observed in purified protein derivative (PPD)-stimulated splenocytes from intralymphatic-vaccinated animals compared to s.c.-vaccinated animals. Direct vaccination of lymph nodes also resulted in significantly reduced Mtb burden (up to 12 weeks post infection) against Mtb challenge in the lungs

and spleen compared to s.c. and unvaccinated control mice [83]. These data suggest that direct vaccination of lymph nodes could improve the efficacy of BCG in eliciting an immune response and protection against Mtb challenge.

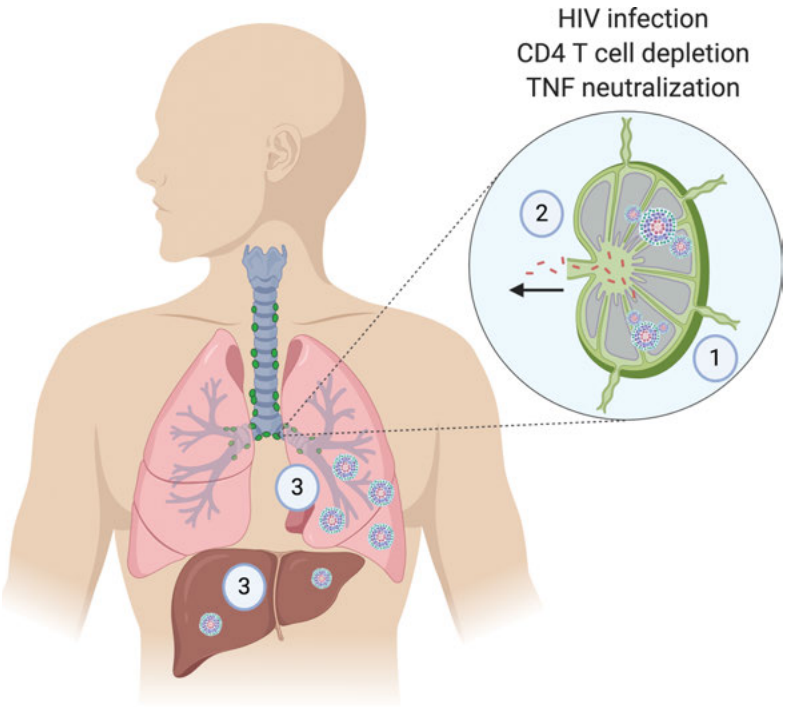

Figure 2.4 Latent TB reactivation can start in the lymph nodes. Mtb resides undetected in lymph nodes during latent Mtb infection or as a result of inadequate drug concentration in lymph nodes during treatment. (1) After latent TB reactivation is induced by HIV infection, CD4 T-cell depletion, or TNF neutralization, non-necrotizing granulomas form adjacent to established and often mineralized granulomas in the lymph nodes. (2) Mtb exits lymph nodes through unknown mechanisms, probably carried by lymph into the subclavian vein and then spreading hematogenously or when the lymph node structure breaks down and delivers bacilli to the airways. (3) Mtb travels to and forms new granulomas in the lungs and other organs (extrapulmonary TB). *Abbreviations*: Mtb, *Mycobacterium tuberculosis*; TB, tuberculosis; TNF, tumor necrosis factor.

Since lymph nodes are sites of Mtb infection and persistence [8], it is worth considering protection against Mtb infection in lymph nodes in preclinical vaccine studies. Two vaccination strategies have shown protection in both lungs and lymph nodes in macaques after Mtb infection. Vaccination with attenuated Mtb [84] and a cytomegalovirus vector encoding Mtb-specific antigens [85] in rhesus macaques have shown that macaques protected against Mtb challenge (e.g., mild disease and significantly lower Mtb burden in lungs) also showed an approximately 100-fold decrease in culturable Mtb in lung-draining lymph nodes. Complete protection against Mtb infection (i.e., no lung granuloma formation or formation of sterile granulomas) was best achieved in the context of Mtb reinfection

in cynomolgus macaques [86] and intravenous (IV) BCG vaccination in rhesus macaques [87]. Using DNA barcoded Mtb libraries, cynomolgus macaques were infected with Mtb library A and after 16 weeks, rechallenged with Mtb library B. Macaques initially infected with Mtb library A developed significantly fewer library B lung granulomas, most of them sterile, compared to macaques only infected with Mtb library B (naïve controls). Importantly, Mtb library B only disseminated to the lymph nodes in 1 of 8 Mtb library A-infected macaques, and this was only in 1 lymph node. In contrast, 5 out of 6 naïve controls had one or more CFU+ lymph nodes [86]. IV BCG vaccination protected 6 out of 10 rhesus macaques from forming lung granulomas after Mtb challenge. Three of the remaining IV BCG vaccinated macaques were protected and formed ≤3 granulomas and had significantly lower lung CFU compared to the standard ID vaccination route. Overall, IV-BCG macaques had a 100,000-fold reduction in thoracic bacterial burden compared to the ID BCG group. Similar to the Mtb reinfection study, 9 out of 10 rhesus macaques in the IV-BCG vaccinated group did not grow Mtb in any of their lymph nodes examined. Based on these studies, a successful vaccine should induce rapid killing of Mtb when it enters the lungs and prevent Mtb from reaching the lymph nodes. When macaques were analyzed only 4 weeks after BCG vaccination (without Mtb challenge), only IV-BCG vaccinated animals had BCG in BAL, spleen, lung lobes, and peripheral and thoracic lymph nodes, while ID-vaccinated animals only harbored BCG in skin and draining axillary lymph nodes. Aerosol-vaccinated animals had BCG in lung lobes and BAL. At 2–4 weeks post-IV BCG vaccination, increased inflammation (FDG activity) in lung-draining lymph nodes, lung lobes, and spleen was observed by PET-CT; this was not seen in the other routes. IV-BCG vaccinated animals also had transient enlargement of the spleen and enlarged lymph nodes that contained non-necrotizing granulomas and increased proliferation in the B cell region, often with active germinal centers [87]. It seems that BCG infiltration and metabolic activity (probably activation of immune response) in thoracic lymph nodes and spleen sets IV BCG vaccinated macaques apart from other vaccination routes and may be important contributors to the astounding protection that IV BCG vaccination conferred against Mtb challenge. In both mice and NHP studies, BCG colonization of lung-draining lymph nodes is correlated with protection against Mtb challenge [83, 87]. In addition, prevention of Mtb infection in lymph nodes and not just the lungs should also be targeted when designing a vaccine. The role of lymph nodes in vaccine efficacy is worthy of additional study.

2.1.8 Lower Drug Penetration in Lymph Nodes

Lymph nodes are sites where Mtb can persist, disseminate, and reactivate [51, 78–81]. Therefore, it is imperative that anti-TB drugs be tested for their ability to eliminate Mtb bacteria in the lymph nodes. Short-course drug treatment studies in cynomolgus macaques show that reduction in Mtb burden in lymph nodes is significantly impaired compared to lung granulomas (55-fold reduction in lymph nodes versus 181-fold reduction in lung granulomas [8]) in

drug-treated versus untreated controls. Thus, anti-TB drugs are more effective in killing Mtb in lung granulomas compared to lymph nodes [8, 88]. There is only one study that the authors are aware of that examined concentrations of rifampicin (RIF) and isoniazid (INH), both first-line anti-TB drugs, in the blood, lungs, granulomas, and lymph nodes in humans [89, 90]. RIF had the highest concentration in the blood (6.95 μg/ml) followed by tuberculous foci (2.43 μg/g) and healthy lung tissue (2.22 μg/g). Thoracic lymph nodes (1.41 μg/g) had lower RIF concentration compared to blood and lung granulomas. Interestingly, the lowest RIF concentration was found in caseous lymph nodes (0.03 μg/g). In contrast, although INH concentration was also highest in the blood (4.11 μg/ml), its concentration in healthy lungs (0.58 μg/g), bronchopulmonary lymph nodes (0.53 μg/g), cavities (0.59 μg/g), tuberculous foci (0.6 μg/g), and caseous lymph nodes (0.21 μg/g) were all relatively similar [89, 90]. Remarkably, caseous lymph nodes had once again the lowest INH concentration. No information about Mtb burden in the different tissues was provided. Although this is just one study, it provides a glimpse of lower RIF and INH penetration in lymph nodes compared to lung granulomas. This lower drug penetration in lymph nodes could explain the reduced efficacy of anti-TB drugs in killing Mtb in the lymph node compartment.

HIV also uses lymph nodes as latent reservoirs. Similar to the findings above, studies of drug penetration in lymph nodes in HIV patients show that concentrations of antiretroviral drugs in lymph nodes are significantly lower compared to the blood [91–93]. By sequencing HIV DNA and RNA from blood and inguinal lymph nodes of HIV-infected patients at different time intervals post antiretroviral treatment, Lorenzo-Redondo and colleagues discovered that despite undetectable levels of viral RNA in plasma during treatment, low-level viral replication still occurs in lymph nodes and this phenomenon was attributed to low antiretroviral drug penetration in these organs [91, 92]. Similar findings were reported in a study of 12 HIV-infected patients after antiretroviral drug initiation. Fletcher and colleagues showed that the antiretroviral drug concentrations in lymph nodes were significantly lower compared to the blood, and this correlated with continuous HIV replication [93]. Thus, lower drug penetration in lymph nodes is not unique to TB.

2.2 Concluding Remarks

Lymph nodes are underappreciated in the study of TB. It is clear that aside from their main function of initiating and shaping adaptive immune responses, lymph nodes also serve as niches for Mtb growth and persistence. Although lymph nodes mount an immune response to Mtb infection, data support that they are poor killers of Mtb [8]. In addition, latent TB reactivation can originate from lymph nodes [51, 78–81]. As such, eliminating Mtb in these organs requires closer attention. Current anti-TB drug regimens are less efficient in reducing Mtb burden in the lymph nodes compared to lung granulomas, which may be attributed

to poor penetration of drugs in the involved lymph nodes. Vaccine and anti-TB drug trials should examine efficacy of preventing Mtb infection or eliminating Mtb in lymph nodes and not just the lungs. Tools (e.g., nanomaterials) and efficient antigen or drug design can also be used to target anti-TB drugs and vaccines to the lymph nodes. Further studies in improving vaccine and drug delivery to the lymph nodes is warranted.

2.3 Methods

2.3.1 Mtb Dissemination from Lungs to Lymph Nodes Analysis

Cynomolgus macaques (*Macaca fascicularis*) (n = 74) that served as controls (no vaccine or drug treatment) for other studies from 2011 to 2018 were selected for this study. These macaques were infected with 2–92 CFU (median = 8 CFU) of Mtb Erdman using a bronchoscope. The animals used in this study are summarized in Table S1. Existing records were reviewed for the location of granulomas in the lungs and CFU positivity of lymph nodes in the same animal. Data were graphed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA). All procedures and protocols were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC).

2.3.2 Histology

Histological examination was performed by an experienced veterinary pathologist (E. Klein) as previously described [39]. Lymph nodes obtained during necropsy were cut (4–6 mm) and stained with hematoxylin–eosin. Characteristics of granulomas, such as size, type (caseous, non-necrotizing, suppurative, or mixed), distribution pattern (focal, multifocal, coalescing, focally extensive, and locally invasive), and cellular composition were noted. The list of animals used in this review are summarized in Table S2.

2.3.3 Ethics statement

All experimental manipulations, protocols, and care of the animals were approved by the University of Pittsburgh School of Medicine IACUC. The protocol assurance number for our IACUC is A3187-01. Our specific protocol approval numbers for this project are as follows: 1105870, 11090030, 11110045, 12060181, 12080653, 12090832, 13122856, 14023305, 14043492, 15055811, 15066174, 15126588, 16017370, 17029987, and 17060529. The IACUC adheres to national guidelines established in the Animal Welfare Act (7 U.S.C. Sections 2131–2159) and the *Guide for the Care and Use of Laboratory Animals* (8th Edition) as mandated by the US Public Health Service Policy.

All macaques used in this study were housed at the University of Pittsburgh in rooms with autonomously controlled temperature, humidity, and lighting.

Animals were singly housed in caging at least 2 m^2 apart that allowed visual and tactile contact with neighboring conspecifics. The macaques were fed twice daily with biscuits formulated for NHPs, supplemented at least 4 days/wk with large pieces of fresh fruits or vegetables. Animals had access to water ad libitum. Because our macaques were singly housed due to the infectious nature of these studies, an enhanced enrichment plan was designed and overseen by our NHP enrichment specialist. This plan has 3 components. First, species-specific behaviors are encouraged. All animals have access to toys and other manipulata, some of which will be filled with food treats (e.g., frozen fruit, peanut butter, etc.). These are rotated on a regular basis. Puzzle feeders, foraging boards, and cardboard tubes containing small food items also are placed in the cage to stimulate foraging behaviors. Adjustable mirrors accessible to the animals stimulate interaction between animals. Second, routine interaction between humans and macaques are encouraged. These interactions occur daily and consist mainly of small food objects offered as enrichment and adhere to established safety protocols. Animal caretakers are encouraged to interact with the animals (by talking or with facial expressions) while performing tasks in the housing area. Routine procedures (e.g., feeding, cage cleaning, etc.) are done on a strict schedule to allow the animals to acclimate to a routine daily schedule. Third, all macaques are provided with a variety of visual and auditory stimulation. Housing areas contain either radios or TV/video equipment that play cartoons or other formats designed for children for at least 3 h each day. The videos and radios are rotated between animal rooms so that the same enrichment is not played repetitively for the same group of animals.

All animals are checked at least twice daily to assess appetite, attitude, activity level, hydration status, etc. Following Mtb infection, the animals are monitored closely for evidence of disease (e.g., anorexia, weight loss, tachypnea, dyspnea, coughing). Physical exams, including weights, are performed on a regular basis. Animals are sedated prior to all veterinary procedures (e.g., blood draws, etc.) using ketamine or other approved drugs. Regular PET-CT imaging is conducted on most of our macaques following infection and has proved very useful for monitoring disease progression. Our veterinary technicians monitor animals especially closely for any signs of pain or distress. If any are noted, appropriate supportive care (e.g., dietary supplementation, rehydration) and clinical treatments (analgesics) are given. Any animal considered to have advanced disease or intractable pain or distress from any cause is sedated with ketamine and then humanely euthanatized using sodium pentobarbital.

Supporting Information

S1 Table. List of animals used in studying Mtb dissemination from lungs to lymph nodes. Mtb, *Mycobacterium tuberculosis*. https://doi.org/10.1371/journal.ppat.1008632.s001.

S2 Table. List of animals used for histology. https://doi.org/10.1371/journal.ppat.1008632.s002.

Abbreviations

BAL: bronchoalveolar lavage
BCG: Bacille Calmette-Guerin
CEQs: chromosomal equivalents
CFU: colony forming units
DC: dendritic cells
FDG: ^{18}F-fluorodeoxyglucose
Foxp3: forkhead box protein 3
GMCSF: granulocyte-macrophage colony-stimulating factor
IL-10: interleukin-10
IFNα: interferon alpha
INH: isoniazid
IV: intravenous
Mtb: *Mycobacterium tuberculosis*
NHPs: nonhuman primates
PET-CT: positron emission tomography coupled with computed tomography
PPD: purified protein derivative
RIF: rifampicin
SIV: simian immunodeficiency virus
TB: tuberculosis
TBLN: TB lymphadenitis
TGFβ: transforming growth factor beta
Th1: T helper type 1
Th17: T helper type 17
TLR: toll-like receptor
TNF: tumor necrosis factor
TST: tuberculin skin test

Disclosures and Conflict of Interest

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Chapter 3

Chronic Kidney Disease in the United States, 2021

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Keywords: albuminuria, angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors, chronic kidney disease (CKD), end-stage renal disease (ESRD), heart disease, nephrologist, serum creatinine, stroke

When people develop chronic kidney disease (CKD), their kidneys become damaged and over time may not clean the blood as well as healthy kidneys. If kidneys do not work well, toxic waste and extra fluid accumulate in the body and may lead to high blood pressure, heart disease, stroke, and early death. However, people with CKD and people at risk for CKD can take steps to protect their kidneys with the help of their health care providers.

3.1 CKD Is Common Among US Adults

Fast Facts

- More than 1 in 7, that is 15% of US adults or 37 million people, are estimated to have CKD.*
- As many as 9 in 10 adults with CKD do not know they have CKD.
- About 2 in 5 adults with severe CKD do not know they have CKD.

*How the estimates were calculated:
Percentage of CKD stages 1–4 among US adults aged 18 years or older using data from the 2015–2018 National Health and Nutrition Examination Survey and the CKD Epidemiology Collaboration (CKD-EPI) equation. CKD stage 5 (that is, kidney failure) was not included. These estimates were based on a single measure of albuminuria or serum creatinine; they do not account for persistence of albuminuria or levels of creatinine that are higher than normal as indicated by the kidney disease Improving Global Outcomes recommendations. Thus, CKD in this chapter might be overestimated. Estimates by sex and race/ethnicity were age-standardized using the 2000 US census population; the overall percentage is unadjusted. The number of adults with CKD stages 1–4 was estimated by applying the overall percentage to the 2019 US Census population aged 18 years or older. Blood pressure-lowering medications included angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers; diagnosed diabetes was self-reported.

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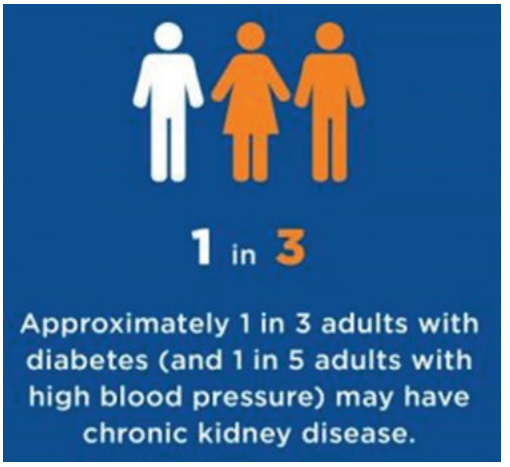
3.2 CKD by Age, Sex, and Race/Ethnicity

According to current estimates*:

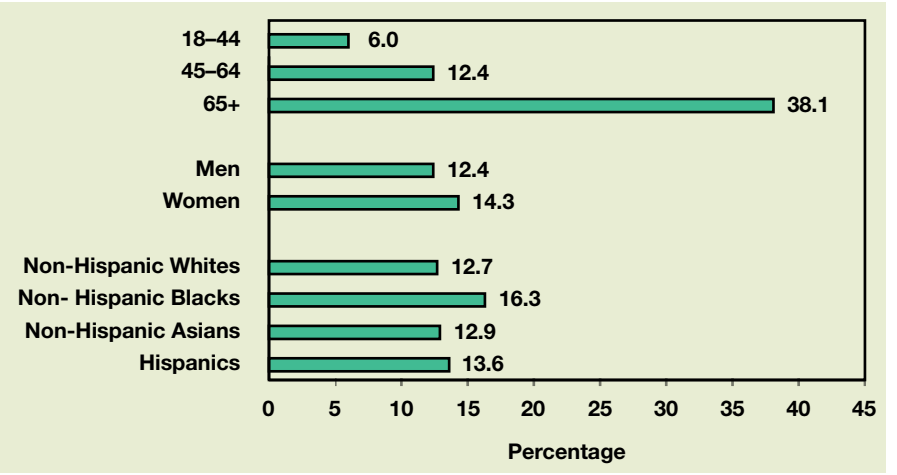
- CKD is more common in people aged 65 years or older (38%) than in people aged 45–64 years (12%) or 18–44 years (6%).
- CKD is slightly more common in women (14%) than men (12%).
- CKD is more common in non-Hispanic Black adults (16%) than in non-Hispanic White adults (13%) or non-Hispanic Asian adults (13%).
- About 14% of Hispanic adults have CKD.

3.3 CKD Risk Factors



Diabetes and high blood pressure are the more common causes of CKD in adults. Other risk factors include heart disease, obesity, a family history of CKD, inherited kidney disorders, past damage to the kidneys, and older age.

Managing blood sugar and blood pressure can help keep kidneys healthy.



Percentage of US adults aged 18 years or older with CKD.*

3.4 Ways to Prevent CKD

- *Manage* risk factors for CKD:
  - High blood pressure.
  - High blood sugar levels.

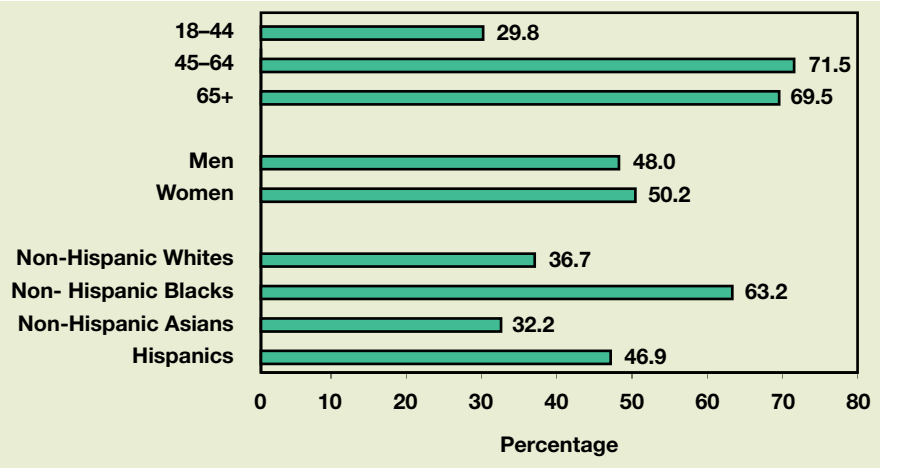
Keeping a healthy body weight through a balanced diet and physical activity may help manage blood pressure and blood sugar levels in people with diabetes or in people at risk of developing type 2 diabetes.

Preventing type 2 diabetes can help prevent CKD and kidney failure.

3.5 Treatment to Lower Blood Pressure

- Blood pressure–lowering medications are recommended for people with diabetes and CKD. However, the percentage of adults with CKD and diagnosed diabetes who are prescribed blood pressure–lowering medications is less than ideal.
  - Prescription of blood pressure–lowering medications is higher in people with CKD and diagnosed diabetes aged 45 years or older (about 70%) than in those aged 18–44 years (30%).
  - Prescription of blood pressure–lowering medications is similar in adult women and men with CKD and diagnosed diabetes (about 50%).

- Prescription of blood pressure–lowering medications is higher in non-Hispanic Black adults with CKD and diagnosed diabetes (63%) than in non-Hispanic White adults (37%) or non-Hispanic Asian adults (32%).
- About 47% of Hispanic adults with CKD and diagnosed diabetes are prescribed blood pressure–lowering medications.



Percentage of US Adults Aged 18 Years or Older With CKD and Diagnosed Diabetes Who Were Prescribed Blood Pressure–Lowering Medications,† by Age, Sex, and Race/Ethnicity.

*Angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers using data from the 2015–2018 National Health and Nutrition Examination Survey. For more details on the methods, see 'How the Estimates Were Calculated.'

3.6 Testing and Treatment: Find It Early, Treat It Early

- Test for CKD regularly in people who have diabetes, high blood pressure, or other risk factors for CKD. People with CKD may not feel ill or notice any symptoms until CKD is advanced.

- The only way to find out if people have CKD is through simple blood and urine tests. The blood test checks for the level of creatinine, a waste product produced by muscles, to see how well the kidneys work. The urine test checks for protein, which may indicate kidney damage.
- Following a healthy diet and taking medicine for diabetes, medicine for high blood pressure, and other medications to protect the kidneys may keep CKD from getting worse and may prevent other health problems such as heart disease.

3.7 CKD Related Health Problems

As CKD worsens over time, related health problems become more likely. However, CKD-related health problems can improve with treatment.

Heart Disease and Stroke

- Having CKD increases the chances of having heart disease and stroke.
- Managing high blood pressure, blood sugar, and cholesterol levels—all factors that increase the risk for heart disease and stroke—is very important for people with CKD.

Early Death

Adults with CKD are at a higher risk of dying earlier than adults of similar age without CKD.

Health Problems Due to Low Kidney Function

- Anemia or low red blood cell count, which can cause fatigue and weakness.
- Extra fluid in the body, which can cause high blood pressure, swelling in the legs, or shortness of breath.
- A weakened immune system, which make it easier to develop infections.
- Loss of appetite or nausea.
- Decreased sexual response.
- Confusion, problems with memory and thinking, or depression.
- Low calcium levels and high phosphorus levels in the blood, which can cause bone disease and heart disease.
- High potassium levels in the blood, which can cause an irregular or abnormal heartbeat and lead to death.

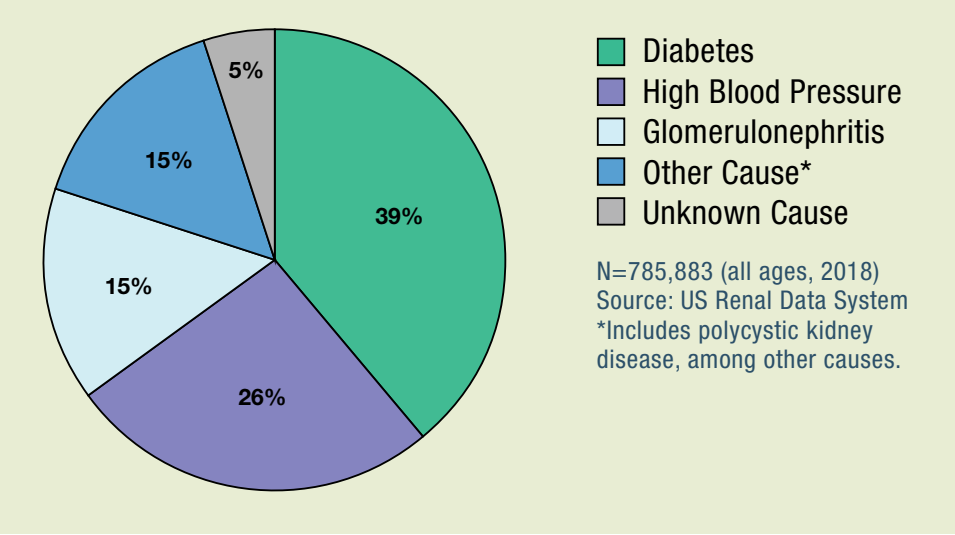
Kidney Failure

Kidney failure happens when kidney damage is severe and kidney function is very low. Dialysis or a kidney transplant is then needed for survival. Kidney failure treated with dialysis or a kidney transplant is called end-stage renal disease

(ESRD). CKD is more likely to lead to kidney failure, especially in older adults, if the kidneys are damaged by the inability to manage risk factors, repeated kidney infections, or drugs or toxins that are harmful to the kidneys. Social factors such as lower income and related factors of food insecurity and poorer access to quality health care are also associated with worsening CKD. However, not everyone with CKD develops kidney failure. If CKD is detected early, treatment may slow the decline in kidney function and delay kidney failure. In some cases, kidney failure develops even with treatment.

Facts about ESRD

- In 2018, about 131,600 people in the United States started treatment for ESRD.
- Nearly 786,000 people in the United States, or 2 in every 1,000 people, are currently living with ESRD: 71% are on dialysis and 29% are living with a kidney transplant.
- For every 2 women who develop ESRD, 3 men develop ESRD.
- For every non-Hispanic White person who develops ESRD, 3 non-Hispanic Black people develop ESRD.
- For every 3 non-Hispanic people who develop ESRD, 4 Hispanic people develop ESRD.
- Among adults aged 18 years or older in the United States, diabetes and high blood pressure are the main causes of ESRD.
- Among children and adolescents younger than 18 years in the United States, polycystic kidney disease and glomerulonephritis (inflammation of the kidneys) are the main causes of ESRD.



Reported causes of end-stage renal disease in the United States.

3.8 People with CKD Can Lower Their Risk for Kidney Failure

Learn about CKD from a primary care doctor or a kidney doctor (nephrologist) to better understand treatment options and protect the kidneys. People with glomerulonephritis, polycystic kidney disease, or other kidney disease should talk about specific treatment options with a kidney doctor.

Monitor and Manage Blood Sugar and Blood Pressure

- Have blood sugar and blood pressure checked regularly.
- Use medicines if prescribed to lower blood sugar and blood pressure.
- *Manage* CKD
  - Make lifestyle changes (e.g., healthy eating, physical activity) to prevent more kidney damage. Meet with a dietitian to create a kidney-healthy eating plan that is low in salt and fat and has the right amount and source of protein. As CKD gets worse, the plan may also include limiting phosphorus and potassium.
  - Use medicines as directed to slow the decline in kidney function.
  - Stop smoking or do not start smoking.
  - Avoid exposures that can harm the kidneys or cause kidney function to suddenly get worse:
    - Certain medicines:
      - Over-the-counter pain medicines like ibuprofen and naproxen, which are also called non-steroidal anti-inflammatory drugs.
      - Some antibiotics.
    - Certain herbal supplements.
    - Excessive alcohol intake.
  - Review with health care providers all prescription and over-the-counter medications to make sure they are safe for the kidneys. Always talk to a doctor before taking any supplements.
  - Check with a doctor about other behaviors or substances that can harm the kidneys or about special precautions to take when doing medical tests or procedures, such as imaging studies or colonoscopies.

People with diabetes, high blood pressure, or CKD need to talk to their doctor about how to protect their kidneys.

Disclosures and Conflict of Interest

The following organizations collaborated in developing and reviewing this chapter. Check their websites for CKD online resources for patients or providers:

Centers for Disease Control and Prevention (www.cdc.gov/kidneydisease)

Centers for Medicare & Medicaid Services (www.cms.gov)

US Department of Defense (www.health.mil)

US Department of Veterans Affairs (www.va.gov/health)

US Food & Drug Administration (www.fda.gov)

Kidney Interagency Coordinating Committee (www.niddk.nih.gov/about-niddk/advisory-coordinating-committees/kuh-icc/kicc)

National Heart, Lung, and Blood Institute of the National Institutes of Health (www.nhlbi.nih.gov)

National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (www.niddk.nih.gov)

United States Renal Data System (www.usrds.org)

American Association of Kidney Patients (www.aakp.org)

American Society of Nephrology (www.asn-online.org)

National Kidney Foundation (www.kidney.org)

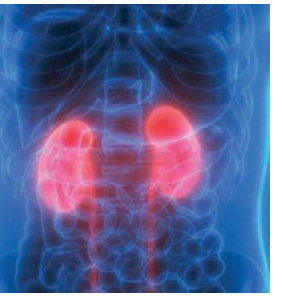
University of California, San Francisco, and University of California, San Francisco Center for Vulnerable Populations (www.ucsf.edu)

University of Michigan, Division of Nephrology, Department of Internal Medicine, and University of Michigan Kidney Epidemiology and Cost Center (www.med.umich.edu/intmed/nephrology)

CHRONIC KIDNEY DISEASE

COMMON • SERIOUS • COSTLY

Chronic kidney disease (CKD) causes loss of kidney function over time and may lead to kidney failure or end-stage kidney disease (ESKD).



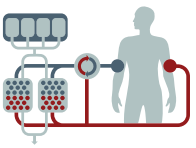
37 MILLION

MORE THAN **1 IN 7**

US adults may have CKD

90% don't know they have it

Kidney diseases are the **9TH LEADING CAUSE OF DEATH** in the United States



EVERY DAY MORE THAN 340 people begin treatment for kidney failure (dialysis or a kidney transplant)

RISK FACTORS

- Diabetes
- High blood pressure
- Heart disease
- Obesity
- Family history of CKD
- Older age

1 IN 3 adults with diabetes may have CKD

1 IN 5 adults with high blood pressure may have CKD

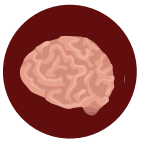
TREATMENT LOWERS RISK FOR ESKD

CHRONIC KIDNEY DISEASE

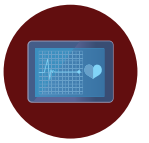
COMMON • SERIOUS • COSTLY

CKD **INCREASES RISK** FOR:

Heart disease and heart failure



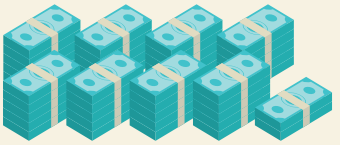
Stroke



Early death

MEDICARE **COSTS**

CKD =



$84 BILLION

ESKD =

$36 BILLION

REDUCE COSTS BY PREVENTING:

- CKD in people at risk
- CKD progressing to ESKD
- Other chronic conditions, including type 2 diabetes and heart disease

CDC's CKD Initiative collaborates with other government agencies, universities, and national organizations to:

- **Prevent and manage** risk factors for CKD
- **Raise awareness** of CKD and its complications
- **Promote early diagnosis and management** of CKD
- **Improve outcomes** for people living with CKD

The **CKD Surveillance System** documents CKD and its risk factors in the United States and tracks progress in preventing, detecting, and managing CKD. These efforts align with Healthy People objectives for CKD.

TAKE CARE OF YOUR **KIDNEYS** AND THEY WILL **TAKE CARE** OF **YOU**.

CHRONIC KIDNEY DISEASE

Your Kidneys May Not Work Well If You Have Diabetes.

Diabetes can cause kidney disease, also known as chronic kidney disease (CKD). The good news is that there is a lot you can do to prevent kidney problems, including keeping your blood sugar and blood pressure under control.

Having kidney disease increases the chances of having heart disease, heart attacks, and strokes.

Keeping your kidneys healthy will help take care of your heart.

Urine Albumin-to-Creatinine Ratio (UACR)

In Evaluating Patients with Diabetes for Kidney Disease

The two key markers for chronic kidney disease (CKD) are urine albumin and estimated glomerular filtration rate (eGFR).

Assess urine albumin excretion yearly to diagnose and monitor kidney damage in patients with type 1 diabetes for five years or more or with type 2 diabetes.

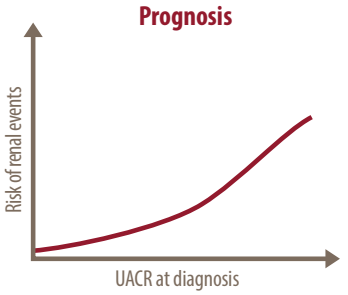
- More frequent monitoring may be indicated in patients with changing clinical status or after therapeutic interventions.
- Use a spot urine albumin-to-creatinine ratio (UACR). UACR estimates 24-hour urine albumin excretion. Twenty-four-hour collection and timed specimens are not necessary.

$$\frac{\text{Urine albumin (mg/dL)}}{\text{Urine creatinine (g/dL)}} = \text{UACR in mg/g} \approx \text{Albumin excretion in mg/day}$$

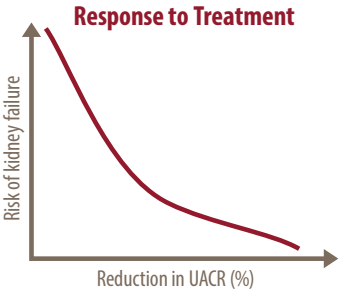
UACR is a ratio between two measured substances. Unlike a dipstick test for albumin, UACR is unaffected by variation in urine concentration.

Albuminuria[1] is present when UACR is greater than 30 mg/g and is a marker for CKD.

Albuminuria is used to diagnose and monitor kidney disease. Change in albuminuria may reflect response to therapy and risk for progression. A decrease in urine albumin may be associated with improved renal and cardiovascular outcomes.



In a large cohort of CKD patients, a higher UACR at time of diagnosis was associated with increased risk for renal events—loss of half of eGFR, dialysis, or death. (Chronic Renal Insufficiency Cohort study)



A randomized trial of diabetes patients with CKD found that the greater the reduction of UACR in response to treatment (with ARBs), the lower the risk of progression to kidney failure. (De Zeeuw D, et al. *Kidney International*, 2004:65:2309-2320)

[1]Albuminuria is a term that describes all levels of urine albumin. *Microalbuminuria* is a term used to describe urine albumin levels not detected by a dipstick test, i.e., 30 mg/g—300 mg/g. *Macroalbuminuria* is sometimes used to describe albumin levels more than 300mg/g.

Estimated Glomerular Filtration Rate (eGFR)

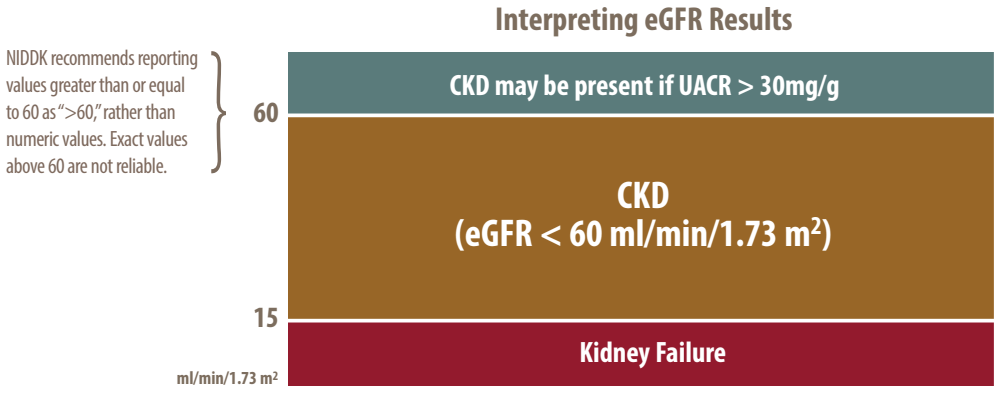
In Evaluating Patients with Diabetes for Kidney Disease

The two key markers for chronic kidney disease (CKD) are estimated glomerular filtration rate (eGFR) and urine albumin.

Calculate eGFR from stable serum creatinine levels at least once a year in all patients with diabetes.

- eGFR is more accurate than serum creatinine alone. Serum creatinine is affected by muscle mass, and related factors of age, sex, and race.
- eGFR is not reliable for patients with rapidly changing creatinine levels, extremes in muscle mass and body size, or altered diet patterns.

See if your lab reports eGFR routinely. If it does not, ask your lab to do so. You can also calculate an eGFR yourself by using GFR calculators available on NIDDK's website at *www.niddk.nih.gov.*



If CKD is detected, it should be addressed as part of a comprehensive approach to the treatment of diabetes.

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What Happens If You Have Kidney Damage?

Changes or damage to your kidneys may cause your kidneys to fail. If your kidneys fail, your blood must be filtered (dialysis treatments) several times a week.

You may also need to have a kidney transplant.

How Will You Know If You Have Kidney Problems?

- Ask your doctor to test your blood and your pee.
- If the doctor finds protein (albumin) in your pee, it is a sign of the start of kidney disease caused by diabetes.
- Get tested yearly.
- Get tested more often if:
  - » **Your test shows protein in your pee or;**
  - » **Your kidneys are not working as they usually do.**

If You Have Diabetes, Take These Steps:

- Meet blood sugar targets as often as you can.
- Get tested for your average level of blood sugar over the past three months (A1C test).
- Get your A1C test at least twice a year, but ideally up to four times a year.
- If your blood pressure is high, check it regularly and get it under control to make sure your kidneys stay healthy.
- Talk to your doctor about medicines that harm your kidneys and other ways to lower your blood pressure.

What is the Best Way to Keep Your Kidneys Healthy?

- Keep your blood pressure below 140/90, or ask your doctor what the best blood pressure target is for you.
- Stay in your target cholesterol range.
- Eat foods lower in salt.
- Eat more fruits and vegetables.
- Stay active.
- Take your medications as directed.

Who is More Likely to Develop Kidney Disease?

- Approximately 1 of 3 adults with diabetes and 1 of 5 adults with high blood pressure may have CKD.
- In addition to diabetes and high blood pressure, other problems that put you at greater chance of kidney disease include: heart disease, obesity (being overweight), and a family history of CKD. Kidney infections and a physical injury can also cause kidney disease.

What Can You Do to Prevent Kidney Failure?

- Get tested for CKD regularly if you are at risk.
- Find it early. Treat it early.
- Ask your doctor to test your blood or pee. If you have diabetes, get tested yearly.
- If you have diabetes, stay in your target blood sugar range as much as possible.
- Lose weight if you are overweight.
- Get active. Physical activity helps control blood sugar levels.
- Quit smoking.
- Getting a checkup? Make sure to get your kidneys checked too.
- Take medications as directed.
- If you have CKD, meet with a dietitian to make a kidney-healthy eating plan.

Learn more: www.cdc.gov/ckd

Chapter 4

Combatting Sepsis: A Public Health Perspective

Raymund B. Dantes, MD,[a,b] and Lauren Epstein, MD[b]

[a]*Division of Hospital Medicine, Department of Medicine,*
Emory University School of Medicine, Atlanta, Georgia, USA
[b]*Division of Healthcare Quality Promotion,*
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Raymund.dantes@emoryhealthcare.org

Keywords: epidemiology, Gram-negative rod bacteremia, microbiome, sepsis, Sepsis-3 criteria, sequential organ failure assessment (SOFA)

Sepsis is defined as a syndrome of life-threatening organ dysfunction due to a person's dysregulated response to infection [1]. Practically speaking, it is the final common pathway for how infections cause death. Sepsis is a frequent cause of severe disease and death globally [2], and in the United States an estimated 1.7 million adult cases occur annually, contributing to 265000 deaths each year [3]. This broad syndrome encompasses diverse presentations, such as a newborn in the neonatal intensive care unit with a group B streptococcal bloodstream infection, a 7-year-old hospitalized with severe influenza, a 60-year-old undergoing chemotherapy for lymphoma who acquires mucositis and multidrug-resistant Gram-negative rod bacteremia, and an 80-year-old with severe pneumonia without an identified pathogen.

Public health organizations have worked for decades to track and prevent infections that can lead to sepsis and to reduce the burden of chronic diseases that increase the risk of sepsis. A growing recognition of sepsis burden has prompted state and national initiatives to improve and benchmark the quality of care within healthcare facilities. Public health professionals have the opportunity to go further, establishing a comprehensive approach to sepsis that extends beyond the hospital by integrating prevention, early recognition, treatment, and tracking of sepsis into public health initiatives.

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4.1 National Trends in Sepsis Burden

A thorough understanding of the burden and epidemiology of sepsis is essential to guide development of a comprehensive strategy for prevention and early recognition. Sepsis burden has been difficult to quantify because there is no definitive diagnostic test and sepsis diagnosis and identification can vary widely, even among critical care experts [4]. Previous estimates of the incidence of sepsis and the related mortality rate have relied on administrative coding and/or death certificates, with each approach presenting significant limitations [5] and resulting in a wide estimated mortality rate range [6]. To better inform policy makers, public health officials, clinicians, and the public, the Centers for Disease Control (CDC) Prevention Epicenters Program recently led a large collaborative study to measure national sepsis trends and burden, using objective clinical data obtained from the electronic health records of adult patients in 409 hospitals.

The clinical data used to identify patients with sepsis was adapted from the Sepsis-3 criteria, which relies on suspicion of infection and associated organ dysfunction, based on the sequential organ failure assessment (SOFA) score [3]. In contrast to previous estimates based on administrative coding data that have suggested increasing incidence and decreasing mortality rates, this study found no change in the incidence of adult sepsis and associated mortality rates from 2009 to 2014 [3]. The estimated adult prevalence of 1.7 million sepsis cases and contribution to 265000 deaths falls within the broad range of previous estimates and confirms the immense public health impact of sepsis [3]. In March 2018, the CDC released its "Hospital Toolkit for Adult Sepsis Surveillance" [7], a document that contains specifications and guidance for hospitals using this validated approach to objectively track sepsis outcomes for internal quality improvement initiatives and measure the effectiveness of sepsis prevention efforts. This approach will allow healthcare facilities to innovate sepsis care using objective definitions of sepsis, rather than just strictly assessing adherence to sepsis protocols.

4.2 Comprehensive Sepsis Prevention Framework

Public health infection prevention efforts have been particularly effective when focused on specific pathogens or settings where transmission of pathogens are likely to occur (e.g., vaccination, outbreak response, food safety, and prevention of healthcare-associated infections). However, the identity and source of the infection-causing pathogen are unknown in 30%–70% of patients with sepsis [8–10], which makes it more difficult to design specific infection prevention strategies. Primary prevention strategies that focus on chronic diseases or exposures that confer increased risk of infection are particularly important, and accounting for the immune response to severe infection is a crucial component in the development of sepsis [11]. Therefore, a sepsis prevention framework is

needed that recognizes patient risk factors and prevention opportunities, before the onset of sepsis and before the patient presents to the hospital (Fig. 4.1).

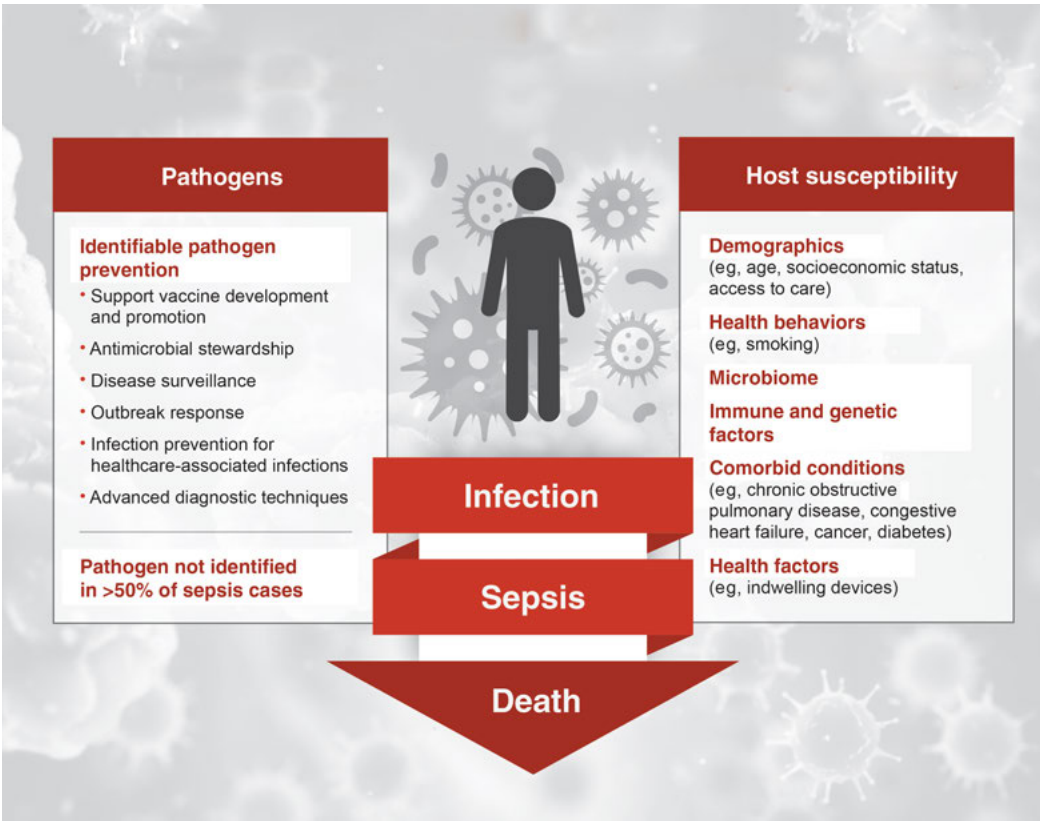

Figure 4.1 Infections and sepsis: risk factors and opportunities for prevention.

Sepsis prevention is already built into many public health interventions. Public health organizations play an important role in supporting programs that prevent infections or reduce infection risks. However, public health efforts can also help clinicians identify opportunities for preventing sepsis and educating patients about how to avoid infections that can lead to sepsis. Explicitly framing existing interventions and programs that reduce infections as providing the additional benefit of sepsis prevention would increase perceived value to patients and clinicians, especially in an era where there is growing public awareness of sepsis burden. For example, in a 65-year-old man with lung cancer undergoing chemotherapy and radiation who presents with sepsis due to influenza pneumonia, one can identify many missed opportunities for prevention of sepsis-related morbidity effects.

Such opportunities included smoking cessation to reduce the risk of lung cancer, annual vaccinations to prevent influenza and other infections, proper infection control practices during visits to healthcare providers to prevent transmission of influenza in the clinic, and early detection and treatment of influenza.

To prevent morbidity effects in a child with sepsis due to Salmonella gastroenteritis, appropriate efforts would include a broad range of food safety steps, in addition to prompt identification and treatment of infection and sepsis in outpatient, urgent care, and emergency department settings.

4.3 Building Partnerships and Increasing Awareness

Strong partnerships among clinical professional organizations, patient advocacy groups, and public health organizations are critical to developing successful initiatives to increase sepsis awareness and early recognition among both patients and healthcare providers. In August 2016, a CDC Vital Signs report [12] highlighted the importance of integrating public health prevention efforts and sepsis. In September 2017, the CDC launched a national educational campaign, "Get Ahead of Sepsis" [13], which aims to raise awareness and knowledge about prevention, early recognition, and timely treatment of sepsis among the public and among healthcare providers. In addition, this campaign aligns with antibiotic stewardship efforts by emphasizing the importance of rapid appropriate antibiotic treatment when sepsis is suspected, reminding clinicians to reassess antibiotic therapy in 24–48 h to adjust or stop therapy when additional clinical information is available.

4.4 Future Public Health Opportunities and Challenges

Future public health initiatives should encompass the spectrum of sepsis, from prevention and early identification in the outpatient setting to treatment and management in the hospital and postsepsis care. To reduce the burden of sepsis, public health organizations should partner with clinical communities to create initiatives that prevent infections that can lead to sepsis and promote clinician knowledge about recommended early sepsis recognition and care. Prevention and early infection identification programs that span the spectrum of increasingly integrated outpatient and inpatient healthcare systems could prevent the onset of sepsis or reduce associated deaths. Antibiotic stewardship programs that reduce unnecessary antibiotic use could reduce the sepsis burden; studies have demonstrated that recent antibiotic exposure increases the risk of sepsis, an effect hypothesized to result from alterations in the microbiome [14].

Sepsis detection and treatment could be improved with the development of new diagnostic tools that could rapidly identify the causative organisms and guide more effective and specific antibiotic therapy. Such technologies would complement antibiotic stewardship efforts and reduce the risk of antibiotic resistance. New biomarkers that that can rapidly predict the likelihood of sepsis and poor outcomes are also needed. As the treatment of sepsis evolves, there are opportunities for public health organizations to disseminate new research findings and treatment guidelines, in coordination with professional societies'

recommendations and sepsis awareness and prevention programs. In addition, whereas CDC's surveillance definition may be an important initial step toward tracking the impact of efforts to reduce morbidity and mortality rates associated with sepsis, new methods are also needed to better quantify the impact of specific interventions. Sepsis survivors often suffer long-term health problems [15]; additional research should measure the public health impact and other long-term outcomes among these survivors.

Finally, our understanding of pediatric sepsis lags behind. Studies that estimate pediatric sepsis burden and trends using an objective surveillance definition (analogous to the study by Rhee et al. [3]) would provide a better baseline for measuring the impact of new initiatives, because prior studies were based on administrative codes [16]. Others have also argued that the pediatric definitions of sepsis are in need of an update analogous to the adult Sepsis-3 definition, which could lead to improvements in clinical care, research, coding practice, and advocacy [17].

In conclusion, sepsis remains an important public health challenge. As our understanding of sepsis improves, more persons can survive sepsis or avoid sepsis entirely through the partnership of public health professionals, clinical experts, patient advocacy groups, and the public.

Disclosures and Conflict of Interest

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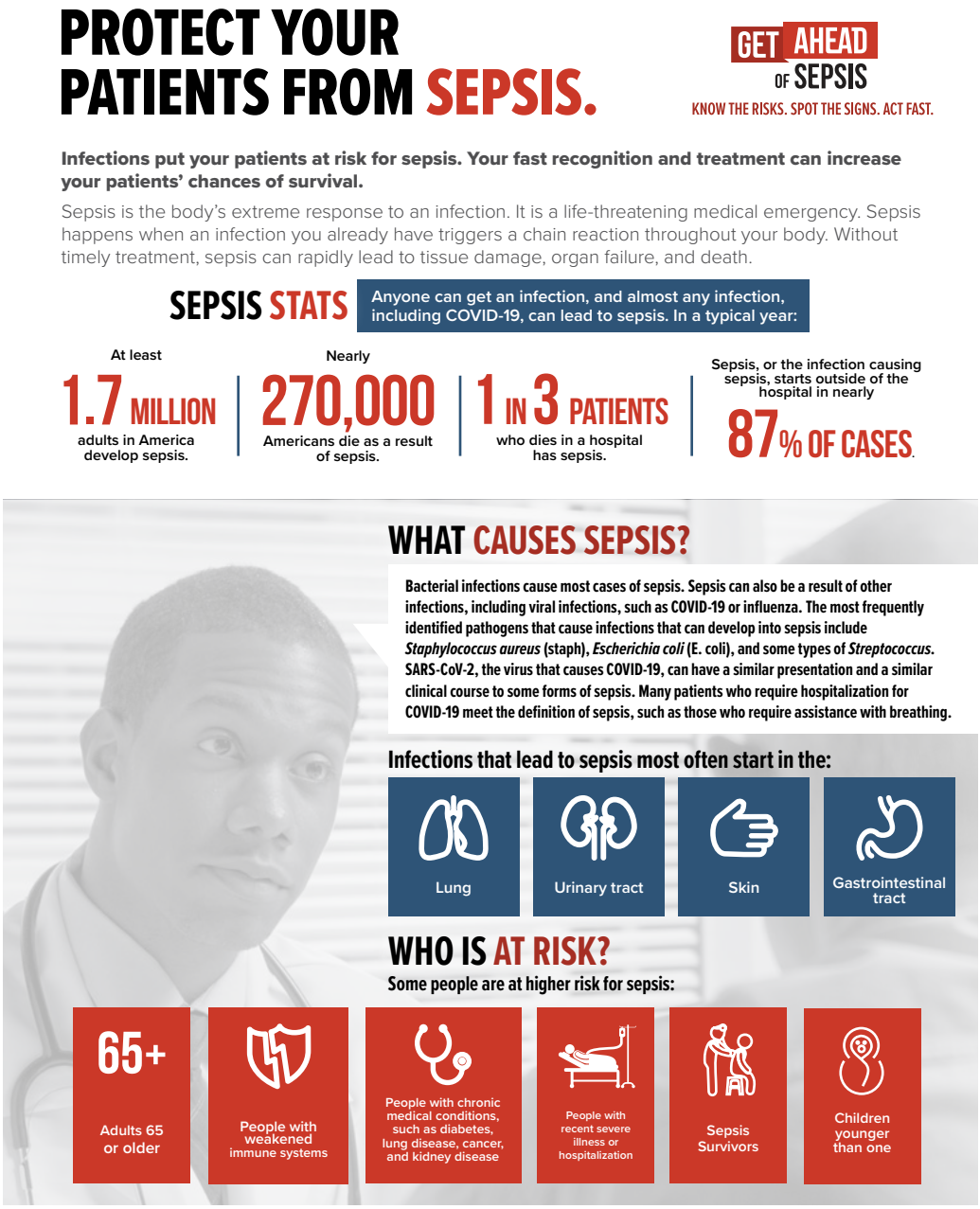
Appendix*



*The Appendix has been compiled by the Series Editor, Raj Bawa, PhD, MD. The materials have been kindly provided to Dr. Bawa by the Centers for Disease Control and Prevention, the Society of Critical Care Medicine and European Society of Intensive Care Medicine.

WHAT ARE THE SIGNS AND SYMPTOMS OF SEPSIS?

A patient with sepsis might have one or more of the following signs or symptoms:

Patients with sepsis should be urgently evaluated and treated by a healthcare professional.

HOW CAN I GET AHEAD OF SEPSIS?

As a healthcare professional you can:

- **Know sepsis signs and symptoms** to identify and treat patients early.
- **ACT FAST** if you suspect sepsis.
- **Prevent infections** by following infection control practices (e.g., hand hygiene, appropriate catheter management) and ensuring patients receive recommended vaccines.
- **Educate your patients and their families about:**
  - Preventing infections
  - Keeping cuts clean and covered until healed
  - Managing chronic conditions
  - Recognizing early signs and symptoms of worsening infection and sepsis, and seeking immediate care if signs and symptoms are present

Sepsis is a medical emergency. You play a critical role. Protect your patients by **ACTING FAST.**

WHAT SHOULD I DO IF I SUSPECT SEPSIS?

Know your facility's existing guidance for diagnosing and managing sepsis.

- **Immediately alert the clinician in charge if it is not you.**
- **Start antibiotics as soon as possible in addition to other therapies appropriate for the patient.** Once the specific cause of sepsis is known, such as a positive test for COVID-19, therapy can be targeted, and empiric broad-spectrum antibiotics might not be needed.
- **Check patient progress frequently.** Always remember to prescribe the right antibiotic, at the right dose, for the right duration, and at the right time. Reassess antibiotic therapy to stop or tailor treatment based on the patient's clinical condition and diagnostic test results as appropriate.

Chapter 5

Hypertension and Atrial Fibrillation: Closing a Virtuous Circle

Ying X. Gue, PhD,[a] and Gregory Y. H. Lip, MD[a,b]

[a]*Liverpool Centre for Cardiovascular Science,*
University of Liverpool and Liverpool Heart & Chest Hospital, Liverpool, UK
[b]*Aalborg Thrombosis Research Unit, Department of Clinical Medicine,*
Aalborg University, Aalborg, Denmark

Gregory.lip@liverpool.ac.uk

Keywords: atrial fibrillation (AF), Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events (ACTIVE-I), blood pressure (BP), calcium channel blockers (CCB), hypertension, individual-participant data (IPD), major adverse cardiovascular event (MACE), meta-analysis, renin-angiotensin-aldosterone system inhibitor (RASS-I), systolic blood pressure

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with an increased risk of major adverse cardiovascular events (MACE) [1]. Patients with AF typically have other concomitant cardiovascular risk factors—hypertension being one of the commonly associated conditions with a prevalence of up to 90% in major clinical trials of AF [2]. Not only is hypertension common in AF, but it is also an independent risk factor for ischaemic and haemorrhagic strokes, thus bearing important implications for patient prognosis [3]. Therefore, optimal management of hypertension in patients with AF is vital to prevent future MACE. In the accompanying individual-participant data (IPD) meta-analysis [4], the authors from the Blood Pressure Lowering Treatment Trialists' Collaboration (BPLTTC) aimed to investigate the effects of blood pressure

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(BP) lowering treatment on MACE when comparing patients with and without AF at baseline. They aimed to address 4 main questions: firstly, whether AF at baseline modifies BP treatment effects; secondly, whether associations between intensity of BP reduction and outcomes are similar with or without AF; thirdly, whether treatment effect is dependent on baseline systolic BP; and lastly, whether classes of antihypertensives have different treatment effect in AF.

A total of 22 trials were included with a total of 188,570 participants and 13,266 patients with a history AF at baseline. Baseline characteristics were different between the 2 groups, with AF patients being older (mean age 70 years versus 65 years), had lower baseline BP (mean 143/84 mmHG versus 155/88 mmHg), and were more commonly prescribed diuretics (50.5% versus 23.8%), angiotensin converting enzyme inhibitors (59.6% versus 44%), beta-blockers (51.3% versus 36%), and alpha-blockers (10.7% versus 4.4%) at baseline. This reflects the more commonly associated cardiovascular comorbidities (hypertension, heart failure, and older age) in patients with AF at baseline [3, 5, 6].

Among the authors' findings, firstly, the mean difference in Systolic blood pressure (SBP) reduction was 7.2 mmHg in placebo-controlled studies (8 studies), 2.3 mmHg in drug–drug comparisons (12 studies), and 10.9 mmHg in more-versus-less intensive treatment trials (2 studies) with an overall difference of 3.7 mmHg. When comparing differences in SBP reduction, the authors reported no difference between patients with or without AF (3.3 mmHg versus 3.7 mmHg).

Secondly, meta-regression showed that each 5 mmHg reduction in BP equated to a 10% reduction in MACE in patients with and without AF. Thirdly, authors found no evidence of difference in treatment effects at different baseline systolic BP. And lastly, there was no difference between classes of antihypertensives (renin-angiotensin-aldosterone system inhibitor (RAAS-I) and calcium channel blockers (CCB)), although this conclusion was limited by small numbers of participants with AF in these studies.

The authors conclude that due to the higher risk of MACE in AF patients compared to those without AF, the same relative risk reduction with BP control translates to greater absolute risk reduction in AF patients and, therefore, more focus should be placed on addressing the associated cardiovascular risk factor such as hypertension to better improve the outcomes in patients with AF.

We congratulate the authors for performing this highly relevant IPD meta-analysis to highlight the importance of the holistic management of patients with AF and the need for more evidence in this area. This thought process is echoed in the most recent European Society of Cardiology (ESC) guideline on the management of AF with a shift from managing AF, from the CC (Confirm AF and Characterise AF) to ABC ("A" Anticoagulation/Avoid stroke, "B" Better symptom control, and "C" Comorbidities/Cardiovascular risk factor management) approaches of managing AF [7].

The association of BP control and reduction in MACE in patients with AF does not come as a surprise as hypertension has been linked not only with adverse cardiovascular outcomes but also with an increased risk of AF [8]. The

importance of BP control has previously been shown in a large meta-analysis of 61 prospective observational studies involving 12.7 million person-years, i.e., that there is a linear relation between BP and vascular (and overall) mortality, starting from values of 115/75 mmHg [9]. BP control reduces mortality from ischaemic vascular events and haemorrhagic complications from anticoagulation treatment in patients with AF [3]. The reduction in mortality was reflected in the present study [4], although there was no differentiation between haemorrhagic and ischaemic stroke in the outcomes.

One limitation of this work is the inclusion of trials involving only patients with AF. AF status being the inclusion or exclusion criteria prior to randomisation could add to the risk of selection bias within the analysis. In addition, the majority of AF participants are from the Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events (ACTIVE-I) trial dataset [10], which can bias the effect seen given the difference in patient characteristic at baseline. Similarly, including trials with only patients without AF may dilute the effects of BP lowering that could be seen in patients with AF. The authors have addressed this by performing sensitivity analyses excluding these studies which have shown comparable results, reassuring us that the impact of selection bias is not significant on the study conclusions.

In the new 2020 ESC guidelines on the "ABC" approach to the management of AF [7], the management of other associated cardiovascular risk factors has become an integral component of optimal management of AF. The shift in the management of AF towards a more holistic approach is one step in the right direction as has been shown by the improvement in outcomes [11, 12] and reduction in healthcare-associated costs [13]. Compliance with the "ABC" management approach requires clear, evidence-based guidelines in terms of treatment targets. With regard to hypertension, the currently recommended BP target (≤130/80) is based upon the current ESC hypertension guidelines [14] and observational data showing greatest benefit of BP between 120 and 129 systolic [15–17]. Whether this target is optimal for the reduction of future MACE in patients with AF is unknown.

This IPD meta-analysis by the BPLTTC has shown that the presence of AF does not alter the treatment effects of antihypertensives. BP lowering in patients with and without AF shows a corresponding reduction in MACE to a similar extent. Owing to the higher absolute risk of MACE in patients with AF, BP lowering in these patients would result in greater absolute risk reduction. This should provide sufficient evidence to convince clinicians regarding the benefits of strict BP control in patients with AF, and the consultation for patients with AF should always involve a conversation about managing hypertension, be it lifestyle modification or pharmacological treatment. However, the potential benefits (or harms) of a much lower BP target (below the recommended 130/80 mmHg) and ideal choice or combination of antihypertensives remain unanswered and would require future studies to provide further insight.

Abbreviations

AF:	atrial fibrillation
ACTIVE-I:	Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events
BP:	blood pressure
BPLTTC:	Blood Pressure Lowering Treatment Trialists' Collaboration
CCB:	calcium channel blockers
ESC:	European Society of Cardiology
IPD:	individual-participant data
MACE:	major adverse cardiovascular event
RASS-I:	renin-angiotensin-aldosterone system inhibitor
SBP:	Systolic blood pressure

Disclosures and Conflict of Interest

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Chapter 6

Cardiovascular Events after Community-Acquired Pneumonia: A Global Perspective with Systematic Review and Meta-Analysis of Observational Studies

António Tralhão, MD,[a,b] and Pedro Póvoa, PhD[a,c,d]

[a]*Polyvalent Intensive Care Unit, Hospital de São Francisco Xavier, Centro Hospitalar Lisboa Ocidental, Estrada do Forte do Alto do Duque, Lisbon, Portugal*
[b]*Cardiology Department, Hospital de Santa Cruz, Centro Hospitalar Lisboa Ocidental, Avenida Professor Doutor Reinaldo dos Santos, Carnaxide, Portugal*
[c]*NOVA Medical School, CHRH, New University of Lisbon, Lisbon, Portugal*
[d]*Center for Clinical Epidemiology and Research Unit of Clinical Epidemiology, OUH Odense University Hospital, Odense C, Denmark*

pedrorpovoa@gmail.com

Keywords: acute cardiogenic pulmonary edema, acute coronary syndromes, angiotensin receptor blockers (ARB), angiotensin-converting enzyme inhibitors (ACEi), arrhythmias, atrial fibrillation (AF), blood urea nitrogen (BUN), cardiovascular (CV), chronic obstructive pulmonary disease (COPD), community-acquired pneumonia (CAP), confusion, urea, respiratory rate, blood urea nitrogen, age > 65 (CURB-65), congestive heart failure (CHF), coronary artery disease (CAD), Egger's test, Funnel plot analysis, heart failure, intensive care unit (ICU), left atrial area index (LAAi), low-density lipoprotein receptors, myocardial infarction (MI), pneumonia, pneumonia severity index (PSI), Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA), respiratory rate (RR), stroke, troponin leak

6.1 Introduction

Community-acquired pneumonia (CAP) and cardiovascular (CV) disease are two major public health issues. CAP alone results in 1.7 million hospital admissions and almost 50,000 deaths every year in the USA [1, 2], with a similar adjusted incidence observed in Europe [3, 4]. In developed countries, CV disease is the

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leading cause of morbidity and mortality. Together, these conditions weigh heavily on national health systems and efforts to mitigate their global burden should be a priority for policy makers [5].

Although seemingly unrelated beforehand, the interplay between CAP and acute CV events has been elucidated in the last decades [6]. Myocardial infarction, heart failure, arrhythmias and stroke were witnessed to increase following a CAP episode [7]. The risk is maximal during the acute phase but persists in time after the infection has abated [8]. Moreover, a higher likelihood of a poorer outcome in a patient with CAP complicated by a CV event exists when compared to one with an uncomplicated course [7]. At the population level, the practical implications of such an association are immense. The widespread use of influenza and pneumococcal vaccination is an attractive option. When considering treatment possibilities, drugs with proven efficacy in the CV arena could theoretically be initiated to modulate outcomes, both acutely but also in the context of long-term CV prevention. Definite evidence-based guidance on how to perform these interventions at a patient level is, however, lacking.

In this chapter, we provide a broadened description of the current evidence associating CAP and acute CV disease. From bench to bedside, we begin with mechanistic links of the pathophysiologic repercussion of CAP on the cardiovascular system, proceeded to an updated systematic review of and meta-analysis of observational clinical studies and end with the impact of potential interventions and future avenues of research.

6.2 Materials and Methods

6.2.1 Search Strategy

We searched Medline from inception to 1 November 2019 for articles in English, French, Spanish, and Portuguese languages evaluating the incidence of subsequent CV events after a first CAP episode (community-acquired pneumonia" AND ("complications" OR "acute coronary syndromes" OR "myocardial infarction" OR "heart failure" OR "arrhythmia" OR "atrial fibrillation" OR "stroke"). To ensure the completeness of our review, we also retrieved previously published reviews on the subject and scanned their references for any additional missed publications in the primary search.

6.2.2 Population and Outcomes

We restricted our search to adult non-immunocompromised patients with clinical and radiological evidence of CAP (as a means to improve specificity) for whom outcomes were explicitly reported in the methods section. Consecutive enrolment of patients and the quantification of CV events in the entire cohort were also prerequisites for inclusion. Studies focusing on nosocomial or health care–associated pneumonia, antibiotic efficacy trials (due to selection bias) and articles

dealing primarily with pediatric patients or patients infected with the human immunodeficiency virus were excluded.

If assessed, independent predictors for CV complications after CAP were collected and tabulated. To minimize the influence of patient heterogeneity in the estimation of effect sizes, we stratified patients according to treatment setting and clinical severity, when available. Patients initially treated in an ambulatory setting were defined as outpatients while patients admitted to a hospital on presentation were considered as inpatients. High risk patients were defined as having a pneumonia severity index (PSI) class IV or V or and/or requiring admission to the intensive care unit (ICU). Cardiovascular events were categorized as overall cardiac complications, acute coronary syndromes, new or worsening heart failure, and new or worsened arrhythmia and stroke. We collected data on all-cause mortality occurring either during hospital admission on during the first 30 to 90 days.

6.2.3 Study Selection, Data Extraction and Synthesis

After the initial search, the first author extracted all abstracts, excluded irrelevant studies and if deemed adequate, proceeded to full-text reading and data tabulation. Study flowchart is depicted in Fig. 6.1. The methodologic quality of available studies was graded according to Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) guidelines (Supplementary Table 6.S1).

6.2.4 Statistical Analysis

In contrast to a traditional meta-analysis aiming to describe an effect size related to a therapeutic intervention, a meta-analysis of proportions has the goal of obtaining a more precise estimate of the overall proportion for a certain case or event [9]. In order to achieve this, raw proportional data need to be conformed to the normal distribution by employing one of several validated statistical approaches. We chose Freeman and Tukey's double-arcsine transformation, a method which stabilizes study variance and reduces the probability of inaccurate weighting of each study when the inverse of the variance of the transformed proportion is used as a study weight [10]. To further minimize population differences between studies, patient cohorts were divided by event category, setting, and clinical severity before estimating pooled event rates using a random-effects model.

We anticipated that the identification of more studies spanning the eight-year time frame after the last published systematic review would justify an analysis by year of publication. Therefore, a meta-regression using year of publication as a moderator variable was undertaken. To predict the effect of a hypothesized moderator, a weighted linear regression model was performed, in which the effect sizes (i.e., transformed proportions of CV events) are regressed onto the moderator, and a linear equation obtained. Finally, we assessed for publication bias by funnel plot inspection [11] and formally by Egger's test [12]. In a funnel plot,

each study is inscribed around the summary effect size (a vertical line) bounded by two converging slopes defining the 95% confidence interval around it. Imprecision is decrementally displayed on the y-axis and is zero at the triangle vertex. Absence of bias would result in a perfectly symmetrical plot. All analyses were performed using R (version 3.6.1, Vienna, Austria).

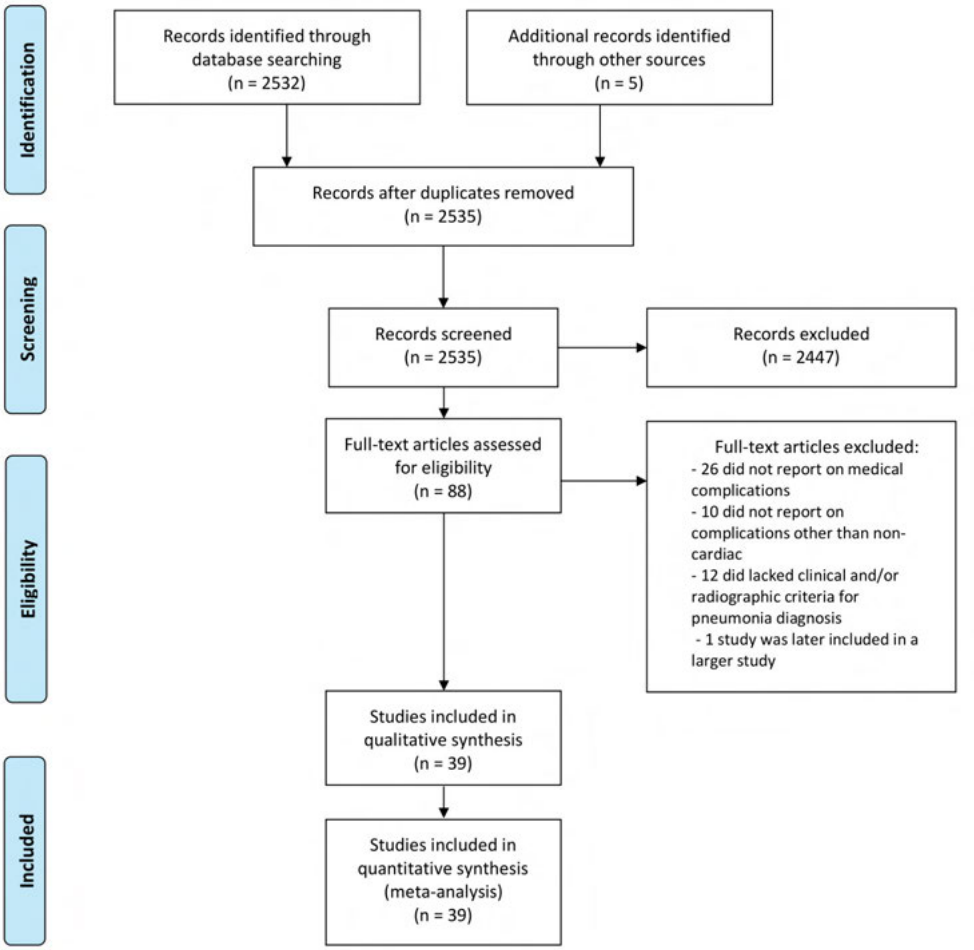


Figure 6.1 Study flowchart.

6.3 Results

6.3.1 Basic Science and Clinical Insights Associating CAP to CV Events

6.3.1.1 Coronary arteries and myocardial infarction

Theoretically, three different mechanisms prompted by pneumonia can contribute to a coronary event: (1) plaque rupture or fissuring leading to superimposed thrombus formation and cardiac troponin rise and/or fall, corresponding to

type 1 myocardial infarction (MI); (2) type 2 MI, due to an imbalance between oxygen delivery and consumption in the myocardium, together with ischemic symptoms, especially if fixed stenoses are present, and (3) "myocardial injury", when a troponin leak is detected without evidence of ischemia [13]. The most accepted and important pathway involves inflammation driven plaque instability [14]. By experimentally inducing sepsis in animal models, investigators have found post-mortem pathologic proof of atheromata growth and vascular inflammation. In mice fed with an atherogenic diet and subsequently developing provoked secondary fecal peritonitis, Kaynar et al. found increased abdominal aorta atheroma size, plaque neutrophil count and circulating proinflammatory cytokines versus a sham procedure, all persisting five months after the index intervention [15]. In humans, similarities are quite striking. In a post-mortem study, Mauriello et al. performed a detailed histologic analysis of coronary segments of sixteen patients who died after acute MI and compared them with controls who died of non-cardiac causes and had either stable angina or no CAD suspicion [16]. Significantly increased inflammatory infiltrates (macrophage and T-lymphocyte) were noticed not only in the culprit lesion but also in non-culprit vulnerable plaques and stable plaques in MI patients, suggesting underlying inflammation of the entire coronary tree even without sepsis. In another small sized study, fourteen patients who died from systemic infection (six of which with either upper or lower respiratory tract infection) were found to have more leucocytes in both plaque and adventitia than controls [17]. Inflammation that persists after the acute infectious episode could be causally linked increased mortality witnessed after hospital discharge following CAP [6].

Other proposed pathophysiologic pathways include hypercoagulability and hampered vascular tone. A significant proportion of pneumonia patients exhibited increased levels of coagulation markers in a study by Milbrandt et al., which persisted up to a week after hospital admission and were associated with increased mortality [18]. Although coronary perfusion is preserved or even increased in human septic shock [19], vasomotor disturbances resulting from changes in vasoactive mediators and capable of compromising myocardial oxygen delivery have been reported in animal [20] studies and could play a role in severe Gram positive infections.

6.3.1.2 Myocardium and heart failure

Direct myocardial involvement by pathogens has been shown for viral and bacterial myocarditis caused mainly by influenza virus, adenovirus, respiratory syncytial virus and enterovirus but also bacteria including *Streptococcus pneumoniae, Chlamydophila pneumoniae, Mycoplasma pneumoniae, Staphylococcus aureus and Legionella* spp. [7]. Molecular studies have further suggested that up to a third of CAP cases in adults result from viral infection [7]. Kotaka et al. demonstrated that influenza virus inoculation in a murine model led to cytotoxic effect in cardiomyocytes and coronary thrombosis [21]. In the human heart, necropsy studies have similarly revealed edema, sarcomeric disarray and viral particle isolation

from fatal influenza infection. Following invasive pneumococcal disease, Brown et al. disclosed for the first time how *Streptococcus pneumoniae* translocates into the myocardium of primates and patients who succumbed to CAP [22]. In their study, microlesions containing bacteria were described inside cardiomyocytes, which if treated with antibiotics led to local inflammation and scarring. The authors speculate that these foci may generate future myocardial dysfunction or cardiac arrhythmias.

6.3.1.3 Cardiac rhythm disturbances

Atrial fibrillation is the most common sustained arrhythmia encountered in clinical practice [23]. Known transient risk factors include fever, hypoxia and hemodynamic disturbances, all of which can be triggered by infections including pneumonia [24]. An increased incidence in AF as well as other specified arrhythmias following CAP is a phenomenon well described in the literature. In a study conducted in critically ill patients, sepsis and acute respiratory failure were the most powerful predictors of AF onset [25]. Proposed mechanisms include direct toxic effect on the cardiac electric system, augmented loading imposed on cardiomyocytes, inflammation, sympathetic hyperactivity, ischemia and pharmacological agents, including antibiotics. AF occurrence is associated with worse short and long term outcomes in patients with infections [26, 27], directly influencing patient management, namely the decision to initiate anticoagulation, and conditioning rhythm or rate control strategies.

6.3.2 Clinical Studies of Short Term Incident Acute CV Disease after CAP

We found 39 studies (Table 6.1, see also Supplementary Table 6.S2 for detailed study description) reporting the incidence of CV events following CAP. Retrieved studies spanned a thirty-five-year period and included mostly North American patients. The mean weighted age of patients was 72 ± 11 years and most were male (79%).

6.3.2.1 Overall cardiac complications

Twelve studies provided data on inpatients [31, 39, 40, 46–48, 54, 56, 58, 59, 64], four on low-risk inpatients [45, 55, 57, 61] and five on high-risk inpatients [35, 55, 57, 61, 63]. Pooled event rates were 13.9% (95% confidence interval (CI) 9.6–18.9), 5.7% (95% CI 3.1–9.0) and 15.6% (95% CI 6.1–28.4), respectively (Fig. 6.2).

6.3.2.2 Acute coronary syndromes

Thirteen studies reported data on inpatients [48–56, 58, 60, 61, 64], two on low-risk patients [44, 57] and high-risk [57, 63] patients and one on outpatients [54]. Pooled event rates were 4.5% (95% CI 2.9–6.5), 0.2 (95% CI 0–0.7), 1.6 (95% CI 0.1–4.9), and 0.9 (95% CI 0.5–1.8), respectively (Fig. 6.3).

Table 6.1 Characteristics of included studies

	Year	Country	Study type	Setting	*n*	CV events[a] (%)	ACS[b] (%)	Heart failure (%)	Stroke[c] (%)	Arrhythmias[d] (%)
Allen et al. [28]	1984	Zambia	Retrospective single center	Inpatients	502	—	—	—	—	0.40
Esposito et al. [29]	1984	USA	Prospective single center	Inpatients	38	—	—	7.9	—	—
Marrie et al. [30]	1989	Canada	Prospective single center	Inpatients	583	—	—	11	—	—
Ortqvist et al. [31]	1990	Sweden	Prospective, single center	Inpatients	277	13	—	—	—	—
Venkatesan et al. [32]	1990	UK	Prospective single center	Inpatients	73	—	—	—	—	11
Fine et al. [33]	1990	USA	Prospective single center	Low-risk[e] inpatients Outpatients	170	—	0.6	—	—	0.6
Woodhead et al. [34]	1992	UK	Retrospective multicenter	High-risk[f] inpatients	72	—	—	—	—	23
Leroy et al. [35]	1995	France	Retrospective single center	High-risk[f] inpatients	299	2.3	—	—	—	—
Janssens et al. [36]	1995	Switzerland	Prospective, single center	Inpatients	99	—	—	33	—	—
Musher et al. [37]	2000	USA	Prospective single center	Inpatients	100	—	—	4.0	—	—
Férnandez-Sabé et al. [38]	2003	Spain	Prospective single center	Inpatients	1474	—	—	7.2	—	—
Fine et al. [39]	2003	USA	Prospective multicenter	Inpatients	608	22	—	—	—	—

(*Continued*)

Table 6.1 (*Continued*)

	Year	Country	Study type	Setting	*n*	CV events[a] (%)	ACS[b] (%)	Heart failure (%)	Stroke[c] (%)	Arrhythmias[d] (%)
Martínez-Moragón et al. [40]	2004	Spain	Prospective single-center	Inpatients	91	5.0	—	—	—	—
Menéndez et al. [41]	2004	Spain	Prospective multicenter	Low-risk[e] inpatients	1424	—	—	8.7	—	—
Querol-Ribelles et al. [42]	2005	Spain	Prospective single-center	Low-risk[e] inpatients	459	—	—	8.6	—	—
Díaz et al. [43]	2005	Chile	Prospective Single-center	High-risk[f] inpatients	113	—	—	24	—	15
Marrie et al. [44]	2005	Canada	Prospective single center	Low-risk[e] inpatients	586	—	0.3	1.4	—	—
McAlister et al. [45]	2005	Canada	Prospective multicenter	Low-risk[e] inpatients	2471	5.9	—	—	—	—
O'Meara et al. [46]	2005	USA	Prospective multicenter	Inpatients	582	24	—	—	—	—
Musher et al. [47]	2007	USA	Retrospective single-center	Inpatients[g]	170	19	7	15	—	6
Becker et al. [48]	2007	Canada	Retrospective multicenter	Inpatients	391	17	8	12	—	3
Ramirez et al. [49]	2008	Spain	Retrospective single-center	Inpatients	500	—	5.8	—	—	—
Cabré et al. [50]	2008	Spain	Prospective single-center	Inpatients	117	—	0.9	12	—	4.4
Corrales-Medina et al. [51]	2009	USA	Retrospective single-center	Inpatients	206	—	11	—	—	—

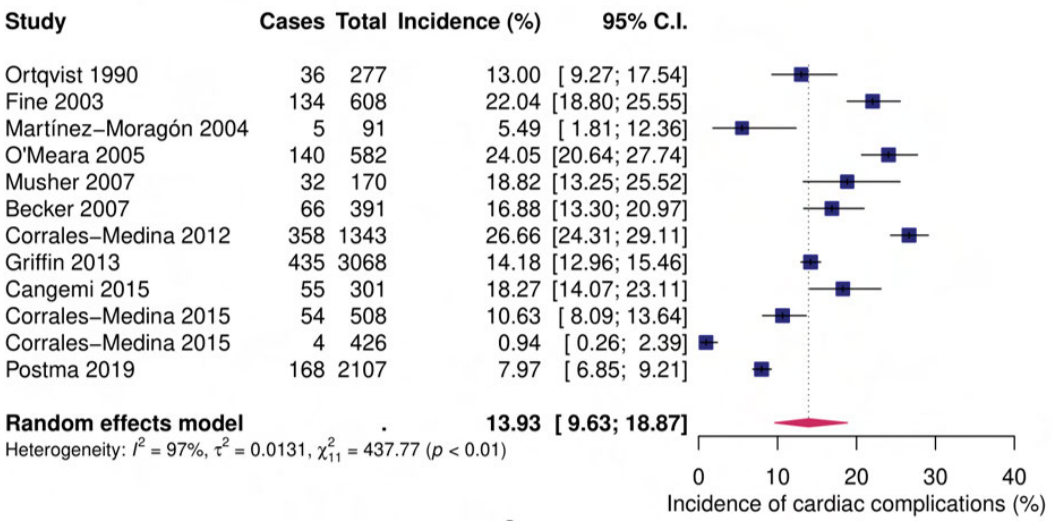
	Year	Country	Study type	Setting	*n*	CV events[a] (%)	ACS[b] (%)	Heart failure (%)	Stroke[c] (%)	Arrhythmias[d] (%)
Mandal et al. [52]	2011	Scotland	Retrospective multicenter	Inpatients	5034	—	5.0	—	2.2	9.3
Perry et al. [53]	2011	USA	Retrospective multicenter	Inpatients	50119	—	2.3	9.1	0.1	8.4
Corrales-Medina et al. [54]	2012	USA and Canada	Prospective multicenter	Inpatients Outpatients	1343 944	27 2.1	3.6 0	67 65	—	22 35
Viasus et al. [55]	2013	Spain	Prospective single center	Low-risk inpatients High-risk inpatients[f]	1621[h] 2300	3.0 11.6	0.76	3.0	—	5.1
Griffin et al. [56]	2013	13 countries	Retrospective multicenter	Inpatients	3068	14	1.3	2.1	—	3.6
Aliberti et al. [57]	2015	Italy, Switzerland	Retrospective multicenter	Inpatients	905	—	2.3	3.7	1.1	19
Cangemi et al. [58]	2015	Italy	Prospective, single center	Inpatients	301	18	11	—	—	10
Corrales-Medina et al. [59]	2015	USA	Retrospective multicenter	Inpatients	508	11	—	—	—	—
Corrales-Medina et al. [59]	2015	USA	Retrospective multicenter	Inpatients	426	0.90	—	—	—	—
Chen et al. [60]	2015	Taiwan	Single-center retrospective	Inpatients	746	—	2.3	—	—	—
Violi et al. [61]	2017	Italy, Canada	Prospective multicenter	Low-risk inpatients High-risk inpatients[f]	355[h] 827	12 41	8.4	24	0.1	9.2

(*Continued*)

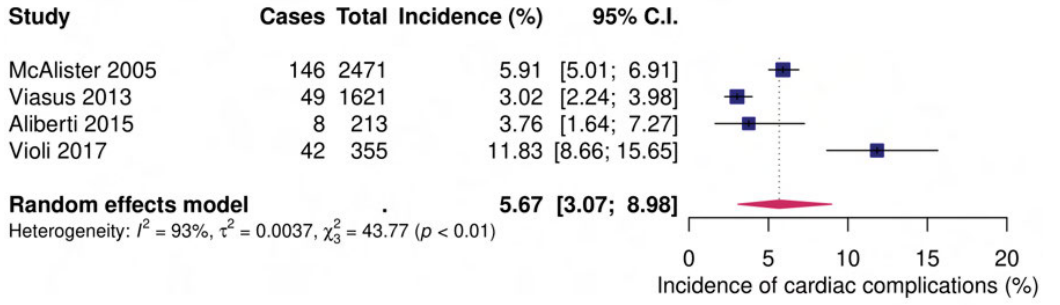
Table 6.1 (*Continued*)

	Year	Country	Study type	Setting	*n*	CV events[a] (%)	ACS[b] (%)	Heart failure (%)	Stroke[c] (%)	Arrhythmias[d] (%)
Eurich et al. [62]	2017	Canada	Prospective multicenter	Inpatients Outpatients	4988	—	—	12	—	—
Cilli et al. [63]	2018	Turkey	Retrospective multicenter	High-risk[f] inpatients	373	15	0.54	2.9	—	12
Postma et al. [64]	2019	Netherlands	Retrospective multicenter	Inpatients	2107	7.9	0.7	4.8	—	2.5
Pieralli et al. [65]	2019	Italy	Retrospective single-center	Inpatients	468	—	—	—	—	10.3
Cangemi et al. [66]	2019	Italy	Prospective single center	Inpatients	545	—	—	—	—	9.5

[a]Cardiovascular (CV) events: congestive heart failure, atrial fibrillation, severe angina or myocardial infarction or stroke [31]; acute coronary or ventricular insufficiency [35]; cardiovascular complications likely to necessitate continued hospitalization [39]; cardiac complications without further specification [40]; acute coronary syndrome and/or heart failure [45]; myocardial infarction, angina pectoris, revascularization by angioplasty/coronary artery bypass graft (CABG) or death secondary to coronary heart disease, cerebrovascular accident, congestive heart insufficiency or claudication [46]; myocardial infarction, atrial fibrillation or ventricular tachycardia or incident heart failure [47]; myocardial infarction, atrial fibrillation, congestive heart failure or stroke [48]; new or worsening heart failure, new or worsening arrhythmias or myocardial infarction [54]; new-onset or worsening cardiac arrhythmias, new-onset or worsening congestive heart failure or myocardial infarction [55]; acute pulmonary edema, new onset cardiac arrhythmia, exacerbation of a preexisting arrhythmia, or myocardial infarction [56]; acute myocardial infarction, acute cardiogenic pulmonary edema, new arrhythmia, acute worsening of a long-term arrhythmia, cerebrovascular accident or pulmonary embolism [57];cardiovascular death, non-fatal myocardial infarction or stroke [58]; non-ST elevation myocardial infarction or ST elevation myocardial infarction, stroke, new episode of atrial fibrillation or deep venous thrombosis and/or pulmonary embolism, new or worsening HF or cardiovascular death [61] new onset or worsening arrhythmia, new onset or worsening heart failure or myocardial infarction [63]: new or worsening arrhythmia, heart failure or myocardial ischemia [64]. [b]Acute coronary syndromes (ACS): myocardial infarction [33, 37, 39, 47–49, 53, 54, 55–58, 60, 61, 63, 64]; unstable angina [44]; acute coronary syndrome [50, 51]; acute coronary syndrome or ST segment elevation myocardial infarction [52]. [c]Stroke: new-onset neurological deficit [53]; unspecified stroke [52, 61]; cerebrovascular accident [57]. [d]Arrhythmias: incident atrial fibrillation [28, 32, 33, 48–50, 52–58, 61, 64–66]; cardiac dysrhythmias/arrhythmias [34, 43]; atrial flutter or fibrillation, and ventricular tachycardia, but excluding terminal arrhythmias [47]. [e]Inpatients without severe vital signs or metabolic abnormalities, altered mental status, suppurative complications or coexisting medical conditions requiring hospitalization [33]; inpatients who survived the first 48 h of hospitalization [41], inpatients not initially admitted to the intensive care unit [42, 45]; inpatients with pneumonia severity index (PSI) risk classes I–II [44]. [f]Inpatients admitted to the intensive care unit (ICU). [g]For ACS, patients from Musher et al. (2007) [47] were included in Corrales-Medina et al. (2009) [51]. [h]Data available for low-risk or high-risk patients if overall cardiac events are considered.



(A)



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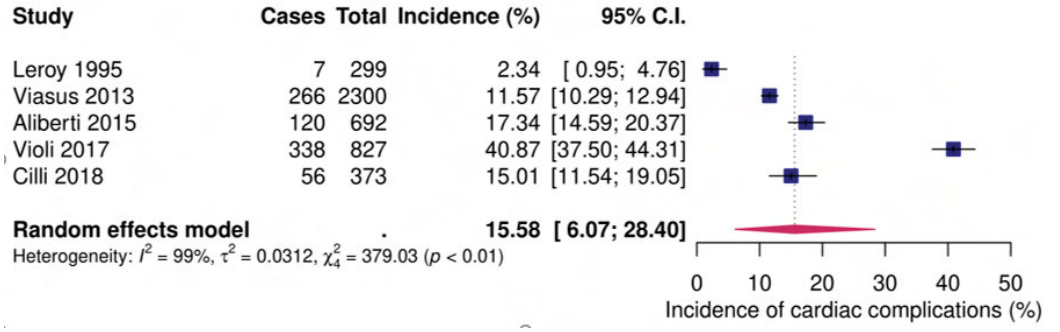

(C)

Figure 6.2 Forest plots of incident overall cardiac complications after community-acquired pneumonia. (**A**) Inpatients (**B**) Low-risk inpatients. (**C**) High-risk inpatients. CI: confidence interval.

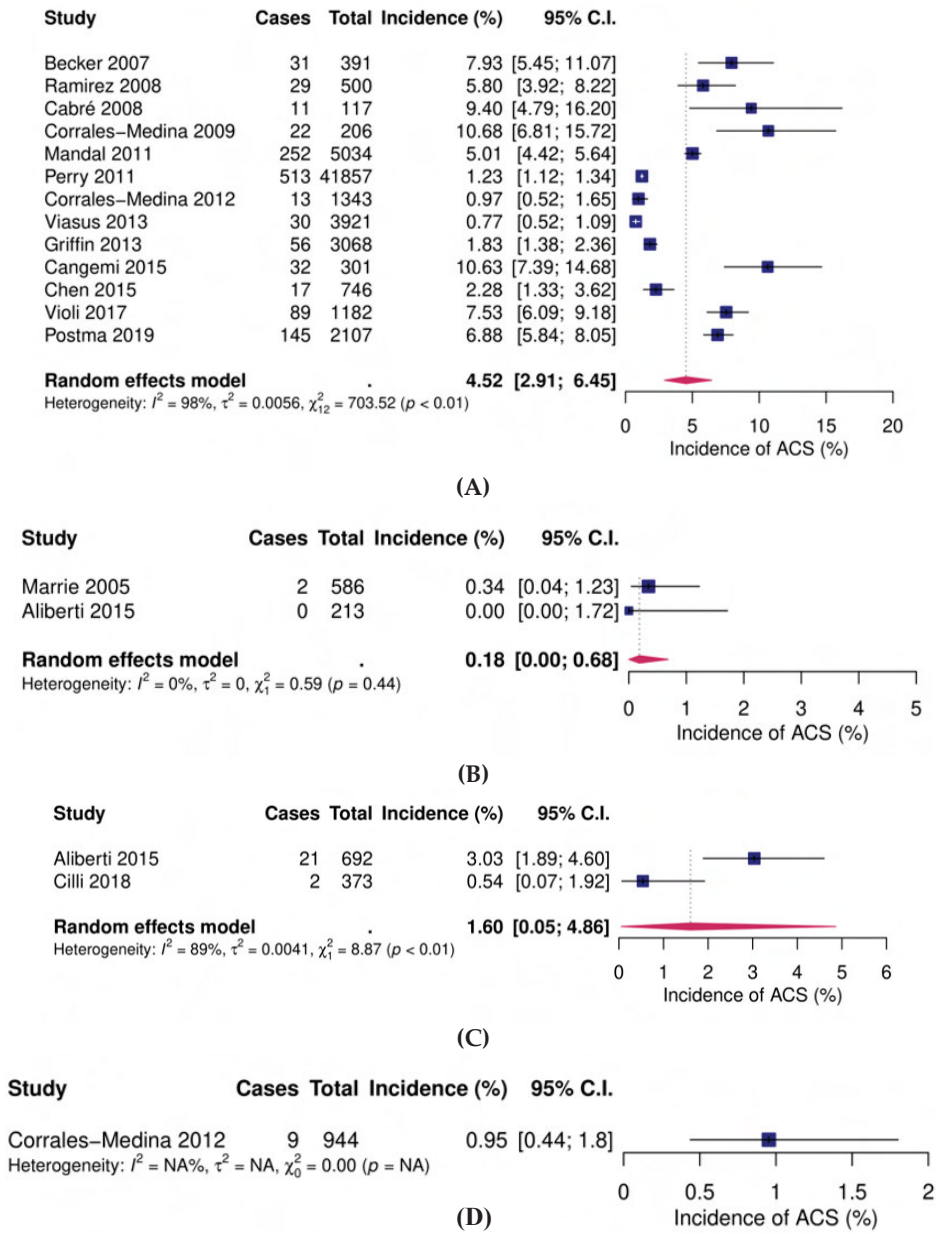

Figure 6.3 Forest plots of incident acute coronary syndromes (ACS) after community-acquired pneumonia. (**A**) Inpatients (**B**) Low-risk inpatients (**C**) High-risk inpatients (**D**) Outpatients. CI: confidence interval.

6.3.2.3 Heart failure

Sixteen studies included inpatients [29, 30, 36–38, 47, 48, 50, 53–57, 61, 62, 64], three focused on low-risk patients [41, 42, 44], two focused on high-risk patients [43, 63], and two on outpatients [54, 62]. Pooled event rates were 9.2%

(95% CI 6.7–12.2), 5.6% (95% CI 1.5–11.9), 11.1% (95% CI% 0–39), and 1.0% (95% CI 0.6–1.6), respectively (Fig. 6.4).

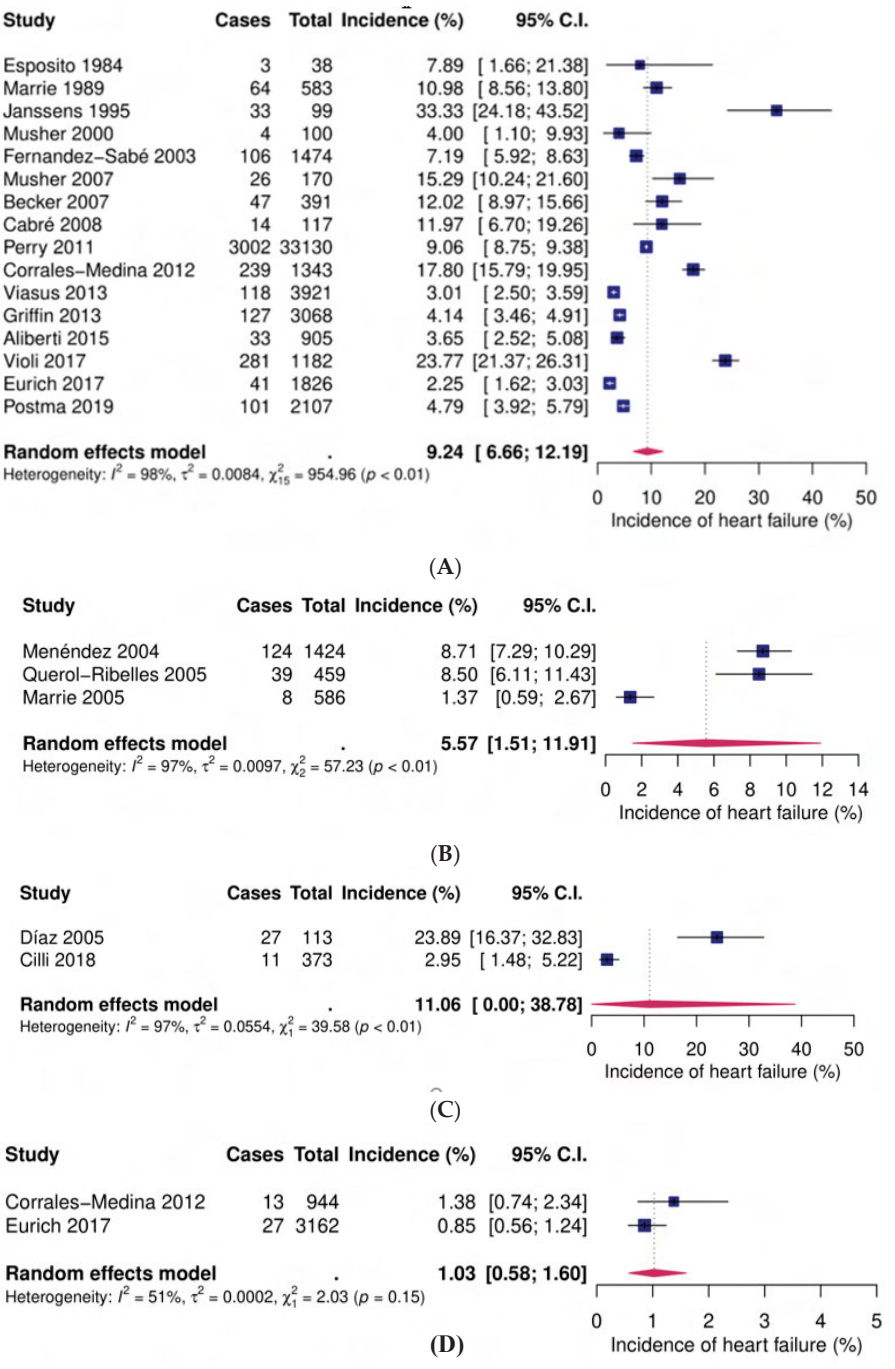

Figure 6.4 Forest plots of incident heart failure after community-acquired pneumonia. (**A**) Inpatients (**B**) Low-risk inpatients (**C**) High-risk patients (**D**) Outpatients. CI: confidence interval.

6.3.2.4 Arrhythmias

Incident arrhythmias were mentioned in twenty studies. Seventeen studies analysed inpatients [28, 32, 34, 47, 48, 50, 52–58, 61, 64–66], two focused on high-risk inpatients [43, 63] and one on outpatients [54]. Pooled event rates were 7.2% (95% CI 5.6–9.0), 12.7% (95% CI 9.8–15.8), and 0.7% (95% CI 0.3–1.5), respectively (Fig. 6.5).

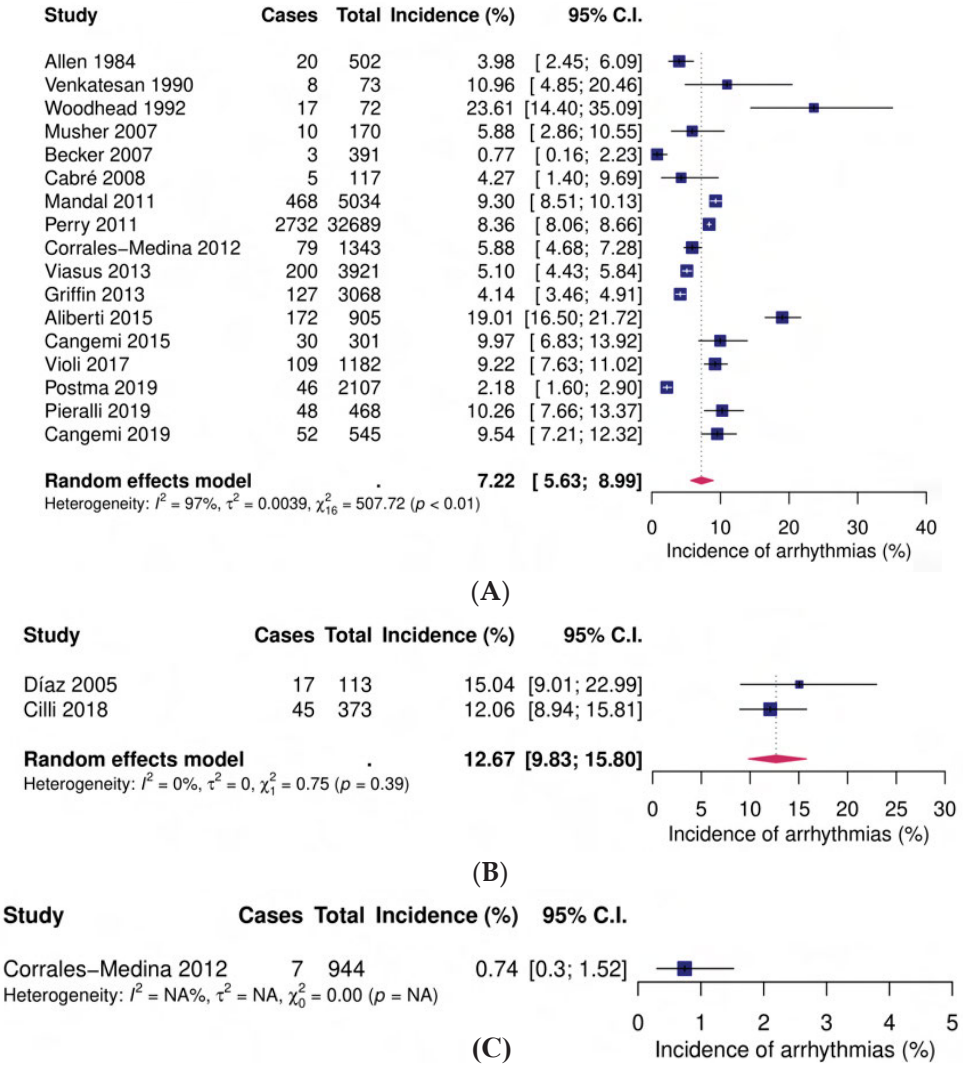

Figure 6.5 Forest plots of incident arrhythmias after community-acquired pneumonia. (**A**) Inpatients (**B**) High-risk inpatients (**C**) Outpatients. CI: confidence interval.

6.3.2.5 Stroke

New-onset stroke following CAP was reported in four studies [52, 57, 61, 63], all on inpatients. Pooled event rate was 1.7% (95% CI 1.0–2.6; Fig. 6.6).

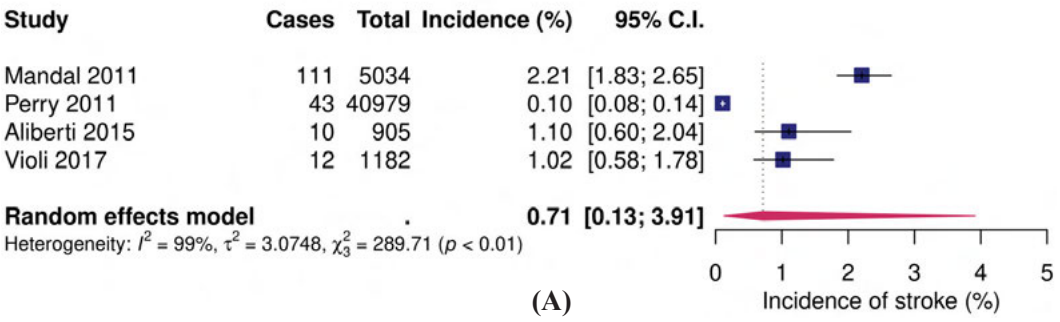

Figure 6.6 Forest plot of incident stroke of inpatients after community-acquired pneumonia. CI: confidence interval.

6.3.2.6 Meta-regression analysis and publication bias assessment

Meta-regression analysis (Fig. 6.7) by study year revealed a decrease both in the overall proportion of cardiac complications and also individually when each complication was considered separately, more notably for incident heart failure. Funnel plot analysis and Egger's test showed significant asymmetry for overall cardiac complications only (z = −3.7562, p = 0.0002; Supplementary File 6.S1).

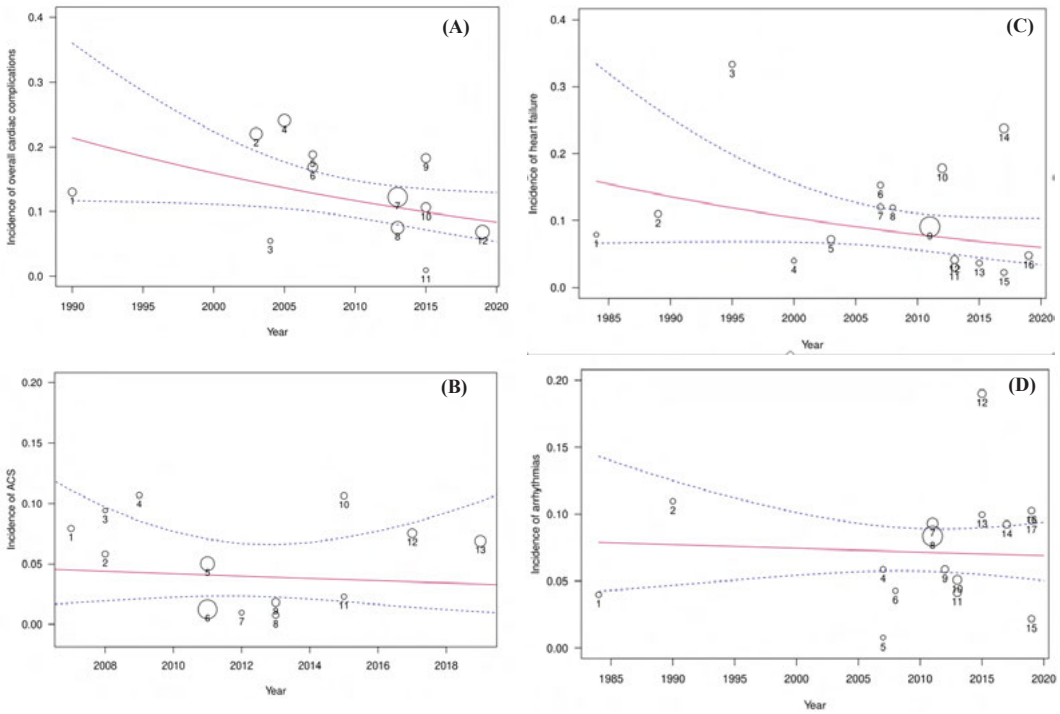

Figure 6.7 Meta-regression for (**A**) overall cardiac complications, (**B**) acute coronary syndromes ACS, (**C**) heart failure and (**D**) arrhythmias after community-acquired pneumonia, moderated by study year. Circle size is proportional to study sample size.

6.3.3 Risk Factors and Impact of Cardiac Complications on CAP Outcomes

Risk factors for the occurrence of CV events were reported in thirteen studies [51–58, 61, 63–66]. After adjustment for covariates, several clinical, microbiologic, laboratory, and imaging features were found to be independent predictors of an adverse CV event (Table 6.2).

Table 6.2 Included studies presenting independent predictors of cardiovascular (CV) events after community-acquired pneumonia (CAP)

Author	Year	*n* with event	Outcome	Independent predictors
Corrales-Medina et al. [51]	2009	206	ACS	Age Congestive heart failure
Mandal et al. [52]	2011	252	ACS	Age ≥ 65 Previous MI COPD Chronic kidney disease
		468	Arrhythmias	Age Previous MI Diabetes
		111	Stroke	Prior stroke COPD
Perry et al. [53]	2011	2002	CHF	Age Admission to ICU Previous MI COPD Diabetes Chronic kidney disease Cancer
		2732	Arrhythmias	Age Admission to ICU
Corrales-Medina et al. [54]	2012	378	Overall cardiac complications	Age Nursing home Hypertension Previous CAD Previous arrhythmias Previous CHF RR ≥ 30/min pH < 7.35 BUN ≥ 30 mg/dL Sodium < 130 mmol/L Hematocrit < 30% Pleural effusion Inpatient
Viasus et al. [55]	2013	315	Overall cardiac complications	Age ≥ 65 Chronic heart disease Septic shock Tachycardia Albumin < 3 g/dL Multilobar pneumonia Streptococcal pneumonia

Author	Year	*n* with event	Outcome	Independent predictors
Griffin et al. [56]	2013	376	Overall cardiac complications	Hyperlipidemia Statin therapy[1] Staphylococcus aureus Klebsiella pneumoniae PSI
Aliberti et al. [57]	2015	21	ACS	Female sex Severe sepsis Liver disease
Cangemi et al. [58]	2015	55	Overall cardiac complications	Age Hypertension Diabetes Baseline troponin
Violi et al. [61]	2017	308	Overall cardiac complications	Age CHF PSI
Cilli et al. [63]	2018	56	Overall cardiac complications	Age Hypoalbuminemia Diuretic Vasopressor Haloperidol
Postma et al. [64]	2019	2107	Overall cardiac complications	Erythromycin use
Pieralli et al. [65]	2019	468	Atrial fibrillation	CURB-65 > 2 CHA_2DS_2-VASc > 3
Cangemi et al. [66]	2019	545	Atrial fibrillation	Prior paroxysmal AF, Enlarged LAAi Left ventricular hypertrophy

[1]Protective effect. ACS: acute coronary syndrome.

Abbreviations: AF, atrial fibrillation; BUN, blood urea nitrogen; CAD, coronary artery disease; CHA_2DS_2-VASc, congestive heart failure, hypertension, age-doubled, diabetes, stroke-doubled, vascular disease, age, sex-category; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; CURB-65, confusion, urea, respiratory rate, blood urea nitrogen, age > 65; ICU, intensive care unit; LAAi, left atrial area index; MI, myocardial infarction; PSI, pneumonia severity index; RR, respiratory rate.

Seven studies evaluated whether CV events were independently associated with mortality in CAP patients [52, 54, 55, 57, 58, 61, 63]. Multivariate analysis and propensity score matching were used to correct for confounders when assessing the impact of composite and individual CV events. Both overall and individual cardiac complications were associated with increased mortality after CAP (Table 6.3).

6.3.4 Long Term Outcomes after CAP

Soon after the onset of infection, a plateau of maximum risk for CV events is reached and persists for the following 30 days [67]. Afterwards, it does not fall abruptly but gradually diminishes, conferring a long term increased risk that goes well beyond the acute phase [67]. By retrospectively analysing data from two large

observational cohorts of patients without known CV disease, Corrales-Medina et al. have estimated almost a doubling of CV events during a ten-year period when compared to controls [59].

Table 6.3 Association of CV events and mortality after CAP

Study	Year	Event	Mortality	Measure of risk
Mandal et al. [52]	2001	Stroke	90-day	OR 1.79 (1.51–2.12), $p < 0.0001$
		MI		OR 2.93 (1.60–2.33), $p < 0.0001$
		AF		OR 1.39 (1.65–2.19), $p < 0.0001$
Corrales-Medina et al. [54]	2012	Overall cardiac complications	30-day	OR 1.6 (1.04–2.5), $p < 0.001$
Viasus et al. [55]	2013	Overall cardiac complications	30-day	OR 2.18 (1.38–3.42)
Aliberti et al. [57]	2015	ACS	In-hospital	OR 3.57 (1.32–9.69), $p = 0.02$
		Other events		OR 2.63 (1.43–4.84), p = NS
Cangemi et al. [58]	2015	Overall cardiac complications	6-60 months	OR 1.759 (1.099–2.816), $p = 0.019$
Violi et al. [61]	2017	Overall cardiac complications	30-day	HR 5.49, $p < 0.001$
Cilli et al. [63]	2018	Overall cardiac complications	In-hospital	OR 2.18 (1.03–4.61), $p = 0.04$
			90-day	NS

Abbreviations: ACS, acute coronary syndrome; AF, atrial fibrillation; MI, myocardial infarction; NS, not significant; OR, odds-ratio; HR, hazard-ratio.

6.3.5 What Is the Role of Pharmacological Therapies?

Multiple investigators aimed to establish a role for CV and immunomodulating drug therapies in CAP patients. Below, we enumerate pharmacological classes and summarize the available evidence for their potential use in CAP.

6.3.5.1 Antiplatelet drugs

Different receptor pathways serve as potential targets for antiplatelet agents: (1) inhibitors of thromboxane A2 production (aspirin, triflusal), (2) antagonists of adenosine diphosphate (ADP)-activated $P2Y_{12}$ receptors (ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor), (3) antagonists of thrombin-activated proteinase activated receptor 1 (vorapaxar), and (4) GPIIb/IIIa inhibitors (abciximab, tirofiban, eptifibatide). Conflicting evidence regarding the role of aspirin in primary prevention led to the publication of two large scale randomized clinical trials who failed to demonstrate the benefit of aspirin in unselected patients without a previous CV event [68, 69]. To make matters worse, a signal toward increased bleeding was detected, leaving no compelling evidence for the chronic blocking of platelet cyclooxygenase beyond secondary prevention. In CAP patients, two prospective propensity matched studies showed that aspirin may be associated with decreased CV events [70, 71]. Recently, ticagrelor, a reversible $P2Y_{12}$ platelet ADP receptor inhibitor, showed to decrease leukocyte adherent

platelets and inflammatory markers in a small double-blinded trial in CAP [72]. Patients randomized to ticagrelor had faster amelioration of oxygenation deficit and a trend towards better progression in lung function test results. Although using an antiplatelet agent in the acute setting might seem appealing based on heightened inflammation during CAP, this hypothesis remains to be adequately tested in a powered randomized trial and cannot therefore be recommended.

6.3.5.2 Statins and other lipid lowering agents

Impeding cholesterol synthesis is perhaps the most effective drug intervention to reduce the incidence of de novo and recurring CV events. Data from more than 170,000 patients have demonstrated a significant impact of statin therapy in all-cause mortality, CV mortality and CV events, with a reassuring safety profile, in both primary and secondary prevention settings [73]. *In vitro* and observational studies led to the belief that reducing inflammation through statins non-lipid lowering effects could translate into a similar benefit in sepsis, an hypothesis not confirmed in subsequent randomized trials [74]. Specifically, trials that tested statins in ventilator associated pneumonia [75] or acute respiratory distress syndrome [76] have not proved to be better than placebo, leaving no convincing role for their use in this setting. The need for a prolonged exposure to statins in order to obtain clinical benefit might be an explanation to justify failure to improve outcomes. Recently, two other lipid lowering drugs (evolocumab, alirocumab) acting through inhibition of PCSK9 (proproteine convertase subtilisin/kexin type 9, a protein that targets low-density lipoprotein receptors for degradation in the liver) achieved event reduction on top of statin therapy [77, 78]. There are currently no published human studies exploring a potential effect of this new class of molecules on infection related outcomes. In an animal model, Berger et al. did not show a reduction of lipopolysaccharide induced mortality with the administration of anti-PCSK9 antibodies [79].

6.3.5.3 Beta-blockers, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB)

Given their established role in CV disease treatment, investigators have explored a possible benefit of other cardioprotective drugs in CAP patients. Wu et al. employed multilevel regression modeling to examine the association between CV drug classes and either mortality or CV events [80]. They found that while ACEi and ARBs were associated with decreased mortality, there was no significant association with decreased CV events. These results suggest that this decreased mortality is unlikely due to their potential cardioprotective effects. Higher quality studies are needed to confirm or refute this unexpected benefit.

6.3.5.4 Corticosteroids

Corticosteroids have been tested in a wide spectrum of infectious diseases, yielding controversial results. In CAP, one large patient level meta-analysis [81] pointed to a mortality benefit (34% relative risk reduction), while in another meta-analysis [82] a very similar absolute reduction reached statistical significance

with one of two random-effects models. In both studies, shorter length of stay (~1 day) and more hyperglycemic episodes were noted. In the analysis of Briel et al., steroids were associated with an increased incidence of CAP related readmissions for various reasons including CV events. Recently, Cangemi et al. found that using corticosteroids for CAP halved the incident of intra-hospital MI, albeit not reducing either all-cause or CV mortality [83].

6.3.5.5 Novel drugs

Canakinumab, a fully human monoclonal antibody targeting 1L-ß interleukin demonstrated a reduction in recurrent CV events in a randomized clinical trial against placebo [84]. Conversely, the drug was associated with a small but significant increase of fatal infection or sepsis. Pneumonia rates were similar in both treatment arms.

6.3.6 Vaccination

Observational studies and secondary post-hoc analysis of large trials in CV disease have hinted towards a prognostic impact of influenza and pneumococcal vaccination, particularly in coronary artery disease related outcomes [85]. In a meta-analysis of five randomized trials, influenza vaccination was associated with a 36% relative risk reduction in the incidence of major adverse cardiovascular events during a 12-month period, with no influence in mortality [86]. Patients who had a recent ACS seemed to derive the largest benefit (55% relative risk reduction). Inferior quality evidence exists for pneumococcal vaccination. A meta-analysis of observational studies by Ren et al. revealed a 23% reduction in ACS in patients aged 65 or above [87]. The benefit was lost when all patients were considered and was not extensible to other vascular territories. In the heart failure population, a signal towards a reduction in a composite endpoint of CV mortality and HF hospitalization in propensity matched influenza-vaccinated patients from a large trial was identified [88]. There are presently no studies specifically addressing the effect of pneumococcal immunization in HF patients.

6.3.7 Effects of Antibiotics on the Cardiovascular System

While decisive to improve outcomes in patients with CAP, antibiotics are not free from toxicity, which may include the CV system. Although possessing useful anti-inflammatory properties, macrolides have been ascribed an increased risk in arrhythmic events and sudden cardiac death (SCD) through QT interval prolongation and polymorphic ventricular tachycardia facilitation. For azithromycin, the absolute risk was estimated to be an additional 47 deaths per one million five-day courses of therapy (all indications considered) when compared to amoxicillin [89]. In a smaller propensity score matching study, Schembri et al. also found an independent association between clarithromycin use for CAP and CV events during a one-year follow-up (HR 1.68, 95% CI 1.18–2.38), which was not extensible to increased mortality [90].

6.4 Discussion

By analysing epidemiologic data from more than a quarter billion individuals, Collins found an excess all-cause mortality accompanying a pairwise increase in influenza and pneumonia related fatalities during successive early 20th century infection outbreaks [91]. After this seminal report, a burgeoning number of observational studies and reviews has strengthened this association [92–96]. Our updated systematic review and meta-analysis, encompassing 92,188 patients, the overwhelming majority (95%) treated as inpatients, further emphasizes the burden of incident CV disease following CAP. After pooling individual study rates, we found an incidence of overall cardiac complications of 13.9%. The most frequent cardiac complication in inpatients who were not stratified by severity was de novo or worsening heart failure in 9.2% of patients, 7.2% new-onset or worsening arrhythmias, and ACS in 4.5%. Stroke was the least common CV event in CAP patients.

The present review accrues data from fourteen more additional studies (~75,000 patients) to the last review by Corrales-Medina et al. [97], allowing us to perform a supplementary meta-regression moderated by study year. Globally and individually, the incidence of acute CV events following CAP seems to be decreasing, albeit slightly. These findings seem to be in line with reported rates of CV events in the non-CAP population, with the exception of atrial fibrillation. Yearly trends in acute myocardial infarction show that hospital admission is decreasing in both the US and in Europe [5, 98]. This may reflect the improved utilization of protective CV medication in the context of better primary care based prevention including pneumococcal and influenza immunization, along with improved secondary prevention after ACS. For heart failure, statistics indicate that the incidence is diminishing, at the expense of increased prevalence, which can be attributed to demographic shifts, improved quality of care, medical therapy and handling of comorbidities [99]. Finally, regarding atrial fibrillation, data published by Schnabel et al. suggest that both its incidence and prevalence are increasing [100]. In our study, the influence of considering all arrhythmias might have contributed to this apparent discrepancy.

After incorporating more recent studies, the list of independent predictors for cardiac complications is now longer and more complex. The range of predisposing factors includes host (CV and non-CV comorbidities) and pathogen-related features. Whilst the baseline comorbidities may share the same approach and in a way merely denote a sicker patient, different microbes may pose an opportunity for influencing patient outcomes by improving time to diagnosis and starting pathogen specific interventions. For pneumococcal pneumonia, bacterial exotoxin pneumolysin has been identified as key effector of cell and organ damage in murine models [101, 102] and could theoretically become a target for macrolides (or macrolide-like antibiotics), statins or cholesterol rich liposomes [103]. If influenza is being considered, prompt detection may allow the early initiation of neuraminidase inhibitors. Not surprisingly, if a CV event supervenes after CAP, patients will likely fare worse. Our aggregated data from eight studies suggests an up to five-fold increase in mortality, mostly for compiled

cardiac complications but also for individual events. Despite its observational nature, this signal is consistent between studies and possesses biological credibility, anticipating the likely presence of a true effect.

Notwithstanding the strengths provided by the increased number of studies included and a clearer view on the temporal trends in CV events after CAP in our updated review and meta-analysis, some limitations have to be acknowledged. Population heterogeneity, different causal pathogens, asymmetries in study design and imprecise event definitions may be responsible for the observed between study variance and wide confidence intervals around the measured effect, reducing the accuracy estimates of the real effect. Funnel plot analysis is in agreement with such wide dispersion of data. For heart failure, the interpretation of results is confounded by the fact that both diseases are simultaneously risk factors and consequences of each other. Finally, we chose not to perform a meta-regression of stroke due to the paucity of data on acute cerebrovascular events after CAP.

6.5 Future Directions

Despite the apparent reduction in the event rate, the absolute burden of CV disease means there is clearly room for improvement. Hence, from a health system perspective, CV morbidity and mortality after CAP highlight the pressing need for (1) acquiring a better understanding of the pathophysiologic mediators that lead to acute CV events, (2) conducting well-designed randomized clinical trials to assess the effect of targeted drug interventions (e.g., statins) on acute CV outcomes in CAP patients, and (3) implementing a risk stratification model to ameliorate the long-term prognosis of these patients. Finally, and despite common features shared by myocardial infarction, heart failure, arrhythmias, and stroke, gaining deeper insight into the interaction between each event and CAP could prove useful in tailoring preventative and therapeutic strategies at a patient level.

Abbreviations

ACEi:	angiotensin-converting enzyme inhibitors
ACS:	acute coronary syndromes
ADP:	adenosine diphosphate
AF:	atrial fibrillation
ARB:	angiotensin receptor blockers
BUN:	blood urea nitrogen
CABG:	coronary artery bypass graft
CAD:	coronary artery disease
CAP:	community-acquired pneumonia
CHA_2DS_2-VASc:	congestive heart failure, hypertension, age-doubled, diabetes, stroke-doubled, vascular disease, age, sex-category
CHF:	congestive heart failure
COPD:	chronic obstructive pulmonary disease

CURB-65:	confusion, urea, respiratory rate, blood urea nitrogen, age > 65
CV:	cardiovascular
ICU:	intensive care unit
LAAi:	left atrial area index
MI:	myocardial infarction
PICOS:	participants, interventions, comparisons, outcomes, and study design
PSI:	pneumonia severity index
PRISMA:	Preferred Reporting Items for Systematic reviews and Meta-analyses
RR:	respiratory rate
SCD:	sudden cardiac death

Supplementary Materials

The following are available online at: https://www.mdpi.com/2077-0383/9/2/414/s1 Table S1: PRISMA checklist; Table S2: Detailed characteristics of included studies; File S1: Funnel plot and Egger's test for included studies of (a) overall cardiac complications, (b) ACS, (c) heart failure, (d) arrhythmias and (e) stroke after CAP.

Disclosures and Conflict of Interest

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Chapter 7

Risk Factors for Recurrent Arterial Ischemic Stroke in Children and Young Adults

Beata Sarecka-Hujar, PhD, DSc,[a] and Ilona Kopyta, PhD, DSc[b]

[a]*Department of Basic Biomedical Science, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, Sosnowiec, Poland*
[b]*Department of Paediatric Neurology, School of Medicine in Katowice, Medical University of Silesia in Katowice, Katowice, Poland*

beatasarecka@poczta.onet.pl, bsarecka-hujar@sum.edu.pl

Keywords: acetylsalicylic acid (ASA), antithrombotic treatment (AT), arterial ischemic stroke (AIS), arteriopathies, atherothrombosis, cardiac embolism, cardioembolic stroke, cervical artery dissection, childhood stroke, corticosteroids and antithrombotics (CAT), diabetes mellitus, dyslipidemia, encephaloduroarteriosynangiosis (EDAS), focal cerebral arteriopathy of childhood (FCA), genetic polymorphisms, genetic risk factors, hazard ratio (HR), hemorrhagic stroke and cerebral sinuvenous thrombosis (CSVT), hypercholesterolemia, hypertension, methylenetetrahydrofolate reductase (*MTHFR*), mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), Moyamoya disease, novel oral anticoagulants (NOAC), pediatric stroke, plial synangiosis, prothrombotic states, recurrence, recurrent stroke, sickle cell disease (SCD), silent cerebral ischemia (SCI), small vessel disease, thrombophilia, transient ischemic attack (TIA), vasculopathy

7.1 Introduction

Arterial ischemic stroke occurring in children and young adults is a serious medical problem. Taking into account the fact that children and young adults who survive AIS will live longer than older stroke patients, it is important to optimize the medical management for these patients and properly recognize impairments in cognition and mood, because they can be significant barriers in patients' independent life. The incidence rate for pediatric AIS is about 1.2 to 7.9/100,000 children per year [1, 2]. The differences result from ethnic and genetic variability, as well as the age range of the populations examined.

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Most researchers analyzing pediatric AIS exclude newborns. Therefore, the age of pediatric patients with AIS ranges from 29 days of life to 18 years [3–6]. In a group of young adults, the prevalence of AIS varies from 3.4 to 11.3/100,000 persons per year [7, 8]. On the other hand, Groppo et al. [9] estimated the mean annual incidence rate of first-ever stroke in young adults to be 12.1 cases per 100,000 person-years in an Italian population from Ferrara. It was demonstrated that in a group of adult patients with AIS, 10% to 15% of all strokes were experienced by young adults [10, 11]. The data also indicate different percentages of stroke frequency in young adults and adolescents compared to all strokes; a study performed in the USA showed that 5% of all strokes occurred in a young adult population aged between 18 and 44 years [12]. A particularly high prevalence of AIS in young adults was previously observed in developing countries. However, the results were based on a small number of young patients with AIS [13].

The wide range of stroke incidence in young adults may result from the heterogeneity of individual patient populations, as well as contradictions in defining a person as a "young adult". The available data may be confusing in terms of this matter. In the literature, a person between the age of 18 and 45 is considered to be a young adult. The upper age limit has been extended to 50 years by some authors [14, 15]. In turn, Kissela et al. [16] considered people under the age of 54 as younger adults. A similar upper age limit of 55 years was adopted by Ferro et al. [17]. Surprisingly, all stroke patients aged 15 to 60 with radiologically confirmed AIS were recruited as part of The Norwegian Stroke in the Young Study [18]. In turn, according to Prasal and Singhal, studies on stroke in young people should consider cases for those aged 15–49 years (<50 years) [19] since AIS risk factors for people older than 50 years are usually similar to those present in the elderly. As for the lower age limit, 15-year-old patients are often analyzed together with pediatric patients.

The etiologic mechanisms of AIS occurring in adulthood and at a developmental age differ. Similarity may be found in the case of a significant predominance of the male sex, both in pediatric patients with AIS and in young adults with stroke [20–22]. However, some discrepancies have also been noted in this respect as Rasura et al. [23] observed higher incidence rates for stroke in young females than in young males (8.4 vs. 7.6) among stroke patients from Rome. On the other hand, a reliable age-related analysis of 25 studies concerning the incidence of AIS in the young proved the predominance of the male sex in the presence of stroke [24]. The authors analyzed the following age groups: 0–14 years, 15–24 years, 25–34 years, and 35–44 years, obtaining the incidence rates of AIS ranging from 0.99 to 30.66 for men and from 0.73 to 23.99 for women, depending on age. The most important risk factors for AIS identified in children are arteriopathies, and congenital and acquired cardiac defects leading to cardioembolic stroke, as well as thrombophilia and prothrombotic states [25–27]. In turn, the etiologic factors for AIS in young adults include, among others, cardiac embolism, cervical

artery dissection, atherothrombosis, and small vessel disease, whereas in almost 30% of cases, the cause of stroke is undetermined [28].

Despite this, the neurological consequences of AIS, including post-stroke seizures, motor disability, and recurrence of the disease, are detrimental for both age populations. Additionally, they involve huge costs related to many years of care, rehabilitation, and treatment.

According to the latest definition provided by deVeber et al. [29], recurrent stroke is defined as clinically symptomatic AIS events manifested as acute focal neurological deficits with infarction in a vascular distribution in neuroimaging, which began more than 24 h following the onset of the first stroke. The recurrence of stroke was demonstrated as a factor increasing the risk of mortality in patients. Aarnio et al. [30] observed particularly high mortality among stroke patients who experienced a recurrence of the disease. In the analyzed group of patients, stroke recurrence was the most significant risk factor for mortality after first-ever AIS, with a hazard ratio (HR) equal to 16.68 [30]. Since the etiology of AIS is multifactorial, many risk factors may also be related to the appearance of particular outcomes in both children and adults. Nevertheless, patients who suffer from AIS and have no risk factors for the disease are equally frequent. The data regarding the risk factors for AIS recurrence are not common and a number of them are retrospective. However, understanding the predictors (clinical, metabolic, or genetic) for the occurrence of subsequent ischemic stroke may be helpful in building strategies for secondary stroke prevention.

The aim of the present literature review was to discuss and compare the available data regarding the frequency of AIS recurrence in pediatric patients and in groups of young adults, as well as to assess the possible risk factors for recurrent stroke in children and young adults. We also followed data on the secondary prevention of AIS recurrence, both in children and young adults.

7.2 Review Methodology

We searched PubMed, Scopus, and Embase using combinations of the following keywords: “arterial ischemic stroke”, “ischemic stroke”, “stroke”, “recurrent”, “stroke recurrence”, “children”, “pediatric”, and “young adults” (last search early November 2019). As various definitions of “young adults” are available in the literature, we eventually took into account the articles analyzing adult patients younger than 55 years old and pediatric populations aged >one month to ≤18 years. In the present literature review, we included the results analyzing patients suffering from arterial ischemic stroke. For some data on AIS, its recurrence was discussed together with TIA, so we also included the papers. In this chapter, we have excluded data on hemorrhagic stroke and cerebral sinuvenous thrombosis (CSVT), as well as perinatal and neonatal stroke. Finally, we discussed the results which we believed were the most interesting or relevant.

7.3 Prevalence of AIS Recurrence

7.3.1 In a Pediatric Population (Aged >1 Month to ≤18 Years)

Stroke and transient ischemic attack (TIA) recurrence occurs in a pediatric population with different frequencies, mainly dependent on the kind and number of AIS risk factors found in the patient [25, 31]. AIS recurrence is another reason for severe neurological deficits increasing the ones caused by the first brain ischemia and mortality [25, 32]. One of the major obstacles to the proper estimation of the frequency of recurrent AIS is the length of the follow-up period, which can vary significantly among studies.

The lowest frequency (2.7%) of recurrent AIS in children was reported by Steinlin et al. [33], who performed a study of patients from different locations situated far from each other (Switzerland and Australia). In addition, the number of patients was rather low. Per et al. reported a 7% frequency of AIS recurrence, with a median time of 6 months (the distribution interval between the recurrent episodes of AIS was 1–36 months) [34]. This level of prevalence was confirmed by Sultan et al. in a multicenter study including pediatric patients with stroke from 72 sites in 20 countries [35]. In this study, recurrent events comprised symptomatic AIS, silent AIS, or TIA [35]. A study based on pediatric patients from the USA demonstrated a 10.3% stroke recurrence, including 4% for anterior circulation AIS and 19% for posterior circulation AIS [36]. Almost 91% of these patients experienced recurrence within the first 6 months after AIS, whereas 45.5% of cases recurred within the first month [36]. A higher prevalence of recurrent stroke was noted by deVeber et al. [29] and Sfaihi et al. [37], namely 17.9% and 18% from 1 day to 136 months after the first stroke, respectively. In a group of 84 AIS children [38], the median interval to recurrence was 2.3 months and in most observed patients (77%), recurrence occurred within the first 6 months after stroke. Within 5 years of follow-up, AIS recurred in 13 pediatric patients (15.5%), whereas recurrence including TIA was present in 29% of patients (24 out of 84 children) [38].

In a study by Fullerton et al. [39], 42 out of 354 patients (11.86%) exhibited a recurrence of AIS. The cumulative rate of the first recurrent stroke was 6.8% at one month and 12% at one year [39]. The highest frequency of recurrent AIS was observed in Jordanian children after their first ever stroke (46%) in the follow-up, ranging from 1 month to 9 years (median follow-up of 2 years). However, the whole group of analyzed patients was extremely small, which may have influenced the results in a positive or negative way [40]. Surprisingly, no stroke recurrence was demonstrated in a population of 78 children with AIS from Katowice, Poland, during the follow-up, ranging from 1 month to 10 years [41]. The analyzed group of Polish pediatric patients consisted, among others, of children with heart disease treated with antithrombotics for prophylaxis. Moreover, no patients with diagnosed sickle cell disease (SCD) and Moyamoya disease were enrolled in the study. These facts may be an explanation for the lack of AIS recurrence in this group.

Table 7.1 Prevalence of arterial ischemic stroke (AIS) recurrence in different pediatric populations

Study	Type of the study	Population	Age at time of first AIS	No. of analyzed patients/no. of patients with recurrent AIS	Time of the follow-up	Frequency of recurrent AIS
Bohmer et al. [**25**]	Retrospective	Patients admitted to Department of Pediatrics, University Hospital of Muenster, Germany	>29 days and <18 years of age	86/21	Median: 2.1 (interquartile range: 0.7–4.4 years)	21%
deVeber et al. [**29**]	Retrospective	Patients from Canada (Toronto), Germany (Kiel-Lübeck/Münster), and the UK (London/Southampton)	1 month to 18 years	894/160	Median: 35 months (minimum-maximum: 1–256 months)	17.9%
Steinlin et al. [**33**]	Retrospective	Patient cohorts with FCA from Switzerland and Melbourne (Australia)	1 month to 18 years	73/2	(7 months to 31 months)	2.7%
Per et al. [**34**]	Retrospective	Turkish pediatric patients	1 month and 16 years	130/9	5 months to 11 years	7%
Sultan et al. [**35**]	Cross-sectional analysis	Patients from 72 sites in 20 countries	At least 28 days of age and less than 18 years	1652/95	from January, 2003 through April, 2012	5.75% (TIA, AIS, and silent AIS)
Uohara et al. [**36**]	Retrospective	Patients recruited at The Children's Hospital of Philadelphia, USA	Between 29th day of age and 17.99 years	107/11	Median: 20.9 months (interquartile range: 8.7–40.4 months)	10.3%
Stacey et al. [**38**]	Retrospective	Patients admitted to Great Ormond Street Hospital, UK	>28 days and <18 years of age	84/24	Median: 2.4 years (interquartile range: 1.5–4.0 years)	AIS: 15%; both AIS and TIA: 29%
Fullerton et al. [**39**]	Retrospective	Children with AIS from 37 international centers	aged 29 days through 18 years	354/42	Median: 2.0 years (interquartile range: 1.0–3.0)	11.9%
Masri et al. [**40**]	Retrospective	Pediatric patients from Child Neurology Clinic at Jordan University Hospital (Jordan)	1 month to 13 years (median: 5 years).	24/11	Period ranged from 1 month to 9 years	46%

On the other hand, in a study performed using an international multicenter stroke database on children with Moyamoya disease, stroke recurrence was 20% over a median follow-up of 13 months, and 9% of patients had multiple recurrences [42].

Summarizing the data on the prevalence of recurrent stroke in children, the studies concerning this problem present groups of children which differ to a great extent in terms of the number of analyzed patients, their age at stroke onset, their ethnicity, the follow-up period, the etiology, and the stroke lesion location (anterior vs. posterior brain circulation), as well as secondary prophylaxis. For these reasons, an analysis of the data is very difficult and clear information on the number of stroke recurrences in pediatric populations remains largely undefined. Table 7.1 summarizes the data on the frequency of stroke recurrence in different pediatric populations.

7.3.2 In Young Adults (Aged 15–55 Years Old)

For a USA population, Kleindorfer et al. [43] reported a significant decrease in stroke incidence, but only among whites. Simultaneously, another study of this research team indicated that the proportion of all strokes under the age of 55 years increased from 12.9% to 18.6% within a decade [16]. Moreover, a study based on over 4000 young Danish patients (aged 15–30 years) showed rising hospitalization rates for first-ever stroke or TIA [44].

The prevalence of recurrent stroke in young adults may differ between populations. In a group of 428 patients with first-ever stroke, recruited from 46 hospitals in Baltimore City, five central Maryland counties, and Washington, DC, recurrent AIS was demonstrated in four cases (0.93%) during a study period from 1988 to 1991 [45]. A low frequency of stroke recurrence (2%) was found in a study from Rome, Italy, although it was carried out on a small group of patients with AIS (n = 150) within a mean follow-up period of 41.9 months [12]. In a study by Marini et al. [46], performed for patients recruited in seven neurology centers in Italy from April 1984 to March 1988, recurrent stroke occurred in 10 patients (3.29%), with a mean annual incidence rate of 2.36% higher in patients with mixed atherothrombotic and cardioembolic etiology than in patients included in the other diagnostic groups. A comparable group in terms of the number of patients to those studied by Renna et al. [12] was analyzed by Goeggel Simonetti et al. [47]. However, the authors demonstrated a higher frequency of recurrent cerebrovascular events (i.e., in seven patients (5%)), with a median follow-up period of 6.9 years. In a study by Li et al. [22], based on almost 1400 young adults from Northern China, 6.7% patients experienced AIS recurrence; the average duration after the first onset of stroke was 338.7 days. A similar frequency of stroke recurrence (6.26%) was demonstrated in a study by Pezzini et al. [48], who reported recurrent AIS in 32 patients. A study performed in patients with AIS from the Department of Neurology of the University of Iowa revealed a 9% frequency of recurrent stroke, with a mean interval between the initial and recurrent stroke of 4.7 years [49]. Among Estonian stroke patients, recurrence was

Table 7.2 Prevalence of AIS recurrence in different populations of young adults

Study	Type of the study	Population	Age at time of first AIS	No. of analyzed patients/no. of patients with recurrent AIS	Time of the follow-up	Frequency of recurrent AIS
Renna et al. [12]	Retrospective	Patients hospitalized at the stroke unit of Policlinico Gemelli of Rome, Italy	Younger than 50 years (mean age: 41 ± 8.0)	150/3	Mean: 41.9 months	2%
Li et al. [22]	Retrospective	Patients from Northern China	18–45 years	1395/94	At least one year	6.7%
Aarnio et al. [30]	Retrospective	Patients hospitalized at the Department of Neurology, Helsinki University Central Hospital, Finland	15–49 years	970/132	Mean: 10.2 ± 4.3 years	13.6%
Marini et al. [46]	Prospective	Patients recruited at seven departments of neurology (Florence, Genoa, L'Aquila, Milan, Padua, Parma, and Rome)	15–44 years	304/10	Average: 96 months (range: 62–124)	3.3%
Goeggel Simonetti et al. [47]	Prospective cohort study	Cohort was based on two registries: the Swiss Neuropediatric Stroke Registry and the Bernese stroke registry	16.1–45 years	154/7	Median: 6.9 years (interquartile range: 4.7–9.4)	5%
Pezzini et al. [48]	Retrospective	Patients form three Italian centers	Younger than 45 years	511/32	Mean: 43.4 months	6.3%

(*Continued*)

Table 7.2 (*Continued*)

Study	Type of the study	Population	Age at time of first AIS	No. of analyzed patients/no. of patients with recurrent AIS	Time of the follow-up	Frequency of recurrent AIS
Kapelle et al. [49]	Retrospective	Patients hospitalized at the Division of Cerebrovascular Diseases of the Department of Neurology of the University of Iowa, USA	15–45 years	253/23	15 years, from 1st July 1977 to 1st January 1992	9%
Sneider et al. [50]	Retrospective	Patients treated in Tartu University Hospital and North Estonia Medical Centre	18–54 years	837/96	From January 2003 to December 2012	11.5%
Giang et al. [51]	Retrospective	Swedish patients	18–54 years	17149/2432	From 1987 to 2006	14.2%
Schellekens et al. [52]	Prospective	Patients admitted to the Radboud University Medical Centre Nijmegen, The Netherlands	18–50 years	415/29	Mean: 8.9 years	18.6%
Huang et al. [53]	Retrospective	Patients recruited from XuanWu Hospital, China	18–45 years	350/89	Average: 5.8 ± 3.2 years	25.4%
Varona et al. [54]	Retrospective	Patients admitted to the Neurology Department of the University Hospital, Madrid, Spain	15–45 years	240/61	Mean: 12.3 years	25%

observed in 11.5% of patients [50]. In comparison, a study by Aarnio et al. [30] demonstrated 13.6% cases suffering from recurrent stroke in a large group of almost 1000 Finnish patients. In one of the largest study groups, consisting of young Swedish patients, in whom the AIS onset occurred between 1987 to 2006 (over 17,000 patients), the prevalence of recurrent stroke within this period was 14.2% [51]. In turn, a study by Schellekens et al. demonstrated that the frequency of recurrent events in patients from The Netherlands was almost 19% (recurrent AIS was the most frequent) [52].

On the contrary, in Chinese patients with first-ever stroke, an almost two-fold higher frequency of stroke recurrence in comparison to a Finnish study was demonstrated within a mean follow-up period of over five years [53]. Similarly, a high prevalence of recurrent stroke (25%) was found in a Spanish population, with a mean period of 6.5 years between the initial stroke and the first recurrence [54]. The annual stroke recurrence rate during the first year was 3.6%, with a tendency to fall to 1.7% in subsequent years. The authors also observed that 60% of those patients had just one episode of recurrence, whereas others had more than one. In addition, 16% of patients died as a result of the recurrence [54].

Table 7.2 summarizes the data on the frequency of stroke recurrence in various populations of young adults.

7.4 Risk Factors for Recurrent AIS in a Pediatric Population

Arteriopathy, vasculopathies, and some genetic polymorphisms are among risk factors which may increase the risk of recurrent stroke in children. Since the etiology of AIS is multifactorial, the data also indicate that several risk factors may influence the risk of its recurrence. The study by Lanthier et al. [55] demonstrated that children with AIS and multiple risk factors were at a greater risk of recurrent AIS than children with only one risk factor.

7.4.1 Arteriopathies

AIS recurrence in a pediatric population may be related to the presence of arteriopathies. Focal cerebral arteriopathy of childhood (FCA) has been defined as stenosis revealed by brain vasculature imaging, not related to other specific etiologies, i.e., Moyamoya disease and syndrome, dissection, post-varicella arteriopathy, sickle cell disease, vasculitis, or post-radiation vasculopathy [27]. Previously, transient cerebral arteriopathy (TCA) was defined as transient brain middle and/or large artery stenosis; since 2009, it has been included in the definition of FCA [27, 56]. The clinical presentation of FCA is acute and monophasic; the most important risk factor for FCA is a recent upper respiratory tract infection (URI) without any specificity to time between the infection onset and stroke onset, especially in early school-age children [6, 27].

FCA causes a 5-fold increase in the risk of recurrent stroke in comparison with idiopathic AIS [39]. The study by Fullerton et al. [39] did not confirm that other risk factors, i.e., a low socioeconomic status, recent infection, and under-vaccination, are predictors of recurrent AIS in children. At an early stage, FCA may have a tendency to progress, leading to brain ischemia and its recurrence. Another study by Fullerton et al. demonstrated the link between presumed inflammation, FCA progression, AIS, and acute brain ischemic recurrence [57]. The authors observed higher concentrations of high-sensitivity C-reactive protein, as well as serum amyloid A, in children with arteriopathic, but not cardioembolic, stroke, thus revealing that the presence of the first one increased the risk of recurrent AIS [57].

In 2012, CASCADE classification was created to identify, in each AIS pediatric patient at the disease onset, any anatomical abnormality within the brain and neck vessels and the heart [58]. The categories of anatomical abnormalities from 1 to 4 within the acute primary classification correspond to arteriopathies, as follows: 1—small vessel arteriopathy of childhood, 2—unilateral focal cerebral arteriopathy of childhood (FCA), 3—bilateral cerebral arteriopathy of childhood with collaterals (3a, fibromuscular dysplasia, Moyamoya; 3b, without collaterals), and 4—aortic/cervical arteriopathy (e.g., dissection) [58]. Other CASCADE categories are 5—cardioembolic, and then 6 and 7—other and multifactorial, respectively. The CASCADE categories from 1 to 4 may be, at follow-up, classified as progressive, stable, reversible, or indeterminate [58].

In 2018, British authors from the Great Ormond Street Hospital, London, identified 84 AIS children (mostly girls, with a mean age at onset of 4.1 years); the cases were classified according to the CASCADE criteria and followed for a maximum of four years (mean follow-up period was 2.4 years) [38]. As for the CASCADE types, 3a and 3b were significantly associated with the risk of AIS recurrence within the analyzed group of children [38]. In a German study, 57 out of 86 AIS children enrolled between 2004 and 2017 met the criteria of arteriopathic stroke (1–4 CASCADE classification) [25]. The median age at stroke onset was 7.9 years and the median time of the follow-up period was 2.1 years (maximum 4.4 years), which was similar to the British research scheme. Categories 2 and 3 of the CASCADE classification were statistically significant risk factors for stroke recurrence in this group of patients, whereas the patients with unilateral FCA showed early progress and the recurrence of brain ischemia (11 days) and the patients classified as the 3 CASCADE group presented late progress of arteriopathy (124 days) [25]. The above results have proven that CASCADE classification not only helps to unify the definitions and AIS patients' descriptions, but may also be a useful tool for predicting the long-term outcome in pediatric stroke patients. Cases with types 2 and 3 are at a higher risk of vessel wall pathology progress, and, as a result, early or late stroke recurrence [25, 38]. This may be useful and practical information for clinicians for more careful patient observation. Secondary prevention for patients with the CASCADE type 2 category is corticosteroid and antithrombotic treatment, even if the risk for recurrent stroke in these children is low [33, 38]. In the group described by

Stacey et al. [38], two out of 84 children (3a and 3b in the CASCADE classification) did not get any post-stroke treatment and both experienced recurrent AIS.

Until now, one of the confirmed risk factors for recurrent stroke in children has been the presence of vasculopathy, even when Moyamoya is excluded. In a study by deVeber et al. [29], AIS recurrence was significantly more common among children with vasculopathy than in children without vasculopathy (HR = 2.5).

Sickle cell disease (SCD) is one of the most common genetic reasons for anemia, mostly present in sub-Saharan Africa, the Middle East, and India and caused by a mutation in the hemoglobin gene (β subunit) in chromosome 11 [59]. SCD affects different organs and some of the most devastating problems in the course of the disease are central nervous system complications. Progressive arteriopathy mainly concerns the large arteries of the anterior brain vasculature (MCA, middle cerebral artery; ACA, anterior cerebral artery; ICA, internal carotid artery), with progressive narrowing of the vessels and new collaterals similar to Moyamoya vasculopathy. If Moyamoya ("puff of smoke" on digital subtraction angiography) appears in the course of SCD, it is defined as Moyamoya syndrome; in the CASCADE classification, it will meet the criteria of class 3a [58, 59]. The clinical presentation of neurological complications during SCD is represented by both cognitive decline and silent cerebral ischemia (SCI), with radiological findings of brain infarction without clinical symptoms and clinically overt AIS. The last one is present in more than one third of SCD patients before the age of 18 years [60, 61]. Stroke in SCD patients is a leading cause of disability and morbidity, the prevalence of AIS in children and young adults with SCD is 3.75%, and the peak of prevalence occurs at the age of 2 to 5 years [62]. Both Moyamoya syndrome and progressive vasculopathy in children with SCD are risk factors for recurrent silent and clinically overt ischemic strokes; the risk was estimated to be 12.7/100 patient-years. The indirect revascularization procedure is a proven method for reducing recurrent ischemic brain incidents in these patients [63].

7.4.2 Cardiac Defects

Pediatric patients with heart disease are at a high risk of stroke and its recurrence; congenital and acquired cardiological problems are "classical" risk factors for stroke in pediatric populations. In a study by Vazquez Lopez et al. [64], within a 15-year follow-up observation of all children hospitalized for heart disease, mostly congenital cardiomyopathy, 74 suffered a stroke; the age of the children at stroke onset varied from 1.6 months to 51.7 months (mean age of 11.7 months) and the mean age at evaluation was 8.9 years ± 4.4 years. Most children—up to 70% of the examined group—presented an unfavorable neurological outcome (sensimotor disability, as well as cognitive impairment); 20% of the children died; and in 10%, recurrent stroke was observed. On the other hand, in the study by de Veber et al. [31], the only statistically significant risk factor for AIS recurrence was arteriopathy, even if a new ischemic episode was observed in 11 children out of 15 with cardiac disease.

In a study by Rodan et al. [65], which was performed in children with congenital heart disease and AIS, a mechanical valve and a prothrombotic condition were independent risk factors for stroke recurrence. In turn, Per et al. [34] observed recurrent stroke in 7% of cases of the analyzed group, and infection and cardiac catheterization for mostly congenital heart diseases were the most important risk factors for AIS in this group.

7.4.3 Thrombophilia

According to an international study by deVeber at al. [29], the following prothrombotic risk factors, i.e., antithrombin deficiency, elevated lipoprotein (a), and the co-existence of more than one prothrombotic factor, were associated with the increase in AIS recurrence risk when adjusting for vasculopathy. The authors observed a single prothrombotic disorder in 269 children with AIS, whereas in 88 patients, more than one prothrombotic risk factor was detected. In addition, heterozygous antithrombin deficiency, high lipoprotein (a), high fibrinogen, and high fasting homocysteine were related to AIS recurrence [29].

7.4.4 Seizures in the Clinical Presentation of AIS

In a 16-year, prospective, national population-based study, i.e., the Canadian Pediatric Ischemic Stroke Registry by deVeber et al. [31] based on children with AIS recruited during the period from 1992 to 2001, several risk factors which could predict AIS recurrence were analyzed. The authors found that seizures in the clinical presentation of AIS were not risk factors for a poor outcome; the frequency of seizures was comparable in groups of children with a poor and good outcome (49% vs. 47%, respectively). In turn, a higher frequency of seizures was observed among children without recurrent AIS compared to those with recurrent AIS (40% vs. 21%, respectively). Therefore, clinical presentation without seizures was a predictor for stroke recurrence, with HR = 1.96 (p = 0.025) [31].

7.4.5 Genetic Polymorphisms

Recently, several reports indicated relationships between specific genetic polymorphisms and the recurrence of stroke in children. The genetic risk factors, due to the age of the patients, especially pediatric ones, may be of specific importance in the appearance of subsequent AIS. However, studies on the topic are not common and show analyses of various polymorphic variants. Most often, a reliable statistical analysis regarding the relation between a polymorphic variant and stroke recurrence is not possible in pediatric patients due to the fact that recurrent AIS only occurs in a few cases. Therefore, published data on the topic are difficult to interpret and require confirmation based on larger patient populations with AIS.

Coen Herak et al. analyzed 73 children with perinatal and childhood stroke and found no polymorphic variants of thrombotic factors, i.e., factor V (*FV*) Leiden, *FV* HR2, or factor II (*FII*) 20210G>A, among patients with recurrent AIS [66]. On the other hand, the authors found that the frequency of the *HPA* 2a/b genotype was over 2-fold higher in cases with recurrent childhood AIS than in cases with nonrecurrent childhood AIS. Additionally, 75% of patients with recurrent stroke had a combined *APOE* ε2ε3 and *ACE* I/D genotype, whereas in the pediatric group without recurrent stroke, it was only 6%. In this study, it was also demonstrated that one of the children with recurrent stroke was homozygous for methylenetetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism [66]. In turn, in children with AIS from Lebanon, 677C>T polymorphism within the *MTHFR* gene was found to be present in the patients who suffered from recurrent stroke, as well as those who had multiple risk factors for AIS [67]. Previously, the T allele carrier-state of 677C>T polymorphism in the *MTHFR* gene was proved to increase the risk of AIS in children [68], in contrast to another common 1298A>C polymorphism in the *MTHFR* gene [69]. The polymorphic 677TT variant of the *MTHFR* gene was previously linked to a higher level of homocysteine, one of the biochemical risk factors for cerebro- and cardiovascular diseases, both in children and adults.

On the other hand, in a large cohort of almost 900 pediatric patients with first-ever AIS from Canada, the UK, and Germany, a subsequent AIS event was diagnosed in almost 18% of patients and the authors found that the presence of isolated mutations in *FV* at rs6025, as well as in *FII* at rs1799963, were not individually significantly associated with recurrent AIS [29]. In a study by Per et al. [34], *FV* Leiden mutation and homocystinuria were present in patients who experienced subsequent strokes.

7.5 Risk Factors for Recurrent AIS in Young Adults

Risk factors for recurrent stroke observed in children and older patients may differ. However, similar to the observation by Launthier et al. [55], a study by Pezzini et al. [48] based on young adults from Italy demonstrated that the risk of recurrence increased with an increasing number of traditional factors in comparison to patients with no risk factor (HR was 2.29 for subjects with one factor and 5.28 for subjects with two factors). In addition, a significant role of predisposing genotypes was also demonstrated [48]. However, the data on risk factors for stroke recurrence in young adults are not as common as in older patients.

7.5.1 Hypertension

Hypertension was demonstrated to be a risk factor for stroke in young adults [70]. In a study by Putaala et al. [71], hypertension was present in 39% of patients with AIS. It was demonstrated that recurrent ischemic stroke occurred

in 13.6% of young patients with well-documented risk factors, and in only 4.7% of young patients without well-documented risk factors. The presence of ≥4 well-documented risk factors was independently associated with a higher risk for recurrent ischemic stroke [71].

The analysis of traditional risk factors by Pezzini et al. [48] revealed that hypertension and a family history of AIS under the age of 45 years were significantly related to the recurrence of vascular events in young adults. The authors demonstrated a significant difference in the prevalence of patients with hypertension in the group having recurrent vascular events compared to the whole analyzed group of patients (31.5% vs. 20.7%; HR 2.36).

Hypertension was also found to be a risk factor for recurrent stroke in Estonian patients. It was present in almost 69% of AIS cases and in 53% of cases with first-ever stroke [50]. In a study by Xu et al. [72], hypertension was also associated with an increased risk of recurrence in a large cohort of Chinese patients with stroke. However, the age range of the patients was very wide, from 19 to 97 years. In addition, the authors made an important observation that controlling hypertension significantly reduced the recurrent risk [72].

7.5.2 Diabetes Mellitus

Diabetes mellitus is one of the established risk factors for stroke recurrence in young adults. Previous data indicated that patients aged 18–50 years with TIA or ischemic stroke and with diabetes and impaired fasting glucose (IFG) were more likely to experience a vascular event compared to subjects with normal fasting glucose [73]. However, in the case of risk for the recurrence of stroke, it was similar for patients with incident diabetes and IFG compared to those with normal fasting blood glucose values. At follow-up, almost 13% of patients with IFG had at least one stroke compared to 8% of patients with normal fasting glucose [73].

Among cases of patients from Estonia suffering from AIS recurrence, diabetes mellitus and peripheral artery disease were observed with a significantly high frequency compared to the group of patients with first stroke (18.8% vs. 8.7% and 5.5% vs. 1.1%, respectively). Additionally, Schneider et al. [50] demonstrated that patients with recurrent stroke were older than those with first-ever stroke and recurrent AIS was more frequently caused by large-artery atherosclerosis.

An earlier study by Naess et al. [74] including 232 patients with first-ever stroke demonstrated that diabetes mellitus is a factor associated with subsequent vascular events. Other risk factors observed in the study were as follows: myocardial infarction, angina pectoris, intermittent claudication, and smoking, However, in this study, myocardial infarction was analyzed jointly with recurrent stroke as a vascular event. The authors also observed that patients with no risk factor had a low frequency of recurrent vascular events.

An analysis based on 121 Chinese patients with Moyamoya disease and first-ever stroke (aged 18–45 years), who underwent revascularization after the acute phase of initial stroke, proved that diabetes is an independent predictor for stroke recurrences (HR 6.76) [75]. Diabetes was also significantly associated with unfavorable functional outcomes in this study (odds ratio (OR) of 7.87).

7.5.3 Dyslipidemia

Data on relations between lipid abnormalities and the risk of stroke recurrence in young adults are most often contradictory. In older adults, lipid abnormalities were previously associated with a higher risk of ischemic stroke recurrence in patients with a large-artery atherosclerosis subtype of stroke [76]. In young adults with stroke from the US, hypercholesterolemia was associated with a higher cumulative risk for both cardiac events and recurrent stroke [77]. Li et al. demonstrated that abnormal lipid metabolism was associated with AIS recurrence, but only in univariate, and not multivariate, analysis [22]. In turn, Pezzini et al. [48] did not observe a significant difference in the prevalence of patients with hypercholesterolemia in the group with recurrent vascular events compared to the whole analyzed group of patients (27.4% vs. 26%) [49]. This finding was confirmed based on a three-times larger group of patients (n = 1867), also by Pezzini et al. (24.4% of patients with hypercholesterolemia in a group without recurrence vs. 28.2% in a group with recurrence) [78].

Atherogenic dyslipidemia increased the stroke recurrence risk among stroke patients of the large-artery atherosclerosis subtype (HR 2.79), but not in all stroke patients (HR 1.69) [76]. The mean age of the patients with dyslipidemia and AIS was 58.77 years. It was also found that the stroke recurrence rate was significantly higher in patients with atherogenic dyslipidemia than in those without this condition (20.3% vs. 11.9%).

In a study by Schneider et al. [50] in Estonia, both young patients with first-ever stroke and patients with recurrent stroke showed a high prevalence of dyslipidemia (45.5% vs. 47.9%, respectively). However, it was not a risk factor for recurrence.

7.5.4 Thrombophilia

Studies analyzing thrombophilic factors with regard to stroke recurrence are scarce. In the study by Schellekens et al. [52], no relations between the presence of a prothrombotic factor or some recent infections and an increased risk for any recurrent ischemic event or recurrent cerebral ischemia in young patients with cryptogenic stroke were demonstrated after a mean follow-up period of 8.9 years. Similarly, no differences in haemostatic factors (i.e., fibrinogen and D-dimer) were

observed between patients with and without recurrent acute cerebral ischemia in patients from Slovenia aged under 45 years [79]. The case of a young woman with recurrent TIA demonstrated an elevated level of fibrinogen, as well as homocysteine, after methionine loading [80].

7.5.5 Smoking

Cigarette smoking is one of the modifiable risk factors for both primary and secondary stroke prevention in young adults.

In young Estonian patients with AIS, the prevalence of smokers was similar between first-ever stroke and recurrent stroke subgroups (34.7 vs. 28.1, respectively) [50]. Therefore, smoking cessation may have a low efficiency in secondary prevention against recurrent stroke in the young. In turn, smoking was a risk factor for a subsequent vascular event [74]. In this study, the prevalence of smokers in the group with a subsequent vascular event was significantly higher than in the group without these events (78.6% vs. 58.2%, respectively) [74]. Similarly, in a study by Pezzini et al. [78], current smokers were significantly more common among patients with recurrent vascular events than in the group without recurrence (37% vs. 46.6%, respectively). In turn, in young Chinese patients, smoking was only related to stroke recurrence in univariate analysis, while multivariate analysis did not confirm this finding [22].

7.5.6 Substance Abuse

One of the most commonly abused drugs in the USA is cocaine. In a study by Cheng et al. [81], a significant association between acute cocaine use and the risk of AIS in young adults was demonstrated after adjusting for smoking, alcohol consumption, and hypertension (OR = 5.7). As cocaine may increase the risk of AIS, the discontinuation of its use may prevent recurrent stroke [82]. A case of a 39-year-old man who developed occlusion of the frontopolar branches of the left middle cerebral artery 1 h after intravenous cocaine use was described by Sauer in 1991 [83]. Eleven days later, the man developed occlusion of the superior division of the right middle cerebral artery.

7.5.7 Genetic Polymorphisms

As for genetic analysis in relation to stroke recurrence in young adults only, the data are poor. The study by Ou et al. [84] demonstrated that the wild-type G allele of –174G>C polymorphism within the interleukin-6 gene significantly increased the risk of stroke recurrence in comparison to the C allele in young adult patients with moderate internal carotid artery stenosis. In turn, the study by Pezzini et al. [48], which was performed in a large cohort of over 500 patients with AIS younger than 45 years, three of the commonly analyzed polymorphisms (the 20210A variant of the prothrombin gene, the 1691A variant of the *FV* gene,

and the TT677 genotype of the *MTHFR* gene) were assessed in relation to the recurrence of ischemic events. The authors observed that the risk of recurrence increased with an increasing number of predisposing genotypes (from HR equal to 1.96 for cases with one genotype to HR equal to 3.83 for cases with two genotypes).

Almost 40% of ischemic strokes in adults are cryptogenic and the prevalence of persistent foramen ovale (PFO) is two-fold higher in these patients than in healthy patients. In turn, the most frequent cause of embolism in cryptogenic stroke associated with PFO is deep venous thrombosis. One of the earliest studies analyzing genetic variants in the recurrence of thrombotic events was performed by de Stefano et al. [85]. The authors demonstrated that in simultaneous carriers of *FV* Leiden and the 20210G>A polymorphism in the prothrombin gene, the risk of recurrent deep venous thrombosis after the first episode was significantly increased.

Despite the fact that the results of genetic analyzes regarding the onset of stroke recurrence are inconclusive, these are undoubtedly important data which should be considered in the interaction with other, non-genetic risk factors in estimating the risk of recurrence.

7.5.8 Other Predictors

Various other predictors for recurrent ischemic stroke have been reported. Previously, Pezzini et al. [78] demonstrated migraine with aura as a risk factor for recurrence. The Chinese study, which included a sizeable group of young patients, revealed, in a univariate analysis, that AIS recurrence was associated with atrial fibrillation, TOAST type in patients with an unclear cause, and the National Institutes of Health Stroke Scale (NIHSS) score at admission. However, the multivariate analysis only confirmed the NIHSS score to be a predictor of recurrent stroke [22]. In a study by Nedeltchev et al. [28], recurrent stroke was significantly associated with a previous history of TIA. Additionally, the authors observed that age, sex, stroke risk factors other than previous TIA, and stroke etiology, as well as stroke subtypes, were not related to the risk for recurrence.

In turn, among Finnish young adults with stroke, patients with an index stroke caused by high-risk sources of cardioembolism had the highest risk of any subsequent cardiovascular events (HR 3.7), whereas patients with large-artery atherosclerosis had a higher risk of recurrent stroke (HR 2.7) than patients with stroke of an undetermined etiology [30].

In addition, independent predictors for vascular event recurrence in young adults were a familial history of stroke, aPL, and discontinuation of antiplatelet and antihypertensive medications in an Italian population [78].

Figure 7.1 lists the potential risk factors for stroke recurrence in pediatric patients and young adults.

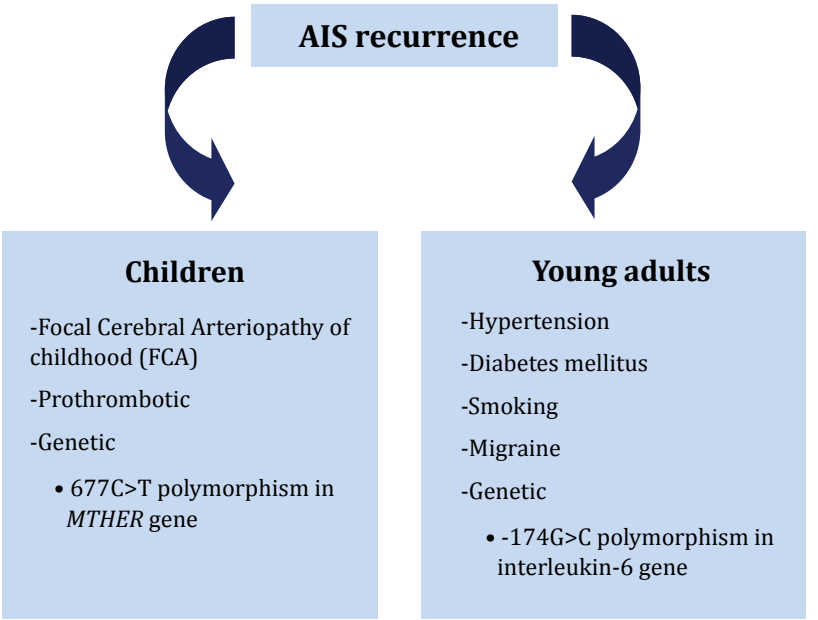

Figure 7.1 Potential risk factors for stroke recurrence identified in different pediatric and young adult populations.

7.6 Secondary Prevention of AIS

7.6.1 In Pediatric Patients

The proposition of secondary prophylaxis in AIS children was published in 2007 [33]. In the group of 73 children diagnosed with AIS and FCA, treatment with a combination of corticosteroids and antithrombotics (CAT) was administered, whereas 52 children received only antithrombotic treatment (AT). Stroke recurrence was observed in each group, but the complete stenosis resolution was found in 81% of CAT patients vs. 59% of AT patients [33]. Even though, at the end of the follow-up, the number of recurrences in these children was the same in both subgroups, the complete resolution of vascular stenosis was more commonly seen in the CAT group, which exhibited a good correlation with a longer AIS recurrence prognosis. For this reason, the proposition of CAT after the first episode of brain ischemia in children seems to be an attractive therapeutic tool, even though a study of a larger group of patients is still needed. As FCA is presumed to be of inflammatory etiology, the proposition of immunosuppressants as secondary prophylaxis was published [86].

In a study by Stacey et al. [38], two patients with the highest risk of AIS recurrence (3a and 3b, according to the CASCADE classification) did not receive any secondary prophylaxis and both of them showed the recurrence of brain ischemia. In total, 82 out of 84 patients from the presented group received

antiplatelet and/or antithrombotic therapy (only antiplatelet in 68 children, and both in seven children); the number of recurrences was 155 within 60 months [38]. Even if antithrombotic therapy is used for secondary AIS prevention in children with specific arteriopathy subtypes, consisting of 3A and 3B of the CASCADE classification, the risk of recurrence is very high [38]. Considering the results of Steinlin et al. [33] and Stacey et al. [38], the most important cause of pediatric AIS recurrence is the type of arteriopathy in the CASCADE classification, where some of the subtypes (FCA) are not so strongly associated with recurrent brain ischemia, whilst other subtypes, 3A and 3B, lead to recurrence, even if secondary prophylaxis is administered.

Preventive treatment for children with SCD is specific for the disease itself, but also for a particularly high risk of stroke in SCD (TAMMV, time-averaged mean of maximum velocities, of ICA or MCA ≥ 200 cm/s). In these patients, chronic red cell transfusions and/or hydroxyurea may be administered as alternative therapy; for the first one, serious side-effects (iron overload, transfusion reactions, infection, alloimmunization) may be the cause of switching to hydroxyurea treatment [59]. Despite receiving secondary prophylaxis with transfusions, approximately 20% of SCD patients had stroke recurrence [87]. Bone marrow transplantation (BMT) in SCD patients below the age of 16 observed in the mean time of 7 years revealed that these patients were protected against stroke and even experienced vasculopathy stabilization [88]. Antithrombotic and especially antiplatelet therapy in SCD children is rather an adjuvant therapy. More promising ways of treating cerebral vasculopathy in SCD patients are surgical procedures like encephaloduroarteriosynangiosis (EDAS) and plial synangiosis [89].

MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) pediatric patients are at a high risk of recurrent stroke-like episodes (in radiological presentation, the locations of stroke areas do not correspond to a typical brain vascular distribution); in 80% of patients, the disease is caused by mutation 3243A>G within the *MT-TL1* gene. The first symptoms occur at the age of 2 years and include muscle weakness, a short posture, learning difficulties, migraine and consciousness disturbances, seizures, hearing impairment, and neuropathy of the peripheral nerves, as well as diabetes and cardiomyopathy. In secondary prophylaxis, after the first ischemic episode, to prevent recurrent stroke-like episodes, arginine should be administered at a 150–300 mg/kg/daily oral dose [90].

In a group of 135 Canadian children with AIS and congenital heart disease analyzed by Rodan et al. [65], 19 patients had stroke recurrence (median follow-up period was 2.15 years). Of them, seven (37%) were not administered with any antiplatelet or antithrombotic medications, three took an anticoagulant at a subtherapeutic level, and the remaining children (n = 9) with stroke recurrence took an antiplatelet agent or an anticoagulant agent at therapeutic levels, or both. At the last follow-up, 32% of the patients without stroke recurrence were on

acetylsalicylic acid (ASA), 15% were on warfarin, 11% were on low molecular weight heparin, and 39% did not take any antiplatelet or antithrombotic medication. In this group, almost 53% of children with recurrent AIS did not receive effective antithrombotic treatment at the time of recurrence [65].

7.6.2 In Young Adults

Since secondary stroke prevention aims to reduce the risk of subsequent AIS, it is important to know the etiologic mechanism of the initial stroke, as well as accompanying risk factors. Together with antiplatelet or anticoagulant therapy, secondary prevention should include the treatment of vascular risk factors (arterial hypertension, hyperlipidemia, diabetes mellitus, and cardiac disease) and/or surgical procedures. However, knowledge on medications used in secondary prevention in young adults is limited. It has therefore been noted that the long-term use of recommended medications raises the greatest problem in preventing secondary stroke, since approximately 30% of stroke patients discontinued one or more drugs within 1 year of hospital discharge [91].

According to the European Stroke Initiative Recommendations for Stroke Management guideline, aspirin should be an antiplatelet drug of first-choice in secondary prevention [92]. In patients with ASA intolerance, clopidogrel at a dose of 75 mg/day may be considered as the first-choice treatment. Where possible, combined aspirin and dipyridamole (25/200 mg twice daily), or triflusal alone, may be used. The combination of ASA and clopidogrel is only recommended in secondary stroke prevention in the case of the coincidence of stroke and recent myocardial infarction or status post-coronary stenting. In cardioembolic AIS, prevention is based on the use of oral anticoagulants [93]. In patients with non-embolic ischemic stroke or TIA, antiplatelet oral agents are more preferable to reduce the risk of recurrent stroke and other cardiovascular events than anticoagulant therapy [94].

In patients with stroke recurrence, novel oral anticoagulants (NOAC; thrombin and factor X inhibitors) can be used for the secondary prevention of cardioembolic stroke, despite appropriate treatment with warfarin [95]. In atherothrombotic IS, antiplatelet therapy and revascularization procedures reduce the risk of recurrence in selected cases of ipsilateral carotid stenosis [93].

A specific role of statins in young adults with AIS has also been discussed. Their benefit in patients with predominantly nonatherosclerotic stroke etiologies is not clear. Putaala et al. [96] demonstrated that patients taking statins at any time during follow-up had a lower risk of outcome events (i.e., stroke, myocardial infarction, other arterial thrombosis, revascularization, or vascular death; HR 0.23). The authors observed that 20% of events occurred among patients who had never been on statins, none occurred among patients with continuous statins, and 11% occurred among patients with discontinuous statins [96].

7.7 Conclusions

Understanding the risk factors for the recurrence of arterial ischemic stroke in the young may help to identify the mechanisms of AIS, especially in children, and in some cases, may contribute to disease prevention. Current knowledge on risk factors for recurrent AIS suggests that multiple risk factors may be related to stroke recurrence, both in children and young adults. Risk factors for stroke recurrence differ between child and young adult populations, which is clearly demonstrated in the present literature review. The best documented risk factors for recurrent AIS in children are arteriopathies, especially FCA, which significantly increase the risk of recurrence. A proposal for secondary prevention in children with FCA to avoid subsequent strokes has even been demonstrated. Treatment of a combination of aspirin and corticosteroids allows for the total resolution of vessel wall stenosis in FCA, and, in this way, this therapy may decrease the risk of AIS recurrence. On the other hand, even if antithrombotic medication is used, pediatric AIS recurrence remains a significant problem, especially in children with specific arteriopathy subtypes of the CASCADE classification, i.e., 3A and 3B.

Risk factors for recurrent stroke such as diabetes mellitus, hypertension, or lipid abnormalities, have been highlighted in young adults. Undoubtedly, many risk factors, including genetic ones, have an impact on AIS etiology, as well as on the presence of its subsequent consequences, including recurrence. Understanding the links between the presence of a specific risk factor and the occurrence of a post-stroke outcome may be of particular importance for practical reasons. Establishing predictors of stroke recurrence may allow for more effective treatment, and may thus reduce the number of cases.

Abbreviations

ACA: anterior cerebral artery
AIS: arterial ischemic stroke
ASA: acetylsalicylic acid
AT: antithrombotic treatment
BMT: bone marrow transplantation
CAT: corticosteroids and antithrombotics
CSVT: cerebral sinuvenous thrombosis
EDAS: encephaloduroarteriosynangiosis
FCA: focal cerebral arteriopathy of childhood
HR: hazard ratio
ICA: internal carotid artery
IFG: impaired fasting glucose
MELAS: mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes

MCA: middle cerebral artery
MTHFR: methylenetetrahydrofolate reductase
NIHSS: National Institutes of Health Stroke Scale
NOAC: novel oral anticoagulants
SCD: sickle cell disease
SCI: silent cerebral ischemia
TCA: transient cerebral arteriopathy
TIA: transient ischemic attack
URI: upper respiratory tract infection

Disclosures and Conflict of Interest

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Chapter 8

Cellular Mechanisms of Human Atherogenesis: Focus on Chronification of Inflammation and Mitochondrial Mutations

Alexander M. Markin, PhD,[a] Igor A. Sobenin, MD, PhD,[b,c] Andrey V. Grechko, PhD,[d] Dongwei Zhang, PhD,[e] and Alexander N. Orekhov, PhD[a,c]

[a]*Laboratory of Infection Pathology and Molecular Microecology, Institute of Human Morphology, Moscow, Russia*
[b]*Laboratory of Medical Genetics, Institute of Experimental Cardiology, National Medical Research Center of Cardiology, Moscow, Russia*
[c]*Laboratory of Angiopathology, Institute of General Pathology and Pathophysiology, Moscow, Russia*
[d]*Federal Research and Clinical Center of Intensive Care Medicine and Rehabilitology, Moscow, Russia*
[e]*Diabetes Research Centre, Traditional Chinese Medicine School, Beijing University of Chinese Medicine, Beijing, China*

alexander.markin.34@gmail.com, a.h.opexob@gmail.com

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8.1 Introduction

Atherosclerosis is a multifactorial disease with a complex pathogenesis. Multiple factors were shown to be involved in atherosclerotic lesion formation, and many

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knowledge gaps still remain. Atherosclerotic lesions can develop in any artery, but are especially dangerous in large vessels that aliment the heart, brain, and other vital organs. Macroscopically, the lesions are seen as local accumulation of fat in the arterial wall (so-called fatty streak), which is followed by local thickening of the innermost arterial wall layer called intima. At the advanced stages, the plaques acquire a thick fibrous cap, which separates them from the vessel lumen and circulating blood. Currently, several key factors in atherosclerotic lesion formation are considered. There is no doubt that impaired endothelial function and increased permeability plays an important role, especially at the initial stages of the lesion development (Mundi et al., 2018). Alterations of lipid metabolism and lipoprotein modifications are prerequisites of lipid accumulation (Summerhill et al., 2019). Chronic inflammation and immune disorders have been extensively studied in relation to atherosclerosis, which is currently regarded as an inflammatory disease (Frostegård, 2013; Rea et al., 2018). During the recent years, the prominent role of genetic factors in atherosclerosis development has been recognized, and numerous identified genes opened new possibilities for novel therapies development (Martínez et al., 2017; Wang W. et al., 2019; González-Becerra et al., 2019; Jha et al., 2019).

Numerous genetic studies were conducted to establish risk factors for the development of atherosclerosis (Björnsson et al., 2019; Li et al., 2019; Rincón et al., 2019). Researchers are constantly discovering new mutations specifically associated with cardiovascular disease. Apart from nuclear genome, mitochondrial genome variants may also be important as risk factors and disease modifiers. It is possible that mitochondrial DNA (mtDNA) mutations are responsible for predisposition to the development of atherosclerotic lesions (Sinyov et al., 2017). Mitochondria are semi-autonomous organelles that bear the genes of many, but not all mitochondrial proteins in their circular genome that resembles a bacterial chromosome. Mitochondrial dysfunction affects the cellular energy balance, metabolism and survival, and results in the development of mitochondrial cytopathies. These diseases can be caused both by nuclear genes encoding mitochondrial proteins or by mtDNA mutations that affect either mitochondrial proteins or transport RNA (tRNA). In this review, we will summarize the current knowledge on mtDNA mutations and mitochondrial dysfunction as pathophysiological factors of atherosclerosis development.

8.2 Cellular Mechanisms of Atherogenesis

Atherosclerotic lesion development takes place in the intimal layer of the arterial wall. Adult intima is a rather thick formation with complex architecture and heterogeneous cellular composition. The intima is separated from the lumen of the vessel by a monolayer of endothelial cells. Endothelium plays a key role in the transport of cells and non-cellular components of blood from the arterial bed to the vascular wall (Krüger-Genge et al., 2019). Endothelial lining is heterogeneous

(Romanov et al., 1995). In addition to cells of normal shape and size, clusters of giant multinucleated endothelial cells are found in the endothelial monolayer. These giant multinucleated cells appear only during the life period, in which atherosclerosis development is most frequent, and are typically not present in young individuals. Their appearance is important for understanding the mechanisms of atherogenesis, since clusters of such cells appear to be more common in the areas predisposed to atherosclerosis. Usually these are hemodynamic stress zones (for example, bifurcation and branching of blood vessels). Presence of such zones may partially explain the mosaicism of atherosclerosis. Lesions are not diffuse, but occur locally or focally, which may be associated with a local disturbance in the permeability of giant multinucleated endothelial cells.

Under the basal membrane, intima is populated by different types of cells (Rekhter et al., 1991). Immune cells such as macrophages (3–5%), dendritic cells (0.3%), and others, are located near the endothelium. Deeper layers contain elongated smooth muscle cells (70%) and pericytes or pericyte-like cells (25–30%) (Orekhov et al., 2014). Pericyte-like cells have a stellate shape and are connected through gap junctions with each other, forming a three-dimensional network, which serves as a kind of second line of immune defense (Ivanova et al., 2015). Pericyte-like cells can perform the functions of phagocytes, are able to secrete pro-inflammatory cytokines (Hill et al., 2014), and can also act as antigen-presenting cells (Ivanova and Orekhov, 2016a). In stimulating the immune response, pericytes are inferior in effectiveness to "professional" immune cells, but due to their abundancy in the arterial wall they are able to actively participate in the innate immunity reactions.

Cellular composition of the arterial wall undergoes profound changes in atherosclerotic lesion areas (Stary, 1990; Xu et al., 1990; Padarti and Zhang, 2018). Lesion development is accompanied by a local increase of the number of cells (cellularity), especially the numbers of macrophages and hematogenous cells. Moreover, the cellular network of pericyte-like cells disintegrates, with loss of intercellular communication and changes of cellular phenotype. Pericyte-like cells, macrophages, and some smooth muscle cells accumulate lipids, turning into foam cells, which have a distinctive appearance due to the lipid droplets accumulating in their cytoplasm. Presence of such cells in the subendothelial space of the arterial wall is a known early manifestation of atherosclerosis.

The primary source of lipids that accumulate in foam cells is atherogenic modified low density lipoprotein (LDL). Particles of LDL circulate in the blood and undergo chemical modifications affecting the glycoconjugate, lipid, and protein moieties (Ivanova et al., 2017; Orekhov and Myasoedova, 2019). Noteworthy, it is the multiply modified LDL that was found in the circulation of atherosclerotic patients, and not specific LDL modifications that have been characterized in *in vitro* experiments, such as oxidized LDL. Atherogenic modification of LDL begins with desialylation, and continues with changes of the lipoprotein particle size, density, and electric charge resulting in the formation of small dense and

electronegative LDL fraction. Oxidation is likely to occur at later stages of atherogenic LDL modification. LDL atherogenicity is enhanced by the formation of self-associates and circulating immune complexes containing modified LDL and anti-LDL autoantibodies (Kacharava et al., 1993).

Native (unmodified) LDL does not cause the accumulation of intracellular lipids in cultured cells, because LDL binds to a specialized LDL receptor (LDLR), and undergoes degradation through the receptor-mediated pathway (Zhang et al., 2016). The excess lipids are eliminated from the cell due to the efflux, in which high-density lipoprotein (HDL) and transporter proteins play a key role (Laatsch et al., 2012).

Associates of modified LDL stimulate the phagocytic activity of subendothelial macrophages and pericytes. Following phagocytosis, inflammatory cytokines are secreted, which attracts monocytes and other immune cells to the emerging site of inflammation. Inflammatory cytokines contribute to further accumulation of intracellular lipids induced by modified atherogenic LDL. Moreover, in some cases, lipid accumulation induction is observed even in absence of modified LDL (Table 8.1).

Therefore, not the accumulation of intracellular lipids caused by LDL, but the immune response to the interaction of the cell with LDL promotes or even induces the formation of foam cells. Intracellular lipid accumulation leads to the rupture of cell contacts in the three-dimensional network of pericyte-like cells (Ivanova et al., 2015). This is accompanied by increased proliferative activity and stimulation of extracellular matrix synthesis (Ponticos and Smith, 2014; Orekhov et al., 2016). Such processes are characteristic of the reparative phase of the inflammatory reaction. Normally, only a small thickening of the intimal tissue remains at the site of inflammation (Fig. 8.1) (Orekhov and Ivanova, 2016). This process may occur in the arteries without causing visible symptoms and lesions can accumulate over time, so that focal formations become a diffuse thickening. Diffuse intimate thickening is not an atherosclerotic lesion, but is considered normal for the arteries of an adult body (Nakashima et al., 2008; Subbotin, 2016).

According to current consensus, the primary event in atherosclerotic lesion development is local endothelial activation and increased permeability, which may be caused by hemodynamic forces occurring at the sites of blood vessel bends and bifurcations. Other known triggering factors of atherosclerosis include circulating mediators of inflammation and vasoactive substances, diet-induced alterations of the levels of circulating lipids, modified LDL, increased blood glucose level, and cigarette smoke. At the sites of local activation, the endothelial cells change their expression pattern, beginning to express cytokines and chemokines (interleukin 1 [IL-1]), tumor necrosis factor alpha (TNF-α), chemokines monocyte chemoattractant factor 1 (MCP-1), growth factors, such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), and adhesion molecules. This leads to the

increased recruitment and adhesion of circulating immune cells that interact with the endothelium and penetrate into the subendothelial space. During this process, circulating monocytes become activated and differentiate into macrophages. This further increases the pro-inflammatory signaling at the emerging lesion site. Macrophages are specialized phagocytes that actively participate in lipid uptake within the lesion and are an important source of foam cells (Koenig, 1999).

Table 8.1 The effect of IL-6, IL-8, IL-12, IL-15, IL-17, IL-18 on the concentration of cholesterol in THP-1 cells

Comparison groups		Relative cell cholesterol concentration,% (SD, %)	P (t-test)		P (M-W)	
			vs control	vs LDL	vs control	vs LDL
1	Control	100.0 (20.4)	—	—	—	—
2	LDL	164.1 (39.4)	<0.001	—	<0.001	—
3	LDL+IL-6	174.8 (28.0)	<0.001	0.134	<0.001	0.121
4	IL-6	116.5 (20.9)	<0.001	<0.001	<0.001	<0.001
5	LDL+IL-8	246.8 (26.5)	<0.001	<0.001	<0.001	<0.001
6	IL-8	117.0 (21.9)	0.012	0.001	0.035	0.001
7	LDL+IL12	180.2 (26.5)	<0.001	0.258	<0.001	0.27
8	IL-12	93.0 (18.5)	0.285	<0.001	0.395	<0.001
9	LDL+IL15	175.8 (28.6)	<0.001	0.05	<0.001	0.096
10	IL-15	100.3 (10.0)	0.943	<0.001	0.631	<0.001
11	LDL+IL17	107.0 (33.8)	0.586	<0.001	0.861	0.001
12	IL17	83.4 (29.7)	0.159	<0.001	0.201	<0.001
13	LDL+IL18	114.1 (76.0)	0.615	0.002	0.948	0.003
14	IL18	100.7 (42.0)	0.967	<0.001	0.928	<0.001

Note: The study was performed on the THP-1 human cell culture obtained from American Type Culture collection (ATCC). Cells were maintained in RPMI with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 mg/ml penicillin/streptomycin. THP-1 monocytes were differentiated into macrophage-like cells by incubation for 3 days in medium supplemented with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml). Total LDL (density 1.019–1.063 g/ml) were isolated from hyperlipidemic plasma of donors by preparative ultracentrifugation as previously described (Chapman et al., 1981). After a 3-day incubation, both LDL (100 mg/ml) and interleukins (Il6 and Il15 each at a concentration of 50 ng/ml) were added simultaneously and incubated for 24 h. After incubation lipids were isolated using the Folch method (Folch et al., 1957), and cholesterol was quantified as previously described (Gamble et al., 1978). Protein was measured in 40 µl aliquots of cell lysate using Lowry method (Lowry et al., 1951) with bovine serum albumin solution as a standard. All measurements were performed in duplicate.

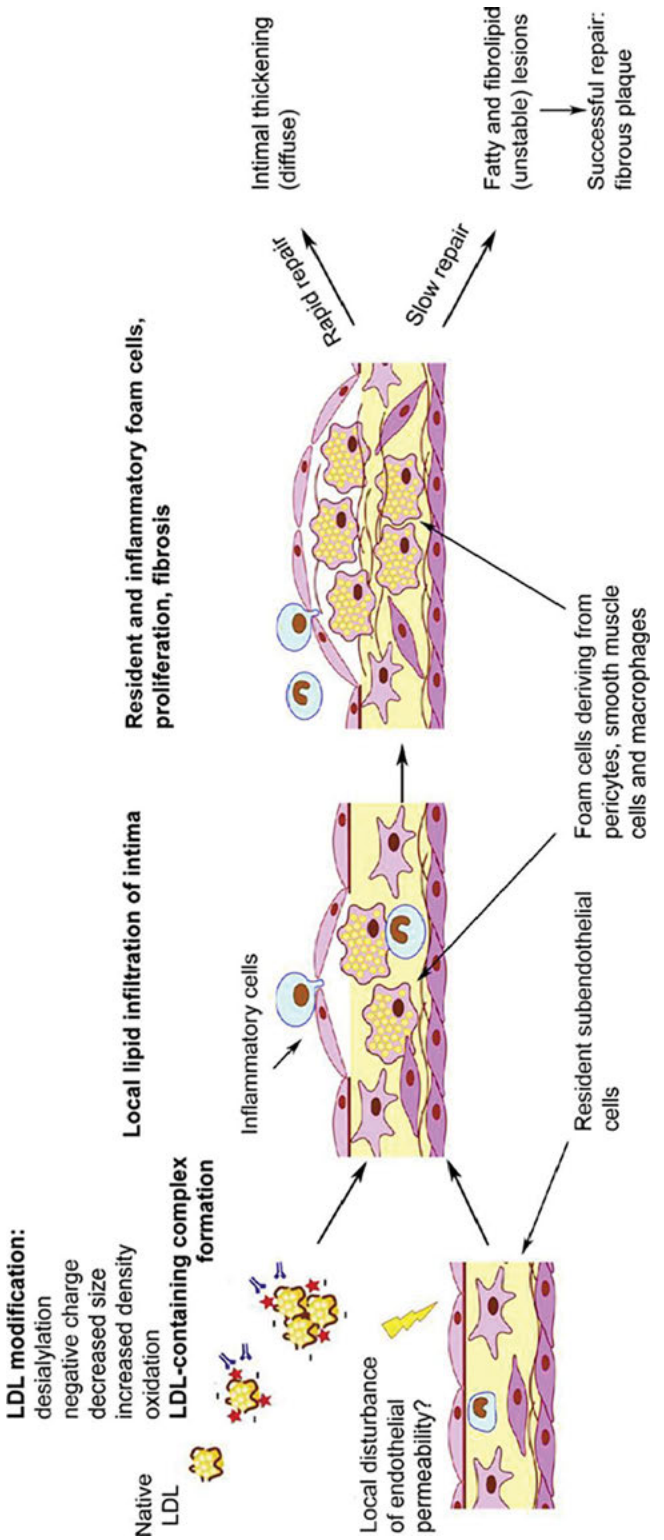

Figure 8.1 Schematic overview of initiation of atherosclerotic lesion formation. Reproduced from Orekhov and Ivanova (2016), with permission.

An increase in the concentration of pro-inflammatory cytokines leads to endoplasmic reticulum stress in the arterial wall cells, which in turn can lead to the initiation of apoptosis (Ivanova and Orekhov, 2016b). Cytokine-induced inflammation disrupts the normal functioning of mitochondria, their synthesis and mitophagy (Gkikas et al., 2018), which also leads to apoptosis. These effects contribute to the prolonged circulation of pro-inflammatory agents in the vascular bed, which can further stimulate the launch of a cascade of inflammatory reactions at the site of vascular damage.

It is plausible that atherosclerotic lesion development occurs when the inflammatory process cannot be resolved in a regular way and becomes chronic (Figure 1) (Orekhov and Ivanova, 2016). Thus, the response of the innate immunity is a trigger for the formation of foam cells, while violation of the normal immune response is the cause of inflammation chronification, which leads to the development of atherosclerotic lesions. It would be interesting to identify the genetic factors that regulate these processes.

8.3 Variants of the Nuclear Genome Associated with Atherosclerosis

It is generally believed that in rare cases, single nucleotide substitute in nuclear genome may greatly affect the clinical phenotype. On the opposite, as a rule, it is impossible to establish a causal relationship between a certain variant of the nuclear genome and a phenotypic manifestation of this change. This is also true for detecting genetic predisposition to chronic pathologies like atherosclerosis and related cardiovascular diseases. In the last decade, the genome-wide associations study (GWAS) approach came into play and was widely applied. In general, this is a targeted search for associations between genomic variants and phenotypic traits, i.e. for the search of associations between single nucleotide polymorphisms (SNPs) and diseases. Such studies provide a great bulk of information, which, however, can hardly be implemented into practical healthcare. On the other hand, systematic approach to the search for such associations is necessary to assess the quality of the results of numerous studies in this area and to identify the most promising sets of markers for further detailed research (Belsky et al., 2013; den Hoed et al., 2015).

Noteworthy, GWAS data are not particularly valuable without modern computational methods. An effective tool for finding the dependencies between mutations and their manifestations are systems biology methods (Huan et al., 2013; Frades et al., 2019). Analysis of molecular networks allows explaining how genetic disorders interact with the environmental factors and why this interaction manifests itself in the form of a pathological phenotype. As an example, Talukdar et al. have analyzed the genetic data and gene expression profiles of several tissues (atherosclerotic arterial wall, internal mammary artery, liver, skeletal muscle, visceral fat, subcutaneous fat, and whole blood from the late-stage patients with coronary artery disease [CAD]). As the result, 30

regulatory gene networks (RGNs) were identified being associated with CAD development and their key mechanisms of action. As a proof of concept, the researchers aimed at key drivers (*AIP, DRAP1, POLR2I*, and *PQBP1* genes) in the RGN of the arterial wall with cross-species verification, including RNA processor genes. This RGN was re-identified in THP-1 foam cells and independently in blood-derived monocytes of CAD patients and macrophages from carotid atherosclerotic lesions. Such studies can help to better identify the candidate genes critical for the development of CAD identified in the framework of GWAS, and this is only the beginning of the path to achieving the goals of personalized medicine (Talukdar et al., 2016).

Currently, about 60 genomic regions associated with coronary heart disease have been identified. However, most cases of hereditary predisposition to atherosclerosis still cannot be explained. Thus, there exists a need for search of other loci associated with atherogenesis. An effective strategy can be a large-scale assessment of promising genomic associations, proposed as a part of various genome research projects. For example, some of the mutation changes detected are associated with endothelial cell dysfunction. Furthermore, genes regulating cellular adhesion, leucocyte migration, coagulation, inflammation, vascular smooth muscle cell differentiation and genes that regulate energy metabolism may prove to be relevant for atherosclerosis. A correlation analysis was performed that linked the identified gene regions with cell type-specific gene expression patterns and phenotypic features such as plasma protein levels (CARDIoGRAMplusC4D Consortium et al., 2013; Howson et al., 2017). Such studies may provide novel and important information that will help developing new therapies.

Numerous genetic studies have been conducted to establish the risk factors of atherosclerosis (Björnsson et al., 2019; Li et al., 2019b Rincón et al., 2019). For example, four new SNPs have recently been found recently to be specifically associated with abdominal aortic aneurysm (Marsman et al., 2019). There is evidence that an increased risk of cardiovascular disease (CVD) exists among bearers of haplogroup I1 (Y-DNA). The Y chromosome of these individuals was enriched in regulatory chromatin variants related to the development of coronary heart disease (Eales et al., 2019; Lusis, 2019). Aortic aneurysm as a whole is characterized by a pathogenesis similar to atherosclerosis, namely: infiltration by the inflammatory cells of the vessel wall, degradation of the extracellular matrix, and dysfunction of vascular smooth muscle cells (Wang Y. et al., 2019).

One way to control the CVD complications is to reduce the intake of saturated fats from food. However, if SNPs in the genes responsible for cholesterol metabolism are present, high levels of total cholesterol and LDL cholesterol and triglyceride, and low HDL cholesterol persist regardless of the diet (Walker et al., 2011).

In addition to mutations in coding regions of genes, changes in the regulatory regions are also capable of affecting gene expression. The long non-coding RNAs (lncRNAs), that are longer than 200 nucleotides, can regulate the expression

of neighboring genes. Recent evidence suggests the role of lncRNAs expression on proliferation of vascular smooth muscle cells and apoptosis, and, consequently, on the risk of aortic aneurysm and atherosclerosis (Brozovich et al., 2016). The likely mechanism of such influence is overexpression of lncRNA-p21 and the increase of p53 downstream target genes *Puma*, *Bax*, *Noxa*, and *MDM2* at mRNA and protein level. This is consistent with the involvement of these genes in the regulation of cell survival, apoptosis, and proliferation (Wu Y. et al., 2014). Importantly, the expression of this lncRNA is suppressed in atherosclerotic plaques in *apoE*$^{-/-}$ mice. In addition, several SNPs at the 9p21 locus that are known to be associated with atherosclerosis were shown to have an impact on the expression of lncRNA ANRIL, which contains at least 21 exons and has multiple linear and circular isoforms. Polymorphisms within ANRIL were shown to be associated with increased risk of different types of cancer and atherosclerosis, obesity, and type 2 diabetes (Kong et al., 2018).

One of the genes implicated in aneurism formation is *LOX*, which encodes for lysyl oxidase, a protein responsible for cross-linking of collagen and elastin molecules during the formation of regular collagen fibers. *LOX* is important for mechanical integrity of the arterial wall, and missense mutations in this gene were shown to cause aneurysm formation and aortic dissection due to insufficient crosslinking of elastin and collagen in the aortic wall. The study that linked *LOX* to aneurism formation offered an algorithm that could be used to search mutational changes associated with the development of coronary heart disease (Lee et al., 2016).

In most cases, familial hypercholesterolemia develops in the presence of pathogenic variants of genes encoding the low-density lipoprotein receptor (*LDLR*), its ligand apolipoprotein B (*APOB*), or the proprotein convertase subtilisin/kexin type 9 (*PCSK9*), which takes part in the regulation of cholesterol metabolism. Binding of PCSK9 to EGF-A (extra-cellular domain of LDLR) leads to degradation of the receptor. A decrease in LDLR causes a decreased LDL metabolism, which can lead to hypercholesterolemia. The PCSK9 is synthesized in a soluble inactive form of proenzyme, which can be activated spontaneously during intramolecular processing in the endoplasmic reticulum (Gu et al., 2013; Cariou and Dijk, 2020). Other identified genes that were less frequently detected in familial hypercholesterolemia encode apolipoprotein E (*APOE*) and the signal-transducing adaptor family member 1 (*STAP1*) (Fouchier et al., 2014). The modern classification of lipid metabolism disorders was developed by Donald Fredrickson in 1965 and is based on changes in the profile of plasma lipoproteins. This classification was adopted by the World Health Organization, although it does not take into account the level of HDL, an important factor that reduces the risk of developing atherosclerosis, as well as the role of genes that cause lipid disorders (Di Taranto et al., 2019; Gomez et al., 2019). However, the genetic disorders described above are generally not specific for typical vascular wall cells, but appear to be important at the level of the whole organism.

8.4 Variants of Mitochondrial Genome Associated with Atherosclerosis

Accumulating evidence indicates that mtDNA mutations play a role in atherosclerosis development alongside with nuclear genome polymorphisms. An important part of mitochondrial proteins that are indispensable for proper functioning of the respiratory chain and energy production are encoded by mtDNA. Therefore, mtDNA mutations can lead to serious consequences, such as altered energy homeostasis, impaired glucose and fat metabolism, elevated oxidative stress and, ultimately, cell damage and death. These processes are tightly implicated in atherosclerotic lesion development (Weakley et al., 2010).

The main function of mitochondria in the eukaryotic cell is oxidation of organic compounds and the use of the released energy for ATP synthesis and thermogenesis (Hu and Liu, 2011). The size of mtDNA, a double-stranded circular molecule, in human cells is about 16,600 nucleotide pairs. It encodes 2 rRNA, 22 tRNA, and 13 subunits of respiratory chain enzymes. Each mitochondrion can contain two to ten copies of mtDNA. If all mtDNA copies contain the same polymorphism, it is called homoplasmic, while presence of mutant and wild-type copies of the mitochondrial gene within the same cell is referred to as heteroplasmy. Mutations in mtDNA are responsible for a number of inherited human diseases. These changes are inherited almost exclusively through the maternal line (Chan, 2006; Sobenin et al., 2013; Tang et al., 2014; Strassheim et al., 2018).

Mitochondrial reactive oxygen species (ROS) generated by dysfunctional mitochondria not only contribute to cell damage during oxidative stress, but also act as intermediate signals, which are modulators of gene expression associated with the development of atherosclerosis. Mutational changes in the mitochondrial genome can partially explain the focal nature of the vascular wall damage in atherosclerosis. Cells of different sections of the vascular wall can vary significantly by the level of heteroplasmy, which leads to differences in cellular metabolism. Therefore, due to the development of mitochondrial dysfunction, and some cells become more susceptible to various pathological influences triggering the atherosclerotic process (Sobenin et al., 2013; Zorov et al., 2014).

Previous studies have revealed several atherosclerosis-associated mtDNA mutations, including m.3256C > T, m.3336T > C, m.5178C > A, m.12315G > A, m.14459G > A, m.15059G > A, and m.13513G > A. It was found that a certain spectrum of pro- and anti-atherogenic mtDNA mutations is characteristic for various types of atherosclerotic lesions of the human aortic intima (Sazonova et al., 2015).

As stated above, uncontrolled ROS production under stress of various origin changes the dynamics of mitochondrial functioning and increases mitochondrial fission and fragmentation at the expense of mitochondrial fusion. Apparently, nuclear respiratory factors (NRF1 and NRF2) play an important role in this process. These factors regulate the expression of the transcription factor of the

mitochondrial genome (Tfam) and many other mitochondrial genes involved in oxidative phosphorylation. Recent studies demonstrated a link between mitochondrial dysfunction and insulin resistance through altered expression of the *PPARGC1A* gene in muscle and liver tissue. It was shown that the suppression of the synthesis of PGC1a protein (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) impacted mitochondrial biogenesis and contributed to the induction of insulin resistance (Siasos et al., 2018). The function of PGC-1α is to stimulate mitochondrial biogenesis and promotes remodeling of muscle tissue into a fibrous composition and is also involved in the regulation of carbohydrate and lipid metabolism (Liang and Ward, 2006).

Study of the $tRNA^{Thr}$ m.15927G> mutation revealed its association with coronary heart disease. The m.15927G > A mutation hindered the highly conserved base-pairing (28C-42G) of anticodon stem of $tRNA^{Thr}$. Molecular modeling study demonstrated that the m.15927G > A mutation resulted in an unstable $tRNA^{Thr}$ structure. The study conducted on cybrid lines bearing mitochondria with the m.15927G > A mutation showed a significant decrease in the efficiency of aminoacylation, which led to a decrease in the number of polypeptides encoded by mtDNA, respiratory failure, a decrease in membrane potential, and an increase of ROS production. The increased release of cytochrome c into the cytosol and caspase 3, 7, 9 and PARP proteins indicated that the presence of this mutation contributes to the development of apoptosis. These observations confirm the data published by different groups supporting the important effect of mitochondrial mutations on the pathophysiology of coronary heart disease (Jia et al., 2019).

Mitochondrial dynamics, fission, and fusion were first detected and studied in yeast. Over the past 10 years, it has become apparent that these processes are common to all cells containing mitochondria. Impairment of fission and fusion contributes to the disease development (Diot et al., 2016). The processes of mitochondrial dynamics determine the morphology of mitochondria, their qualitative and quantitative indicators, which, as it turned out, is critical for the development of cardiovascular diseases. These processes are related to the balance between energy requirements and nutrient intake. Changes in the morphology of mitochondria can be regarded as bioenergetic adaptation during pathological remodeling of cells of the cardiovascular system (Vásquez-Trincado et al., 2016). For example, aging processes are directly related to changes of mitochondrial dynamics. It was found that in old age, the volume, integrity, and functionality of mitochondria decreases due to the accumulation of mutations in mtDNA after oxidative damage caused by ROS. In elderly organisms, mitochondria are characterized by a decrease in the efficiency of oxidative phosphorylation, ATP production, an increase in the formation of ROS and a decrease in antioxidant protection. Moreover, the regulation of mitophagy and autophagy is impaired, preventing the removal of dysfunctional mitochondria. Similar processes enhance mitochondria-mediated apoptosis (Chistiakov et al., 2014).

It is likely that a large part of mtDNA mutations occurs due to the increased ROS production in the proximity of the mitochondrial genome. Accumulation

of mtDNA mutations and impaired mitochondrial function contribute to atherogenesis, as has been confirmed by several studies. That, in turn, confirms the link between increased oxidative stress and cardiovascular risk (Yu and Bennett, 2014). However, oxidative stress cannot be considered as the main mechanism of mtDNA mutagenesis (Trifunovic et al., 2005; Itsara et al., 2014). The key factors in mtDNA mutation generation are replication errors by the mitochondrial DNA polymerase γ and spontaneous base hydrolysis (Kennedy et al., 2013).

Therefore, impaired mitochondrial function, biogenesis, and dynamics disrupt the cell homeostasis. Mitochondrial damage contributes to aging and a number of age-related pathologies. Thus, in the fight against aging and age-related diseases, it is possible to use strategies that effectively improve or eliminate defects in mitochondrial dynamics. To achieve this goal, it is necessary to develop small molecules capable of enhancing mitochondrial biogenesis and inducing mitophagy for patients with age-related disorders. Consequently, effective new therapeutic strategies should include coordinated induction of both mitophagy and mitochondrial biogenesis to maintain a healthy mitochondrial population. Further intervention studies are needed to test how mitophagy and mitochondrial compounds that induce biogenesis affect human physiology. Therefore, the knowledge of the mutation spectrum of the mitochondrial genome may be useful for attending physicians and medical geneticists for early detection of atherosclerosis and analysis of predisposition to its development.

8.5 Role of Mitochondrial Mutations in Cellular Mechanism of Atherosclerosis; Chronification of Inflammation

Some of the above mentioned pro-atherogenic mtDNA mutations associated with atherosclerosis also correlated with pro-inflammatory activation of monocytes in primary culture (Orekhov et al., 2015). Two homoplasmic mutations, m.1811A > G and m.9477G > A, correlated with the degree of monocyte activation. At least three heteroplasmic mutations (m.14459G > A, m.1555A > G, and m.12315G > A), also correlated with pro-inflammatory activation of circulating human monocytes. Thus, some mutations may alter monocyte activation in atherosclerosis through mitochondrial dysfunction.

Studies on cybrid cell lines carrying variants of the mitochondrial genome obtained from atherosclerotic patients revealed the disturbances of mitochondrial functions including impaired mitophagy (Orekhov et al., 2019).

Mitophagy is involved in the innate immune response (Orekhov et al., 2020a). Inhibition of mitophagy in primary culture of human monocyte-derived macrophages increased lipopolysaccharide-induced pro-inflammatory response in the form of up-regulation of the IL-1β gene both in control cells and in the presence of mitophagy inhibitors. Repeated stimulation caused a much smaller pro-inflammatory response in control cells. When mitophagy was suppressed, re-stimulated cells continued to demonstrate a pro-inflammatory response. This

means that suppression of mitophagy leads to a loss of immune tolerance and uncontrolled continuation of the pro-inflammatory response of macrophages.

These and other data allowed to formulate a hypothesis explaining the important role of mitochondrial mutations in atherogenesis (Figure 2). According to this hypothesis, circulating atherogenic multiple modified LDL induces lipid accumulation in the arterial cells (Tertov et al., 1992). Modified LDL particles form self-associates taken up by arterial cell *via* nonspecific phagocytosis (Tertov et al., 1989). Stimulation of phagocytosis activates the pro-inflammatory response of macrophages (Orekhov et al., 2020b). This causes accumulation of intracellular lipids (Table 8.1). In normal response of innate immunity, the pro-inflammatory reaction resolves quickly. However, in the case of macrophages carrying mtDNA mutations associated with defective mitophagy "non-stop" pro-inflammatory response is produced that does not arrest. Local inflammation induced by this "non-stop" response becomes chronic atherosclerotic lesion is formed.

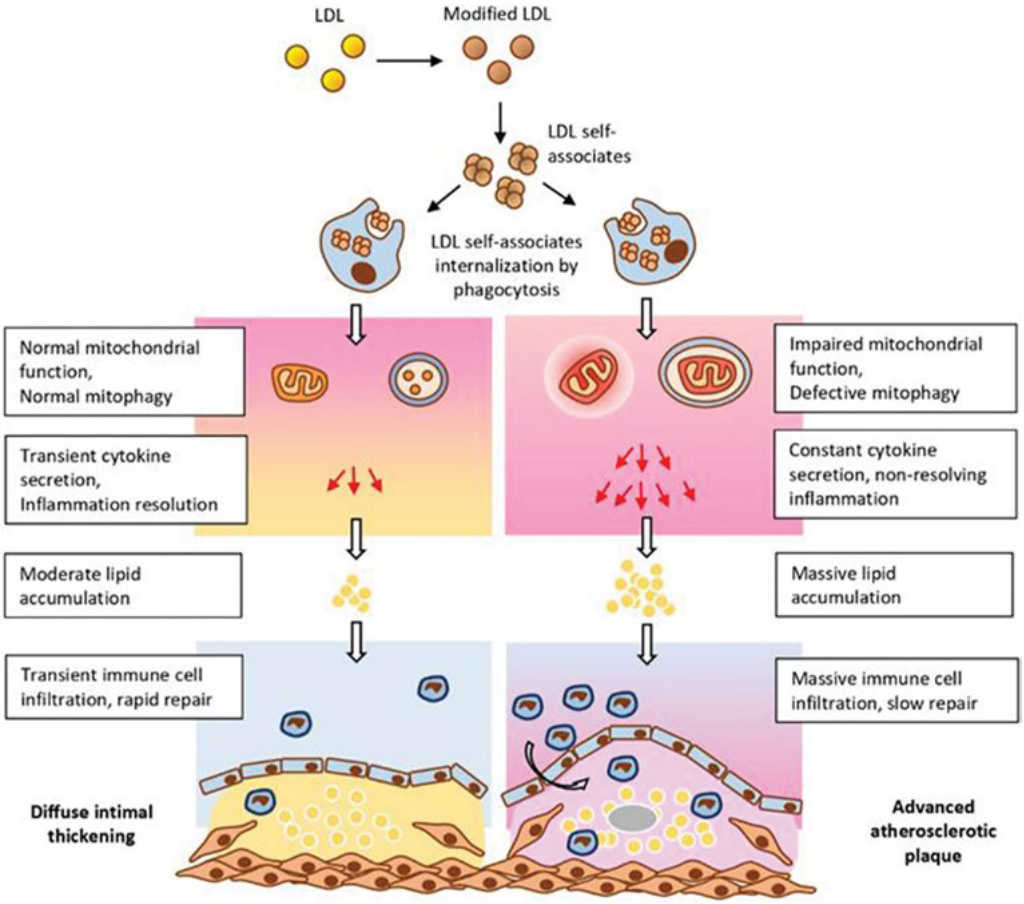

Figure 8.2 Impaired mitochondrial function and deficient mitophagy promote atherosclerotic lesion formation. Multiply modified LDL particles being accumulated and then internalized by macrophages are capable to alter mitochondrial function which ultimately leads to the formation of atherosclerotic plaques. Reproduced from Orekhov et al. (2020a), with permission.

8.6 Conclusions

The genetic aspects of atherosclerosis attract much attention in the current research. As novel genetic methods evolve, a huge amount of data becomes available, including identification of mtDNA mutations. While genomic mutations associated with atherosclerosis are relatively well studied, mtDNA mutations provide new opportunities for development of novel diagnostic and therapeutic tools. The discovered set of mutations in the mitochondrial genome has a clear connection with the likelihood of the development of the disease, its course, and prognosis. The acquired knowledge on the mitochondrial involvement in the development of atherosclerosis and chronic inflammation will be used for designing more selective, mitochondria-targeting treatments.

Abbreviations

APOB: apolipoprotein B
ATCC: American Type Culture collection
bFGF: basic fibroblast growth factor
CAD: coronary artery disease
CVD: cardiovascular disease
GWAS: genome-wide associations study
HDL: high-density lipoprotein
IL: interleukin
IL-1: interleukin 1
LDL: low-density lipoprotein
LDLR: low-density lipoprotein receptor
lncRNA: long non-coding RNA
MCP-1: monocyte chemoattractant factor 1
mtDNA: mitochondrial DNA
PCSK9: proprotein convertase subtilisin/kexin type 9
PDGF: platelet-derived growth factor
RGN: regulatory gene networks
ROS: reactive oxygen species
SNP: single nucleotide polymorphism
TNF: tumor necrosis factor
TNF-α: tumor necrosis factor alpha
tRNA: transport RNA
Y-DNA: haplogroup I1

Disclosures and Conflict of Interest

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Chapter 9

The Microbiota of the Human Gut and Cardiometabolic Health

Angelos K. Sikalidis, PhD, Adeline Maykish, and Aleksandra S. Kristo, PhD

Department of Food Science and Nutrition, California Polytechnic State University, San Luis Obispo, California, USA

asikalid@calpoly.edu

Keywords: Bacteroidetes, *Bacteroidia*, Body Mass Index (BMI), branched-chain amino acids (BCAA), cardiovascular disease (CVD), chronic disease, C-reactive protein (CRP), *Cytophagia*, dysbiosis, Firmicutes, *Flavobacteria*, glucose transporter type 4 (GLUT4), glycemic control, gut health, insulin resistance, medical nutrition therapy, metabolic health, metabolic syndrome, microbial metabolites, microbiome, microbiota, nutrigenomics, oxidative stress, prebiotics, precision nutrition, probiotics, reactive oxygen species (ROS), *Sphingobacteria*, toll-like receptor 4 (TLR4), type 2 diabetes mellitus (T2DM)

9.1 Introduction

In the United States alone, approximately 39.8% of the population is considered obese, as defined by a Body Mass Index (BMI) over 30.0 [1]. This includes 35% of all people under 40 and over 40% of middle-aged adults, while most alarmingly the trend for these figures is clearly increasing [2]. Obesity is a global epidemic interestingly observed in both developed and developing countries [2]. Unhealthy, often hypercaloric, diets combined with sedentary lifestyle typically coinciding from a behavioral standpoint, have been identified as major contributors to the development of the obesity epidemic in America and globally. Obesity can, over time, contribute significantly to the manifestation of various health complications, such as cardiovascular disease (CVD), hypertension, and type 2 diabetes mellitus (T2DM). While obesity has historically been considered the result of primarily external factors, there is evidence to suggest that a significant component of obesity may be strongly associated with a person's gut environment. Recent work on

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the human microbiome strongly supports the notion that an individual's microbiota profile could favor a series of phenotypes such as obesity, inflammation, insulin resistance and eventually induce T2DM and CVD.

The human microbiome is comprised of two primary phyla, namely Bacteroidetes and Firmicutes, typically in a ratio favoring Bacteroidetes over Firmicutes (B/F > 1). Nonetheless, numerous studies have demonstrated that in obese individuals, this ratio is altered, in a manner leading to a higher prevalence of Firmicutes over Bacteroidetes [3, 4]. Research has also validated that transplanting microbiota from obese mice to germ-free (GF) mice resulted in significant weight gain in the latter group compared to control [6], suggesting that the B/F ratio difference could well contribute significantly to the development of the obese phenotype observed. From a mechanistic perspective, it is proposed that the specific demography of the gut microbiome in obese individuals induces increase of energy harvest by the host organism, with any caloric surplus essentially resulting in an overall significant increase of adiposity [5]. This function of gut bacteria is largely attributed to the increased presence of Firmicutes, which are able to metabolize undigestible/insoluble carbohydrates resulting in a higher energy harvest. The particulars of such link and the exact mechanism remain largely elusive [6, 7]. Nevertheless, the evidence available suggest a favorable strong link between the microbiome in terms of demography and obesity, rendering the investigation of the microbiome and by extension its role in T2DM and CVD interesting fields of inquiry with notable potential for therapeutic applications.

9.2 The Microbiome

In order to gather data regarding the microbiome, stool samples are typically collected and analyzed via 16S rRNA sequencing. This approach has estimated the human gut microflora to be containing over 35,000 species, and over 10 million non-redundant genes [7]. The species identified are categorized into one of six major phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Of these, the two most common are Firmicutes and Bacteroidetes, comprising 90% of the microbiome, while both have been associated to obesity.

The Bacteroidetes phylum consists of four major classes: *Bacteroidia, Flavobacteria, Sphingobacteria,* and *Cytophagia*, all of which play a role in fermenting otherwise indigestible carbohydrates [8]. Within those 4 classes, the most frequently found genera are *sphingobacterium, bacteroides, tannerella, parabacteroides, alistipes,* and *prevotella*, all of which are interestingly Gram negative [9]. In humans, dietary fibers and fructooligosaccharides (FOS) are indigestible in the sense that humans do not produce the enzymes for the digestion of these compounds [10]. Conversely, bacteria present in the gut can ferment these compounds for their own benefit, releasing short-chain fatty acids (SCFA) within the host (gut environment) as part of their metabolic processes [11]. This outcome can be beneficial for humans from an energetics standpoint, as SCFAs

can constitute a significant source of energy [7]. Firmicutes also play an important role in the generation of SCFAs, the main SCFA produced being butyrate (some Bacteroidetes also produce butyrate) [12, 13]. The Firmicutes phylum can be grouped into three major classes: *Clostridia, Negativicutes,* and *Bacili*. It consists of over 200 genera, including *Staphylococcus, Lactobacillus, Ruminococcus*, and *Clostridium* [9]. The Firmicutes phylum consists primarily of Gram-positive bacteria, with the exception being those in the class *Negativicutes. Negativicutes* are interesting because of the presence of an outer membrane with lipopolysaccharides, making them stain Gram negative [14]. The Firmicutes phylum has not been fully investigated, and therefore a comprehensive understanding regarding its benefit to the human body remains elusive and largely non-conclusive currently. Nonetheless, the Firmicutes population is clearly positively associated with dysbiosis, with lower numbers of Firmicutes to be considered more favorable. The focus of most microbiome studies center around decreased numbers of Firmicutes, derived from an observational standpoint, thus leaving a knowledge gap regarding Firmicutes' function and mechanism(s) of action. However, it is believed that their primary role lies in metabolic degradation of an energy source/fuel [15] and can therefore be associated with calorie bioavailability and utilization. Firmicutes have also been found to raise lipid droplet numbers in zebrafish, which positively correlates with fatty acid uptake [16]. This is important, as fatty acids can store energy in the body to be used when glucose is not available [17] and have established health benefits in certain cases depending on the type, such as a decreased risk of heart disease, cancer, and arthritis [18, 19]. The issue with Firmicutes and metabolic deregulation has perhaps mostly to do with the degree of accumulation leading to significant lipid amount stored. In a healthy individual, the low number of Firmicutes present, results in adequate energy uptake for the host that does not lead to high calorie availability, thus limiting positive energy balance. Overabundance of Firmicutes in contrast, results in increased energy harvest, a higher caloric bioavailability, positive energy balance, all eventually promoting weight gain/obesity [7]. While this notion has not been proven indisputably, it has been proposed as a biologically plausible and reasonably probable mechanism via which Firmicutes can play a role in weight gain and eventually the development of obesity over time.

9.2.1 Eubiosis versus Dysbiosis

Eubiosis (from Greek ευ/eu: good and βίος/bios: life) refers to the normal/healthy profile of gut microflora, as opposed to dysbiosis (from Greek δυς/dys: non-favorable/difficult and βίος/bios: life) which produces a demography that induces risk for certain diseases (Fig. 9.1).

In a state of eubiosis, the microbiome plays several roles in producing SCFAs and branched-chain amino acids (BCAA), affecting lipid metabolism, and generating other key metabolites. Eubiosis in the gut typically is a condition in

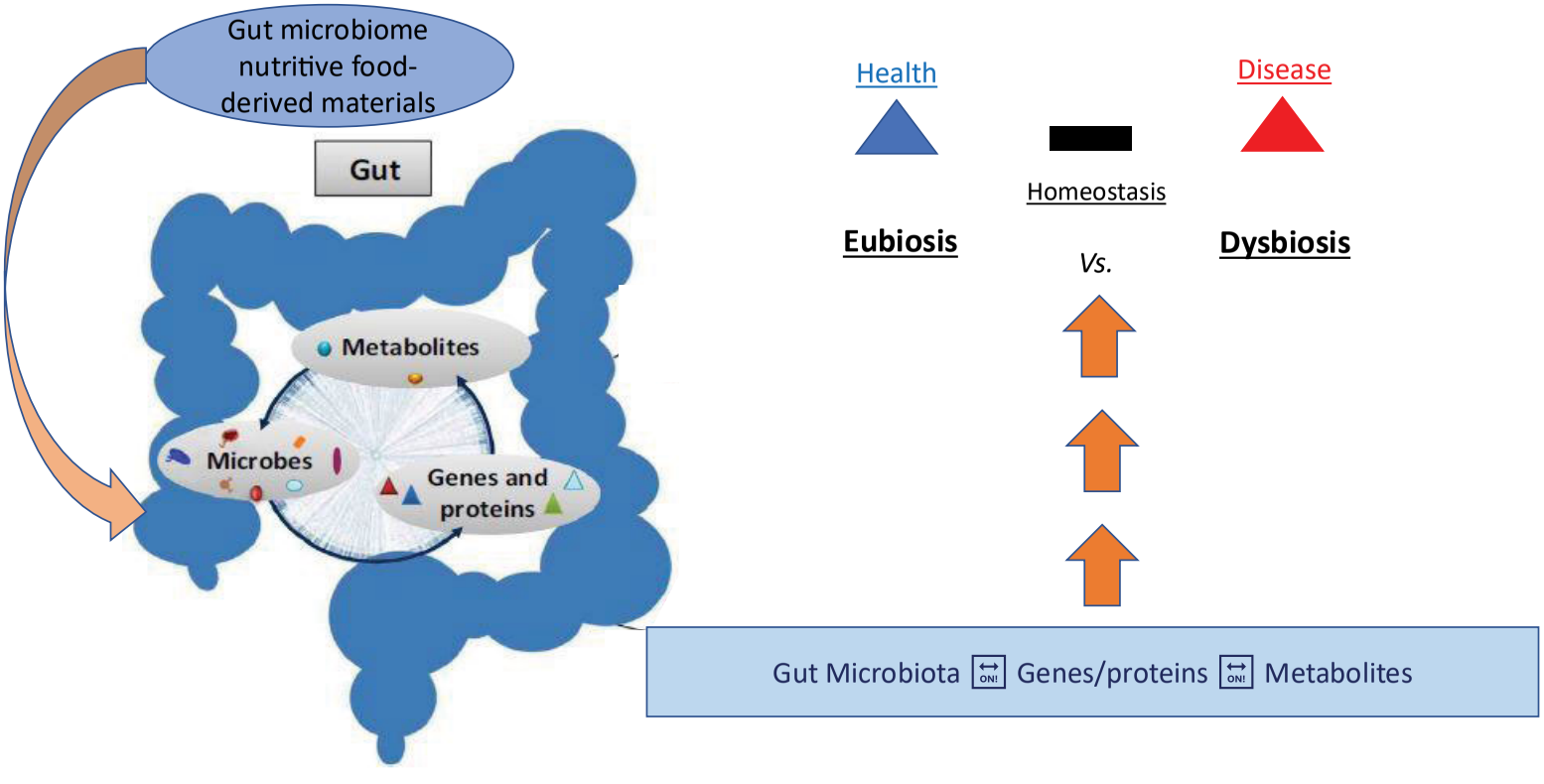

Figure 9.1 Conceptual schematic pictogram elucidating the relationship axis of microbes–metabolites–gene expression vs. eubiosis/dysbiosis equilibrium and disease risk.

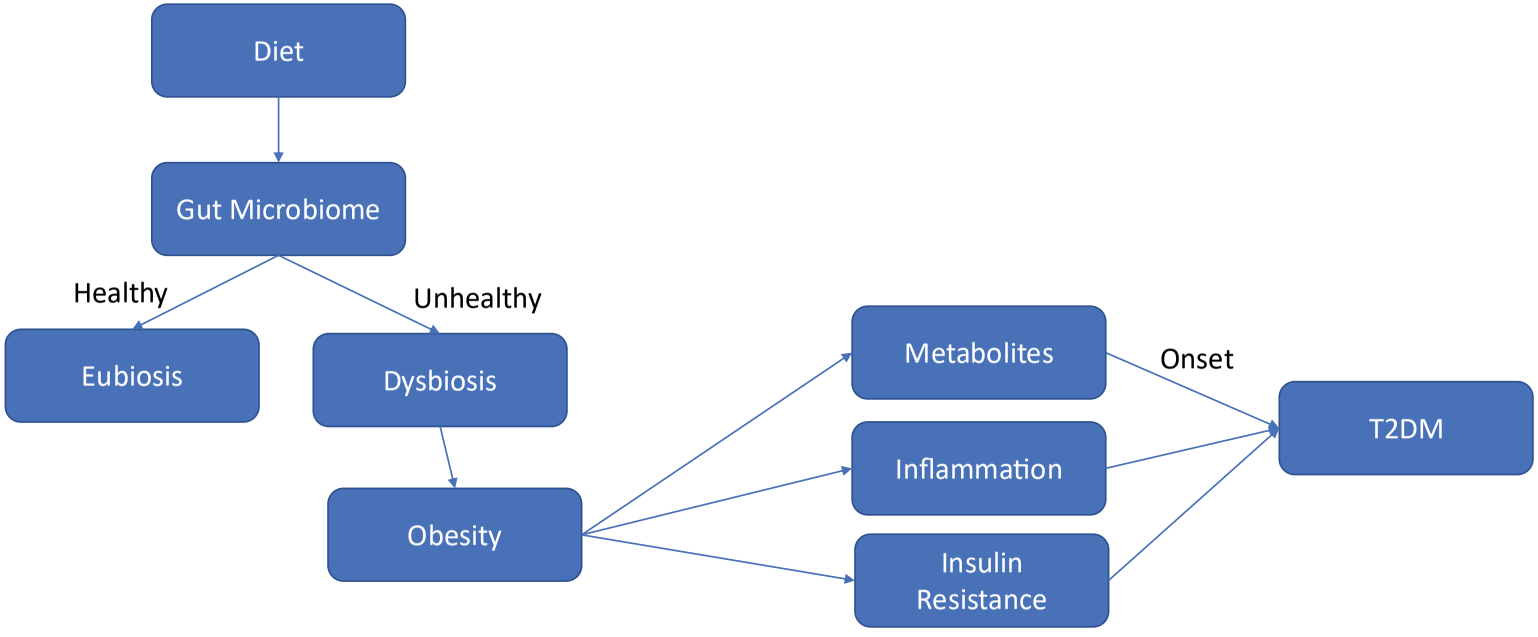

Figure 9.2 Chart exemplifying the effect of diet on the microbiome and the relationship leading to increased risk for T2DM development.

which there is a vibrant gut bacterial population composed of 95% Bacteroidetes, and 5% Firmicutes, forming an ideal B/F ratio. When B/F ratio is such, the microbiome extends optimized gut health, regulates and controls opportunistic pathogens, and contributes to systemic good health. [20]. Dysbiosis, on the other hand, can be defined as any change in the normal/desirable flora in an otherwise healthy gut [20]. In the vast majority of cases, dysbiosis has a negative impact, and can lead to obesity and the onset of several diseases, typically of chronic nature, including T2DM and ensuing CVD (Fig. 9.2) [20].

In a groundbreaking experiment by Turnbaugh et al., GF mice were colonized with gut microbiota from conventionally raised (CONV) mice and were monitored for changes in weight. Within 10-14 days, the GF mice displayed increased body fat, despite a decrease in food consumption [6]. This change was shown to be attributed to the microbial fermentation of undigestible polysaccharides, absorption of monosaccharides, and genes in the microbiome that promote the growth of adipocytes. These findings led researchers to support that obese individuals are typically more efficient at energy harvest, by means of their microbiome, compared to lean counterparts, which may provide some explanation for weight gain.

In another set of experiments by Liou et al., diet-induced obese mice underwent Roux-en-Y gastric bypass (RYGB), which resulted in a decreased body weight and loss of fat mass. Standard 16s ribosomal sequencing was performed on fecal samples to study changes in the mouse microbiome after surgery. Mice that underwent RYGB displayed altered gut microbiota, especially by *Clostridiales*, a family in the Firmicutes phylum [21]. By the end of 12 weeks, the number of *Clostridiales* decreased significantly compared to pre-surgical numbers. This correlates with the variable B/F ratio, where lean individuals displayed lower numbers of Firmicutes than their obese counterparts [21]. This study shows that it is not only the microbiome itself that is responsible for weight fluctuation, but also the B/F bacterial composition of the microbiome.

In a separate study, Lund et al. fed CONV raised mice and GF mice either a high-fat diet (HFD) or low-fat diet (LFD). All mice were then monitored over the course of 2, 6 or 16 weeks for changes in weight. Weight monitoring interestingly revealed that the CONV mice fed the HFD displayed significant weight gain, as opposed to the GF mice fed the HFD, where the mice gained little to no weight. This points towards the idea that microbiome balance disturbance contributes to weight gain [22]. Taken together, these findings importantly suggest that dysbiosis within the microbiome of HFD feeding regime is a contributor towards weight gain and not a consequence of it.

9.3 Major Metabolic Contributors to Microbiome Profile Identity (SCFAA, BCAA, LPS)

It is well established that gut microbiota is responsible for the fermentation of otherwise indigestible carbohydrates, a byproduct of this process being SCFA.

Typically, SCFA play an important role in protecting the gut, where they line the epithelium and help form tight junctions between cells preventing intestinal permeability. When the B/F ratio is altered, the proteins that form the junctions are reduced, resulting in potential lipopolysaccharide (LPS) translocation. Translocation of LPS is an important first step in triggering the immune response. LPS can then bind to toll-like receptor 4 (TLR4), resulting in activation and dimerization of the two [23]. Once this dimerization occurs, downstream adaptor molecules are recruited, activating IL-1 receptor associated kinase, tumor necrosis factor (TNF) receptor-associated factor, transforming growth factor B-associated kinase, c-Jun N-terminal kinases (JNK) and IκB kinase (IKK). This activation of JNK and IKK can also induce insulin receptor substrates (IRSs) serine phosphorylation, an important step in establishing insulin resistance [23]. The newly formed IKK complex then meets and activates Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). The complex is then tagged for degradation. Then, NF-κB is in turn translocated into the nucleus, activating the inflammatory response [23].

9.3.1 LPS

LPS translocation is considered one of the first steps in the pro-inflammatory cascade response. To further emphasize the importance of dysbiosis, LPS, and inflammation, Whelan et al. fed mice either a high-fat diet, a diet supplemented with LPS (a low dose), or a control diet. The mice fed LPS developed obesity in a similar way as those who were fed a high-fat diet. However, when mice missing CD14, an immunoprotein responsible for inflammatory reactions, were fed LPS, no weight gain was observed [24]. In both the HFD and the diet supplemented with LPS, binding to TLR-4 was able to occur. However, in the absence of CD14, the inflammatory response was never initiated. Similar results have been obtained in mice not expressing TLR-4 [25].

9.3.2 Short-Chain Fatty Acids

As previously mentioned, SCFAs are highly important in the regulation of the inflammatory response, and a decrease in Bacteroidetes results in a decrease of SCFA. Butyrate, a type of SCFA, is a major metabolite and important for gut health. While its role is not completely understood, its known importance is highlighted by a series of experiments. In a study by Gao et al., mice were given sodium butyrate as a dietary supplement. Their insulin sensitivity and energy metabolism were both monitored over the course of 16 weeks. It was observed that mice which consumed a HFD along with butyrate supplements did not develop insulin resistance or obesity [26].

Interestingly, in a study that explored the gut microbiota of urban Italians versus a community of hunter-gatherers called Hadza located in Tanzania, to analyze how gut microbiota in urban settings compares to that of a foraging lifestyle, one that all human ancestors took part in. Fecal samples from 27 Hadza and 16 Italians were analyzed, and while much of this study focused on microbiota

demographics, SCFA profiles were also analyzed. Conclusively, it was found that urban Italians generate significantly more butyrate, whereas Hadza generate more propionate [27]. This is particularly interesting, as butyrate is typically associated with Firmicutes, and propionate with Bacteroidetes [27], while excess Firmicutes are associated with weight gain. Based on this association, it could be argued that butyrate supplementation in the discussed study would not be beneficial. However, by the same token it can be argued that this further emphasizes the importance of SCFA balance. Even in adverse conditions, the phenotype is still improved, showing that SCFA function in a corrective way in the gut. It is also important to note that some Bacteroidetes produce butyrate as well, meaning that in an ideal B/F ratio, the butyrate producing Bacteroidetes do produce ample butyrate to compensate for the lack of Firmicutes, thus restoring a metabolite balance in the gut environment [12].

Based on current knowledge, the microbiome seemingly plays an important role in inflammatory responses, both in its own right, as well as in an interplay with the diet [28]. Mackay and colleagues studied colitis in GF and CONV raised mice. The mice were treated with dextran sulfate sodium (DSS) to chemically induce colitis. The GF mice faired significantly worse than the CONV mice, displaying much worse colonic inflammation. Additionally, when GF mice were then colonized with CONV gut microbiota, their inflammatory response was reduced. To identify the cause of this reduction in inflammation, GF mice that were not colonized were treated with acetate, a SCFA known to be produced by Bacteroidetes. This also caused a decrease in colitis symptoms, further emphasizing the importance of SCFA in the inflammatory response [29]. This again underscores the importance of SCFA, especially those produced by Bacteroidetes and argues in favor of the proposition that SCFA production is a plausible mechanism for salvaging desirable phenotypes as per the gut health and related metabolism. Furthermore, numerous studies have indicated that the gut environment is highly responsive to a variety of bioactive compounds found in food sources (typically in fruits and vegetables and their products) in ways that reduce risk for several chronic diseases including T2DM, CVD and cancers [30].

9.3.3 Branched-Chain Amino Acids

While increased levels of SCFAs may be beneficial in the prevention of T2DM, this is not necessarily the case with BCAAs. Three of the nine essential amino acids that must be obtained through diet in humans are BCAAs (leucine, isoleucine, and valine) [31]. Elevated levels of BCAAs have been observed in obese individuals and those with T2DM [32], while obese individuals demonstrate increased BCAAs catabolism [33].

The effects of BCAAs on insulin resistance are fairly complex. BCAAs have been shown to interfere with insulin signaling by stimulating mTOR, a kinase that plays an important role in protein synthesis [34], S6K1, a kinase important for cell growth [35], and phosphorylation of Insulin Receptor Substrate 1 (IRS1) [33, 36].

To better understand this process, Newgard and co-workers, fed rats a HFD, a HFD supplemented with BCAAs, or a ND (Normal Diet; control). The rats were then fasted for 48 h, then re-fed their original diet. Upon re-feeding, there was an evident increase in the amount of phospho-mTOR^{Ser2448}, phospho-S6K1^{Thr389}, and phospho-IRS1^{Ser302} in the rats on the HFD supplemented with BCAAs group in comparison to the other two groups [33]. In the same study, the HFD/BCAAs rats also demonstrated a lower food intake and less weight gain than the ND (control) rats but were equally as insulin resistant as those on the HFD [33]. This helps to illustrate the role of BCAAs on the insulin signaling pathway, and how elevated levels of BCAAs help to upregulate this pathway.

In a cohort of 2,422 normoglycemic individuals followed for 12 years, 201 developed diabetes. The amino acids, amines, and other metabolites were assessed initially with liquid chromatography tandem mass spectrometry (LC/MS) and used as a baseline. Isoleucine, leucine, and valine exhibited higher concentrations during fasting, while levels were elevated up to 12 years prior to the development of T2DM. These observations correlated with a four-fold increase in the development of T2DM [37].

The results from the aforementioned studies are particularly interesting, as the recent shift in health trends contradict what has been observed by experimentation. There has been an increasing emphasis on dietary supplements, probiotics, and overall heath beyond diet and exercise. A popular type of dietary supplement is BCAAs, recommended before or after weightlifting exercise to build or restore muscle mass and produce energy [38]. While the intention of supplementation is to provide a series of essential amino acids, and specifically those inducing protein synthesis, in order to promote anabolism and muscle growth, significant results may not be seen if the exercise stimuli is insufficient to elicit muscle hypertrophy. Furthermore, while anabolism is induced, so is the production of pro-anabolic hormones such as insulin, leading to an orchestration of the metabolic signaling that maintains insulin input for longer periods of time. This condition, while it may induce anabolic processes, plausibly also induces insulin resistance over time [39, 40]. Notably, in individuals with adequate protein intake, the supplementation of BCAAs is most likely not necessary, or advantageous [41]. Furthermore, looking at evidence produced by a series of studies discussed, it seems that elevated levels of BCAAs inducing anabolism, lead to increased insulin resistance and the onset of T2DM.

9.4 Dysbiosis and the Development of T2DM

T2DM develops when, systematically, the pancreas is forced to produce gradually increasing amounts of insulin needed for glucose clearance, reaching eventually a point of such low insulin responsiveness from peripheral tissues (insulin resistance) that normoglycemia cannot be achieved [42]. The exact mechanism of this malfunction is unknown, however many factors, such as obesity, sedentary lifestyle, genetics, diet and other environmental factors, and now, the microbiome,

seem to influence the onset and development of this disease [43]. Insulin postprandially stimulates cells to uptake glucose by binding to insulin receptor on cellar membrane initiating a signaling cascade that normally leads to the translocation of glucose transporter type 4 (GLUT4) to the cellular membrane, thus initiating glucose clearance, as GLUT4 transports glucose into the cell down a concentration gradient [44]. While critical in the attempt to control onset of T2DM, the precise mechanism of how glucose undergoes this transportation is not entirely understood. Once inside the cell, glucose is either used for energy production or stored as glycogen within specific cells (hepatocytes and myocytes). Notably, if insulin is not present, there is no effective alternative mechanism for glucose clearance, resulting in hyperglycemia [45].

All responses described above, appear to be linked to the microbiome as well, while more specifically dysbiosis in the gut appears to be a risk factor for T2DM development. In a metagenome-wide study of 345 Chinese individuals with T2DM, 60,000 T2DM associated markers were validated, and all correlated with gut dysbiosis, decrease in butyrate producing bacteria, and an increase in oxidative stress [46]. This pioneering study provided solid evidence to suggest that the microbiome plays an important role in the development of T2DM, and dysbiosis is a contributor to the disease.

9.4.1 Inflammation

There is no clear, direct, known pathway by which inflammation relates to T2DM, while increasing evidence supports a definite relationship between induced inflammation and increased risk for insulin resistance, which in turn leads to T2DM. Individuals in a pre-diabetic state compensate for insulin resistance by β-cells insulin hypersecretion [47], but as the disease progresses, β-cells progressively become less able to supply the needed amount of insulin, they gradually become exhausted, and eventually die. In this context, β-cells dedifferentiation is being investigated as a means of β-cells failure in T2DM [48], but this pathway is not confirmed. Dietary modifications, including foods that reduce inflammation, are considered to help reduce risk of diabetes [49]. Several inflammatory cytokines, such as IL-1, IL-6, NF-κB, and TNF-alpha have been linked to obesity and diabetes. Specifically IL-6 biosynthesis functions as an initial state of inflammation. Upon generation, it moves to the liver triggering the rapid protein synthesis of C-reactive protein (CRP), which will be discussed further. IL-1 inhibits β-cell function by inducing the destruction of β-cells hence reducing β-cell mass over-time, which is primarily seen in T2DM development at the late stages of the disease. Higher levels of IL-1 have also been commonly observed in obese individuals. TNF-alpha IL-6, IL-1, are all adipokines, a subset of cytokines. They are secreted by adipose tissue and can function as pro-inflammatory signaling agents. As a result, dysregulation has been linked to obesity and T2DM onset, especially considering inflammation. In obese individuals, it has been consistently observed that

expression of pro-inflammatory cytokines is commonly followed by insulin resistance as well [50], hence making cytokines an important area of investigation when considering T2DM risk, onset and disease management. Based on this approach, the microbiome and inflammation have been a focus of study when looking for causes and treatments regarding obesity and T2DM.

The effect of RYGB on mice microbiomes was previously illustrated as an example [21], but such effect on human microbiomes and the body as a whole, is an important area of investigation for fully understanding how the microbiome and T2DM development are dynamically interrelated. In a study by Bornstein et al., five individuals with T2DM and one obese individual who had all undergone RYGB were studied for changes in microbiota as an effect of RYGB and how observed changes influenced disease management [51]. Researchers showed that RYGB procedure resulted in a decrease in both Firmicutes and Bacteroidetes, and a concurrent increase in Proteobacteria. It is important to note that while Bacteroidetes decreased, the phylum was still present in higher amounts than in Firmicutes, and the ideal B/F ratio was actually more closely achieved towards desirable post-operation, suggesting a favorable effect of the RYGB surgical operation [51]. The RYGB and subsequent microbiota change was also observed, when association of inflammatory state was tested. Out of all the detected species, 9 of the 22 species were significantly correlated to C-reactive protein, a biomarker for systemic inflammation commonly tested to assess inflammatory status [52]. Since 9 bacterial species demonstrated significant correlation with CRP levels in the blood, it was suggested that the gut microbiome closely relates to the inflammatory state. Furthermore, a significant correlation of inflammatory state as assessed by CRP levels was seen with BMI, suggesting that a lower BMI correlates with a lower inflammatory state [48]. This is consistent with the proposition that obesity, due to increased cytokine excretion, induces a chronic mild pro-inflammatory state.

In a study investigating the effect of HFD on inflammatory markers, both CONV and GF mice were fed either HFD or ND for 16 weeks [22]. Intestinal inflammation was evaluated by observing TNF-α mRNA levels and activation of a NF-κB reporter gene. Both TNF-α and NF-κB are important in the activation and sustenance of inflammatory responses [22]. Results showed that CONV mice, but not GF mice, on the HFD demonstrated weight gain and upregulated TNF-α mRNA levels. The TNF-α mRNA induction also directly preceded obesity onset in these animals. The same pattern was also observed for NF-κB activation, occurring in epithelia, immune, and endothelial cells in CONV mice. Furthermore, when fecal slurries from the HFD CONV mice were used to inoculate GF mice, it was observed that this trans-inoculation was enough to activate NF-κB in GF mice [22]. These results repeatedly highlight the importance of the microbiome associated to the inflammatory response, suggesting that the inflammatory response activation can be mediated by the microbiome.

9.4.2 Insulin Resistance

While insulin resistance is a metabolic condition that typically leads to T2DM, the microbiome appears to extend significant influence over the course of events and ultimately risk towards T2DM outcome. Work by Nieuwdorp et al. investigated the effects of microbial infusion in men with metabolic syndrome (MS), defined as: "a cluster of conditions that occur together… including increased blood pressure, high blood glucose, excess abdominal fat, and abnormal cholesterol or triglyceride levels" [53]. Intestinal microbiota from lean donors were transferred to male recipients with MS, and recipient microbiota and glucose metabolism were monitored post-transfer. Six weeks after trans-inoculation, insulin sensitivity of the recipients almost doubled, along with a significant increase in desirable butyrate producing bacteria [54].

Moreover, in a recent study, 291 non-diabetic Danish participants underwent microbiome analysis, and results were compared to 75 individuals with T2DM [55]. After analysis, insulin resistance levels and MS related metabolites by analyzing the serum metabolome profile were investigated and compared between the two groups of focus [56]. The microbiome composition of both groups was then clustered based on metabolite production, where it was found that 19 of the 74 clusters were significantly associated with insulin resistance and metabolic syndrome. The correlated clusters were consistent across all 291 individuals and were also confirmed in the T2DM patients [55]. This suggests that certain metabolites produced by microbial clusters are strongly associated with higher insulin resistance, reinforcing the idea that certain microbiome configurations contribute to the development of insulin resistance.

Metformin is commonly prescribed medication to help manage T2DM, where it functions to suppress glucose production and increase insulin sensitivity. In a study evaluating the effects of metformin on metabolic improvement and the microbiome, mice on HFDs were evaluated. Mice were fed: (i) HFD, (ii) HFD and then switched to a ND, or (iii) HFD supplemented with metformin. These dietary regimes were provided to induce obesity; hence obesity was the desired outcome, and not the development of T2DM. Results showed that upon administration of metformin to the HFD mice, the number of Bacteroidetes increased significantly, from 43% in the HFD group to 77% in the HFD-met group. Additionally, 18 metabolic pathways were also upregulated as a result of metformin administration [56]. While metformin is used primarily because of its positive effect on insulin sensitivity, interestingly it is shown to also alter the microbiome significantly in a desirable fashion. It cannot be ruled out that a potential mechanism via which insulin sensitivity is improved upon metformin administration is mediated by metformin-induced changes in the microbiome.

While the direct connection between the microbiome and insulin resistance is not clear, it is evident that the microbiome plays an important role in regulating insulin resistance. These discussed findings provide a foundation for understanding this pathway, although more work needs to be done in the field

to elucidate potential mechanistic pathways and series of events establishing how metformin, or other factors, may be influencing the microbiome leading to improved insulin sensitivity.

9.4.3 Oxidative Stress

Human cells naturally produce free radicals when exposed to agents including food substances, alcohol, and air pollutants [57]. Reactive oxygen species (ROS) form as a result of metabolism, and transfer unpaired electrons causing in turn oxidation of cellular machinery [47]. In healthy individuals, antioxidants, to a large extent, counteract this process, neutralizing ROS and defending homeostasis [58]. Imbalance, due to ineffective antioxidant defense, results in oxidative stress, which is closely related to glycation phenomena and diabetes onset [59]. Sedentary lifestyle and Western-type diets have been associated with overabundance of glucose and fatty acids, resulting in excess ROS. Glucose also reacts with plasma proteins to form glycation end-products, further producing ROS [59]. Oxidative stress induces inflammation which in turn increases risk for T2DM among other pathologies.

A recent study aimed to further understand the association between the microbiome and oxidative stress examining mice on HFD [61]. Mice were either fed HFD or HFD supplemented with lipoic acid, an antioxidant known to decrease oxidative stress [60]. ROS and total antioxidant capacity were assessed, as well as the microbiome of all mice in the study. Interestingly, in the mice supplemented with lipoic acid, Lactobacilli were present in much lower numbers than in the mice on the HFD with no lipoic acid supplementation group. This constituted an important finding, as lactobacilli are members of the Firmicutes phylum. Thus low numbers of lactobacilli observed also corresponded with decreased oxidative stress and better ROS levels, suggesting that antioxidants can ameliorate microbiome profile and subsequently oxidative stress, hence lowering risk for associated chronic disease such as T2DM [60].

While the correlation between the microbiome and onset of T2DM appears strong, there are several aspects of the microbiome that influence the development of disease. More specifically, microbiomes of patients with T2DM have begun to be evaluated in an attempt for a new search treatment. It has been revealed that the microbial composition of T2DM patients is quite different compared to non-T2DM individuals. The importance of diet in combination with disease state is critical in the establishment of microbiome's demography. As such, lifestyle and dietary intake factors need to be considered when evaluating the microbiome, in addition to disease state and medication. In a 2010 study, 36 men, 18 diagnosed with T2DM, with a wide range of BMI, underwent gut microbiota analysis [61]. Bacterial composition was analyzed using 16s rRNA sequencing, and it was found that the diabetic patients had significantly less Firmicutes present than their non-diabetic counterparts, specifically of the class *Clostridia*. Additionally, T2DM patients also displayed a higher B/F ratio (Bacteroidetes to Firmicutes) [61].

These results taken together may appear surprising at first, as they contradict the available literature as a whole. However, lifestyle and diet were not considered in this study. Commonly, T2DM patients must follow a strict diet, low in simple carbohydrates and refined sugars, rich in complex carbohydrates and low glycemic index foods/meals [62], whereas non-diabetic individuals are typically not on as strict of a dietary regime. The improved B/F ratio and overall lower numbers of Firmicutes observed, may be attributed to differences in the dietary regime plausibly followed by T2DM patients, as well as medication effects.

Overall, the available evidence to date underlines a clear relationship between the type and state of the microbiome and the onset of chronic diseases including T2DM. Further investigation considering the microbiome as a target for treatment towards chronic disease particularly T2DM and ensuing CVD is important and potentially highly valuable. The food industry and health-care industry need to be involved in the development of potential foods [62] or systems [63] to provide potential therapeutic solutions enhancing and/or positively modifying the microbiome's profile into the optimally desirable that would minimize risk of disease.

9.5 Gut Microbiota and CVD

9.5.1 Dysbiosis and the Development of CVD

A variety of human studies particularly case-control ones have described the changes in microbial demography in the human gut pertinent to the development of atherosclerosis, by using fecal samples and assessing the microbiota. More specifically, certain microbiota taxa were positively associated with the development of atherosclerosis. However, whether the installation of the taxa identified constitutes contributors to CVD or outcomes due to risk factors and/or medication associated with the disease has not been yet clearly delineated [64]. Recent studies have recorded characteristic changes in CVD patients who demonstrated significant increase in Lactobacillales (Firmicutes) coupled with a decrease in Bacteroidetes (Bacteroides and Prevotella), which was not observed in a comparative cohort of patients with diabetes [64, 65]. In an extensive and very large metagenome-wide association set of studies, Jie et al. [64, 66] reported an increased abundance of Enterobacteriaceae and oral cavity-associated bacteria and relatively depleted butyrate-producing bacteria in patients with atherosclerotic CVD versus those in healthy control participants. Reduced diversity and depletion of core intestinal microbiota have both been seen consistently in patients with heart failure [64]. Subsequently and rather interestingly, patients with heart failure have long been observed to be more prone to *Clostridium difficile* infections. Moreover, detailed characterization of intestinal luminal surfaces has shown significantly bacterial overgrowth with mucosal biofilm as well as increased bacterial adhesions in patients with heart failure versus healthy controls.

Bowel wall edema during splanchnic congestion typically is seen with heart failure. In this context, intestinal barrier function is impaired, while structural components of microbiota could develop enhanced interaction with host intestinal mucosa. When surface intestinal epithelial cells-mediated interactions take place by means of pattern recognition receptors, stimulation of host immune responses and subsequent vascular inflammation are induced. This mechanism was detected in cases of heart failure patients in which acute exacerbations were associated with increased circulating lipopolysaccharide recognition and downstream inflammatory responses. Interestingly, enhanced abundance of pathogenic microbial colonies of fungi (Candida spp) and bacteria (Campylobacter, Shigella, and Yersinia) were isolated from fecal samples of heart failure patients, especially in those with elevated right atrial pressure and impaired intestinal barrier function [67]. Furthermore, three independent heart failure patient cohorts, in which sequencing techniques were employed to characterize intestinal microbial compositions, demonstrated a consistent and persistent decrease in microbial diversity along with a depletion of several butyrate producers (*Faecalibacterium prausnitzii*, Lachnospiraceae family, *Eubacterium hallii*), all inversely associated with inflammatory biomarkers [64, 68-70]. Perturbations of the intestinal microbiome in response to high sodium dietary intake were recently reported by *in vivo* work with mouse models [71]. High sodium dietary intake resulted in depletion of *Lactobacillus murinus*. Subsequently, treatment of mice with *Lactobacillus murinus* prevented sodium sensitive hypertension partly by modulating TH17 cells [71]. These interesting observations merit further investigation, particularly involving humans.

9.5.2 Mechanistic Approaches Linking Gut Microbiota to CVD

Gut dysbiosis can mediate pro-atherosclerotic effects by influencing metabolism regulation through various metabolites generated. Among the various gut microbiota-derived metabolites, trimethylamine N-oxide (TMAO) is a major contributor to the development of atherosclerosis. Trimethylamine (TMA), a TMAO precursor, is produced by two distinct types of bacterial enzymes, essentially carnitine-specific and choline-specific lyases, that cleave specific carbon–nitrogen bonds to generate TMA [72]. Once in circulation, TMA is metabolized into TMAO in the liver by the flavin-containing monooxygenase family of enzymes [72]. A conceptual discussion of the role of gut mediated TMA/TMAO production and the subsequent contribution to CVD is graphically depicted in Fig. 9.3. As a proof of concept, when microbial TMA production was inhibited, attenuated intermittent hypoxia (IH)-induced pulmonary artery atherosclerosis was reported in an obstructive sleep apnea-associated mouse model [73]. Dysbiosis-associated changes in bacterial composition can increase intestinal permeability, leading to significantly elevated LPS levels in the blood. Circulating LPS complexes with TLR4 producing signaling events transduced by myeloid differentiation primary response 88 (MYD88) to promote inflammation and foam cell formation [74].

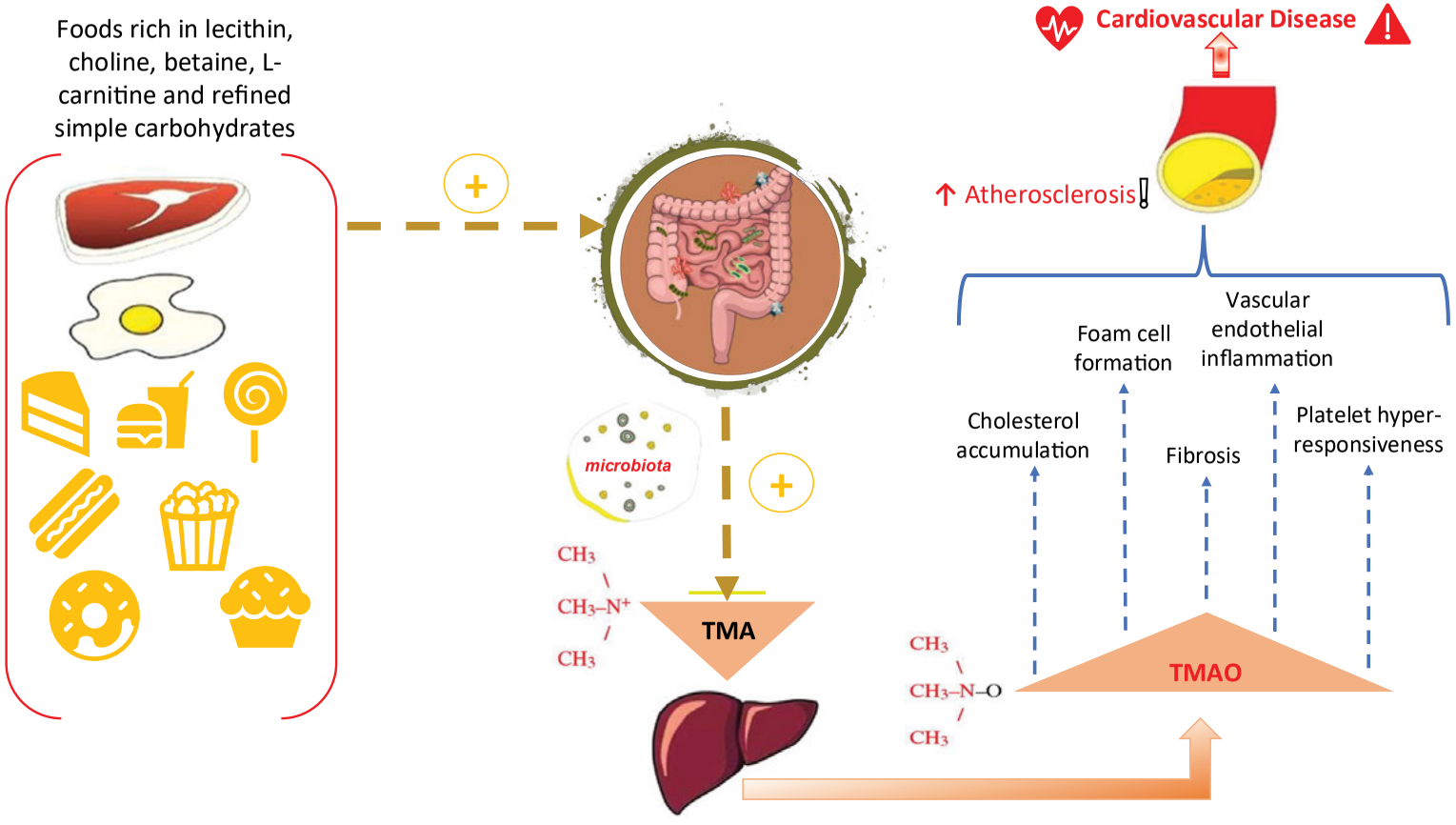

Figure 9.3 Conceptual schematic of the relationship between diet and CVD risk through the mediation of the gut microbiota via TMA/TMAO production.

Moreover, TLRs-sensed signals induce B2 cell activation in the spleen, hence modifying IgG production to stimulate atherosclerosis [75]. TMAO-dependent upregulation of macrophage scavenger receptors and CD36 expression, synergistically impair cholesterol metabolism in macrophages, thus promoting foam cell production [76]. Additionally, TMAO strongly inhibits the hepatic bile acid synthetic rate-limiting enzymes Cyp7a1 and Cyp27a1, hence resulting in decreased cholesterol elimination and compromised reverse cholesterol transport (RCT) [77]. Moreover, TMAO produces vascular endothelial dysfunction via NF-κB and inflammasome activation, therefore inducing the expression of vascular endothelial inflammation factors [78].

Intestinal barrier impairment disrupts host immune homeostasis and is associated with various inflammatory-derived pathologies [79], including CVD. As far as lipid profile is concerned, several microbes, including Turicibacter, Roseburia, Lachnospira, and Romboutsia, have been demonstrated to extend positive correlations with abnormal serum triglyceride, total cholesterol and low-density lipoprotein (LDL) levels, and a negative correlation with serum high-density lipoprotein (HDL) levels, collectively promoting a unfavorable blood lipid profile especially as it relates to CVD risk. On the contrary, the abundance of Ruminococcus-1, Rikenella, Bacteroides, Butyrivibrio, and Alistipes was negatively associated with serum triglyceride, total cholesterol and LDL levels, while positively associated with HDL levels [80].

Although the molecular mechanisms of how gut microbiota-derived TMAO can increase the incidence of atherosclerosis- and thrombosis-related cardiovascular events are not entirely understood, the observations discussed above strongly suggest that TMAO could serve as a reasonable promising therapeutic target to decrease the risk for development and progression of atherosclerosis while also attenuate pro-thrombotic tendencies without bleeding complications [72].

9.6 Concluding Remarks

It becomes increasingly evident that the microbiome is linked to obesity, insulin resistance and subsequently T2DM onset, as well as CVD. Therefore, microbiome emerges as a significant target for treatment and prevention of disease, constituting an area of focus for both the food and healthcare industry, preferably in combined efforts through the development of novel therapeutic foods optimizing gut demography, thus maximizing the capitalization on the microbiome's potential to extend health benefits pertinent to obesity, T2DM and CVD.

The so-termed "fad" diets are certainly not of new news. Keto-diets, juice cleanses, and the Atkins diet are just a mere of three examples out of the numerous diets claiming to support rapid weight loss, interestingly this being the main argument and/or "selling point" for any "fad" diet as opposed to health promotion in its own right for instance. Recently, the "Microbiome Diet (MD)" has come into play, a term coined by Dr. Raphael Kellman, and as it has been increasingly gaining

visibility it has become somewhat trendy. The MD as a dietary approach is claimed to restore gut health (promoting eubiosis), increase metabolism, and decrease inflammation. It runs in three parts, with the overarching idea of eating less processed foods and more foods rich in prebiotics. Dr. Kellman has published a book describing the diet in its entirety, with testimonies by individuals who have followed it, certainly granted that these constitute simply anecdotal evidence. It is, however, important to note that it is challenging to identify published research on the effects of the diet described and published by Dr. Kellman himself (interestingly with the exception of his book) [81].

Probiotics have also been trending in recent years, again with emphasis on their effects on the microbiome. In a study done by the Cambridge Cardiac Care Centre in Ontario, Canada, the Dietary Approaches to Stop Hypertension (DASH) diet was investigated. Eighty participants either consumed a diet typical of DASH, with emphasis on vegetables, fruits, and low-fat dairy items, or a DASH diet supplemented with probiotics. 15% of these individuals were in the pre-diabetes stage. Hemoglobin A1C, fasting blood glucose, and blood pressure were all measured at the beginning and at the conclusion of a three-month dietary intervention experiment. Hemoglobin A1C levels, a measure of plasma glucose concentration profile over time [82] used to evaluate the quality of glucose management, were reduced in both groups suggesting an improvement in glucose management, although the probiotic group was significantly lower compared to the non-supplemented group. In fact, the DASH only group showed a decrease of 3.4%, but the probiotic-supplemented group showed a decrease of 8.9%, a significant difference. Fasting plasma glucose levels behaved similarly, 10.7% decrease in the supplemented group, compared to a mere 3.3% in the DASH only group [83].

While the microbiome diet and probiotic supplements are both somewhat new concepts, it does seem that there is significant scientific evidence mounting to support their claims. While research regarding the microbiome diet itself is minimal, it is evident that decreasing the B/F ratio does make a notable difference concerning disease management. Probiotic supplement also extended a significant impact on health when paired with the DASH diet. As a result, it may be beneficial for both the food and healthcare industry to market towards the microbiome enhancement.

Recent research on the microbiome has caused a shift of focus on health and disease highlighting the idea that eating a healthier diet reduces disease risk at levels as basic as the microbiome. In fact, there have been several reports on other diseases triggered by obesity, such as heart disease and colon cancer. In an earlier study by Sikalidis et al., mice were fed a diet to induce obesity, and it was found that the obese mice had significantly more aberrant crypt foci and higher proliferation rate levels of colonocytes than their lean counterparts [84]. This further illustrates the importance of healthy diet and a healthy microbiome. Eating LFD improves the B/F ratio, decreasing risk of obesity, and therefore decreasing the risk of disease as a whole. The role of the microbiome in the onset

of disease, in this case specifically T2DM, has been increasingly understood, resulting in investigation of this otherwise, until recently, relatively overlooked or underestimated factor with significant health implications. Onset of T2DM is much more complicated than addressing one particular factor, but by exploring the microbiome and how it modulates risk of T2DM, new answers and areas of research promoting more effective T2DM management could arise.

Diet is shown to extend a significant effect on gut health, and a healthy gut is responsible for more optimally regulating numerous pathways at a systemic level. In the case of dysbiosis, many of these pathways are negatively impacted, contributing to the eventual onset of chronic disease. There is evidence supporting the idea that a plant-based diet results in decreased inflammation, a better B/F ratio, and an overall lower risk of disease [85]. Recently, however, the effect of cooked versus raw food on the microbiome has been investigated, with cooked or raw beef or sweet potato fed to mice. Both of the cooked diets resulted in increased body mass, despite a lower caloric intake, supporting the idea that cooking food results in an increased net energy gain [86]. Interestingly, the mice fed the cooked and raw beef diets both displayed similar microbial composition, but the mice fed the raw or cooked sweet potato displayed significant differences within their microbiome. The cooked sweet potato resulted in decreased diversity, and the mice fed the raw potato displayed increased weight loss. It is believed that this is due to the previously mentioned idea that cooking results in an increased energy harvest. The mice fed the raw potato diet also displayed better starch digestibility and degradation of plant compounds [86]. Therefore, it can be argued that while humans evolved to cook their food because of the beneficial energy gain, for weight loss and decreased risk of obesity, a raw and plant-based diet may be advantageous. On a different research approach, nutrient delivery/sequestration systems utilizing edible minerals have been investigated to attenuate CVD risk by reducing cholesterol levels through bile sequestration, thus modulating the GI environment [87]. Significant work with zeolites has been done to identify it as a pro-health dietary supplement in pigs [88]. The incidence, severity and duration of diarrhea is reduced in pigs fed clay materials such as clinoptilolite. The potential mechanisms include increases in Bifidobacteria and Lactobacillus, along with decreases in Clostridia and *E. coli* [89].

It is evident that there is a link between diet and the microbiome, including the microbiome's role in obesity. There also appears to be a link between the microbiome and T2DM [90] as well as CVD [64, 73]. Of particular interest are polyphenols and similar bioactive compounds which have been shown to alter desirably the gut microflora thus providing a potential mechanism for improved status of inflammation and associated conditions including CVD [91, 92]. Conclusively, further research on the relationship between gut microbiota and T2DM/CVD is essential to determine mediators and mechanisms of action thus yielding a potentially effective treatment for T2DM/CVD involving solutions utilizing the potential of the gut microbiome.

Abbreviations

BCAA: branched-chain amino acids
BMI: body mass index
CONV: conventionally raised
CRP: C-reactive protein
CVD: cardiovascular disease
DASH: dietary approaches to stop hypertension
DSS: dextran sulfate sodium
FOS: fructooligosaccharides
GF: germ-free
GLUT4: glucose transporter type 4
HDL: high-density lipoprotein
HFD: high-fat diet
IH: intermittent hypoxia
IKK: IκB kinase
IRS1: insulin receptor substrate 1
IRSs: insulin receptor substrates
JNK: c-Jun N-terminal kinases
LDL: low-density lipoprotein
LFD: low-fat diet
LPS: lipopolysaccharide
MD: microbiome diet
MS: metabolic syndrome
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
RCT: reverse cholesterol transport
ROS: reactive oxygen species
RYGB: Roux-en-Y gastric bypass
SCFA: short-chain fatty acids
T2DM: type 2 diabetes mellitus
TLR4: toll-like receptor 4
TMA: trimethylamine
TMAO: trimethylamine N-oxide
TNF: tumor necrosis factor

Disclosures and Conflict of Interest

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Chapter 10

Pharmaceutical Strategies for Reducing LDL-C and Risk of Cardiovascular Disease

Alberico L. Catapano, PhD,[a] Lale Tokgözoğlu, MD,[b] Alberto Mello e Silva, MD,[c] and Eric Bruckert, MD[d]

[a]*Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy*
[b]*Department of Cardiology, Hacettepe University, Ankara, Turkey*
[c]*Hospital Egas Moniz, Centro Hospitalar de Lisboa Ocidental, Serviço de Medicina I, Lisbon, Portugal*
[d]*Hopital Pitié Salpêtrière et Université Paris Sorbonne, Paris, France*

eric.bruckert@aphp.fr

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10.1 Introduction

Lowering levels of low-density lipoprotein cholesterol (LDL-C) is a key strategy in preventing cardiovascular (CV) disease (CVD) [1, 2]. When lifestyle interventions prove ineffective in lowering LDL-C levels, a variety of pharmaceutical therapies can be considered. While statins are most often prescribed as a primary treatment, other therapies that may be considered include cholesterol absorption inhibitors, bile acid sequestrants and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitory monoclonal antibodies [1]. These monotherapies are generally efficacious and well tolerated but may be associated with low compliancy rates, heterogeneous efficacy and the occurrence of adverse events [3].

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Monotherapy is often adequate in achieving LDL-C goals, although patients with very high LDL-C or at very high risk of CVD may require additional treatment, and in these situations, combination therapy is often recommended [1]. This overview will discuss monotherapies and combination therapies further.

10.2 The Importance of Potency in Statin Monotherapy

The joint European Society of Cardiology and European Atherosclerosis Society (ESC/EAS) 2016 guidelines dictate that the greatest LDL-C reductions lead to the greatest reductions in CVD risk, with the highest potency statins proving the most effective at decreasing LDL-C levels [1, 4]. Statin potency differs according to the type of statin and the dose used. Equal doses of atorvastatin and rosuvastatin lead to the greatest reductions in LDL-C compared with those of other statins, such as fluvastatin [5, 6]. Atorvastatin and rosuvastatin are both recommended for use in patients with the highest CVD risk [4]. In pairwise dose-to-dose comparisons with atorvastatin, rosuvastatin leads to a significantly greater decrease in LDL-C (p = 0.001) and an increase in high-density lipoprotein cholesterol (HDL-C) (p = 0.001) [7], while changes in triglyceride (TG) levels are similar (with the exception of 10 mg atorvastatin vs. 10 mg rosuvastatin, p < 0.001) [8]. Contrary to LDL-C, high levels of HDL-C may provide protection against CVD, while high TG levels are considered a direct cause of CVD [1].

The greatest reductions in the occurrence of CVD can be achieved by decreasing LDL-C in patients who have the greatest CVD risk (Table 10.1) [9]. High-potency statins should be used in patients with the greatest CVD risk [4]. Furthermore, prolonged statin therapy leads to the greatest reduction of CVD risk [9]. However, prolonged use of high-potency statins may not be possible for every patient. Response to statins is heterogeneous [3], and elevated dosage of high-potency statins may increase the likelihood of statin intolerance and statin-related adverse events, including myalgia [1]. In these patients, the highest tolerable dose or an alternative dosing schedule may be considered [1]. Alternative therapies can also be employed to achieve and maintain a desired LDL-C goal.

Table 10.1 Reduction in major vascular events as determined by LDL-C reduction and 5-year risk of major vascular event

Major vascular events avoided, per 10,000 patients treated for 5 years		5-year risk of major vascular event, %			
		5–9	10–19	20–29	≥30
LDL-C reduction achieved with statin treatment, mmol/L	**1.00**	170	370	540	730
	1.50	250	540	800	1130
	2.00	310	680	1010	1440

Note: Adapted from Collins et al. (2016) [9]. *Abbreviation*: LDL-C, low-density lipoprotein cholesterol.

Great variation in the level of LDL-C reduction achieved exists between individuals on the same fixed-dose statin regimen [3]. Consequently, prescribed doses can vary across a 64-fold range, with great variation between individual statins [10]. Another source of heterogeneity in response to statin therapy is the patient demographic. Small but significant differences in LDL-C reduction have been reported in female patients compared with male patients treated with atorvastatin ($p < 0.05$) and rosuvastatin ($p = 0.02$) monotherapies [11, 12]. Differences in rosuvastatin efficacy have also been observed between different ethnic populations [13].

10.3 Alternative Strategies to Achieve LDL-C Goal

Many patients with a high CVD risk or very high LDL-C levels are not able to achieve LDL-C goals through statins alone. Additionally, a number of patients are either statin intolerant, or unable to tolerate high doses of statins [1]. The EURIKA study found that only 41.2% of patients from 12 European countries achieved target total cholesterol and LDL-C goals when treated with lipid-lowering drugs [14]. Although full adherence is associated with a reduced rate of CV-related morbidity and mortality, non-adherence is commonplace [15].

For patients who are unable to achieve LDL-C goal, the two main recommended strategies are to: (1) increase statin dose to the maximum tolerated, or (2) use a combination of therapies after statin monotherapies have been unsuccessful [1].

10.3.1 Intensive Statin Therapy

By using uptitrated doses of high-potency statins, lower LDL-C goals can be achieved compared with standard doses of lower-potency statins, decreasing the occurrence of major CV events [16, 17]. However, due to the heterogeneity in individual response to intensive statin therapy, >40% of patients still do not achieve an LDL-C goal of <70 mg/dl, with non-adherence a major factor due to dose-related adverse events and patient-related issues [3].

10.3.2 Combination Therapy

The ESC/EAS 2016 guidelines recommend that "If the [LDL-C] goal is not reached [with statin monotherapy], statin combination with a cholesterol absorption inhibitor should be considered" [1]. Combination therapy can increase efficacy and reduce the occurrence of adverse events [18]. Multiple combination therapies have been developed for the improvement of lipid profiles (Fig. 10.1).

Statins are often combined with cholesterol absorption inhibitors, most commonly ezetimibe, due to their synergistic mechanisms of action [1]. The IMPROVE-IT trial found that the addition of ezetimibe to simvastatin led to a significant additional decrease in LDL-C levels of 24% compared with simvastatin alone ($p < 0.001$), and significantly lowered the risk of myocardial infarction (MI) ($p = 0.002$) and ischaemic stroke ($p = 0.008$) [19].

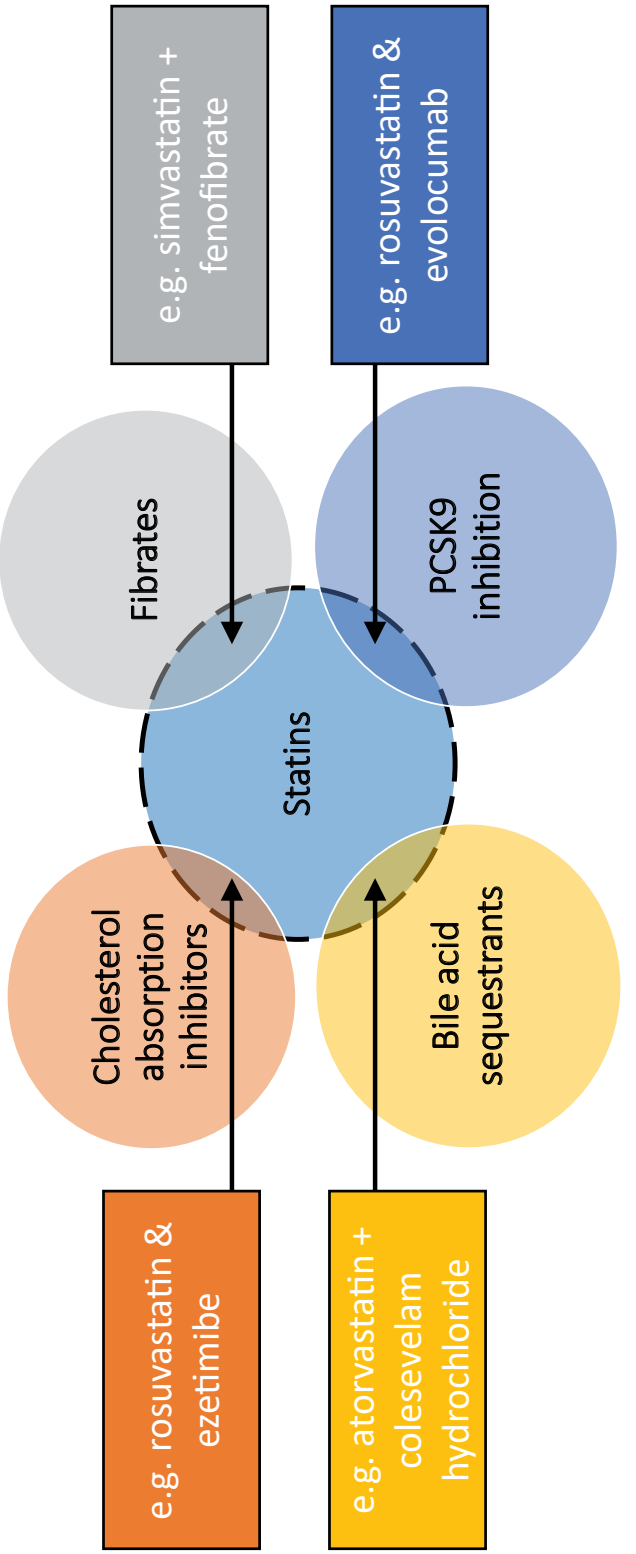

Figure 10.1 Combination therapies for the management of LDL-C. *Abbreviations*: LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9.

High-potency statins can also be combined with other therapies, as demonstrated by the EZ-PATH trial, where the addition of ezetimibe to atorvastatin led to a significant increase in the number of patients achieving an LDL-C goal of <70 mg/dl compared with those receiving atorvastatin alone ($p < 0.001$) [20]. Significant improvements in coronary plaque regression have been observed with combined ezetimibe–atorvastatin therapy compared with atorvastatin alone ($p = 0.001$) [21]. Other combined therapies include combinations of statins and bile acid sequestrants, which have been shown to lead to a significant decrease in LDL-C of 17.5% compared with co-administration of simvastatin and placebo ($p < 0.001$) [22].

Alternative lipids and other CV-related molecules can be targeted in the management of lipid profiles. Fibrate monotherapy has been shown to decrease serum TG and increase HDL-C levels in patients with hypercholesterolaemia [23], and decrease levels of C-reactive protein [24]. In addition, combination therapy with fenofibrate–simvastatin decreased the rate of non-fatal MI, stroke or CV death by 31% in a subgroup of patients with type 2 diabetes with elevated TG and low HDL-C levels in the ACCORD Lipid trial [25]. Combined use of fibrates and statins, therefore, can be considered for use in patients with high LDL-C, low HDL-C and high TG levels [2].

Low rates of adherence can greatly affect the efficacy of therapy, leading to increased CV-related morbidity and mortality [26]. One solution is a combined treatment of multiple CV drugs, in the form of a fixed-dose 'polypill', in order to increase adherence in a cost-effective manner [1]. The UMPIRE trial showed that the use of a polypill containing simvastatin (alongside other molecules) significantly increased adherence (treatment effect = 1.33 [95% confidence interval 1.26, 1.41], $p < 0.001$) and significantly decreased LDL-C (treatment effect = −4.2 [95% confidence interval −6.6, −1.9], $p < 0.001$) compared with separate, concomitant treatments [27].

10.4 PCSK9, a New Therapeutic Target

Loss of function mutations within the PCSK9 gene are associated with reduced plasma LDL-C levels and large reductions in the risk of coronary heart disease [28]. Inhibition of PCSK9 represents a new therapeutic target to help achieve LDL-C goals in patients at high risk of CV events [29]. Meta-analysis on the efficacy of PCSK9 inhibition in patients with hypercholesterolaemia demonstrated a mean LDL-C level decrease from baseline of approximately 50%, and a decrease in MI event rate [30]. A separate meta-analysis demonstrated a reduced incidence of all-cause mortality, but found a higher rate of neurocognitive adverse events [31].

Three monoclonal antibodies have been developed as therapies to lower LDL-C levels in patients with hypercholesterolaemia: bococizumab, alirocumab and evolocumab. Bococizumab, a humanised monoclonal antibody that inhibits PCSK9, demonstrated no benefit over placebo with respect to major adverse CV events in a randomised trial involving lower-risk patients (SPIRE-1), but did

have a significant benefit in a parallel trial of higher-risk patients (SPIRE-2) [32], although the studies were terminated early after the sponsor discontinued development of bococizumab. The GAUSS-3 trial demonstrated a significantly greater reduction in mean LDL-C in patients intolerant to statins following treatment with evolocumab for 24 weeks compared with ezetimibe (52.8% and 16.7%, respectively; $p < 0.001$) [33]. The ODYSSEY ALTERNATIVE trial produced similar efficacy results in patients with statin intolerancy, with alirocumab leading to greater reductions in mean LDL-C compared with ezetimibe after 24 weeks compared with baseline (54.8% and 20.1%, respectively) [34].

The ODYSSEY FHI and FHII trials described similar efficacy levels in patients with heterozygous familial hypercholesterolaemia (FH) treated with alirocumab, leading to significant LDL-C reductions from baseline of 48.8% (FHI; $p < 0.0001$ vs. placebo) and 48.7% (FHII; $p < 0.0001$ vs. placebo) [35]. The TAUSSIG study in patients with homozygous FH showed that PCSK9 inhibition therapy lead to LDL-C reductions of 20.6% and 23.3% after 12 and 48 weeks, respectively, compared with baseline ($p < 0.0001$ for both timepoints) [36].

The first PCSK9 inhibition therapy clinical outcome study, the FOURIER trial, examined the efficacy of evolocumab in reducing the risk of CV events in high-risk patients. Evolocumab significantly reduced the composite risk of CV death, MI, stroke, hospitalisation for unstable angina or coronary revascularisation by 15%, and specifically reduced the composite risk of CV death, MI and stroke by 20%, compared with placebo ($p < 0.001$ for both composite risks) (Fig. 10.2) [37].

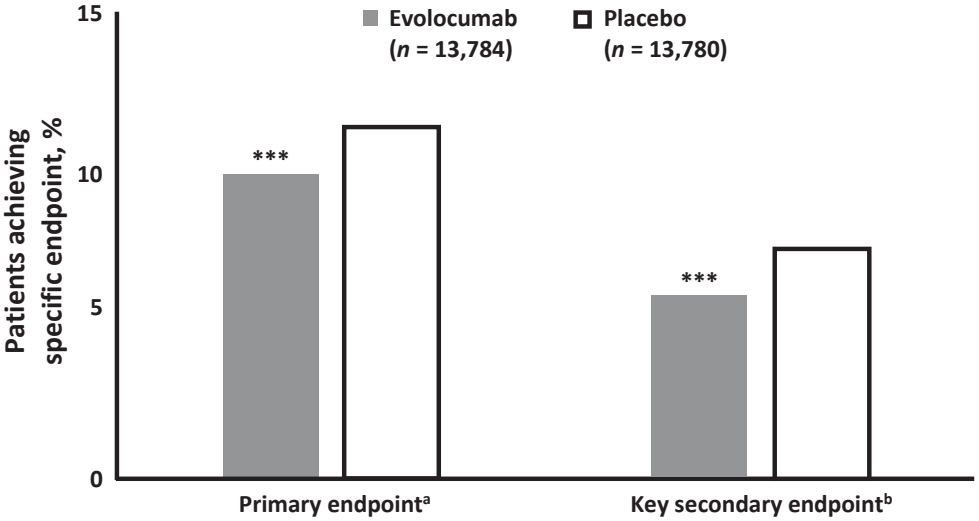

Figure 10.2 The percentage of patients achieving the primary and key secondary endpoints from the FOURIER clinical trial. Data from Sabatine et al. (2017) [37]. ***$p < 0.001$. [a]The primary endpoint was the occurrence of major cardiovascular events, defined as the composite of cardiovascular death, myocardial infarction, stroke, hospitalisation for unstable angina or coronary revascularisation. [b]The key secondary endpoint was the composite of cardiovascular death, myocardial infarction or stroke.

The ESC/EAS 2016 guidelines recommend PCSK9 inhibition therapy for use in patients who are statin intolerant, patients with heterozygous FH and homozygous FH, and patients at very high CV risk, as a potential means for achieving and maintaining LDL-C goals [1].

The ESC/EAS Task Force updated clinical guidance for the use of these novel agents and recommends consideration of PCSK9 inhibition therapy in very high-risk patients with atherosclerotic CVD and with FH without a prior clinical event, specifically in those not adequately controlled with maximally tolerated statin with or without ezetimibe therapy, or those who do not tolerate appropriate doses of at least three statins [29].

10.5 Conclusion

The greatest reductions in LDL-C are most needed in patients at the highest risk of CVD [1]. Statin potency differs according to type of statin and dose [5, 6], which can be strategically exploited in treating patients of varying CVD risk. However, the vast heterogeneity in patient statin responses and the possibility of developing statin intolerance often means that elevated statin monotherapy may not be optimal [3]. Combination therapy is recommended in patients not achieving LDL-C goal at the highest tolerated statin dose, and in patients who are statin intolerant [1, 18]. Multiple combination therapies can be recommended, which may not only increase efficacy in lowering LDL-C due to synergistic mechanisms of action (statin–ezetimibe combination therapy) but also reduce levels of other molecules involved in hypercholesterolaemia (statin–fibrate combinations in lowering TG) [1, 2, 19, 23]. Following the observed decrease in CV events in patients with type 2 diabetes and low HDL-C and elevated TG levels (a prespecified subgroup with atherogenic dyslipidaemia) with fenofibrate–simvastatin combination therapy in the ACCORD Lipid trial [25], the PROMINENT study (NCT03071692, https://clinicaltrials.gov) is currently evaluating a new selective peroxisome proliferator-activated receptor alpha modulator, pemafibrate, in addition to statin therapy. Additional benefits of combination therapy include increased adherence [27], which is a key strategy in increasing therapeutic efficacy [1].

PCSK9 inhibition therapy is capable of reducing LDL-C levels by 50% [30]. The ESC/EAS Task Force recommend consideration of PCSK9 inhibition therapy for very high-risk patients with atherosclerotic CVD or FH with inadequately controlled LDL-C levels [29]. Clinical data released after the authors' workshop provides further evidence regarding the efficacy of PCSK9 inhibition therapy. Alirocumab treatment has recently been shown in the ODYSSEY OUTCOMES trial to lead to a significant 15% decrease in major adverse cardiac events compared with placebo ($p = 0.0003$), after 48 months of treatment in patients who experienced an acute coronary syndrome within 1–12 months prior to randomisation. This was accompanied by significant reductions in all-cause mortality ($p = 0.026$), MI ($p = 0.006$), ischaemic stroke ($p = 0.01$), ischaemia-driven coronary revascularisation ($p = 0.02$) or unstable angina ($p = 0.02$) compared with placebo. Furthermore,

LDL-C was reduced by 62.7% at 4 months and 54.7% at 48 months, compared with placebo [38]. The ORION-1 study has demonstrated that a single dose of inclisiran, a small interfering ribonucleic acid (RNA) that produces PCSK9-specific RNA silencing, produced a 27.9–41.9% reduction in LDL-C after 180 days, compared with baseline ($p < 0.001$ for all doses). ORION-1 showed inclisiran could reduce LDL-C safely but also maintain reductions consistently over time [39].

Therapies that decrease the risk of major CV events by mechanisms independent of LDL-C reduction present alternative therapeutic options, including anti-inflammatory therapies. Canakinumab, a monoclonal antibody targeting interleukin-1β, has recently been shown in the CANTOS trial to significantly reduce the rate of recurrent CV events and decrease the levels of C-reactive protein, compared with placebo ($p = 0.02$ and $p < 0.001$, respectively), in patients with previous MI and elevated C-reactive protein levels [40]. Low-dose methotrexate, a dihydrofolate inhibitor used as an anti-inflammatory agent, is currently being evaluated in lowering the risk of CV events in patients with diabetes and previous MI [41].

Abbreviations

CV: cardiovascular
CVD: cardiovascular disease
EAS: European Atherosclerosis Society
ESC: European Society of Cardiology
FH: familial hypercholesterolaemia
HDL-C: high-density lipoprotein cholesterol
LDL-C: low-density lipoprotein cholesterol
MI: myocardial infarction
PCSK9: proprotein convertase subtilisin/kexin type 9
RNA: ribonucleic acid
TG: triglyceride

Disclosures and Conflict of Interest

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Chapter 11

Atherogenic Markers in Predicting Cardiovascular Risk and Targeting Residual Cardiovascular Risk

Alberico L. Catapano, PhD,[a] Lale Tokgözoğlu, MD,[b] Alberto Mello e Silva, MD,[c] and Eric Bruckert, MD[d]

[a]*Department of Pharmacological and Biomolecular Sciences, University of Milan, Multimedica IRCCS, Milan, Italy*
[b]*Department of Cardiology, Hacettepe University, Ankara, Turkey*
[c]*Hospital Egas Moniz, Centro Hospitalar de Lisboa Ocidental, Serviço de Medicina I, Lisbon, Portugal*
[d]*Hopital Pitié Salpêtrière et Université Paris Sorbonne, Paris, France*

alberico.catapano@unimi.it, laletok@gmail.com

Keywords: acute coronary syndrome (ACS), apolipoprotein B (apoB), atherogenic lipoproteins, cardiovascular (CV), cardiovascular disease (CVD), coronary heart disease (CHD), discordant markers, dyslipidaemia, evolocumab, low-density lipoprotein (LDL), low-density lipoprotein cholesterol (LDL-C), metabolic syndrome, non-high-density lipoprotein cholesterol (non-HDL-C), randomised controlled trial (RCT), residual cardiovascular risk, simvastatin–ezetimibe combination, statin–fenofibrate combination, triglyceride (TG), type 2 diabetes mellitus, very low-density lipoproteins (VLDL)

11.1 Introduction

The association between elevated levels of low-density lipoprotein (LDL) cholesterol (LDL-C) and increased risk of cardiovascular (CV) disease (CVD) is the basis for guidelines recommending LDL-C as the primary target in CVD prevention. This is supported by a large body of genetic, biochemical and epidemiological evidence demonstrating a causal role of LDL in CVD, and clinical evidence demonstrating that reduction of LDL-C is associated with a reduction in the risk of CVD [1, 2]. Although there is overwhelming evidence for the causality of LDL-C, there are other potential markers that also influence CVD risk through atherosclerosis and other mechanisms [3]. Non-high-density lipoprotein cholesterol (non-HDL-C) and apolipoprotein B (apoB) are recommended as secondary targets

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in the joint European Society of Cardiology and European Atherosclerosis Society (ESC/EAS) 2016 guidelines, and should be considered as alternatives in risk estimations when LDL-C is not appropriate, for example, when triglyceride (TG) levels are high [1]. Conflicting evidence on the associations between these lipid markers and CV events raises the question of the most appropriate measure for CV risk. An overview of the clinical evidence for these associations will be discussed here.

11.2 Markers of Atherogenic Risk

11.2.1 Non-High-Density Lipoprotein Cholesterol and Apolipoprotein B

Non-HDL-C (total cholesterol minus HDL-C) represents a measure of the atherogenic lipoproteins very low-density lipoproteins (VLDL), VLDL remnants, intermediate-density lipoprotein, LDL and lipoprotein(a). Apolipoprotein B relates well to non-HDL-C and represents a measure of atherogenic lipoproteins VLDL, intermediate-density lipoproteins and LDL particles [1, 4]. There is evidence to demonstrate that these markers of atherogenic lipoproteins are associated with CV risk; lowering non-HDL-C reduced the risk of coronary heart disease (CHD) in a meta-analysis of studies with various lipid-modifying therapies, and apoB was strongly associated with onset of ischaemic heart disease in a population-based study [5, 6]. The strength of these associations in comparison with those of other lipids and lipoproteins can vary widely between reports. The AMORIS study identified apoB as an important risk factor for fatal myocardial infarction with stronger predictive power than LDL-C [7]. Similarly, Sniderman et al. (2011) [8] supported this finding in a meta-analysis of epidemiological studies, which indicated that apoB is a stronger predictor of CV risk than LDL-C, followed by non-HDL-C, with LDL-C being the least strong. These studies suggest apoB may be, therefore, a superior marker for predicting CV risk than the current guideline-recommended LDL-C. Another meta-analysis by Boekholdt et al. (2012) [9] also demonstrated associations between LDL-C, non-HDL-C and apoB with the risk of major CV events, but determined a stronger association for non-HDL-C than for LDL-C and apoB. The proportions of treatment effect that are explained by changes in lipid or apo B levels are shown in Fig. 11.1. The proportion of treatment effect explained by non-HDL-C was larger than by LDL-C and by apoB [9].

Conversely to studies indicating superiority of apoB or non-HDL-C for predicting CV risk, other studies have not observed any differences in the associations between LDL-C, non-HDL-C and apoB. For example, Parish et al. (2012) [3] found no difference in the strength of association with major coronary events or revascularisation between these lipoproteins, and The Emerging Risk Factors Collaboration found a similar prediction of CHD risk with non-HDL-C and apoB [10].

Based on the evidenced association between different lipoproteins and CV events, it may be beneficial to consider markers other than LDL-C when

estimating risk. The ESC/EAS 2016 guidelines recommend non-HDL-C and apoB lipid analyses should be considered in CV risk estimation, particularly in patients with high levels of TG. The advantages and disadvantages of LDL-C, non-HDL-C and apoB as predictive markers for CV risk are summarised in Table 11.1 [1, 2].

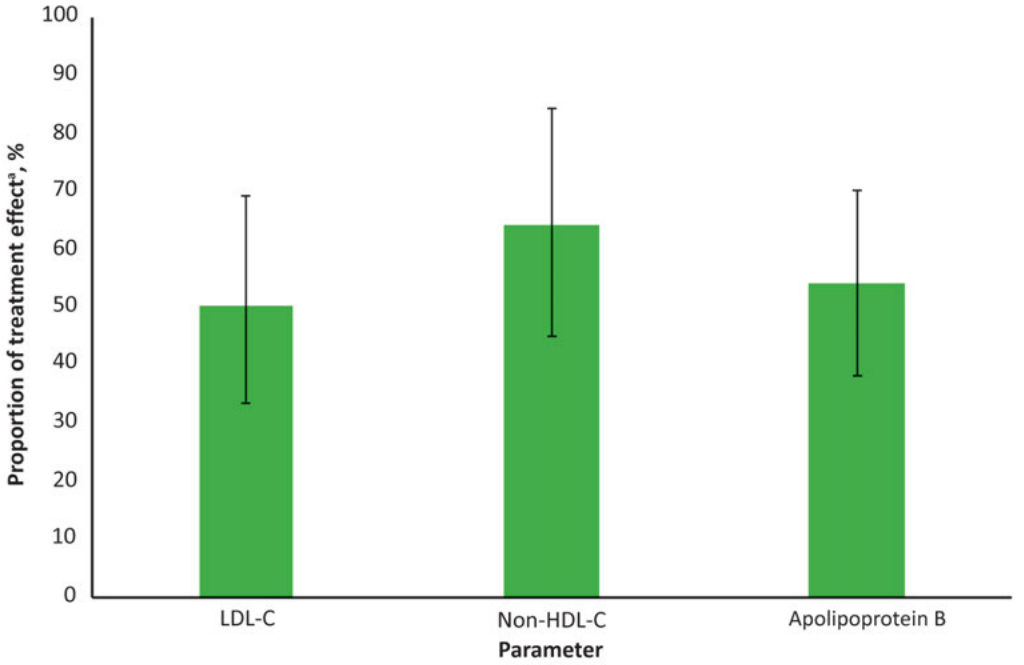

Figure 11.1 Proportion of treatment effect explained by lipid or apoB levels. Adapted from Boekholdt et al. (2012) [9]. [a]Indicates the proportion of treatment effect explained by a lipid or apoB parameter. *Abbreviations*: HDL-C, non-high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 11.1 Advantages and disadvantages of LDL-C, non-HDL-C and apoB as markers for predicting CV risk [1, 2]

Marker	Advantage	Disadvantage
LDL-C	Causality for CVD proven and well-studied Primary target in guidelines	Less reliable when TGs are high
Non-HDL-C	Associated with CV risk in some studies More reliable in patients with high TGs Secondary target in most guidelines	Not evaluated as a primary target in RCTs
apoB	Associated with CV risk in some studies Reliable analysis, even with high TGs Secondary target in most guidelines	Not evaluated as a primary target in RCTs Analysis not always available

Abbreviations: apoB, apolipoprotein B; CV, cardiovascular; CVD, cardiovascular disease; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; RCT, randomised controlled trial; TG, triglyceride.

11.2.2 Discordance in markers of cardiovascular risk

Discordant markers may contribute to the conflicting evidence for non-HDL-C and apoB as predictors of risk in studies performing analyses with them as independent markers. Markers LDL-C, non-HDL-C and apoB are concordant when the apoB particles contain an average amount of cholesterol, and become discordant when they contain more or less cholesterol than average. When these markers are concordant, LDL-C, non-HDL-C and apoB can predict risk equally well; whereas, when markers are discordant (e.g. metabolic syndrome or diabetes mellitus) their predictive pattern can differ [11]. When using discordance analysis, where variables were analysed by concordance or discordance, apoB was more strongly associated with CV risk than LDL-C and non-HDL-C [12].

11.3 Residual Cardiovascular Risk

The LDL-C lowering effects of statins and the substantial reduction in CV morbidity and mortality has been well documented [13–18]. Despite this, there remains a residual risk of CV events with statin treatment in clinical trials, even in patients achieving target LDL-C levels [19, 20]. Combination therapy can further reduce CV risk; treatment with statin plus an additional LDL-C-lowering therapy results in a reduced number of CV events compared with statin monotherapy [21, 22]. In the IMPROVE-IT trial in patients who had recently had an acute coronary syndrome (ACS), simvastatin–ezetimibe combination lowered LDL-C by 24% compared with statin monotherapy and, additionally, reduced non-HDL-C, apoB, total cholesterol and TGs to a greater extent [21]. In the FOURIER trial in patients with atherosclerotic CVD, evolocumab added to statin therapy further lowered LDL-C levels by 59% compared with statin monotherapy, non-HDL-C levels by 52% and apoB by 49% [22]. Therefore, high-risk patients may benefit by lowering lipid levels beyond current target levels through combination therapy.

Atherogenic dyslipidaemia, characterised by abnormalities in LDL, HDL-C and TGs, is very common in patients with type 2 diabetes mellitus or metabolic syndrome [23, 24]. The DYSIS study indicated that these abnormalities can persist in statin-treated patients; 58.1% of patients did not achieve target LDL-C goals. Additionally, 22.7% of patients had low HDL-C levels and 47.3% of patients had elevated TG levels, rising to 24.0% and 54.3%, respectively, in those with diabetes. The number of patients with abnormal levels of atherogenic lipoproteins, despite being treated with statins, was substantial [24]. Residual abnormal levels of lipoproteins other than LDL-C could be one potential cause of residual CV risk.

The PROVE IT-TIMI 22 trial identified that in statin-treated patients after an ACS, those with lower TG levels (National Cholesterol Education Program [NCEP] cut-point of <150 mg/dl) had fewer CHD events than patients with higher TG levels (≥150 mg/dl; 13.2% vs. 17.6%, respectively). The CHD risk was lowest when both LDL-C and TG levels in statin-treated patients were low (NCEP cut-points of <70 mg/dl and <150 mg/dl, respectively), compared with when levels were

high (NCEP cut-points of ≥70 mg/dl and ≥150 mg/dl, respectively; CHD event rate 11.7% vs. 17.9%) (Table 11.2). Therefore, targeting TG in addition to LDL-C in patients with a residual CV risk after an ACS may be beneficial in further reducing the risk of CVD [25].

Table 11.2 Risk of death, myocardial infarction and recurrent acute coronary syndrome with LDL-C and TG cut-points

Rate of recurrent events, %		
Lipid cut-point	LDL-C <70 mg/dl	LDL-C ≥70 mg/dl
TG < 150 mg/dl	11.7	15.0
TG ≥ 150 mg/dl	16.5	17.9

Abbreviations: LDL-C, low-density lipoprotein cholesterol; TG, triglyceride.

Source: Adapted from Miller et al. (2008) [25].

In the ACCORD Lipid trial, no CV benefit was observed in patients with type 2 diabetes mellitus treated with a statin–fenofibrate combination compared with those treated with statin monotherapy (CV event rate 10.1% in both groups). There was, however, a reduced major CV event rate in a subgroup of patients with low HDL-C and high TG levels treated with a statin–fenofibrate combination compared with statin monotherapy (12.4% vs. 17.3%, respectively) [26].

Currently, the ESC/EAS guidelines primarily approach lipid management by targeting LDL-C. Recommendations are to reduce LDL-C as much as possible, particularly in high-risk patients. As less extensively studied lipids, non-HDL-C and apoB are recommended as secondary targets; however, more clinical evidence is needed before recommendations can be made for targeting HDL-C or TGs [1].

11.4 Conclusions

The evidenced association of elevated LDL-C with increased CV risk is well documented and, consequently, LDL-C is the primary target for CVD prevention [1, 2]. In addition, there is clinical evidence, although conflicting, to suggest that non-HDL-C and apoB are also strongly associated with CV risk [3, 5–10]. These markers could, therefore, be considered in analyses for estimations of CV risk, especially when LDL-C is not an appropriate marker, for example in the presence of elevated TG levels [1].

Although CV risk is reduced substantially by the LDL-C lowering effects of statins, patients still have a residual CV risk [20]. Combination therapy to further reduce CV risk linked with elevated LDL-C and targeting other lipids, mainly TGs, may be beneficial for further reducing the residual risk [21, 22]. TG levels are a marker of TG-rich lipoproteins and their remnants, and lower levels are often associated with lower levels of remnant lipoproteins. More clinical evidence is needed before making definitive recommendations, but the authors suggest that the current focus on TG levels should be shifted to focus on remnant lipoprotein levels and their implications for CV risk [27].

Additionally, two randomised controlled trials (REDUCE-IT) [28] [NCT01492361, https://clinicaltrials.gov] and the ongoing STRENGTH [NCT02104817, https://clinicaltrials.gov]) have been designed to evaluate the efficacy of Omega 3 fatty acids (ethyl eicosapentaenoic acid or ethyl eicosapentaenoic acid/docosahexaenoic acid) in reducing major adverse CV events in patients at high risk of CVD, when added to LDL-C-lowering therapy. As reported by the REDUCE-IT trial, among statin-treated patients with elevated TG levels, the risk of ischemic events was significantly reduced following treatment with icosapent ethyl [28].

Abbreviations

ACS:	acute coronary syndrome
apoB:	apolipoprotein B
CHD:	coronary heart disease
CV:	cardiovascular
CVD:	cardiovascular disease
EAS:	European Atherosclerosis Society
ESC:	European Society of Cardiology
LDL:	low-density lipoprotein
LDL-C:	low-density lipoprotein cholesterol
non-HDL-C:	non-high-density lipoprotein cholesterol
RCT:	randomised controlled trial
TG:	triglyceride
VLDL:	very low-density lipoproteins

Disclosures and Conflict of Interest

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Chapter 12

The Continuous Quest for More Effective and Safer Thromboprophylaxis Protocols

Mario Ganau, MD, PhD, MBA,[a] Sibel E. Huet, MD,[a] Marco Meloni, MD,[b] Salvatore Chibbaro, MD, PhD,[c] and Nikolaos Syrmos, MD, PhD[d]

[a]*Department of Neurosciences, John Radcliffe Hospital, Oxford University, UK*
[b]*Department of Neurosurgery, 'Moriggia-Pelascini' Hospital, Gravedona ed Uniti, Italy*
[c]*Hopital Universitaire Strasbourg, France*
[d]*Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece*

mario.ganau@alumni.harvard.edu

Keywords: anti-phospholipid syndrome (APS), arterial thromboembolism (ATE), biomarkers, coagulation cascade, D-dimer, deep venous thrombosis (DVT), direct oral anticoagulants (DOACs), Doppler ultrasound, inferior vena cava (IVC), low-molecular-weight heparin (LMWH), mechanical prophylaxis, oral anticoagulants, pharmacological prophylaxis, postthrombotic syndrome (PTS), P-selectin, pulmonary embolism (PE), statins, thromboprophylaxis, traumatic brain injury (TBI), unfractionated heparin (UFH), venous thromboembolism (VTE), vitamin K antagonists (VKA)

12.1 Thrombosis: A Preventable Healthcare Burden

Although the thrombosis is by definition the formation of a blood clot within any type of vessel, this pathological event is more common in veins than arteries. This process can lead to the vessel occlusion as a result of local formation of the blood clot, or its transport along the bloodstream. As such, we usually further categorize the more common venous thromboembolism (VTE) into deep venous thrombosis (DVT), which represents about two thirds of the cases, and pulmonary embolism (PE) [1].

Thrombosis usually forms as a result of a tri-factorial etiology, more commonly known as Virchow's triad, which includes venous stasis, hypercoagulable state, and vessel wall damage [2, 3]. In modern medicine, we tend to define as

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healthcare-acquired VTE all episodes of thrombosis resulting from surgical interventions or occurring within 90 days from a recent hospitalization [4]. DVT and PE are major public health concerns because they act as significant preventable contributors to nosocomial morbidity and mortality [1].

Overall, the incidence of VTE is thought to be ~29–78 new cases/100.000 residents annually, but those figures may vary widely in different countries and among different ethnicities [5]. What seems to be common among various healthcare systems is that VTE tends to rank as the third most common vascular diagnosis after myocardial infarcts and stroke [3]. Although the most common risk factors for VTE are all linked to Virchow's triad, their specific role may be slightly different and do not carry the same weight [5, 6].

Table 12.1 Risk factors for VTE

• Old Age
• Obesity/increased body mass index (BMI) ≥ 30 kg/m^2
• Male gender
• Immobility (bed rest, long travel)
• Previous history of thrombosis
• Family member with a history of VTE
• Surgery
• Hospitalization
• Cancer
• Trauma
• Varicose veins
• Pregnancy/post-partum state
• Hormonal therapy (post-menopausal, estrogen modulating drugs, oral contraceptives)
• Anti-phospholipid Syndrome (APS)
• Genetic factors determining thrombophilia or altering the coagulation cascade (Factor V Leiden, protein C deficiency, anti-thrombin deficiency, elevated D-dimer, elevated homocysteine)

For this reason, patients must be stratified according to their individual risk level: For instance, it should be noted that above all a previous medical history of VTE and an underlying malignancy represent by far the most important factors in the development of hospital-acquired VTE [7].

While the chapter will mainly focus on current prophylaxis protocols for VTE, it will also touch upon the prevention of arterial thromboembolism (ATE). The former is known to cause chronic pulmonary hypertension, post-thrombotic syndrome, and venous insufficiency-induced lymphedema [7]. However, the latter can also be an avoidable source of complications, such as myocardial infarct, ischemic stroke, or acute limb ischemia, in patients hospitalized for

surgical interventions or chemotherapy. Expanding the discussion to those two conditions makes sense because many unspecific risk factors such as hypertension, hypercholesterolemia, and diabetes are common to both VTE and ATE. The identification of those risk factors (Table 12.1) is fundamental to the implementation of the strategies meant to mitigate the risk of DVT, PE, and whenever reasonable even ATE.

Whereas in the past ATE and VTE have always been considered as two different conditions, they share many pathological pathways; hence, recent studies have suggested that they should be discussed together [7, 8]. For both conditions, most lifestyle changes can be remarkably effective; nonetheless, the use of pharmacological and mechanical prophylaxis represents the hallmark of all preventive and therapeutic measures [8, 9].

12.2 Implementation Strategies and Risk Stratification

The first and foremost step in preventing thrombosis requires the identification of patients most at risk of developing it. Only by doing so, clinicians and healthcare managers can maximize the return on the investment needed to implement costly preventive measures. However, there are five main challenges that slow down the effectiveness of this apparently logical process**:**

1. **Risk assessment** is one of the main challenges in determining and executing a safe prophylaxis protocol. Initially guidelines failed to contemplate precise risk assessment tools and methods, and even now some are solely focused on recommending prophylaxis for the type of procedure rather than the type of patient. It should be also noted that most of the trials to validate prophylaxis protocols were designed to exclude patients with a high risk of thrombosis from enrollment; as such, the shortcomings of *blindly following consensus guidelines without first determining whether the patients in studies used to develop the guidelines fit real patients needs* started to be discussed by the scientific community [7]. Consequently, scientific bodies, such as the European Society of Anesthesiology, have started to mention in their recommendations for surgery in the elderly that underlying co-morbidities increasing the risk of VTE must be assessed and corrected if possible by treating clinicians [10]. In order to meet the current gap in clinical needs more explicit risk-stratification tools have been proposed. The Padua and Caprini models represented pioneering answers to the quest for risk assessment questionnaires [11, 12]. Subsequently, in the United Kingdom, the NICE-NG89 checklist went a step further by providing a tool to estimate not only the risk of VTE but also that of bleeding [4]. A similar approach was taken in Switzerland, by the Geneva University Hospitals and Faculty of Medicine to validate a scoring system for acutely ill patients that is shorter and simpler than the Caprini questionnaire [13]. Finally, in an attempt to move toward personalized medicine, the American College of

Chest Physicians (ACCP) have simplified the risk stratification process by offering a model based on a statewide research that calculates and predicts the risk of developing VTE within 90 days, and attempts to assess the relative weight of each risk factor [14].

2. **Lack of consensus on protocols** for VTE relates to the need to evaluate thrombosis in the context of different underlying diseases and comorbidities. For this, many associations and societies representing different medical and surgical specialties have developed their own guidelines to establish safe and effective thromboprophylaxis protocols. Determining the superiority of a given protocol over the others is difficult though; In fact, many guidelines lack agreement on a number of relevant parameters, such as determination of need for prophylaxis, risk assessment methods, advise on prophylactic drugs and their dosage, duration of prophylaxis before, during and after surgery, etc. [15, 16].
3. **Low-level evidence** is another challenge is that most guidelines fail to achieve. In fact, many of them only give conditional recommendations with low evidence. Such limitation highlights the need for more quality information and to redefine also precise risk stratification methods. Moreover, as the current guidelines are also based on trials and meta-analyses that are older, and *"surgical practice has changed considerably over the decades, aimed at improving the patient experience. In most circumstances, these innovations would be expected to reduce the overall risk of postoperative VTEs"* [17]. All these new and current practices could alter the outcome and prophylaxis procedure.
4. **Compliance with guidelines** has not always been good, on both clinical teams and patients' perspectives. On one hand, healthcare workers might have issues with the application of guidelines whenever they feel that benefits are outweighed by the risks. Clinical management can only be as good as the level of compliance to guidelines from clinicians, leading to either under-, over-, or misuse of prophylaxis [16, 18–21]. In fact, overuse of prophylaxis can be as harming to the patient as underuse because it can lead to extremes such as bleeding or thrombosis [22]. On the other hand, patient compliance is also key for prophylaxis to be effective, especially after discharge from the hospital, which is when adherence to recommendations seems to drastically decrease [23]. Some studies have shown that the compliance rate of patients was particularly affected by their limited understanding of the consequences of poor adherence to recommended treatments [24]. Interestingly, Gao et al. [25] stated that "fewer than 1 in 5 patients maintained compliant [*sic*] with thromboprophylaxis guidelines after discharge," which significantly affected the risk for development of VTE. For this, the most recent NICE guidelines recommend patients engagement and education prior to discharge from the hospital, to prevent misunderstanding and misjudgment of the situation by patients and families [4].

5. Another challenge is related to the **dynamic course of VTE**. In fact, while we can optimize the identification and weighting of risk factors, we still find it difficult to predict the course of this condition, the increase or decrease of patients mobilization in response to VTE complications, the duration of hospital stay, etc. Clinicians should therefore be aware that the VTE risk score is not a static parameter but will fluctuate over time. This shows that patients must be constantly re-appraised, and the treatment plan should be updated accordingly [16].

12.3 Prophylaxis Options with Comparison of Their Efficacy and Risks

As outlined above, when it comes to patient assessment and tailored management, it is crucial to make a clear distinction regarding individual needs for prophylaxis, which will factor in when choosing the appropriate approach. For instance, most guidelines distinguish between protocols for surgical and non-surgical inpatients: This is due to the fact that surgery, especially major surgery, is one of the biggest risk factors for thrombosis. In fact, immobilization during general anesthesia, blood loss, damage to vessels walls, and activation of coagulation cascade can all trigger the formation and release of blood clots, and even fat emboli, in bloodstream**.**

The most commonly prescribed medications for thromboprophylaxis are Low-molecular-weight heparin (LMWH), unfractionated heparin (UFH), direct oral anticoagulants (DOACs), vitamin K antagonists (VKA), and aspirin. Although each of these drugs is associated with specific indications, risks, and benefits, the most relevant side effect of any anticoagulant is bleeding. Given the better profile of LMWH, whose effect spontaneously weans off within 12 h from the last administration, this class has been the leading choice over the other drugs for most patients until recently [26]. Thanks to the latest progress in pharmacology, DOACs have slowly started to catch up and compete with LMWH as recent studies show that both share similar risks of bleeding and efficacy [17].

- Some considerations are particularly useful to optimize the management of **surgical patients:**

  1. The type of procedure changes the approach to prophylaxis. Since major surgical interventions constitute a greater risk factor for the development of VTE, most guidelines recommend the use of anticoagulants in the postoperative period [17, 27–29].
  2. The assumption that risk of developing VTE is the highest in orthopedic patients compared to other surgical specialties [30] is a topic of controversy and disagreement [15]. Orthopedic patients are more at risk of fat embolism and more prone to postoperative immobilization, hence their higher risk profile. While the ACCP and the American Academy of Orthopedic Surgeons (AAOS) recommend LMWH as a first choice

for prophylaxis [28, 29], the American Society of Hematology (ASH) recommends the use of DOACs over LMWH, amid the superior administration route and comparable efficacy [17, 31].

3. Trauma patients are a vulnerable subgroup. It is known that 20% to 90% of trauma patients will develop DVT [32], and views are divided on this area as well. The ASH recommends the use of anticoagulants in trauma patients with low risk of postoperative bleeding but does not recommend it in the case of major trauma with high risk of bleeding.
4. Neurosurgical patients, especially those with traumatic brain injury (TBI), intracranial hemorrhages, and brain tumors, require a dedicated pre-, intra-, and post-operative prophylaxis [32]. Our group [32, 34, 35] and others [33] investigated the optimal timing for pharmacological and mechanical prophylaxis in this class of patients and stressed the importance of adapting the timing of and type of prophylaxis to patients' risk stratification. Our recommendations are in keeping with the Neurocritical Care Society guidelines, which also support the use of mechanical prophylaxis and chemoprophylaxis within 24 h and 24–48 h from admission, respectively [36].
5. In situations where chemoprophylaxis is not feasible, mechanical prophylaxis is always recommended as it is believed to decrease the risk of DVT by around two thirds [32]. Intermittent/pneumatic compression socks are believed to be superior to graduate compressions socks [17, 27, 28, 35, 37], as they are also linked to better compliance [38]. Therefore, they confer better protection from incidence of VTE. Mechanical prophylaxis and chemoprophylaxis can also be used at the same time [17, 37]; this aspect is particularly relevant for patients who require post-surgical admission to intensive care units.

- In addition to the statements above, **non-surgical patients** might have different requirements:

1. Underlying malignancy is believe to increase the VTE by about 4–7.5 times and constitutes 20–30% of VTE associated thrombosis events [42, 43]; this increased risk is related not only to the biology of cancer but also to chemotherapy, which constitutes an independent risk factor for VTE. For this reason, chemotherapy has been incorporated into models for risk stratification of oncological patients, such as the Vienna Prediction and the Khorana Model [44, 45].
2. There is still lack of consensus regarding dosage, frequency of administration, and length of prophylaxis for patients who require long-term hospitalization. The general agreement is that for patients at significant risk of VTE, long-term prophylaxis is more beneficial than the short-term one; however, its recommended length varies, ranging from 19–42 days to more than 35 days [17, 28].

3. While there is an increasing use of inferior vena cava (IVC) filters for prophylaxis filters, evidence of their superiority to pharmacological and mechanical prophylaxis is lacking [39]. IVC are currently recommended only in patients with known DVT [17, 27, 28, 40]. A recent systematic review by Bikdeli et al. (2017) [41] states that filters decrease the risk of PE but do not seem to lead to any significant difference in overall mortality.
4. Even in non-hospitalized patients undergoing ambulatory chemotherapy, most guidelines will follow the same principle of weighing the benefit of thromboprophylaxis versus risk of bleeding. While some consider ambulatory chemotherapy patients to have a low-risk of VTE and therefore do not recommend any prophylaxis, other studies deem also ambulatory chemotherapy to confer a high-risk of VTE, hence suggesting prophylaxis with LMWH, and if needed with UFH or DOACs [46, 47].

12.4 Screening Methods and Secondary Prophylaxis

Even when all the precautions are taken, the development of DVT and PE can still occur. Therefore, it is important to implement a strict monitoring even in patients undergoing pharmacological and mechanical thromboprophylaxis. Such approach is commonly known as secondary prophylaxis, and all the strategies meant to tackle the detection and management of an already formed DVT fall under its umbrella.

The most commonly adopted screening method for secondary prophylaxis is Doppler ultrasound [48]. Its reliability can vary according to the clinical picture and the class of patients, being, for instance, more useful in neuro-oncology over other neurosurgical patients [32, 49]. The specificity ranges around 96% and the sensitivity 95%; however, the latter drops to around 73% for DVT in the calf vein [50].

However, about 85–90% of all Doppler scans for signs or symptoms of DVT result in false-negative studies [16]; hence, other radiology and laboratory methodologies are to be taken into account [51, 52]. In this regard, some biomarkers deserve a mention:

- **D-Dimer** is a known fibrin degradation product and commonly used in clinical practice, especially for its strong negative predictive value for PE but found to have a lower level of specificity [53]. It is now also researched for its role in the prediction of recurrence of and risk stratification power for DVT and seems to be giving optimistic results [54, 55].
- **P-Selectin** is a recently discovered cell adhesion molecule that is shown to be promising as a routine DVT detecting biomarker, especially in cancer patients [56, 57]. Some authors demonstrated that it could be used to confirm or exclude the presence of DVT with better specificity than D-Dimer testing [55]. However, such effectiveness is still a matter of debate, especially in non-cancer populations [58].

12.5 New Horizons: Emerging Prophylaxis Methods, Screening Strategies and Treatment Policies

The constant attention to the costs of VTE has led to a series of approaches to public health meant to reduce the overall risk in our population.

For instance, **statins** are a group of medication used for years in cardiovascular diseases for their lipid-lowering properties. Reducing hypercholesterolemia is, in fact, fundamental to limiting vessel wall damage, one of the pillars of Virchow's triad [59]. Recent studies have hypothesized that they could have an antithrombotic and anticoagulant effect as well [60], by lowering endogenous levels of thrombin [61, 62].

Computerized risk-assessment represents another innovative approach that has been recently emerging for improving the effectiveness of programs for VTE prevention. Technology and computers are omnipresent in every aspect of our lives, and healthcare is no different. Computer-alert programs have been introduced into hospital wards and tested to see if they have a positive impact on thromboprophylaxis risk stratification. Several studies have found such approach particularly useful in increasing the awareness of the medical staff and lowering the rates of VTE [18, 47, 63]. Given the diffusion of digital health in our hospitals computerized risk-assessment is emerging an easy and practical method to increase clinical team's awareness and compliance hence addressing one of the five challenges listed above.

Finally, **interventional studies** will show how treatment of VTE will continue to change and improve with the advent of new screening methods and new pharmacological regimens. It is therefore important to conclude this chapter with the latest evidence provided by a recent systematic review with meta-analysis on the use of thrombolytics for VTE events. The study by Izcovich et al [64] indicates that thrombolytics probably reduce mortality in patients with submassive- or intermediate-risk PE and may reduce postthrombotic syndrome (PTS) in patients with proximal DVT at the expense of a significant increase in major bleeding. Pooled estimates of PE studies indicate probable reduction in mortality with thrombolysis (risk ratio [RR], 0.61; 95% confidence interval [CI], 0.40–0.94) (moderate certainty) and possible reduction in nonfatal PE recurrence (RR, 0.56; 95% CI, 0.35–0.89) (low certainty). Pooled estimates of DVT studies indicate the possible absence of effects on mortality (RR, 0.77; 95% CI, 0.26–2.28) (low certainty) and recurrent DVT (RR, 0.99; 95% CI, 0.56–1.76) (low certainty), but possible reduction in PTS with thrombolytics (RR, 0.70; 95% CI, 0.59–0.83) (low certainty). Pooled estimates of the complete body of evidence indicate increases in major bleeding (RR, 1.89; 95% CI, 1.46–2.46) (high certainty) and a probable increase in intracranial bleeding (RR, 3.17; 95% CI 1.19–8.41) (moderate certainty) with thrombolytics.

12.6 Conclusion

To conclude, defining a high-evidence-based thromboprophylaxis protocol is extremely difficult; however, better is often the enemy of good. VTE is a preventable disease that inflicts a great burden in terms of morbidity and mortality on our healthcare systems [65]. The lack of sufficient evidence is slowly being addressed by the increasing number of observational studies based on newly established registries and well-designed multinational intervention trials comparing different parameters such as compliance or effectiveness and risks of prescribed agents [66–68]. It is therefore possible to foresee how future guidelines will be able to provide stronger recommendations for more precise categories of patients and hopefully reduce the impact of VTE and ATE on our patients.

Abbreviations

AAOS: American Academy of Orthopedic Surgeons
ACCP: American College of Chest Physicians
APS: anti-phospholipid syndrome
ASH: American Society of Hematology
ATE: arterial thromboembolism
DOACs: direct oral anticoagulants
DVT: deep venous thrombosis
IVC: inferior vena cava
LMWH: low-molecular-weight heparin
PE: pulmonary embolism
PTS: postthrombotic syndrome
TBI: traumatic brain injury
UFH: unfractionated heparin
VKA: vitamin K antagonists
VTE: venous thromboembolism

Disclosures and Conflict of Interest

The opinions and perspectives here reflect the current views of the authors. The authors declare that they have no conflict of interest and have no affiliations or financial involvement with any organization or entity discussed in this chapter. No writing assistance was utilized in the production of this chapter and the authors have received no payment for its preparation.

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Chapter 13

Wound Healing: Cellular Mechanisms and Pathological Outcomes

Holly N. Wilkinson, PhD, and Matthew J. Hardman, PhD

Centre for Atherothrombosis and Metabolic Disease, Hull York Medical School, The University of Hull, Hull, UK

h.n.wilkinson@hull.ac.uk

Keywords: advanced glycation end products (AGEs), angiogenesis, antibiotics (ABs), chronic wounds, damage-associated molecular patterns (DAMPs), diabetes, extracellular matrix (ECM), extracellular polymeric substance (EPS), fibrin matrix, fibroblasts macrophages, haemostasis, histamine, hypoxia-inducible factors (HIFs), inflammation neutrophil, interferon-gamma (IFN-γ), interleukin 1 (IL-1), keratinocyte, lipopolysaccharide (LPS), macrophage, mast cell, matrix metalloproteinases (MMPs), mesenchymal stem cells (MSCs), metformin, monocyte, myofibroblast, pathogen-associated molecular patterns (PAMPs), platelet plug, platelet-derived growth factor (PDGF), quorum sensing (QS), reactive oxygen species (ROS), re-epithelialization (re-ep), regulatory T cells (Tregs), senescence-associated secretory phenotype (SASP), senolytics, stromal-derived factor 1 (SDF-1), tissue repair, toll-like receptors (TLRs), transforming growth factor-beta (TGF-β), tumour necrosis factor-alpha (TNF-α), type I diabetes mellitus (T1DM), type II diabetes mellitus (T2DM)

13.1 Introduction

Millennia of evolution have created our skin, a highly adaptive, multifunctional organ that protects us from a daily onslaught of chemical, physical and ultraviolet radiation challenge. This harsh external environment often results in injury to the skin, and it will therefore come as no surprise that our skin possesses sophisticated reparative processes that allow it to heal quickly and efficiently. Despite considerable innate reparative ability, multiple cellular aspects of an individual's injury response can become attenuated, compromising wound closure. This attenuation is most often a result of pathological systemic changes, such as those associated with advanced age or uncontrolled diabetes. Indeed, age and

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diabetes are primary risk factors for developing a chronic wound (i.e., a wound that takes longer than 12 weeks to heal). Unfortunately, these chronic wounds (primarily venous ulcers, pressure sores and diabetic foot ulcers) are a major area of unmet clinical need, increasing significantly on a global scale [1]. In this chapter, we discuss the current understanding of skin repair and illustrate impaired cellular behaviours that underpin chronic wound healing pathology. Application of emerging research technologies will be essential in further elucidating the underlying cellular and molecular basis of acute and pathological repair.

13.2 Cellular Aspects of Acute Wound Repair

Our skin is specialized to interface with the external environment and provides a variety of important homeostatic functions, from regulating thermostability to sensing extrinsic stimuli. Crucially, the skin acts as a primary defence barrier, preventing desiccation and mechanical, chemical, thermal and photic damage to internal structures [2]. This defence extends to a sophisticated immune barrier response that protects against pathogenic infection, while supporting commensal microorganisms via an elegantly adapted host–microbiota axis [3]. The skin has also evolved efficient and rapid mechanisms to close breaches to its barrier in a process collectively known as the wound healing response. Wound repair is classically simplified into four main phases: haemostasis, inflammation, proliferation and dermal remodelling [4], which result in architectural and physiological restoration following damage (Fig. 13.1). The following sections describe these stages in detail.

13.2.1 Haemostasis

Immediately after injury, damaged blood vessels rapid contract and a blood clot forms preventing exsanguination from vascular damage [5]. Platelets, principle contributors to haemostasis and coagulation, are activated when they encounter the vascular subendothelial matrix. Platelet receptors (e.g. glycoprotein VI) interact with extracellular matrix (ECM) proteins (e.g. fibronectin, collagen and von Willebrand factor), promoting adherence to the blood vessel wall. Thrombin subsequently triggers platelet activation, inducing a conformational change, and release of alpha and dense granules containing bioactive molecules which reinforce coagulation (reviewed in [6]). An insoluble clot (eschar) of fibrin, fibronectin, vitronectin and thrombospondin forms [7], primarily serving to plug the wound and prevent bleeding. The eschar also fulfils a number of secondary functions, including shielding against bacterial invasion, providing a scaffold for incoming immune cells and harbouring a reservoir of cytokines and growth factors to guide the behaviour of wound cells in early repair [8].

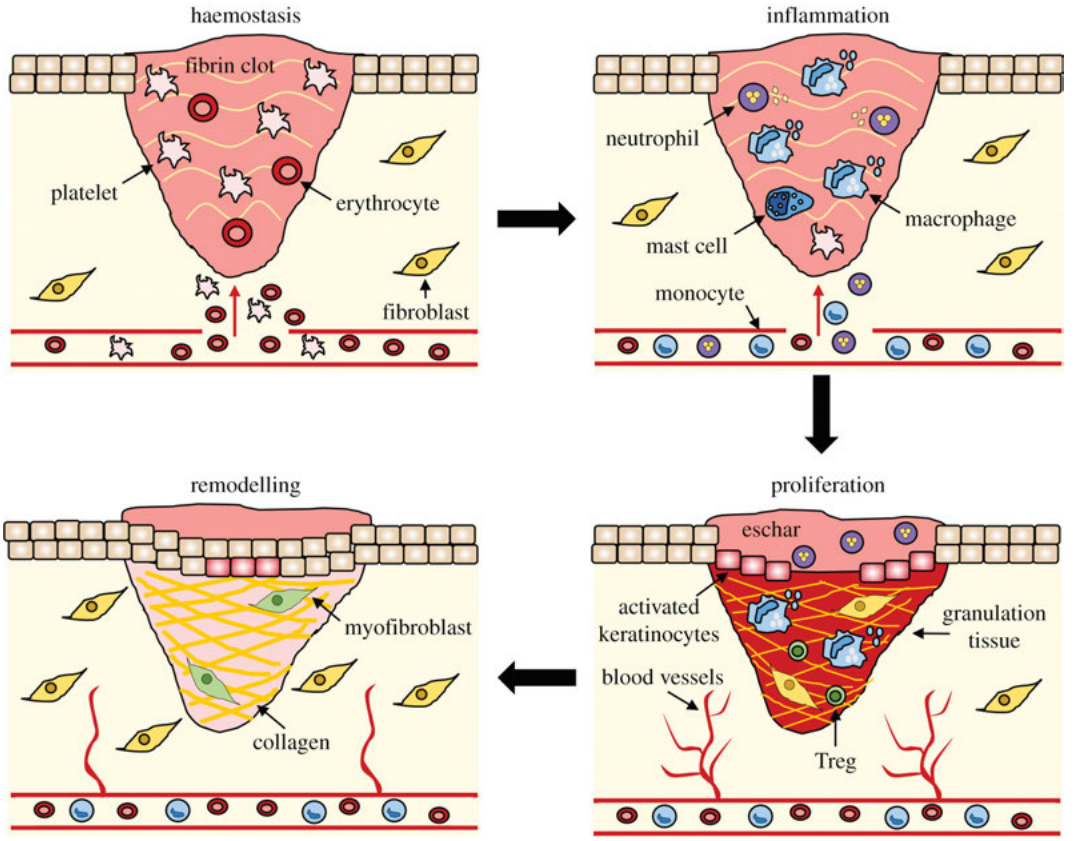

Figure 13.1 The stages of wound repair and their major cellular components. Wound repair begins with haemostasis, where a platelet plug prevents blood loss and a preliminary fibrin matrix is formed. Inflammation then ensues to remove debris and prevent infection, commencing with neutrophil influx, which is promoted by histamine release from mast cells. Monocytes arrive later and differentiate into tissue macrophages to clear remaining cell debris and neutrophils. During the proliferative phase, keratinocytes migrate to close the wound gap, blood vessels reform through angiogenesis, and fibroblasts replace the initial fibrin clot with granulation tissue. Macrophages and regulatory T cells (Tregs) are also vital for this stage of healing. Finally, the deposited matrix is remodelled further by fibroblasts, blood vessels regress and myofibroblasts cause overall wound contraction.

Platelets are crucial in the recruitment of immune cells to the injury site, by either directly capturing immune cells in the eschar, or by releasing a secretome of chemokine attractants upon degranulation [6]. In fact, the platelet secretome also contains growth factors that stimulate resident skin cells, including fibroblasts and keratinocytes [9]. As the most abundant cell type during early repair, platelets play an active role in the early inhibition of bacterial infection. They express a number of toll-like receptors (TLRs) [10, 11], which regulate the production of antimicrobial peptides [12]. Once a sufficient clot has formed,

the coagulation process is switched off, preventing excessive thrombosis. Here, platelet aggregation is inhibited by prostacylin, thrombin inhibited by antithrombin III, and coagulation factors V and VII degraded by activated protein C [13]. At the same time, the injured vessel wall is repaired by smooth muscle cells and endothelial cells that proliferate in response to released platelet-derived growth factor (PDGF) [14]. Endothelial progenitors are also recruited to aid this process as mature endothelial cells show limited proliferative capacity [15].

13.2.2 Inflammation

Innate inflammation evolved as the primary defence against pathogenic wound invasion. This immune response is initiated by injury-induced signals; damage-associated molecular patterns (DAMPs) released by necrotic cells and damaged tissue, and pathogen-associated molecular patterns (PAMPs) from bacterial components. These PAMPs and DAMPs activate resident immune cells, such as mast cells, Langerhans cells, T cells and macrophages, by binding pattern recognition receptors to elicit downstream inflammatory pathways [16]. A subsequent release of pro-inflammatory cytokines and chemokines attracts circulating leucocytes to the site of injury (reviewed in [17]). Pro-inflammatory molecules also stimulate vasodilatation, which, along with the expression of endothelial cell adhesion molecules, such as selectins, facilitates neutrophil and monocyte adhesion and diapedesis [18]. In fact, the importance of selectins in immune cell recruitment has been clearly demonstrated, with genetic [19] and pharmacological [20] blockade of E- and P-selectin significantly impairing both immune cell infiltration and wound healing.

Neutrophils, which arrive early after injury, are recruited into the wound from damaged vessels, attracted by chemoattractants, including interleukin 1 (IL-1), tumour necrosis factor-alpha (TNF-α) and bacterial endotoxins, such as lipopolysaccharide (LPS) [21]. In response to pro-inflammatory signals, and activation of inflammatory signalling pathways (e.g. NF-κB [21]), neutrophils (and other wound cells) release their own cytokines. Neutrophils remove necrotic tissue and pathogens via phagocytosis and the release of reactive oxygen species (ROS), antimicrobial peptides, eicosanoids and proteolytic enzymes [22]. They also trap and kill pathogens in an extruded web of DNA coated with antimicrobial peptides and cytotoxic histones, termed extracellular traps [23].

The inflammatory response is complex, modulated by a multitude host of intrinsic and extrinsic factors. Uncontrolled and excessive inflammation promotes tissue injury and delays healing (as in diabetic mice [24]). However, insufficient immune cell recruitment, for example in TLR3 knockout mice, also hinders repair [25]. Thus, immune cell responses must be situational, increasing to respond appropriately to infection, yet clearing effectively to allow wound resolution. In the absence of infection, wound neutrophils decline within a few days of injury onset [26]. Most neutrophils are extruded from the wound site as they adhere to the fibrin scab, while others are removed by innate clearance mechanisms such as macrophage efferocytosis [17]. Remaining neutrophils are

cleared by apoptosis, necrosis or phagocytosis, or may leave inflamed tissue and return to the circulation through reverse transendothelial migration, as observed in zebrafish [27], mice [28] and human neutrophils *in vitro* [29].

Circulating monocytes enter the wound tissue where, in response to the local milieu, they differentiate into macrophages. Although it is generally suggested that macrophages are recruited following neutrophils, an initial wave of monocytes has been observed entering the wound simultaneously with neutrophils [30]. Macrophages are master effector cells in tissue repair, displaying both versatility and high plasticity (reviewed in [31]). They reach peak wound infiltration 72 h after injury in mice and 7 days post-injury in humans [32]. Like neutrophils, macrophages engulf necrotic cellular debris and pathogenic material through evolutionarily conserved receptors, but also exhibit differential behaviours and morphological changes in response to cytokines [33].

Wound macrophages are traditionally separated into two main subsets: M1-stimulated and M2-stimulated. However, this dichotomous classification has become outdated, with both human [34] and murine [35] macrophages now known to show diverse transcriptional and phenotypic responses to different stimuli (reviewed in [36]). Hence, the macrophage repertoire should be viewed as a spectrum of phenotypes governed by tissue status and environmental signals [37, 38]. For simplicity, we will herein refer to classically activated (pro-inflammatory) and alternatively activated (anti-inflammatory) groups.

Classically activated macrophages are induced by pro-inflammatory stimuli, such as LPS and interferon-gamma (IFN-γ), and promote inflammation by releasing ROS, inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and growth factors (e.g. vascular endothelial growth factor, VEGF and PDGF). These macrophages phagocytose apoptotic neutrophils, replacing them as the main inflammatory mediator [8]. Later stages of inflammation are characterized by a transition to alternative activation, which occurs through neo-differentiation of newly recruited monocytes, or via switching of existing macrophages *in situ* to an anti-inflammatory phenotype. Although not widely characterized, this phenotypic switch can be stimulated by environmental changes in cytokines [39] and efferocytosis [40]. It may additionally be driven by miRNAs [31], transcription factors [41], and modulation of pro-inflammatory and anti-inflammatory receptors [41, 42].

Alternatively activated macrophages express pro-resolutory cytokines (IL-4, IL-10, IL-13 [43, 44]) and arginase, a key factor for effective wound repair [45]. Anti-inflammatory macrophages also release a myriad of growth factors to promote re-epithelialization, fibroplasia [8] and angiogenesis [46]. More recently, macrophages have been shown to be crucial in the stabilization and remodelling of blood vessels in mice and fish [47].

The importance of macrophages is further demonstrated in selective ablation studies, where *Cd11b*-specific deletion of macrophages leads to delayed wound repair and increased inflammation [48]. Similarly, inducible knockdown of macrophages during early healing caused delayed re-epithelialization, angiogenesis and granulation tissue formation, while knockdown of macrophages mid-way through healing led to endothelial cell damage, severe haemorrhage and

immature granulation [49]. Thus, the collective behaviours of macrophages promote scavenging of debris, bacteria and pro-inflammatory cells, while also stimulating reparative processes to allow effective wound resolution.

The overwhelming presence of neutrophils and macrophages in wounds has potentially masked the importance of other myeloid cells in wound repair. However, recent studies have revealed that resident T cells are critical for the early injury response, while circulating T cells are recruited to resolve inflammation [50]. Indeed, aged and diabetic mice show reduced resident dendritic epidermal T cells and a delayed healing phenotype, whereas subcutaneous administration of dendritic epidermal T cells can restore healing [51, 52]. Moreover, the removal of anti-inflammatory regulatory T cells delays tissue repair in mice [50]. Mast cells also play a role in wounds, releasing histamine to aid neutrophil recruitment during early inflammation [53].

13.2.3 Proliferation

The proliferative phase of healing is characterized by extensive activation of keratinocytes, fibroblasts, macrophages and endothelial cells to orchestrate wound closure, matrix deposition and angiogenesis. As early as 12 h post-injury, keratinocytes are activated by changes in mechanical tension and electrical gradients, and exposure to hydrogen peroxide, pathogens, growth factors and cytokines [54]. This activation causes keratinocytes at the wound edge to undergo partial epithelial–mesenchymal transition, where they develop a more invasive and migratory phenotype [55]. Front-to-rear polarity replaces top-to-bottom polarity, allowing the leading-edge keratinocytes to migrate laterally across the wound to reform the epidermal layer, a process termed re-epithelialization [56]. Keratinocytes behind the leading edge modulate their cell adhesion via PCKα-mediated changes in desmosome adhesiveness [57] and Eph-mediated changes in adherens junctions [58], allowing them to rearrange their order with the migrating epithelial sheet [54]. Keratinocytes in the neo-epidermis release matrix metalloproteinases (MMPs) to aid their path of migration, while laying down new ECM proteins to reconstitute the basement membrane [59].

Hair follicle stem cells are induced to proliferate, with progeny epidermal cells streaming out of the follicle to meet the cellular demand required to resurface the wound [60]. These cells sprout from damaged appendages in shallow wounds or arrive from the epidermal edge in full-thickness wounds. Only specific stem cell compartments are activated or recruited to the re-epithelialization process [61]. For example, Krt15+ve [62] and Krt19+ve [63] bulge region stem cells appear dispensable for re-epithelialization, while Lgr5- and Lgr6-expressing cells from the follicle and interfollicular epidermis respond to wound cues, contributing to re-epithelialization [64]. A key characteristic of full-thickness wounds in mice is that appendages, including follicles, are absent from re-formed scar tissue [2]. However, under specific circumstances wound-induced follicle neo-genesis can occur, seemingly via re-activation of developmental Wnt and Shh signalling [60].

Keratinocytes negotiate through debris and necrotic tissue of the wound bed through their interactions with structural proteins of the preliminary matrix via integrin receptors [65]. MMPs, particularly MMP-1 and MMP-9, are vital for keratinocyte migration as they aid integrin receptor dissociation [56]. The production of other proteases, such as plasmin, further facilitates keratinocyte migration by degrading the provisional fibrin-rich wound bed [59]. When keratinocytes from opposing edges meet, migration terminates (via an undetermined mechanism), a thin epithelial layer is established, and keratinocytes form new adhesions to the underlying matrix. Keratinocytes then fully reform the basement membrane and undergo terminal differentiation, to stratify and regenerate the epidermis [32].

Fibroblasts are the main cell type responsible for replacing the provisional fibrin-rich matrix with a more substantial granulation tissue. Resident and mesenchymally derived fibroblasts respond to a milieu of signalling molecules from platelets, endothelial cells and macrophages, including transforming growth factor beta (TGF-β) and PDGF. These signals direct fibroblasts to either become pro-fibrotic, laying down ECM proteins, or differentiate into myofibroblasts which drive wound contraction [55]. It is important to note that this is again a simplification, as in reality fibroblasts exhibit functional diversity, assisting dermal repair in different ways. In a seminal study Driskell et al. [66] demonstrated that skin fibroblasts originate from two distinct lineages, where the upper lineage aids re-epithelialization while the lower lineage contributes to ECM deposition. Recent findings have further challenged conventional understanding of wound fibroblast origin, showing that two-thirds of granulation tissue fibroblasts are actually myeloid derived [67], and are thus likely to stem from wound macrophages. Fibroblasts degrade the provisional matrix by producing MMPs and replace it with a granulation tissue rich in fibronectin, immature collagens and proteoglycans [68]. This granulation tissue acts as a scaffold for the migration and differentiation of wound cells, supporting both the formation of new blood vessels and the deposition of mature ECM.

New blood vessels are created during the process of angiogenesis to meet the metabolic demands of the highly proliferative healing tissue. Angiogenesis is triggered by hypoxia, which in turn drives the expression of hypoxia-inducible factors (HIFs) and cyclooxygenase 2, and subsequent release of VEGF and other factors [69]. In response to these changes, microvascular endothelial cells proliferate and migrate into the wound bed, sprouting new vessels that fuse with others to develop stable, tubular networks [70]. VEGF prevents endothelial cell apoptosis by upregulating anti-apoptotic proteins such as BCL-2 [71], while the fibrin matrix promotes angiogenesis by triggering phenotypic changes in endothelial cells to stimulate their migration [72].

Macrophages play a significant role in angiogenesis by aiding microvascular endothelial cell behaviours. They produce proteases such as MMPs to degrade the dense fibrin network and chemotactic factors (e.g. TNF-α, VEGF and TGF-β) to drive endothelial migration (reviewed in [73]). Willenborg et al. [74] demonstrated the importance of macrophage-derived factors in angiogenesis, where myeloid-specific deletion of VEGF-A reduced capillary formation in murine wounds.

Macrophages also participate in the remodelling of new vasculature, by guiding vessel tips together [75], phagocytosing superfluous vessels [47, 76] and dampening the angiogenic response to prevent excessive vascularization [77].

The skin houses a dense network of sensory and autonomous nerve fibres which allow sensation and movement. Nerve fibre regeneration is therefore essential following injury. Despite the principle role of diabetic skin denervation in wound pathogenesis (reviewed in [78]), wound innervation *per se* remains an understudied area. Neuropeptides, such as substance P, are known to be released from sprouting neurons and immune cells during repair, influencing diverse cellular processes (e.g. proliferation and angiogenesis [79, 80]). Notably, substance P is reduced in delayed healing in diabetic wounds, where topical restoration restores healing [81, 82] and contributes to nerve regeneration [83]. Wound-activated glial cells are also an important component of the repair response, shown to express factors important for chemotaxis, while the loss of glial cells delays healing in wild-type mice [84]. These and other studies suggest that innervation plays a substantial role in effective repair.

13.2.4 Matrix Remodelling

Remodelling of the ECM spans the entire injury response, beginning with the initial deposition of a fibrin clot, and ending several years later with the formation of a mature, type I collagen-rich scar [55]. Fibroblasts are the major cell type responsible for wound ECM remodelling, replacing the initial fibrin clot with hyaluronan, fibronectin and proteoglycans, and forming mature collagen fibrils later in repair [85]. Proteoglycans aid construction of mature, cross-linked collagen fibrils and act as a conduit for cell migration [86]. The collagen composition of uninjured adult skin is approximately 80% collagen type I: 10% collagen type III. By contrast, granulation tissue predominantly comprises of the embryo-associated collagen type III (approx. 30%), with only 10% collagen type I [87]. As healing progresses, collagen type III is replaced by collagen type I, directly increasing the tensile strength of the forming scar [88]. The integrity and architecture of scar ECM never fully returns to that of unwounded skin. Collagen fibrils in scar dermis adopt large parallel bundles, while in uninjured skin fibrils adopt a basket weave orientation. Thus, wound scar tissue confers only up to 80% of pre-wounding strength post-injury [87, 89].

These sequential changes in the ECM require a fine balance between collagen degradation and synthesis, achieved through temporal regulation of key MMPs. These collagenases, expressed by anti-inflammatory macrophages, fibroblasts and keratinocytes, cleave native helical collagens throughout repair [85]. Elastin, another key dermal ECM component, must reform elastic fibres to retain skin elasticity. Interestingly, the degradation of normal dermal matrix causes the release of elastin fragments, or elastokines, which act as signalling molecules [90]. Elastin is formed from its precursor, tropoelastin, and early in healing shows the aberrant arrangement. In fact, mature elastin fibres are often only apparent in scar tissue many months after injury [91, 92].

Heightened expression of TGF-β and mechanical tension stimulate myofibroblast differentiation *in vivo* and *in vitro* [93]. Myofibroblasts are characterized by an abundance of alpha-smooth muscle actin (α-SMA), associated with an ability to generate strong contractile forces and focal adhesions [85]. Curiously, mice lacking the gene encoding α-SMA, *Acta2*, heal normally with no obvious change in fibroblast contraction [94]. This apparent redundancy, with compensation by other microfilaments, highlights the importance of wound contraction. Myofibroblast contraction is facilitated by pseudopodial extensions that allow cytoplasmic actin to bind to fibronectin in the matrix scaffold [55]. Myofibroblasts adhere to one another via desmosomes, binding to matrix fibrils and drawing the matrix together by a process termed contracture [95]. The wound healing response abates when macrophages, endothelial cells and fibroblasts undergo apoptosis or exit the injury site, leaving a scar [96].

13.3 When Healing Fails—Factors Influencing Chronic Wound Healing

Acute wound repair is a highly dynamic cascade of cellular signalling and behavioural events that ensures rapid closure of the skin barrier. High levels of redundancy and compensatory mechanisms ensure that small alterations to this response seldom cause problems in healing wounds [97]. For example, the ablation of specific subsets of hair follicle stem cells [63], MMPs [98], fibroblast growth factors [99], TGF-α [100] and VEGFR2 [101] each individually fail to significantly impair wound closure. However, like any biological process, sufficient perturbation to the system leads to aberrations, which in the case of wounds manifest as excessive scarring at one extreme or failure to heal entirely at the other. Wounds that fail to heal (defined as generally remaining unhealed after 12 weeks) are termed chronic wounds. They primarily affect the elderly and diabetic, are highly prevalent and a major socioeconomic burden [102, 103]. More effective clinical management would prevent a proportion of these wounds [104], yet many remain refractory to current treatment, highlighting the need to better understand the cellular basis of wound pathology in order to develop therapeutically viable treatments.

Susceptibility to injury remains understudied. We know that the skin of aged and diabetic mammals is more predisposed to injury, as it undergoes atrophy, with altered skin barrier and reduced hydration [105, 106]. Both ageing and diabetes lead to the gradual loss of dermal matrix, with corresponding changes in tissue mechanics, loss of resilience and increased susceptibility to friction damage [107, 108]. Once an injury occurs, a range of molecular and cellular perturbations contribute to overall healing impairment. One factor widely implicated in aged and diabetic wound pathology is cellular senescence (reviewed in [109]). Mitotic cells become senescent and non-proliferative in response to a host of intrinsic and extrinsic factors. Senescent cells acquire a hypersecretory phenotype, producing a secretome rich in pro-inflammatory cytokines and tissue-degrading proteases

(reviewed in [110]). The chronic wound environment is the perfect platform for senescent cell induction due to the high levels of inflammation and oxidative stress [111]. Indeed, we recently demonstrated that high senescent cell burden contributes to wound pathology, where blockade of the proposed senescence receptor, CXCR2, dampens macrophage senescence and improves healing in diabetic mice [112].

A key contributor to wound pathology is excessive inflammation, which perpetuates chronicity through the continued destruction of wound tissue. Chronic wounds are characterized by high numbers of Langerhans cells [113, 114], neutrophils [115], pro-inflammatory macrophages [116, 117] and proteases [118–120], linked to clinical ulcer severity [121]. Along with elevated infiltration of specific immune cell subsets [122], pathological immune cell function is perturbed and collectively contributes to poor healing. Here, neutrophils are excessively primed to produce neutrophil extracellular traps, which are cytotoxic [123] and delay wound healing [124]. In diabetic mice, neutrophils are more resistant to apoptosis, and less effectively cleared by macrophages [125], furthering their excessive presence in pathological wounds. Diabetic macrophages also exhibit defective efferocytosis of apoptotic cells [126], impaired phagocytosis of bacteria [127, 128] and reduced ability to polarize to an anti-inflammatory state [129]. Interestingly, even prior to ulceration, the skin of diabetic humans and mice exhibits higher numbers of mast cells and macrophages primed to the pro-inflammatory state [130]. By contrast, T cell receptor diversity [131] and the number of CD4+ T cells [116, 131] are reduced in diabetic foot ulcers. Together, these aberrant features of chronic wound immune cells not only prevent the shift from inflammation to resolution, but greatly increase vulnerability to infection. Heightened inflammation may also persist due to chronic wound infection, thus maintaining the wound in a continuous cycle of infection, inflammation and inadequate repair.

Cellular impairment is not only restricted to inflammation, but also extends to re-epithelialization and dermal remodelling. Non-healing diabetic foot ulcers are typically characterized by an epidermal wound edge that is hyperkeratotic and parakeratotic [132]. Keratinocytes at the chronic wound edge show abnormal nuclear presence of β-catenin and elevated c-*myc*, which directly delays migration *in vitro* [132] and prevents healing in mice [133]. Ulcer wound edge epidermis additionally displays the misexpression of a number of cell cycle, differentiation and desmosomal markers [134], impaired growth factor receptor signalling [135], and lacks hair follicles [136]. This aberrant activation phenotype, with seemingly uncontrolled wound edge proliferation, is thought to directly inhibit keratinocyte-mediated chronic wound closure.

At the same time, dermal reconstitution is significantly inhibited by the high wound protease levels, which not only break down dermal ECM components, but also degrade growth factors (e.g. VEGF and TGF-β [137, 138]) and cytokines (e.g. TNF-α [139]). Chronic wound fibroblasts are highly senescent, further compromising ECM deposition [140–142], and are unresponsive to ECM-stimulating factors such as TGF-β [143, 144]. Interestingly, we recently demonstrated that deficiency

in wound iron may underpin reduced ECM deposition in diabetic mice, as iron loading of fibroblasts directly stimulates ECM deposition and remodelling [145]. Macrophages are key to this reparative response, where iron sequestration causes alternatively activated macrophages to produce ECM-stimulating factors [146]. Note that disparities exist in the reported role(s) of iron in wound repair. Sindrilaru et al. [117] suggest that iron deposition caused delayed healing in diabetic foot ulcers, promoting an unrestrained M1-like macrophage phenotype, increased oxidative stress and senescence. Similarly, others have shown that the iron chelator, deferoxamine, improves wound healing in pressure ulcers of diabetic [147] and aged [148] mice. Thus, the cellular effects of iron are probably context-dependent and wound-type-specific, exacerbating tissue damage in an already pro-inflammatory environment, while promoting alternatively activated macrophage- and fibroblast-mediated wound resolution in late-stage repair.

Sustained hyperglycaemia in diabetes directly contributes to defective healing, compromising leucocyte function [149], inducing cellular senescence [150] and causing non-enzymatic glycation of ECM and the formation of advanced glycation end products (AGEs) [151]. AGEs not only alter the dermal structural architecture, but also trigger inflammation and ROS via their receptor, RAGE [152]. These effects impair neovascularization, in part by preventing HIF-1α transactivation and subsequent upregulation of VEGF and stromal-derived factor 1 (SDF-1) [153, 154]. At the macroscopic level, uncontrolled diabetes causes long-term damage to the microvasculature, which results in local tissue hypoxia, arterial vasculopathy and/or lower limb neuropathy—all extreme risk factors for chronic wound development [155].

In diabetes, stem cell populations that would usually participate in vascularization are depleted (e.g. bone marrow [156]) or show impaired neovascular potential (in adipose tissue [157]). A reduction in SDF-1, which aids recruitment of endothelial progenitor cells to wounds, is also observed, while topical administration of SDF-1 accelerates diabetic wound repair [158]. Slowing AGE formation in diabetic mice improves the neovascular potential of bone marrow progenitors [159], confirming functional relevance and further demonstrating the important contribution of uncontrolled diabetes in wound pathology.

It is crucial to note that the causes of delayed healing, while simplified above, are often multifactorial and complex. Wound chronicity is influenced by local and systemic defects [160], along with imbalances in hormones, cytokines and growth factors (e.g. reduced PDGF [161]). However, in recent years, the presence and persistence of wound infection has been widely discussed as a major contributor to chronicity [162]. Indeed, high abundance of common wound pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, is reported in chronic wounds [163, 164], with a wound's microbial profile strongly linked to healing outcome [165]. These pathogens often develop into polymicrobial aggregates (biofilms) encapsulated in a protective matrix of extracellular polymeric substances that confers resistant to traditional antibiotics and host defences (reviewed in [166]).

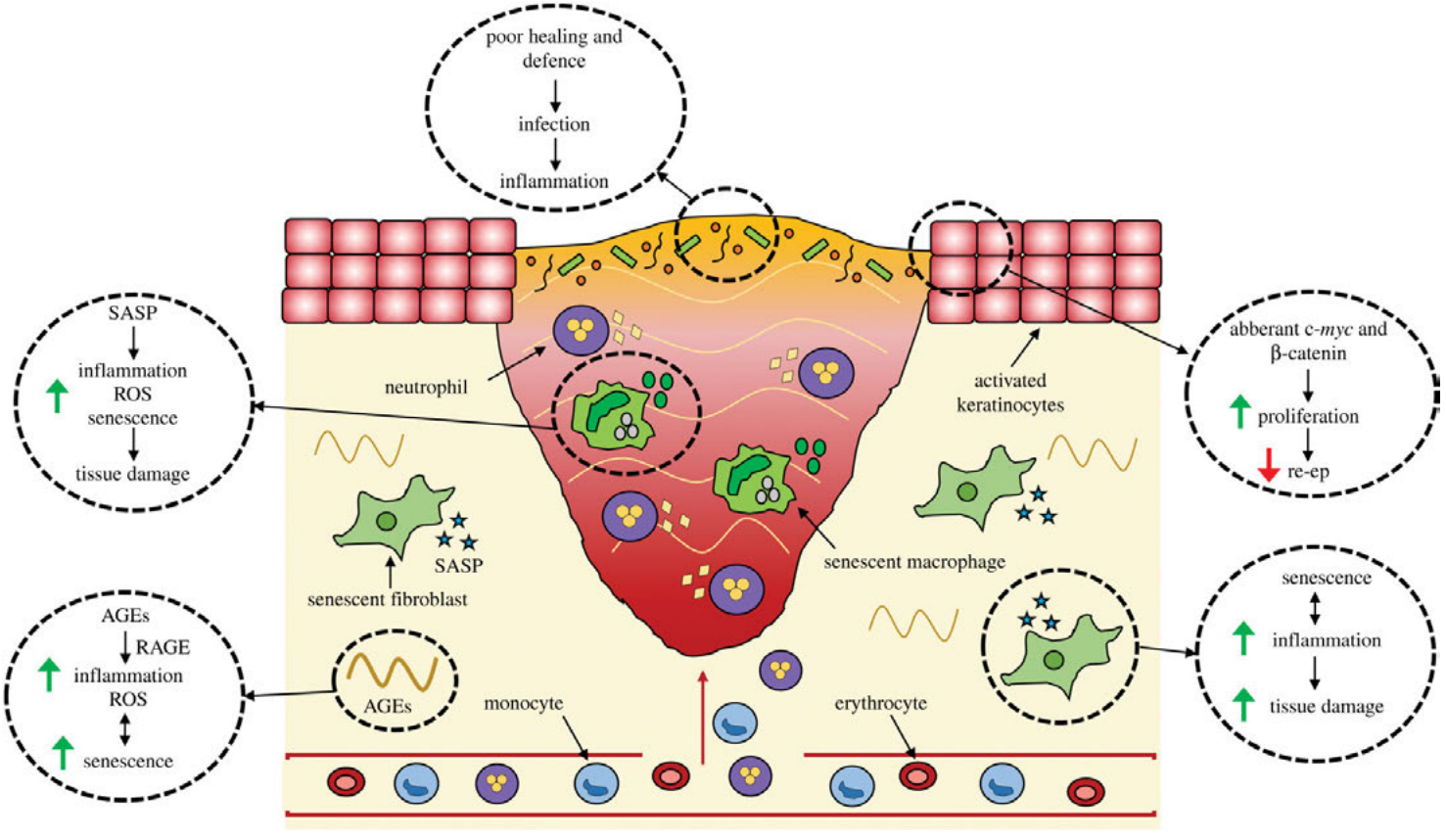

Figure 13.2 Factors contributing to chronic wound healing. Chronic wounds become infiltrated with bacteria that exacerbates inflammation. Chronic wound keratinocytes show aberrant activation causing hyperproliferation and impaired migration. A large proportion of chronic wound cells (e.g. macrophages and fibroblasts) become senescent, producing a senescence-associated secretory phenotype (SASP) that perpetuates senescence, triggers reactive oxygen species (ROS) release and heightens inflammation. High amounts of advanced glycation end products (AGEs) also contribute to inflammation and cellular senescence in the wound environment. Together these features cause excessive tissue breakdown and impair cellular functions to prevent normal healing. re-ep = re-epithelialization.

The microbiome profiles of aged and diabetic skin differ considerably from their young and non-diabetic counterparts, in each case displaying reduced α-diversity [167, 168]. Although critical wound colonization occurs as a result of inadequate immune cell function, poor perfusion and the presence of a persistent open wound, it is likely (though yet to be proven) that aged and diabetic skin is intrinsically predisposed to infection by an altered microbiome. Diabetic wounds also show altered expression of pattern recognition receptors responsible for eliciting a host response, which may link to poor healing [169]. Interestingly, knockout of the pattern recognition receptor, Nod2, impaired wound closure [170] and altered the skin microbiome [171] of mice. Curiously, wild-type mice cross-fostered into Nod2−/− litters adopted an altered microbiome and acquired a delayed healing phenotype [171], therefore directly demonstrating the impact of skin microbiota dysbiosis on repair. Key factors in chronic wound pathology are summarized in Fig. 13.2.

13.4 Translational Techniques to Enhance Clinical Understanding of Wounds

Our knowledge of the mechanisms underlying chronic wound healing is constantly improving, largely due to the development and refinement of wound models and diagnostic tools. For example, until the advent of sequencing technologies, wound bacterial profiling was restricted to simple culture methods, limiting speciation to only organisms capable of expansion in culture. Further analysis was then required to gather complete diagnostic information about a clinical isolate (reviewed in [172]). The emergence of short-read 16S sequencing provided new insight into clinical bacterial communities, but bacterial identification was limited to genus level based on inference from sequence homology [173], with little information about their virulence or clinical significance. Novel genomic technologies are now emerging to allow rapid molecular identification of microorganisms to the sub-species level. Simultaneous characterization of antibiotic resistance and virulence profiles [173, 174] provides unprecedented insight into the role of bacterial, fungal and viral ecosystems in wound pathology. Combining these techniques with host genomic, metabolomic and proteomic approaches promises to deliver in depth understanding of the myriad of factors influencing wound repair, while ultimately facilitating a true “personalised medicine” approach to clinical wound management.

Historically, wound studies have relied on the use of *in vivo* models to address the complexity of the multifactorial wound response. However, it is widely accepted that between-species differences have hindered translational wound research efforts. We are now moving towards the development of more dynamic *in vitro* approaches, such as three-dimensional skin equivalents [175], allowing closer modelling of native human cell behaviours, and moving away from artificial single-cell monolayer culture. While cultured three-dimensional skin equivalents still lack many skin features, such as glands, immune cells and blood vessels,

current research is beginning to address this deficit [176, 177]. The development of three-dimensional-printed skin equivalents is particularly exciting, offering profound implications in translational research. Indeed, a recently developed vascularized three-dimensional-printed skin model reflected many aspects of native skin, including tissue maturation, and epidermal stratification and stemness [178].

Porcine and human *ex vivo* models are also gaining traction, with the advantage that they provide native skin tissue architecture and the full gamut of resident skin cells to recapitulate important aspects of the human chronic wound healing response [179, 180]. *Ex vivo* models are not without their caveats, lacking immune cell infiltration and maintaining viability for a limited time-frame [181]. It is likely that novel culture methods, such as microfluidics [182], will extend tissue viability and allow skin perfusion with biologically relevant factors (and immune cells) to increase the relevance of *ex vivo* wound models.

In vivo models are still widely used, with mice favoured for mechanistic studies [183]. The multitude of available transgenic mouse lines (including reporter lines) allows temporal and spatial investigation of the molecular basis of *in vivo* wound healing. Nevertheless, strain- and species-specific differences must be considered, especially when extrapolating conclusions for translational research purposes. Pigs, though used far less frequently, provide a useful translational model with skin that closely resembles that of humans. Wounding in mice involves full-thickness incisions or excisions, yet variability can be introduced between laboratories by the methods used to apply wounds, the analgesics and anaesthetics used, and how the wounds are treated (e.g. splinted, occluded or left to heal by secondary intention [184, 185]). Continued efforts to standardize *in vivo* methodology will be essential to increase experimental validity and progress current and future wound research.

An array of pre-clinical delayed healing models are used to better recapitulate human chronic wounds, from pressure ulcers in mice using magnets [186], to infected wounds in pigs [187]. As those primarily at risk of developing chronic wounds are elderly or diabetic, it follows that the most widely used chronic healing models involve aged and diabetic rodents [188]. Type I and type II diabetes mellitus (T1DM and T2DM) can be modelled in mice. T1DM-mediated delayed healing is commonly stimulated through streptozocin injection [189, 190], where timing post-injection is critical to the delayed healing phenotype [192]. Genetically altered mice are used to mimic T2DM through leptin or leptin receptor deficiency. These mice are morbidly obese by 6–8 weeks of age, go on to show hallmarks of T2DM (reviewed in [193]), and display substantially delayed healing versus their non-diabetic, heterozygous littermates [194]. There remains some controversy as to whether delayed healing in diabetic mice is a result of hyperglycaemia, leptin deficiency or obesity [184].

To mimic age-associated healing pathology, mice are wounded at 18 plus months of age (reviewed in [195]). Young ovariectomized mice provide an alternative accelerated ageing model, where surgical removal of the ovaries

mimics the human menopause [196]. Here, the loss of circulating sex hormones, particularly 17β-estradiol, produces a delayed healing phenotype that is largely comparable to that of aged mice (reviewed in [197]). Unlike diabetic models, limited to comparison against diabetic wounds, aged models have the advantage that they emulate a more generalized underlying risk factor for all chronic wounds, advanced age [198].

13.5 Current Therapies and Future Opportunities

Wound management begins with an assessment of wound aetiology and a patient-centric approach to managing systemic and lifestyle factors. In the case of diabetic foot ulcers, local management often starts with debridement, the removal of necrotic, infected or hyperkeratotic tissue via surgical or less invasive modalities [5, 199]. Extracting the chronic tissue back to less affected epidermis, while triggering an acute injury response, is thought to kick-start normal reparative healing pathways [200]. Wounds are then irrigated with saline or antibacterial solution and a tailored dressing is applied [201]. Contemporary dressings contain a myriad of material properties to aid tissue repair and incorporate substances with known pro-healing or antimicrobial effects [202, 203]. More advanced solutions are available, including the continually evolving negative pressure wound therapy modality [204]. Despite numerous available treatments, current best practice wound management is almost exclusively aimed at addressing secondary causes of chronicity, while also relying heavily on patient compliance. These two factors result in up to 40% of chronic wounds persisting for many months or years despite extensive treatment [102]. There remains a clinical unmet need to address this shortfall with novel therapies that are financially, physiologically and practically viable for the wound care setting.

A major contributor to chronic wound recalcitrance is persistent, antibiotic-resistant biofilm infection. It is therefore unsurprising that a large proportion of recent wound research has focused on the development of novel antimicrobial and anti-biofilm therapies. Traditional non-antibiotic antimicrobials, such as silver salts, alleviate bacterial burden but are cytotoxic to the host, while modern formulations (e.g. nanoparticles) have lower cytotoxicity and may also promote wound healing (reviewed in [205]). Emerging antimicrobial treatments that may also show beneficial roles in tissue repair include cold atmospheric plasma [206, 207] and bioactive glass [179, 208].

Most antimicrobials display broad effects and are not targeted to specific pathogenic species and strains. This is important, as commensal bacteria have a positive role in skin maintenance and wound repair (reviewed in [209]), and unlike their pathogenic counterparts, commensal biofilms do not cause persistent delayed healing in diabetic wounds [166]. As a result, more directed treatments for pathogenic bacteria, such as phage therapy [210] or pharmacological inhibition of bacterial virulence mechanisms such as quorum sensing [211], may confer higher specificity and efficacy. Moreover, most treatments focus on the bacterial

component of infection, but the fungal diversity of wounds is also linked to healing outcome [212]. Thus, to elucidate the role of host–microorganism interactions in pathological repair, prospective research should acknowledge the wound ecosystem in its entirety.

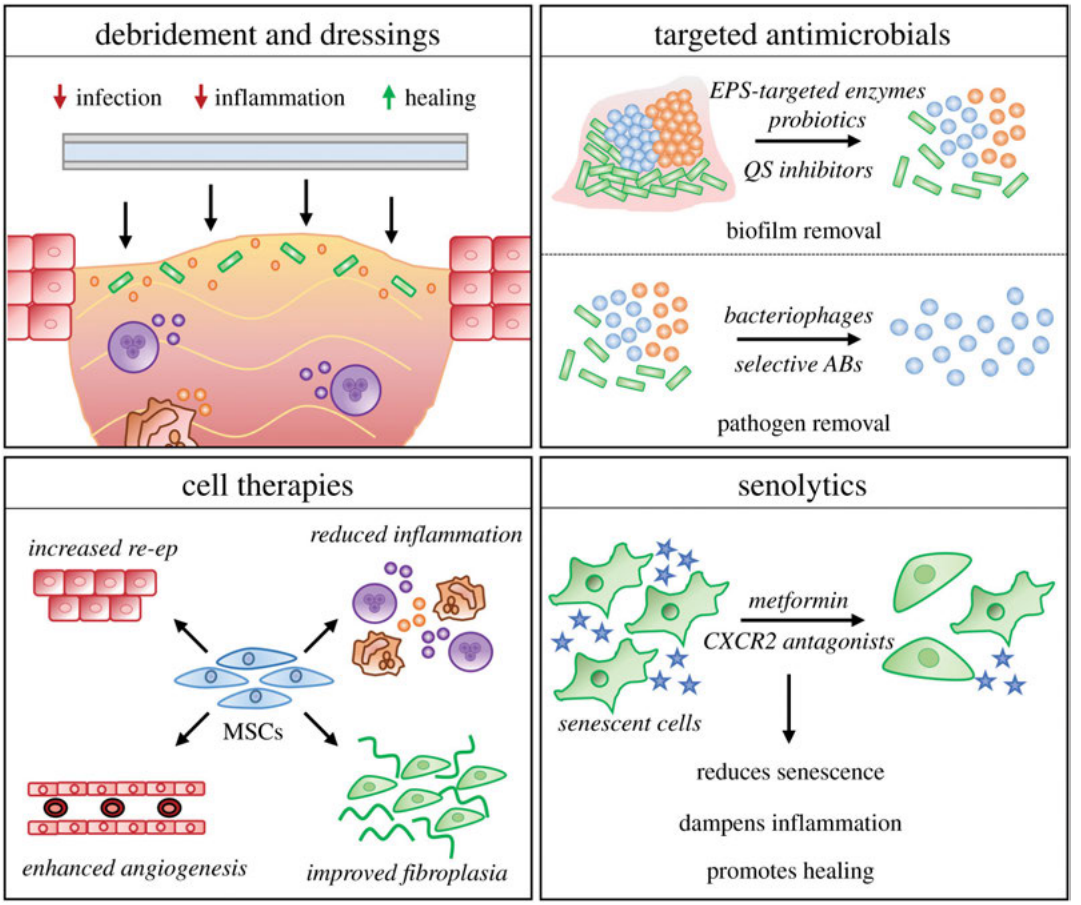

Figure 13.3 Traditional and novel chronic wound treatments and their major tissue effects. Debridement of infected and necrotic tissue, followed by tailored dressing use, is common in wound treatment, with the aim of reducing microbial burden, dampening inflammation and providing a more suitable environment for healing. Antimicrobial therapies are emerging to disrupt biofilms and selectively remove pathogenic, rather than commensal, organisms. EPS = extracellular polymeric substance. QS = quorum sensing. ABs = antibiotics. Cell therapies such as mesenchymal stem cells (MSCs) can benefit multiple aspects of wound repair. re-ep = re-epithelialization. Finally, targeting chronic wound senescence with senolytics (e.g. metformin or CXCR2 antagonists) may be a viable option to reduce inflammation and promote healing.

Experimental studies are providing new insight into the underlying molecular and cellular correlates to chronic wound pathology. This in turn offers exciting new avenues for future therapeutic prevention and intervention. For

example, chronic wounds are burdened by high levels of cellular senescence [141, 142]. Senolytic drugs such as quercetin target senescent cells, and have already shown promise in reducing senescent cell burden in pathology [213, 214] and ameliorating symptoms of diabetes, including inflammation and hyperglycaemia (reviewed in [215]). Further, blockade of the senescence-linked receptor, CXCR2, directly accelerates diabetic wound repair *in vivo* [112]. Repurposing these existing treatments (a number of senolytics drugs and CXCR2 antagonists have been tested in clinical trials [216, 217]) offers an attractive approach for wound management. Other cell-targeted strategies include the administration of stem cells (reviewed in [218]), growth factors (reviewed in [219]) and gene therapies (reviewed in [220]). The major reparative effects of emerging and potential chronic wound therapies are outlined in Fig. 13.3.

13.6 Conclusions

The high cellular diversity, complexity and plasticity of wound healing provide a considerable challenge to comprehensively elucidate. While this remains a perplexing goal, it is essential that we continue to strive to more fully understand the mechanisms that underpin both normal and pathological healing. While not without their limitations, emerging wound models provide an unprecedented opportunity to further explore the molecular and cellular features of wound repair. Combining these approaches with novel tissue, cell and molecular 'omics' technologies will considerably advance our understanding of wound pathology. Indeed, the future holds great promise for the development of innovative new therapeutic strategies for advanced wound care.

Disclosures and Conflict of Interest

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Chapter 14

Advances in Cervical Cancer Prevention: Efficacy, Effectiveness, Elimination?

Karin Sundström, MD, PhD,[a] and K. Miriam Elfström, PhD[a,b]

[a]*Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Stockholm, Sweden*
[b]*Regional Cancer Center Stockholm-Gotland, Stockholm, Sweden*

karin.sundstrom@ki.se

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14.1 Evolution of Human Papilloma Virus Vaccination

Currently, there are 3 commercially available Human Papilloma Virus (HPV) vaccines: the bivalent (targeting high-risk types HPV16 and 18), quadrivalent (targeting HPV16, 18, and low-risk types 6 and 11) and nonavalent (targeting HPV6/11/16/18 and another 5 high-risk types), which have all shown excellent efficacy against cervical cancer precursor lesions and, in the case of the latter 2, against external genital warts. In the future, we are likely to observe efficacy against large portions of the burden of HPV16-dominated anal, oropharyngeal, and penile cancers as well. Early in 2019, the US Centers for Disease Control and Prevention (CDC) increased the recommended upper age limit through 26 years for women and men to receive prophylactic HPV vaccination [1]. This followed an announcement made by the UK's National Health Service (NHS) that free-of-charge HPV vaccination for boys aged 12–13 years would be provided from September 2019 [2]. These 2 landmark decisions can be seen as the natural continuation of a process in which the HPV vaccine has gone from being viewed

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as a "cervical cancer" vaccine, only for adolescent girls who have not made their sexual debut, to a vaccine that prevents infection with a virus transmitted throughout the life span that can also cause carcinomas in men [3].

14.2 Prospects for Cervical Cancer Elimination

A growing body of population-based studies have been key in shaping the globally accepted view of HPV vaccine outcomes, in which evidence of efficacy from randomized clinical trials (reviewed in [4]) is gradually complemented and refined by findings of sustained effectiveness in clinical practice (reviewed in [5]). In a similar development, early cost-effectiveness studies naturally focused initially on the impact on cervical outcomes of reaching high vaccination coverage among preadolescent girls [6]. However, it has since been proposed to extend vaccination to women up to age 30 or even 45–50 years [7], and subsequent modeling work has suggested that similar or greater benefits than those attained by vaccination of preadolescent girls may be achieved if vaccination is extended to older women and boys, if uptake is high in both sexes [8].

Such extended approaches would target the spread of the virus faster while at the same time providing direct cancer prevention in both women and men, and ambitions for disease prevention have grown accordingly. The growing evidence has suggested prospects for so-called disease elimination: when the rate of cervical cancer is reduced to a minimum and is no longer considered prevalent in the population—currently defined as a rate below 4 cases/100,000 woman-years [9]. In 2018, the WHO issued a global call for cervical cancer elimination [10], in which disease containment/control is no longer the main goal but rather the decisive removal from circulation of one of the world's major known carcinogens, as already projected in Australia, where a 20-year plan has been put in place and disease elimination is deemed possible [11].

14.3 Opportunities and Challenges Posed by Resource Levels

The foundation for cervical cancer elimination is a 2-pronged approach in which vaccination in adolescents is complemented by more widespread cervical screening in women, defined in the motto "90:70:90"—i.e., 90% vaccinated, 70% screened, and 90% of those with cervical disease being offered effective treatment. Today, however, few regions worldwide reach near the ideal level of vaccinated girls. Despite carrying 80% of the global cervical cancer disease burden, typically because of an absence of effective screening at the population level, low- and middle-income countries (LMIC) in 2014 only accounted for 1% of the vaccinated girls worldwide [12]. For example, China only recently approved HPV vaccines for use, and coverage is low to nonexistent, especially in lower-resource regions.

Indeed, the need for a more rapid rollout of vaccination in LMIC has been called for as the only way to reduce the risk for worsening global inequalities in prevention [12]. For this to be possible, vaccine delivery needs to be acceptable, available, and affordable. Yet, a systematic review recently found that there is a distinct lack of high-quality studies on HPV vaccine acceptability from these regions [13], and for 90% global coverage to be reached, such gaps in evidence need to be addressed.

Furthermore, although the logistical issues of cold chain storage and vaccine cost have long been anticipated, the most recent challenge that has arisen is a global limitation in the number of HPV vaccine doses available [14]. Timely delivery of vaccines, following international demand on the scale required for global viral elimination, could pose a challenge for manufacturers to meet. It follows that a key focus of future research should be estimating the effectiveness of 1 vaccine dose for children, because if fewer doses than the currently recommended 2 are enough, potential shortage issues would be alleviated. Naturally, a 1-dose schedule would itself lead to large public health gains: a 1-stop vaccination service, perhaps coordinated with cervical screening efforts, would suffice.

By the same logic, it should be equally valuable to show 2-dose vaccine schedule effectiveness in individuals greater than 13 years at age of vaccination, which—if firmly established—could allow a reduction from 3 doses to 2 and ensure that the available doses went even further.

Even in high-resource settings, elimination may prove to be a challenge, as exemplified by the situation in France, where HPV vaccine delivery has been challenging and uptake has remained low, at below 20% [15]. Experience from Sweden shows that despite complete population registers and well-organized delivery, it is still challenging to achieve 90% coverage in population-based prevention programs [16]. Furthermore, the discrepancy in uptake of one preventive strategy may be exacerbated by lack of engagement in another: some girls are not vaccinated despite it being free of charge, and some women abstain from screening despite repeated, renewed invitations. Especially if combined, such choices lead to a particularly high risk for cervical cancer regardless of the overall resource level of the setting. If we do not reach these women, a substantial burden of cervical cancer will remain. Reaching out is now facilitated by HPV-based self-sampling kits, which have been shown to increase participation among previous screening nonattenders [17]. Self-sampling could be more broadly used to reach those for whom the standard model of clinician-based sampling is not acceptable or feasible.

14.4 Cervical Screening in the Presence of HPV Vaccination

Another programmatic challenge will be how to integrate vaccine services with cervical screening delivery, possibly in the HPV-FASTER concept, which suggests

also vaccinating adult women against HPV (because this could eliminate HPV from the population faster while acknowledging that older, sexually active women's benefit from the vaccine will be lower than that for adolescent girls) [7]. A further key issue will be how to adapt screening algorithms for increasingly vaccinated cohorts.

This is critical to achieve the greatest impact of prevention resources and reduce the burden on individuals of repeated vaccinations and screening rounds. Currently, the best evidence indicates that because the underlying probability of cervical lesions will diminish in vaccinated women, the positive predictive value of screening in such women will diminish as well [18, 19]. It may thus be pertinent to consider HPV-based screening strategies specific to both age group and level of birth cohort HPV vaccine coverage, but this may not be trivial to resolve even for highly organized programs. Greater integration and the implementation of HPV-based screening, which has proven to be more effective in preventing cervical cancer than cytology [20], would position us well for achieving stronger preventive effects among groups of women not primarily targeted for vaccination.

14.5 Future Perspectives

Although efficacious strategies have been developed, several public health challenges remain in order to achieve effective global control of cancers driven by HPV. High-income countries should be able to achieve elimination more easily because of the presence of organized screening programs. Strengthening screening in LMIC will be paramount, e.g., through implementing primary HPV-based methods such as self-sampling and rapid HPV testing [21]. When it comes to vaccination uptake, however, we note that there are substantial differences between countries, not necessarily related to resource level. Indeed, several LMIC, including Rwanda and Bhutan, show excellent vaccine uptake of >90%, demonstrating that concerted efforts to match vaccination strategy with country-specific conditions can result in significant success independent of setting [22, 23]. However, for elimination to occur, greater investments and constant vigilance will be required. Gavi, The Vaccine Alliance was successful in reaching its initial goal of 1 million vaccinated girls by 2015 in eligible countries, but the organization has recently stated that the target of 30 million vaccinated girls by 2030 is at risk [24].

The recent announcements from the US CDC, UK NHS, and WHO constitute important but incremental steps on the way to reduce cervical cancer. We must also keep in mind the time frame required to show sizeable reductions in disease, reductions that will take more or less time depending, firstly, on the success of the screening component of the elimination strategy—which, if successful, could detect and treat a large proportion of cervical cancer precursors before they become invasive, thereby leading to an early reduction in the incidence of invasive cervical cancers—and secondly, on the time lag required between mass vaccination being performed and a preventive effect to be observed. Although

much work remains, if complementary and equally ambitious measures in prevention program accessibility and adaptability are implemented and sustained, we believe that the elimination of cervical cancer could move from prospect to reality.

Disclosures and Conflict of Interest

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Chapter 15

Molecular Classification of Breast Cancer: A Retrospective Cohort Study

Fatma Khinaifis Al-thoubaity, MD

Department of Surgery, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

fatmalthoubaity@yahoo.com, fthoubaity@kau.edu.sa

Keywords: Allred (Quick) scoring system, axillary lymph node metastasis, breast carcinoma, carcinoma *in situ*, estrogen receptor (ER), fluorescent *in situ* hybridization (FISH), HER2-positive tumors, histological grade, histopathological subtype, human epidermal growth factor receptor 2 (HER2/neu), immunohistochemical profile, immunohistochemistry (IHC), Luminal A, Luminal B, molecular subtypes, patient-specific prognosis, progesterone receptor (PR), Scarff-Bloom-Richardson (SBR) grade, Surveillance, Epidemiology, and End Results (SEER), triple negative

15.1 Introduction

Breast cancer is a heterogeneous disorder representative of numerous subcategories of several cellular compositions, molecular alterations as well as clinical behavior. A number of factors such as histological grade, type and size of tumor, lymph node metastasis, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu), influence the prognosis and response to the treatment of cancer. One of the most commonly observed malignancies around the world is breast cancer (BC) [1]. In the United States (US), an estimate of about a quarter of million new cases of BC were recorded in the year 2014, which in turn accounted for about 14% of all the new cancer cases [2]. Around 50,285 new cases of BC were diagnosed in the United Kingdom, accounting for nearly 15% of all new cancer cases [3]. Incidence of BC is much lower in the Kingdom of Saudi Arabia (KSA) than the Western world. However, in the Saudi women, breast cancer is the most common malignancy and accounts for about a

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quarter of the newly diagnosed cancer in females [4]. Approximately 27.4% of the newly diagnosed female cancers (5,378) in Saudi were breast cancer patients (ranked first) as reported by the National Cancer Registry, KSA in the year 2010. It is worthy to note that the incidences of breast cancer is lower in KSA (age standardized rate per 100,000 is 29.6) as compared to the worldwide average (age standardized rate per 100,000 is 43.1), but it nonetheless represents a significant fraction of the cancer related fatality in women [1]. In US, median diagnosis age for breast cancer is 61 [5], whereas, in Arab countries breast cancer is diagnosed at a younger age. In KSA, mean age for the diagnosis of breast cancer is 49 years [4, 6], and this cancer is generally found to be aggressive and locally advanced [7]. Despite the fact that breast cancer mortality has moderately reduced due to currently available treatments, it is estimated that more than 450,000 deaths occur annually due to breast cancer worldwide [4, 6]. Molecular subtypes of breast cancer based on histological grade and lymph node metastases, are strong prognostic and predictive factors. Consequently, classifying breast cancer into relevant molecular subtypes is an important aspect of therapeutic decision-making. Classical immunohistochemistry (IHC) markers such as ER, PR and HER2 play a crucial role in molecular subtyping [8]. Newer methods like gene expression profiling using complementary DNA microarrays have been developed, which are therapeutically important for molecular classification. Immunohistochemical analyses of tumors on the basis of status of ER, PR, and HER2 are used in clinical practice, and this method is easier and cost-effective and provides similar results for molecular subtypes [9, 10]. Immunohistochemistry based molecular subtyping of tumors is now considered as the main stay to predict susceptibility of tumor to hormonal therapy and subsequent trastuzumab therapy [9, 11]. Newer classification methods are also being developed that are based on immunohistochemical, genetic and molecular findings [11, 12]. Availability of hormone (estrogen and progesterone) receptor markers marked the beginning of molecular classification about 30 years ago. HER2/neu based determination techniques then followed the earlier developments. Further, a new study mandated the molecular classification of human BC by initially dividing BC into four major classes: luminal-like, basal-like, normal-like, and HER-2 positive [13]. Subsequently, luminal class was divided into luminal A and luminal B classes, thereby resulting in addition of a fifth class of BC [14]. According to the St. Gallen Consensus 2011, molecular subtypes of breast cancer can be classified into Luminal A (ER+/PR+/HER2-/lowKi-67); Luminal B (ER+/PR+/HER2-/+/high Ki-67); HER2-overexpression (ER-/PR-/HER2+) and triple negative breast cancers/TNBCs (ER-/PR-/HER2-) [9]. Basal-like subtype of breast cancer referred to as TNBC was found to be positive for basal marker (CK5/6) expression [15, 16]. Thus, it is important that use of techniques enabling molecular subtyping in clinical practice would provide more accurate information about patient-specific prognosis, risk of relapse and probability for pathological complete response. One of the major advantages would be the ease of identification of patients for whom the benefits of neoadjuvant therapy outweigh the risks, i.e. it will result

in improved risk stratification. Furthermore, aggressive treatment strategy or increased surveillance can be designed for the patients who face an increased risk of relapse. In the present study, we aimed to analyze the prevalence of breast cancer subtypes in the western region of KSA and associated clinico-pathological features, which will eventually increase our understanding of breast cancer and lead to effective healthcare management.

15.2 Materials and Methods

15.2.1 Data Collection

This is a retrospective analysis based on data retrieved from the Pathology Department at the King Abdul Aziz University Hospital across a seven-year study period. All the histopathology reports of patients diagnosed with primary invasive breast cancer from January 2012 to December 2018 were utilized. This analysis was conducted with prior approval from the Institutional Review Board at the King Abdul Aziz University Hospital. The study has been reported in line with the STROCSS criteria [17]. Histological grade was assessed according to the Nottingham modification of the Bloom-Richardson system. The criteria for inclusion of patients into this analysis were as follows: (a) patients with invasive breast carcinoma, (b) patients with available histological grade and lymph node status, and (c) available formalin-fixed, paraffin embedded samples with good quality. Male patients and recurrence cases were excluded from the analysis leading to a total of 740 finally approved cases. We obtained the following parameters for each patient: age at the time of diagnosis, tumor size, histopathological subtype, Scarff-Bloom-Richardson (SBR) grade, presence or absence of carcinoma in-situ component, lymph node status, immunohistochemical profile of the hormonal receptors ER and PR, and immunohistochemical profile of HER2 in the invasive malignant cells. The tumor size measurement was retrieved from ultrasound reports of the breast prior to the biopsy or using reports from other radiological modalities. After size assessment, tumors were grouped into three categories: ≤2 cm, >2 but ≤5 cm, and >5 cm. Tumor grade evaluation was carried out based on the established Elston-Ellis modification of the SBR system, which relies on histochemical features such as the percentage of tubular differentiation, the presence of nuclear atypia/pleomorphism and the number of mitoses [18]. The status of the lymph node metastasis was determined either using radiological modalities or from the evaluation of axillary lymph nodes obtained at mastectomy. Thereafter, the number of lymph nodes was determined along with the number of lymph nodes positive for metastasis. The ER, PR and HER2 tests were scored according to the Guidelines of the College of American Pathologists [18]. Positive ER or PR is considered when ≥1% of invasive malignant cells that exhibit nuclear staining or immunoreactivity. Additionally, for ER and PR, another semi-quantitative scoring system called the Allred (Quick) scoring system was employed to ascertain the proportion of stained cells and assess the intensity of the nuclear staining [18].

The HER2 test was scored from 0 to 3+ in which: score 0 or 1 is negative; 2+ is equivocal; and 3+ is positive. A 3+ score is given when an intense full circumferential cytoplasmic membrane staining is observed in more than 10% of invasive malignant cells. Specimens showing equivocal HER2 staining were sent for further examination with the help of fluorescent *in situ* hybridization (FISH) and their results were documented. We classified breast cancer (Fig. 15.1) into four molecular subtypes according to ER, PR, and HER2/neu status: (1) luminal A (ER and/or PR positive and HER2/neu negative), (2) luminal B (ER and/or PR positive and HER2/neu positive), (3) HER2-positive (ER and PR negative and HER2/neu positive), and (4) triple negative (ER, PR, and HER2/neu negative) [9, 10].

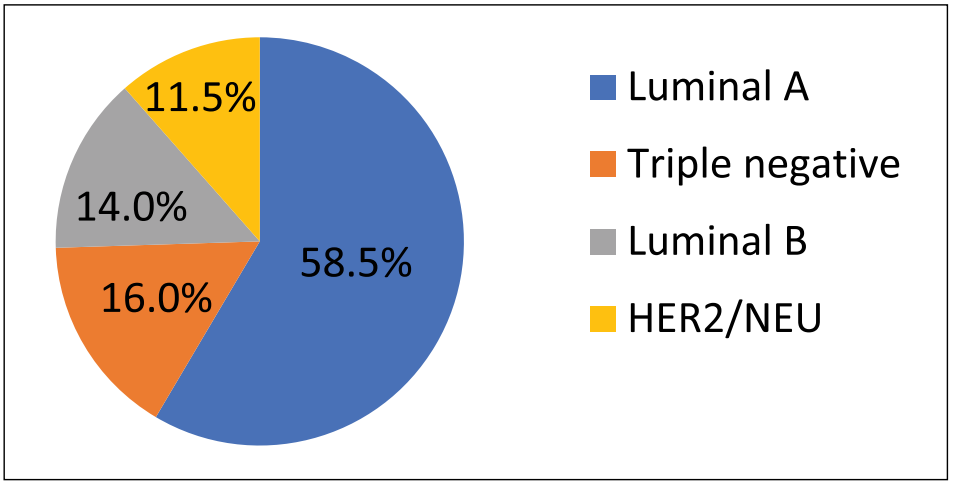

Figure 15.1 Molecular subtype of breast carcinoma in KAUH (2012–2018).

15.2.2 Statistical Analysis

The Statistical Package for Social Sciences software version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Descriptive statistics, frequency, and percentages of categorical variables have been reported. We examined the association between the molecular subtypes and age at diagnosis, tumor size, histopathological subtype, grade, presence of foci of *in situ* carcinoma, and nodal status using Chi-squared test for categorical variables. We computed the odds ratio (OR) where appropriate and constructed the 95% confidence interval (CI). The results were considered statistically significant if the *p*-value was <0.05.

15.2.3 Ethical Considerations

There were minimal ethical implications and issues since it is a retrospective study. Patient identity and confidentiality were protected by assigning each patient a specific serial number. Moreover, no one except the investigating research team accessed the patients' records. We obtained prior approval from

the Institutional Review Board since a consent form was not applicable to our study. The study is registered with Research Registry 5151.

15.3 Results

In the present study, a total of 740 breast cancer cases were examined, and the average age of the patient at time of diagnosis was 49 years (standard deviation of 12.28). Most cases (n = 629, 85%) were ductal, a few of them (n = 84, 11.4%) were lobular, while the rest of the cases were of other histological types that included medullary, tubular, mucinous, metaplastic, adenoid cystic, and encysted papillary carcinoma. Most of the cases of cancer that were detected were mildly differentiated (n = 274, 37.1%) followed by moderately differentiated (n = 248, 33.5%). The average size of the tumor at the time of diagnosis was found to be 3.2 cm (standard deviation of 1.92). Most patients presented a tumor size between 2 and 5 cm (n = 362, 48.9%), while some of the patients (n = 315, 42.6%) exhibited a tumor size <2 cm (Table 15.1). At diagnosis, most breast cancer tumors exceeded 2 cm in maximum dimension. The patients whose age was less than 50 years had higher probability of displaying greater tumor size (p = 0.036, OR = 1.613, 95% CI, 1.030–2.526) than the patients who were in their sixties or older. More than half of the cases (n = 493, 66.6%) that were investigated showed lymph node metastases (Table 15.1). Positive ER immunostaining was found in 70.8% of the cases and the PR in 63.8%. HER2 immunostaining was found positive in 18.7% of the cases and equivocal in 22.8%. FISH testing was performed for the equivocal cases, and it was established that 34.2% of the equivocal cases were HER2 positive. Luminal A was the most prevalent subtype (n = 434, 58.5%) followed by, triple negative (n = 117, 16%), luminal B (n = 104, 14%), and HER2-positive (n = 85, 11.5%) (Fig. 15.1). Tables 15.1 and 15.2 showed the distribution of various clinical and pathological characteristics among different molecular subtypes. Higher frequency (66–70.5%) of HER2-positive and triple negative tumors was observed as compared to luminal tumors, in the patients whose age was less than 50 years. But, these results were statistically insignificant (p = 0.124). HER2-positive (n1) samples showed tumor mass size greater than 2 cm in 82.5% of the cases. On the other hand, triple negative tumors (n2) had a tumor mass size greater than 2 cm in 75% patients (p = 0.018), where the majority of the tumor sizes ranged between 2 and 5 cm (n1 = 26, 65% and n2 = 31, 59.6%), while the rest showed tumor size of more than 5 cm (n1 = 7, 17.5% and n2 = 8, 15.4%) (p = 0.057). Additionally, these subtypes showed aggressive microscopic features and approximately two-thirds of these subtypes demonstrated poorly differentiated carcinomas. Furthermore, HER2-positive tumors were observed to be displayed least frequently as an *in situ* component (41.2%, p = 0.026). On the other hand, lobular carcinomas were found almost exclusively in the luminal A and triple negative tumor subtype (77%, p = 0.002). Around 69.5% had modified radical mastectomy and 30.5% had breast-conserving therapy. Survival rate was found to be 90%. Patients with recurrence of breast cancer were excluded from study.

Table 15.1 The distribution of clinico-pathological characteristics, according to the hormonal and molecular subtypes in 740 women with invasive breast cancer

Characteristics'	Luminal A	Luminal B	HER-2 positive	Triple negative	Total
Total	434 (58.5%)	104 (14%)	85 (11.5%)	117 (16%)	740(100%)
Age (years)					
≤50	236 (54.3%)	61 (58.7%)	50 (58.8%)	90 (76.9%)	437(59%)
>50	198 (45.7%)	43 (41.3%)	35 (41.2%)	27(23.1%)	303(41%)
Tumor size (cm)					
≤2	221(50.9%)	32(30.8%)	30(35.3%)	32 (27.4%)	315(42.6%)
>2 – ≤ 5	200 (46.1%)	52 (50%)	40(47.1%)	70 (59.8%)	362(48.9%)
>5	13 (3%)	20(19.2%)	15 (17.6%)	15(12.8%)	63(8.5%)
Lymph nodes metastasis					
Negative	192(44.2%)	20(19.2%)	15(17.7%)	20(17.1%)	247(33.4%)
Positive					
1–3	52(12%)	50(48.1%)	50(58.8%)	50(42.7%)	202(27.3%)
>4	190(43.8%)	34(2.7%)	20(23.5%)	47(40.2%)	291(39.3%)

Table 15.2 The distribution of histopathological characteristics, based on hormonal and molecular subtypes in 740 women with invasive breast cancer

Characteristics'	Luminal A	Luminal B	HER-2 positive	Triple negative	Total
Total	434 (58.5%)	104(14%)	85 (11.5%)	117 (16%)	740(100)
Histology					
Ductal	343 (78.6%)	88 (84.6%)	84 (98.8%)	114 (97.4%)	629 (85%)
Lobular	73 (16.7%)	10 (9.6%)	1 (1.2%)	0	84 (11.4%)
Others	18 (4.7%)	6 (5.8%)	0	3 (2.6%)	27 (3.6%)
Tumor grade					
Grade 1	270 (62.2%)	4 (3.8%)	0	0	274 (37.1%)
Grade II	116 (26.7%)	60 (57.7%)	35 (41.2%)	37 (31.6%)	248 (33.5%)
Grade III	48 (11.1%)	40 (38.5%)	50 (58.8%)	80 (68.4%)	218 (29.4%)
Carcinoma in situ					
Percent	334 (77%)	54 (51.9%)	35 (41.2%	90 (76.9%)	227 (30.7%)
Absent	100 (23%)	50 (48.1%)	50 (58.8%)	27 (23.1%)	513 (69.3%)

15.4 Discussion

In this study, we investigated the distribution of various molecular subtypes of breast cancer from patients at King Abdul Aziz University Hospital, and also evaluated the differences in clinico-pathological features between these subtypes. Our study found that the average age of the patients was 49 years, which was in accordance with national average as reported by the Saudi Arabian Cancer Incidence Report [4, 19]. Most of our cases (54.3%) were detected in women who were younger than 50 years of age, which is again similar to a recently reported study from Oman [20]. These results were in contrast to the observations in US where 65.1% of the reported cases were found in women older than 55 years of age, as evident from the Surveillance, Epidemiology, and End Results (SEER) Cancer Statistics Review [5]. This difference in age group and earlier onset may be a consequence of a lack of adequate healthcare systems in the Middle East compared to the US. It is also important to note that in the present study 42.6% of the patients had a tumor size <2 cm, while in countries like the US and Poland it is 58.4% and 51.9%, respectively [2, 21]. This implies that there is aggressive presentation and delayed diagnosis in the Saudi population, which may be due to lack of awareness in the community about breast cancer as well as absence of a comprehensive screening program. The findings in our study relating to the distribution of molecular subtypes was found to be in concurrence with the results obtained from studies originating from various Asian and Western countries (Table 15.3). In most of the studies pertaining to the distribution of breast cancer, luminal A was found to be the most prevalent subtype (Table 15.3). Even so, any minor geographical variations of tumor subtypes proportions could be related to environmental factors, genetic factors and/or technological disparity. In contrast to our study, about half the cases (52.8%) in the study by Tamimi et al. [22] were found to be triple negative, while luminal tumors represented 28.5%. Furthermore, in our study we observed that occurrence of lobular carcinomas was majorly found in the luminal A and triple negative group (77%, $p = 0.002$), which in a way matched the findings of Tamimi et al. [22] and Yang et al. [21]. However, according to the data presented in the Egyptian [15] and Norwegian studies [23], only 55% of lobular carcinomas were luminal A. Moreover, poorly differentiated carcinomas, the HER2-positive and triple negative tumors were observed in greater frequency [14, 24]. In comparison with the luminal A subtype, these subtypes are found to be associated with an increased frequency of a larger tumor size [25, 26], and with a young age group [25, 27]. In the current study, we identified a strong association between the different molecular subtypes and lymph node status, with 82.3% positive lymph node involvement in HER2-positive cases. Although, there were multiple studies that failed to detect such an association [25, 28], there were other studies that identified a high degree of association between lymph node metastasis with HER2-positive tumors and lower frequency with basal-like tumors [18, 29]. This contradiction may be due to the fact that there are studies that indicate the tumor subtype may be intrinsic

Table 15.3 The distribution of molecular subtypes of breast carcinomas by immunohisto-chemistry in various regional and Western countries

Variables	Mehdi et al.	Yang et al.	Cheng et al.	Vallejos et al.	Fourati et al.	Carey et al. African American	Carey et al. Non-African American	Riyadh	Jeddah
Setting	Oman	Poland	China	Peru	Tunisia	California, USA		Saudi Arabia	
Number of patients	452	804	628	1198	966	196	300	357	740
Years	2006–2010	2000–2003	2007–2010	2000–2002	2007–2009	1993–1996	1993–1996	2010–2014	2012–2018
Luminal A	34.7%	69.0%	46.5%	49.3%	50.7%	47.4%	54.0%	58.5%	58.5%
Luminal B	15.9%	6.0%	17.0%	13.%	13.4%	12.7%	17.3%	14.5%	14%
HER2/NEU	24.1%	8.0%	15.0%	16.2%	13.4%	8.2%	5.6%	12.3%	11.5%
Triple negative	25.3%	18.0%	21.5%	21.3%	22.5%	31.6%	23.0%	14.8%	16%

and therefore only loosely associated with lymph node status. In contrast to 434 patients with luminal A tumors (77%) and 117 patients with triple negative tumors (76.9%), only 41.2% of HER2-positive tumors (p = 0.026) displayed an in-situ component. In another study, 45 cases of the luminal tumors (n = 124) showed an in-situ component [30]. The role of mammography in the detection of the various molecular subtypes has also been suggested in a recent study [31]. 69.5% had modified radical mastectomy and 30.5% had breast-conserving therapy; a higher percentage of patients had mastectomy because of advanced cancer. In our study, the survival rate was 90%. Despite providing many interesting observations, our study has certain limitations. One of the limitation are due to the unavailability of Ki67 [32], a cellular marker that differentiates between non-HER2 expressing luminal B from luminal A tumors [9]. Similarly, limitations are in the detection of Basal-like tumors, a subset of triple negative tumors, due to the absence of cytokeratin 5/6 [29]. Moreover, there is a discrepancy rate of 39% in the molecular classification of tumors by immunohistochemistry and gene expression [33].

15.5 Conclusion

Our study has revealed that the most common tumor subtype are the luminal A tumors, followed by triple negative tumors. Luminal A and triple negative tumors were found to be closely linked with increased frequency of lobular carcinomas. The HER2-positive and triple negative tumors were associated with an increased frequency of large tumor size and poorly differentiated carcinomas as well as more aggressive manifestation of cancer. Additionally, HER2-positive tumors were less frequently observed in carcinoma, *in situ*. We also observed a strong correlation between lymph node status and molecular subtypes. This phenomenon needs to be examined, urgently addressed, and early screening mammography should be established in KSA. We also recommend in-depth investigation into the risks factors associated with different molecular subtypes of breast carcinoma in KSA. Further, it is also important to investigate the effect of different breast cancer subtypes on the prognosis and survival of the patient.

Abbreviations

BC: breast cancer
CI: confidence interval
ER: estrogen receptor
FISH: fluorescent *in situ* hybridization
IHC: immunohistochemistry
OR: odds ratio
PR: progesterone receptor
SBR: Scarff-Bloom-Richardson
SEER: Surveillance, Epidemiology, and End Results

Disclosures and Conflict of Interest

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Author contribution: It is a single-author chapter and the author performed all the tasks.

Trial registry number:

1. Name of the registry: Research Registry.
2. Unique Identifying number or registration ID: researchregistry5151.
3. Hyperlink to the registration (must be publicly accessible): https://www.researchregistry.com/browse-the-registry#home/

Consent: No consent obtained as consent form was not applicable to our study.

This is retrospective study, collecting data from file of patients.

Declaration of competing interest: Author has no conflict of interest. This is retrospective study data collected from files of patient from King Fahad research Centre in King Abdul-Aziz University Hospital, Jeddah, Saudi Arabia.

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Chapter 16

Cutaneous Squamous Cell Carcinoma: From Biology to Therapy

Roberto Corchado-Cobos, MSc,[a,b] Natalia García-Sancha, MSc,[a,b] Rogelio González-Sarmiento, MD, PhD,[b,c] Jesús Pérez-Losada, MD, PhD,[a,b] and Javier Cañueto, MD, PhD[a,b,d]

[a]*Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, Salamanca, Spain*
[b]*Instituto de Investigación Biomédica de Salamanca, Complejo Asistencial Universitario de Salamanca, Hospital Virgen de la Vega, Salamanca, Spain*
[c]*Molecular Medicine Unit, Department of Medicine, University of Salamanca, Salamanca, Spain*
[d]*Department of Dermatology, Complejo Asistencial Universitario de Salamanca, Salamanca, Spain*

jcanueto@usal.es

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16.1 Introduction

Cutaneous squamous cell carcinoma (CSCC) is the second most frequent cancer in humans, with an estimated incidence of 1 million cases each year in the United States. This figure continues to rise, and is an underestimate [1, 2]. The number of CSCCs has increased from 50% to 300% in the last three decades [3], and by 2030 its incidence in European countries will be twice the current level [4]. It is estimated that the risk of developing a CSCC at some point in life is 7% to 11% in

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the Caucasian population [5] (from 9% to 14% in men and from 4% to 9% in women) [6].

While it usually exhibits benign clinical behavior, it can be locally invasive and metastatic. Ten-year survival after surgery exceeds 90% for CSCC, but drops dramatically when metastases occur [7]. The frequency of lymph node metastases is around 4%, and mortality rates are nearly 2%. Given its high frequency, CSCC has a significant impact on overall mortality [8]. It is the second most common cause of death from skin cancer after melanoma and is responsible for the majority of deaths from skin cancer in people older than 85 years [3]. In some areas of the United States, it has a mortality comparable to that of renal, oropharyngeal, and melanoma carcinomas [3].

CSCC arises from the malignant proliferation of epidermal keratinocytes. There are environmental and constitutional risk factors for its development. With respect to the former, older age, male sex, fair skin, immunosuppression, and a previous history of actinic keratosis (AK) are of known importance. Chronic sun exposure is the most important and well-known environmental factor associated with CSCC [9–14]. Solid-organ transplant recipients, who have a human papillomavirus infection or chronic lymphocytic leukemia, have a higher risk of developing CSCC than the general population [15–18]. AK is considered a premalignant lesion that may progress to an invasive CSCC, and is the most significant predictive factor of CSCC [19].

Several molecular pathways have been implicated in CSCC development. Ultraviolet-induced *P53* mutations are early events in CSCC, and are responsible for great genomic instability [10, 20]. CSCC has the greatest mutational burden of all solid tumors, which, as we will see later, has therapeutic implications [21]. Other genetic changes occur subsequently in other suppressor genes, such as *CDKN2A* and *NOTCH* [22, 23], and in oncogenes, such as *RAS* [24]. The accumulation of mutations ultimately involves various signaling pathways [25], including the activation of the NF-kB, MAPK, and PI3K/AKT/mTOR pathways [26, 27], which mediate epidermal growth factor receptor (EGFR) overexpression. Epigenetic changes may also occur [28].

Surgery is the cornerstone of the management of CSCC, and radiotherapy is sometimes also implemented. However, a subset of patients with locally advanced and metastatic CSCC may benefit from systemic treatments [29]. The signaling pathways involved in CSCC development have given rise to targetable molecules in recent decades. Moreover, the high mutational burden and increased risk of CSCC in patients under immunosuppression were part of the rationale for developing the immunotherapy for CSCC that has changed the therapeutic landscape in recent years [30]. This review focuses on the molecular basis of CSCC and the current biology-based approaches of targeted therapies and immune checkpoint inhibitors. Another purpose of this review is to explore the landscape of drugs that may induce CSCC. Beginning with the pathogenetic basis of these drug-induced CSCCs, we move on to consider potential therapeutic opportunities for overcoming this adverse effect.

16.2 Molecular Basis of CSCC

Cutaneous squamous cell cancer is one of the most highly mutated human cancers [21, 31]. A deeper knowledge of the molecular basis of CSCC would be useful for developing better ways of treating this disease.

The mutation of the tumor suppressor gene *TP53* has an important role early in the pathogenesis of CSCC and occurs in 54%–95% of cases [10, 20, 32]. Mutations of *TP53* are induced by ultraviolet radiation (UVR), the most important environmental risk factor for CSCC, and are reported in pre-malignant AK lesions and CSCC [33, 34]. UVR-induced mutagenesis results in characteristic C-T and CC-TT dipyrimidine transitions, which enable tumor cells to prevent apoptosis and to promote clonal expansion of p53 mutant keratinocytes [35]. The role of *p53* in ultraviolet B-induced carcinogenesis has been confirmed in $p53^{-/-}$ mice, which have an increased propensity for developing AK lesions and CSCCs secondary to ultraviolet B (UVB) exposure [36, 37]. Furthermore, several groups have confirmed the presence of *p53* mutations in CSCC cell lines [38, 39]. *P53* mutations are an early event in CSCC development and are ultimately responsible for great genomic instability.

Other mutations subsequently occur in tumor suppressors, such as *CDKN2A* and *NOTCH*, and in oncogenes, such as *RAS*. [22]. The *CDKN2A* gene encodes two alternatively spliced proteins, p16INK4a and p14ARF. The inactivation of the *CDKN2A* locus may be due to loss of heterozygosity, point mutations, and promoter hypermethylation [23]. Loss of function of either p16INK4a or p14ARF may lead to unrestrained cell cycling and uncontrolled cell growth mediating pRB [40] and p53 [41]. On the other hand, loss of function *NOTCH1* and *NOTCH2* mutations are identified in more than 75% of CSCCs [42]. *In vivo* mouse studies show that *Notch1* deletion, a mutation that occurs early in CSCC, results in the development of skin tumors and facilitation of chemically induced skin carcinogenesis [43, 44]. The *Notch1* gene is a direct target of *p53* [45], and keratinocyte-specific ablation of *Notch1* disrupts the balance between growth and differentiation [46]. The upregulation of the Wnt/beta-catenin pathway, which may result from Notch1 loss of function, facilitates skin tumor development and promotion [43], and is at least partly dependent on p21WAP/Cip1 [47]. *In vivo* studies of *Notch1*-deficient mouse skin showed an increase in fibroplasia, angiogenesis, and inflammation, demonstrating the importance of the stromal microenvironment in CSCC development [48].

Loss of the *NOTCH1* gene may have cooperative effects with Ras-activation in keratinocyte transformation [22, 45]. Regarding *RAS* genes, *HRAS* mutations (3%–20% of CSCCs), rather than *NRAS* and *KRAS,* are commonly associated with CSCC [21, 31]. *Ras* has been implicated in the initiation of CSCC in a murine chemical carcinogenesis model [49], and mediating CDK4, in the induction of cell cycle arrest and transformation of primary keratinocytes into invasive

carcinoma [50]. *HRAS* mutations were found at a higher frequency in CSCC lesions arising in melanoma patients treated with BRAF-inhibition [51]. RAS activation promotes upregulation of downstream MAPK and PI3K/AKT/mTOR intracellular signaling. These pathways, in non-*RAS* mutant CSCCs, may also result from alternative mechanisms, including EGFR overexpression or PTEN inactivation.

EGFR overexpression is common in CSCC, and is associated with the acquisition of a more aggressive phenotype and a poor prognosis [26, 52]. EGFR is a member of the ErbB family of tyrosine kinase receptors that transmit a growth-inducing signal to cells that have been stimulated by an EGFR ligand. The union of ligand with EGFR produces a conformational change that allows a homodimerization with another EGFR or heterodimerization with another ErbB family member, both of which induce activation [53]. The pathways affected by the activation of EGFR include RAS-RAF-MEK-MAPK, PLC-gamma/PKC, and PI3K/AKT/mTOR. STAT and NF-kB can also be activated [54]. All these pathways are frequently altered in tumors, including CSCC [55], and trigger increased proliferation, migration, survival, resistance to apoptosis, and altered differentiation. The EGFR and downstream pathways can both be targeted with a variety of drugs to inhibit CSCC progression, as discussed below.

Therefore, epigenetic events play important roles in AK and CSCC [56]. CSCC includes the promoter hypermethylation of previous genes, such as *p16INK4a* and *p14ARF*, as well as *CDH1*, *RB1, MGMT*, and *RASSF1*, among others. These genes are involved in cycle regulation, DNA repair, epithelial adhesion, and signal transduction, while hypermethylation of CpG islands in the promoter regions produces transcriptional silencing [28]. MicroRNAs also have an important role; some act as oncogenes and others as tumor suppressors [57], and some are regulated by epigenetic factors. Recurrent copy number aberration has been noted in the development of CSCC (loss of heterozygosity at 3p, 8p, 9p, 9q, 13q, and 17q and chromosomal gain of 11q and 8q), including the formation of isochromosomes, chromosomal deletions, and whole-arm translocation [58].

Finally, the tumor microenvironment is important in the carcinogenesis of CSCC [59], attracting greater attention as its relevance in tumor development has become apparent [60, 61]. One of the main components of the tumor microenvironment is inflammation [61], which may act as a tumor promoter [62, 63]. The lack of inflammatory response is relevant in tumor progression [64]. Recent studies demonstrate that the CSCC tumor microenvironment is enriched in cancer-associated fibroblasts (CAFs) [65] and tumor-associated macrophages [66]. Tumor stromal cells are implicated in the invasion, metastases, tumor progression, and response to chemotherapy [67, 68]. Cellular and molecular components of the tumor microenvironment are of great importance in the effect of immunotherapy, as described below.

16.3 Treatment of CSCC

16.3.1 Targeted Therapy in CSCC

16.3.3.1 EGFR inhibitors

Current strategies in cancer therapy have pointed towards the interruption of signaling pathways that are involved in its pathogenesis. EGFR inhibitors were one of the first systemic therapies tested to treat CSCC. Some studies demonstrated that EGFR could be relevant to CSCC development, and in the context of the low effectiveness of drugs for treating CSCC, this was a logical and promising pathway to explore. EGFR inhibitors were tested in other cancers and yielded reasonable responses [69–72], and some isolated cases showed an anti-EGFR response in CSCC [73–77], prompting the design of clinical trials.

Targeting EGFR inhibits the PI3K/AKT/mTOR and RAS/RAF/ERK signal transduction pathways [78]. There are two classes of EGFR inhibitors: monoclonal antibodies that block the extracellular domain of the receptor (e.g., cetuximab, panitumumab, nimotuzumab, zalutumumab), and small-molecule tyrosine kinase inhibitors (TKIs), which block tyrosine kinase activity and thereby inactivate downstream cellular pathways (e.g., gefitinib, erlotinib, afatinib, lapatinib, neratinib, dacomitinib). Monoclonal antibodies and TKIs have been evaluated in clinical trials for poor-prognosis CSCC but are currently off label.

Cetuximab is a human-mouse chimeric monoclonal antibody that competitively binds to the extracellular domain of EGFR and inhibits dimerization of the receptor and the subsequent downstream signaling. Cetuximab is a U.S. Food and Drug Administration (FDA)-approved drug for colorectal and head and neck cancers and has shown some clinical efficacy as a first-line treatment in patients with unresectable CSCC [79]. Cetuximab was the first EGFR inhibitor to be evaluated in CSCC in a phase II trial. In that study, cetuximab showed valuable clinical activity with an overall disease control rate of 69% and a response rate (RR) of 28% at six weeks, including two complete remissions (6%) and eight partial remissions (22%). To confirm these results, a larger clinical trial (NCT03325738) is currently underway. Cetuximab is also being tested in combination with radiotherapy (NCT01979211), lenvatinib, which is a TKI (NCT03524326), avelumab, which is an anti-PD-L1 checkpoint inhibitor (NCT03944941), pembrolizumab, which is directed against as programmed cell death 1 protein (PD-1) (NCT03082534), and before surgery, as a neoadjuvant therapy (NCT02324608). Cetuximab is well-tolerated, but skin reactions may develop as side-effects in more than 80% of patients, mainly presenting as an acne-like rash, pruritus, desquamation, hypertrichosis, or nail disorders that must be treated [80–82]. The presence of acne-like eruption in patients under treatment has been associated with better response [79, 83]. Another monoclonal antibody, panitumumab, was evaluated

in 16 patients with incurable CSCC, five of whom (31%) showed a response [84]. Panitumumab is a good alternative to cetuximab when anaphylaxis occurs [85].

Small-molecule TKIs, like gefitinib, erlotinib, and lapatinib, have been partially effective in patients with CSCC. Gefitinib demonstrated modest activity in metastatic and locoregional recurrent CSCC with an overall RR of 16% and a disease control rate of 51% [86] (NCT00054691). Indeed, as neoadjuvant therapy before standard surgery or radiotherapy, gefitinib achieves a 45.5% RR in patients with aggressive or recurrent CSCC [87] (NCT00126555). In a single-arm phase II clinical trial, erlotinib exhibited a RR of 10% and progression-free survival (PFS) of 4.7 months in patients with recurrent or metastatic CSCC [88] (NCT01198028). Erlotinib has been used to inhibit EGFR in a three-dimensional *in vitro* human skin model, in which it resulted in a significant reduction of epidermal thickness [89]. Lapatinib, a dual TKI that blocks the HER2/neu and EGFR pathways, has been used to treat patients with CSCC and AK. It produced tumor reduction in two out of eight patients and AK reduction in seven out of eight patients, encouraging larger clinical trials [90]. *In vitro* studies demonstrate that lapatinib produces cell-cycle arrest, autophagy induction, and epithelial-to-mesenchymal inhibition in the CSCC A431 cell line [91].

The efficacy of EGFR inhibitors was somewhat lower than expected, and a better selection of patients should optimize the drug's usefulness. It should be borne in mind that these targeted therapies, which inhibit signaling pathways that contribute to the CSCC progression, frequently disrupt skin homeostasis and produce side effects.

16.3.1.2 Other targeted therapies in CSCC

The involvement of RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways in cancer has led to the development of several inhibitors that target them [92, 93]. In CSCC, a recent *in vivo* study demonstrated that the inhibition of MEK with trametinib and cobimetinib induces senescence in CSCC cell lines and reduces tumor growth in a mouse model [94]. Moreover, cobimetinib is being studied in combination with atezolizumab, a PD-L1 inhibitor, in metastatic or locally advanced and unresectable CSCCs, and locally advanced CSCCs that are technically resectable but where surgery could produce disfigurement (NCT03108131). mTOR inhibitors such as rapamycin are currently being used to decrease the risk of CSCC development in immunosuppressed patients that receive traditional immunosuppression [95–97]. Combining topical mTOR inhibitors and AKT inhibitors (PHT-427) enhances the chemopreventive effects of rapamycin [98]. Pan-PI3K and selective PI3K inhibitors have been developed to treat other cancers [99]. In CSCC, GDC-0084 and LY3023414, which are novel small-molecule PI3K-mTOR dual inhibitors, inhibit survival and proliferation and promote apoptosis in CSCC cells. Moreover, these drugs inhibit A431 xenograft tumor growth [100, 101]. Thus, targeting pathways downstream of EGFR could be a practical option for attacking CSCC. All the clinical trials that are currently being conducted with targeted therapies are listed in Table 16.1.

Table 16.1 Clinical trials of targeted therapies in cutaneous squamous cell carcinoma (CSCC) (revised until 29 January 2020)

Drug	Treatment	Conditions	Current state	NCT code
Cetuximab	Alone	Locally advanced and metastatic CSCC surgically unresectable	Completed (28% response rate, 6% complete remission, 2% partial remission)	NCT00240682
	Alone	Locally advanced and metastatic CSCC surgically unresectable	Completed	NCT03325738
	Alone (neoadjuvant therapy)	Aggressive locally advanced CSCC	Recruiting	NCT02324608
	Combination with post-operative radiation	Locally advanced head and neck CSCC	Active, not recruiting	NCT01979211
	Combination with pembrolizumab	Recurrent/ metastatic CSCC	Recruiting	NCT03082534
	Combination with lenvatinib	Advanced CSCC	Recruiting	NCT03524326
	Combination with avelumab	Advanced CSCC	Recruiting	NCT03944941
Gefitinib	Alone (neoadjuvant therapy)	Locally advanced/ recurrent CSCC	Completed (45.5% response rate)	NCT00126555
	Alone	Metastatic or locorregional recurrent	Completed (16% response rate)	NCT00054691
Erlotinib	Alone	Recurrent/ metastatic CSCC	Completed (10% response rate)	NCT01198028
	Combination with radiotherapy	Advanced head and neck CSCC	Completed	NCT00369512
	Alone (before surgery)	Head and neck CSCC	Active, not recruiting	NCT00954226
Cobimetinib	Combination with atezolizumab	CSCC	Recruiting	NCT03108131

16.3.2 Immunotherapy in CSCC

Tumor cells produce neoantigens that are recognized and targeted by the immune system. When a T-cell recognizes the antigen expressed by the human leukocyte

antigen (HLA) complex in the tumor cell, co-receptors act as activators and inhibitors of the immune response [102]. Inhibitory receptors, such as programmed cell death 1 protein (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA4), are known as "immune checkpoint" receptors. PD-1 is an inhibitor co-receptor expressed on the surface of T-cells, B-cells, monocytes, natural killer cells, and dendritic cells [103]. This transmembrane protein binds to two ligands, PD-L1 and PD-L2, which are present on the surface of the tumor cell, and their interaction triggers a signal that inhibits the activated T-cells and induces immunological exhaustion via anergy and T-cell apoptosis [102, 104, 105]. The PD-L1/PD-1 axis is a primary mechanism of cancer immune evasion, and this was the rationale for developing new drugs that have emerged in recent years. Targeting the immune checkpoint proteins with monoclonal antibodies has yielded a clinical benefit in cancer [106, 107], and dramatically changed prospects for the treatment of some types of cancer, such as melanoma [108]. An established tumor is composed both by the neoplastic cells and the tumor microenvironment. The latter is composed both by the tumor stroma and the inflammatory infiltrate. The tumor microenvironment, and not only the neoplastic cells, can also be modulated to destroy the neoplastic cells. Indeed, most immune checkpoint inhibitors are directed towards the lymphocytes, which belong to the tumor microenvironment, in order to enhance the immune response [109].

PD-1 inhibitors of several forms of cancer have been released, but given the low responsiveness of CSCC to other systemic treatments, some isolated cases were treated with drugs directed towards this axis and responded well [110, 111]. These preliminary results justified closer examination of this pathway and its potential therapeutic role in CSCC. Some studies demonstrated the presence of cell surface PD-1/PD-L1 in human tumors, and this expression has been linked to poor clinical outcomes in a variety of cancers [112–116], including CSCC [117, 118]. CSCC has the highest mutational burden of all tumors, and is a good candidate for immunotherapy treatment [21]. Tumors with a higher tumor mutational burden are known to be more responsive to immune checkpoint inhibitors [119–121]. In addition, the higher risk of immunocompromised patients developing CSCC indicates the importance of the immune system in this tumor [122, 123]. For these reasons, clinical trials with these drugs for the treatment of CSCC were designed.

Cemiplimab is the first drug approved by the FDA and the European Medicines Agency (EMA) for the treatment of locally advanced and metastatic CSCC [124]. It is a human monoclonal antibody directed against PD-1, and has demonstrated efficacy in immunocompetent patients with advanced CSCC and with metastatic disease, yielding RRs of 50% and 47%, respectively [124]. Cemiplimab is currently being tested in patients with recurrent stage III-IV head and neck CSCC before surgery as neoadjuvant therapy (NCT03565783), and in patients with recurrent CSCC as a pre-operative intralesional injection (NCT03889912). Future trials will focus on cemiplimab as an adjuvant drug versus placebo after surgery and

radiotherapy in patients with high-risk CSCC (NCT03969004), as monotherapy, or in combination with RP1 oncolytic virus in patients with locally advanced or metastatic CSCC (NCT04050436).

Other immunotherapeutic drugs are under evaluation in CSCC. Pembrolizumab is a human PD-1-blocking antibody indicated for the treatment of non-small-cell lung, head and neck, gastric, cervical, hepatocellular, and endometrial cancers, melanoma, Hodgkin's lymphoma, and Merkel cell, urothelial, renal cell, small-cell lung, and esophageal carcinomas [125]. In CSCC, pembrolizumab is being tested in a phase II study of 150 adults with recurrent/metastatic or locally advanced unresectable CSCC (MK-3475-629/KEYNOTE-629, NCT03284424). The interim results of the preview clinical trial (CARSKIN, NCT02883556) presented at the American Society of Clinical Oncology (ASCO) meeting 2018 showed high RRs (42%) and a durable response, with a median of around 7 months in patients with unresectable CSCC [126]. Pembrolizumab is also being examined in participants with locally advanced CSCC versus placebo after surgery and radiation (MK-3475-630/KEYNOTE-630, NCT03833167). It is being investigated as an addition to postoperative radiotherapy in resected cutaneous squamous cell cancer of the head and neck (NCT03057613) to assess safety with dose-limiting responses. Finally, pembrolizumab is being tested in combination with cetuximab (NCT03082534), AST-008 (NCT03684785), abexinostat (NCT035890054), and sonidegib (NCT04007744) in different stages of CSCC.

Nivolumab, another PD-1 inhibitor, is being studied in patients with CSCC in monotherapy (NCT04204837, NCT03834233) or combination with pembrolizumab (NCT02955290), and there have already been case reports demonstrating its clinical efficacy and good tolerability [127]. Nivolumab is also being tested in combination with ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients who are immunosuppressed due to having received a kidney transplant and who have unresectable or metastatic CSCC (NCT03816332). Pembrolizumab and nivolumab are FDA-approved for treating unresectable or metastatic melanoma but have yet to be approved for the treatment of CSCC. The most frequently reported side-effects of immune checkpoint inhibitors are diarrhea and fatigue, and they are usually low-grade side-effects. Immune checkpoint inhibitors can cause inflammation in any organ/system of the body, and thus it is important to take it seriously if the patient presents colitis, pneumonitis, hepatitis, thyroiditis, or hypophysitis. These autoimmune side-effects may sometimes be severe and force a treatment cycle to be discontinued or even withdrawn. Headache, pruritus, and dermatitis may be expected as well [128].

In addition to the evidence from clinical trials, there are several case reports of the efficacy of immunotherapy in CSCC-immunocompetent patients [129–132]. Transplant patients represent a group in which the use of checkpointinhibitors presents a problem because enhanced T-cell activation can lead to allograft rejection [106, 133, 134]. Limited data exist because transplant patients are often excluded from clinical trials, and only data from isolated cases are available [130, 135, 136].

All the clinical trials with immunotherapy that are currently underway are listed in Table 16.2. Figure 16.1 shows the therapeutic landscape of CSCC.

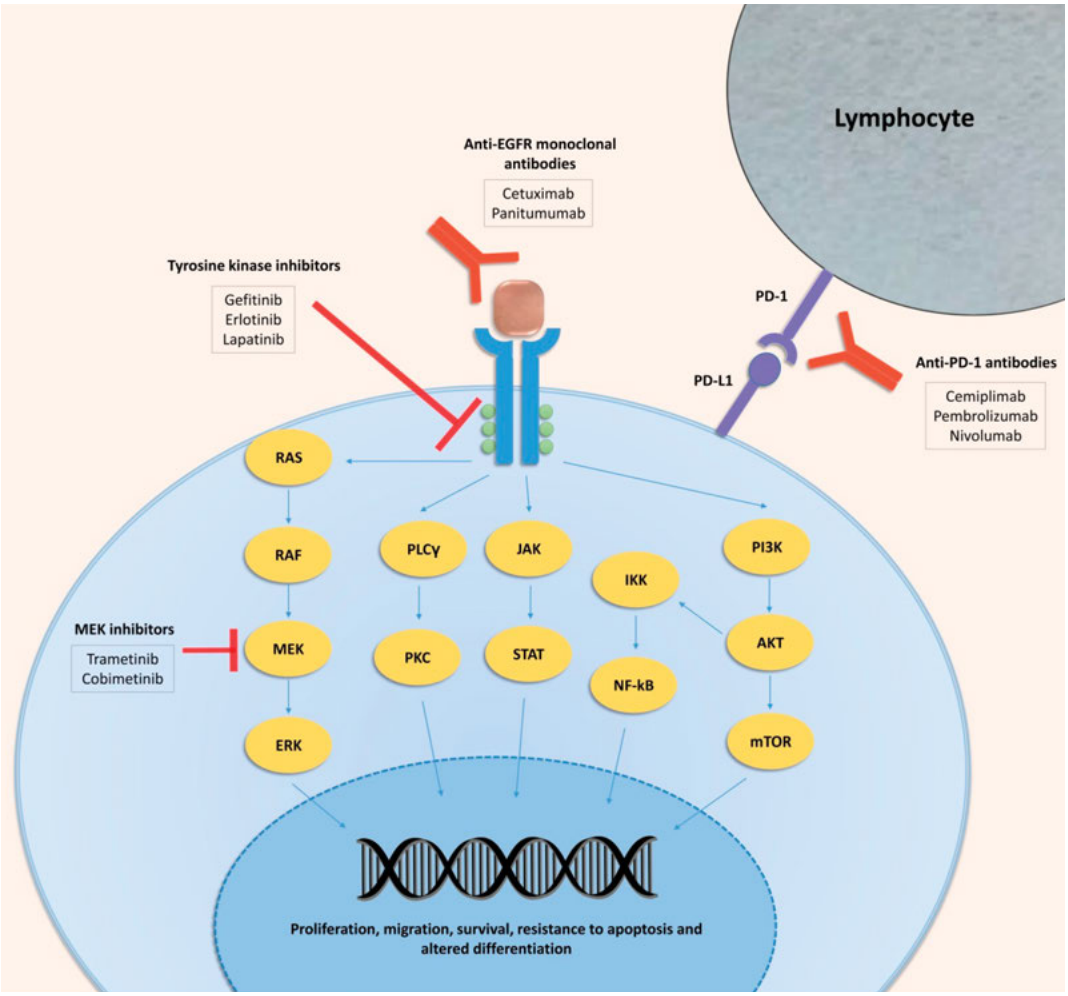

Figure 16.1 Therapeutic landscape of cutaneous squamous cell carcinoma.

16.4 Pharmacologically Induced Cutaneous Squamous Cell Carcinoma

Several drugs have been developed for CSCC treatment, but the disease may actually be induced by drugs as well. Molecular mechanisms underlie pharmacologically induced CSCC, and a sound knowledge of them could help physicians better tackle this tumor. Drug-induced CSCC is poorly covered in the literature, and for this reason, we focus on this CSCC in the last part of this review.

Table 16.2 Clinical trials of immunotherapy in cutaneous squamous cell carcinoma (revised until 29 January 2020)

Drug	Treatment	Conditions	Current state	NCT code
Cemiplimab	Alone	Advanced and metastatic CSCC	Completed (47%–50% response rate) Recruiting next phase	NCT02383212 NCT02760498
	Alone (before surgery)	Recurrent stage III-IV head and neck CSCC	Recruiting	NCT03565783
	Alone (pre-operative therapy intralesional)	Recurrent CSCC	Recruiting	NCT03889912
	Adjuvant therapy after surgery and radiotherapy	High risk CSCC	Recruiting	NCT03969004
	Alone or combination with RP1	Advanced or metastatic CSCC	Recruiting	NCT04050436
	Alone	Unresectable locally recurrent and/or metastatic CSCC	Recruiting	NCT04242173
	Alone (neoadjuvant therapy)	Stage II to IV CSCC	Recruiting	NCT04154943
Pembrolizumab	Alone	Recurrent/metastatic or locally advanced unresectable CSCC	Active, not recruiting	NCT03284424
	Alone	Locally advanced or metastatic CSCC	Active, not recruiting (preview results presented in ASCO show 42% response rate)	NCT02883556
	Alone	Locally advanced and metastatic CSCC	Active, not recruiting	NCT02964559
	Adjuvant therapy after surgery and radiotherapy	High risk locally advanced CSCC	Recruiting	NCT03833167

Table 16.2 (*Continued*)

Drug	Treatment	Conditions	Current state	NCT code
	Combination with postoperative radiotherapy	CSCC of head and neck	Recruiting	NCT03057613
	Combination with cetuximab	Recurrent/metastatic CSCC	Recruiting	NCT03082534
	Combination with AST-008	Advanced/metastatic CSCC	Recruiting	NCT03684785
	Combination with abexinostat	Stage III-IV CSCC of head and neck	Recruiting	NCT03590054
	Combination with sonidegib	Stage IV CSCC of head and neck	Not yet recruiting	NCT04007744
	Combination with nivolumab and CIMAvax vaccine	Stage III-IV CSCC of head and neck	Recruiting	NCT02955290
	Combination with SO-C101	Advanced/metastatic CSCC	Recruiting	NCT04234113
Nivolumab	Alone	Locally advanced/metastatic CSCC	Recruiting	NCT04204837
	Alone	Advanced CSCC	Recruiting	NCT03834233
	Alone or combination with ipilimumab	Metastatic CSCC in immunosuppressed patients	Recruiting	NCT03816332
	Combination with pembrolizumab and CIMAvax vaccine	Stage III-IV CSCC of head and neck	Recruiting	NCT02955290

16.4.1 Immunosuppressive Drugs and CSCC

The immunosuppressive therapy used in organ transplant recipients (OTRs) to prevent allograft rejection promotes cutaneous infection and skin neoplasms [15, 122]. The classic immunosuppressant drugs used for organ transplantation are glucocorticosteroids (prednisone and prednisolone), calcineurin inhibitors (cyclosporine and tacrolimus), and anti-proliferative agents (azathioprine and mycophenolic acid). Here we focus on cyclosporine and azathioprine.

16.4.1.1 Cyclosporine and CSCC

Cyclosporine is a calcineurin inhibitor that increases the risk of CSCC, especially under UVR [137–139]. Cyclosporine A reduces UVB-induced DNA damage repair and inhibits apoptosis in human keratinocytes by inhibiting the nuclear factor of activated T-cells (NFAT) [140]. Calcineurin inhibition is known to selectively induce the expression of activating transcription factor 3 (ATF3), which downregulated p53 expression and increased CSCC formation in a mouse model and in human CSCCs [141]. *In vitro* studies demonstrated that chronic treatment of human HaCaT keratinocytes with cyclosporine enhances AKT activation by suppressing PTEN, and promotes tumor growth of the CSCC A431 cell line in immune-deficient nude mice [142, 143]. Furthermore, cyclosporine enhances epithelial-to-mesenchymal transition involving the upregulation of TGFβ signaling [144].

The increased risk of CSCC in patients under cyclosporine has led physicians to search for different options. Some studies of tacrolimus, a calcineurin inhibitor introduced to replace cyclosporine, demonstrated no difference in a comparison of overall cancer rates of the two drugs [145]; however, more recent data from a clinical trial and from *in vivo* studies indicate a lower skin cancer risk associated with tacrolimus [146, 147]. Nevertheless, the most important drugs for preventing cyclosporine-induced CSCC development are the mTOR inhibitors.

The newest immunosuppressants used for OTRs are sirolimus (rapamycin) and everolimus. Both inhibit interleukin (IL)-2 and IL-15 via mTOR. It is not known whether these inhibitors have anticarcinogenic effects [148]. Preliminary data suggest that conversion from calcineurin inhibitors to sirolimus reduces the incidence of skin cancer in renal graft recipients [95, 97], possibly because sirolimus reduces vascularization and the thickness of post-transplant CSCCs [149]. The change of therapy from calcineurin inhibitors to sirolimus in patients with one CSCC lowered the risk of a new CSCC, and metastasis events only occurred in patients who received calcineurin inhibitors [96], the effect being maintained over 5 years of follow-up [150]. *In vivo* studies of hairless mice show that sirolimus significantly increases the latency of large tumors and reduces their multiplicity. Tumors from the rapamycin group have a lower UV-signature p53 mutation rate [151]. Case reports of conversion to everolimus show a reduced likelihood of CSCC development [152].

Recent studies have shown that cyclosporine exposure upregulates IL-22R1 [153] and causes increased JAK1, STAT1, and STAT3 expression. Using ruxolitinib, an FDA-approved JAK1/2 inhibitor, in human CSCC cells and xenografts reduces proliferation and growth. This could be a feasible option for preventing CSCC in OTRs who face long-term immunosuppression [154].

16.4.1.2 Azathioprine and CSCC

In a cohort study of 361 renal transplant recipients, the immunosuppressant drug azathioprine increased the risk of CSCC 2.4-fold [155]; and in an organ transplantation cohort of 207 patients, post-transplant azathioprine treatment increased the risk of CSCC compared with controls in a dose-dependent manner [156]. A systematic review and meta-analysis of 27 studies confirmed the association of OTRs treated with azathioprine and CSCC [157]. It is clear that azathioprine enhances the effect of UVR on skin cancer risk, and indeed, it strongly induces and promotes CSCC in hairless mice exposed to UVR [158]. Azathioprine photosensitizes the skin to UVR by changing the absorption interval of DNA upon incorporation of 6-thioguanine, the active metabolite of azathioprine. UVR absorption then induces the formation of reactive oxygen species that have been linked to DNA damage and cutaneous malignancies [159–161]. Whole-exome sequencing has revealed a novel CSCC mutational signature, which is associated with chronic exposure to azathioprine [39].

To reduce the risk of CSCC associated with this drug, azathioprine can be replaced by mycophenolate, leading to lower levels of DNA 6-thioguanine, skin ultraviolet A (UVA) sensitivity, and DNA damage, and a lower risk of CSCC [146, 162, 163]. However, another study suggests that the calcineurin inhibitor tacrolimus and mycophenolate mofetil (MMF) inhibit UVB-induced DNA damage repair, demonstrating the tumor-promoting action of these immunosuppressants [164].

16.4.1.3 Voriconazole and CSCC

Voriconazole, an antifungal used to prevent and treat invasive fungal infections after lung transplantation, has been associated with an increased risk of developing CSCC [165]. Voriconazole causes photosensitivity [166] in a dose-dependent manner [167]. The mechanism underlying this may arise from a primary metabolite, voriconazole N-oxide, which absorbs UVA and UVB wavelengths [166, 168]. Expression arrays of *in vitro* cultures of primary human keratinocytes exposed to voriconazole also show that this drug inhibits terminal epithelial differentiation pathways, resulting in poor formation of epithelial layers that are important for photoprotection, favoring its phototoxicity [169]. *In vitro* and *in vivo* assays demonstrated that voriconazole and its product inhibit catalase, raising intracellular levels of UV-associated oxidative stress and DNA damage in keratinocytes to promote skin carcinogenesis [170]. While photoprotection is fundamental for preventing CSCC, this is especially important in patients under voriconazole.

16.4.2 Targeted Therapies

16.4.2.1 Sonic-Hedgehog inhibitors and CSCC

Medications to treat other skin cancers, such as melanoma and basal cell carcinoma (BCC), can paradoxically lead to the development of CSCC. Vismodegib is a smoothened inhibitor (Hedgehog pathway inhibitor) that the FDA and EMA have approved for treating locally advanced and metastatic BCC [171]. The association of vismodegib with CSCC was reported in several case reports [172–174], and a retrospective cohort study highlighted this increased risk [175]. Some researchers disputed the latter study [176], and a subsequent paper failed to replicate such an association [177]. Furthermore, squamous metaplasia has been found in BCCs treated with vismodegib [178]. Nevertheless, there is some evidence to suggest that hedgehog inhibitors may indeed increase the risk of CSCC. The mechanism of action of vismodegib to promote CSCC is thought to be the activation of the RAS/MAPK pathway, which is responsible for CSCC progression [179].

A CSCC may arise from a BCC because both develop from the same target cell, as some authors have suggested. Two studies revealed new roles for *Ptch1* that lie at the nexus between BCC and CSCC formation [180, 181]. *Ptch1* gene is thought to occupy a critical role in determining the basal or squamous cell lineage [181], and its polymorphisms are involved in cell fate decisions. In BCC, loss of *Ptch1* activates the Sonic-Hedgehog pathway, but the overexpression of *Ptch1* promotes an alternative cell-fate decision, leading to increased CSCC susceptibility [180].

16.4.2.2 BRAF inhibitors and CSCC

BRAF is mutated in around 50% of melanomas, and some years ago, the therapeutic landscape of this tumor broadened through the development of BRAF inhibitors [182], specifically vemurafenib and dabrafenib [183]. These drugs provided greater overall survival and PFS compared with dacarbazine [184, 185], but they also increased the risk of CSCC development [186–188]. The effectiveness of these drugs stems from their ability to attenuate the MAPK pathway, which is downstream of constitutive BRAF activation [189]. However, BRAF inhibitors are capable, paradoxically, of activating the MAPK pathway in cells containing non-mutated *BRAF*, and this pathway is essential for CSCC development [51, 190–192]. The inhibition of MEK proved to be effective in preventing CSCC while on BRAF inhibitors, and thereafter BRAF inhibitors were combined with MEK inhibitors to avoid these side effects. Specifically, vemurafenib is combined with cobimetinib [193], and dabrafenib with trametinib [194]. A meta-analysis of five phase III randomized controlled trials, 17 phase II trials, and two phase IV trials [195] demonstrated that combined BRAF and MEK inhibition (trametinib) reduces the incidence of CSCC relative to BRAF monotherapy, as seen in another study [196]. More recent work demonstrated that BRAF inhibitors induce *RAS* mutations that are essential for MAPK activation. *RAS* mutations were detected in 21%–60% of lesions after BRAF inhibitor treatment in contrast to 3%–30%

in normal CSCCs [51, 197]. A mutational signature has been noted in squamous proliferative lesions induced by BRAF inhibitors that differs from the mutation pattern seen in spontaneous CSCCs [198]. Additionally, human papillomaviruses (HPVs) are detected more frequently in BRAF inhibitor-induced CSCCs, which means that HPV might accelerate keratinocyte oncogenesis in this subset of patients [199].

Other than MEK inhibitors, the inhibition of cyclooxygenase (COX)-2 has been evaluated as a strategy to prevent BRAF-inhibitor-mediated CSCC development. Experimental investigations that induce CSCC carcinogenesis by UVR have shown that COX-2 inhibitors (celecoxib and diclofenac) decrease prostaglandin production, thereby mitigating CSCC development [200, 201]. Moreover, celecoxib delayed the onset of CSCC in a mouse model mediated by DMBA/TPA and of CSCC induced by the BRAF inhibitor PLX7420, reducing the tumor burden by 90% [202]. All the drugs that may contribute to the development of CSCC are listed in Table 16.3.

16.5 Conclusions

In recent years, a deeper understanding of the molecular bases of cutaneous squamous cell carcinogenesis (CSCC) has helped identify novel therapies. EGFR inhibitors were found to be promising drugs in CSCC, based on several studies that suggested an important role for this pathway in CSCC development at a time when there was little to offer patients by way of effective treatment. Subsequently, other targets were evaluated and continue to be developed. More recently, the high mutational burden of this tumor and the increased risk of CSCC in immunosuppressed patients have raised the possibility of using immunotherapy to treat CSCC. As the new checkpoint inhibitors are surprisingly effective in other tumors, some CSCC cases have also been treated, with anti-PD-1 yielding particularly good responses. This prompted the design of clinical trials, and cemiplimab was the first inhibitor to be approved for use. It seems likely that other checkpoint inhibitors will be incorporated into the therapeutic arsenal of CSCC in the near future.

It is important to emphasize that patients who are receiving drug treatments that are associated with increased susceptibility to developing CSCC may require dermatological supervision, especially if any suspicious skin lesion arises.

The major message emerging from our review is that we should guard against the view that CSCC is a tumor with a good prognosis simply because it usually has a favorable evolution. In truth, its high incidence means that the absolute frequency of complicated and disseminated cases will also be high.

Metastatic CSCC remains a therapeutic challenge. The new arsenal of drugs that target different signaling pathways, especially immunotherapeutic medications, opens up new possibilities for treating CSCC patients, and we may expect these to be increasingly incorporated into the new wave of personalized and precision medicine protocols.

Table 16.3 Pharmacologically induced CSCC

Drug	Treatment	Mechanisms to promote CSCC	Options to reduce CSCC risk
Cyclosporine	Immunosuppressant	Reduces UVB-induced DNA damage repair and inhibits apoptosis by inhibiting nuclear factor of activated T-cells (NFAT) [**140**]	Sirolimus and everolimus [**95–97,149–152**]
		Induces the expression of ATF3, which downregulates p53 and increases CSCC formation [141]	
		Enhances AKT activation by suppressing PTEN and promotes tumor growth [142, 143]	
		Enhances epithelial-to-mesenchymal transition involving the upregulation of TGFβ signaling [144]	
Azathioprine	Immunosuppressant	Photosensitizes the skin to ultraviolet radiation (UVR) by changing the absorption interval of DNA upon incorporation of 6-thioguanine and induces the formation of reactive oxygen species [159–161]	Mycophenolate mofetil [**146**, **162**, **163**]
Voriconazole	Antifungal	The primary metabolite, voriconazole N-oxide, absorbs UVA and UVB wavelengths and causes photosensitivity [166–168]	Photoprotection
		Inhibits terminal epithelial differentiation pathways resulting in poor formation of epithelial layers that are important for photoprotection [169]	
		Inhibits catalase, raising intracellular levels of UV-associated oxidative stress and DNA damage [170]	
Vismodegib (Sonic-hedgehog inhibitor)	Basal cell carcinoma	Activates RAS-MAPK pathway [179]	Close follow-up
Vemurafenib and dabrafenib (BRAF inhibitors)	Melanoma	Activate, paradoxically, MAPK pathway and induce RAS mutations [51, 190–192, 197]	BRAF inhibitors + MEK inhibitors [**193–196**] or BRAF inhibitors + cyclooxygenase (COX)-2 inhibitors [**200**, **202**]

Abbreviations

AK: actinic keratosis
ASCO: American Society of Clinical Oncology
ATF3: activating transcription factor 3
BCC: basal cell carcinoma
CAFs: cancer-associated fibroblasts
COX: cyclooxygenase
CSCC: cutaneous squamous cell carcinoma
CTLA4: cytotoxic T-lymphocyte antigen 4
EGFR: epidermal growth factor receptor
EMA: European Medicines Agency
FDA: U.S. Food and Drug Administration
HLA: human leukocyte antigen
HPVs: human papillomaviruses
IL: interleukin
MMF: mycophenolate mofetil
NFAT: nuclear factor of activated T-cells
OTRs: organ transplant recipients
PD-1: programmed cell death 1 protein
RR: response rate
UVA: ultraviolet A
UVR: ultraviolet radiation

Disclosures and Conflict of Interest

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Chapter 17

Brain and Testis: More Alike Than Previously Thought?

Bárbara Matos, MSc,[a] Stephen J. Publicover, PhD,[b] Luis Filipe C. Castro, PhD,[c,d] Pedro J. Esteves, PhD,[d,e] and Margarida Fardilha[a]

[a]*Laboratory of Signal Transduction, Department of Medical Sciences, Institute of Biomedicine, University of Aveiro, Aveiro, Portugal*
[b]*School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, UK*
[c]*CIIMAR/CIMAR—Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Porto, Portugal*
[d]*Department of Biology, FCUP—Faculty of Sciences, University of Porto, Porto, Portugal*
[e]*CIBIO-InBIO, Research Centre in Biodiversity and Genetic Resources, Campus Agrico de Vairão, University of Porto, Vairão, Portugal*

mfardilha@ua.pt

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17.1 Introduction

The human body is an orchestrated set of different organs that work together, contributing to the maintenance of overall health and homeostasis. The human brain is the control center of the nervous system, playing a critical coordination role. It receives signals from sensory organs and translates them into functional information to multiple physiological compartments such as muscles and glands.

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In addition, the brain is also responsible for speech production, memory storage, and the elaboration of thought and emotion [1, 2]. The human testis is the male gonad and is of the utmost importance for reproduction and species evolution. It has two main functions: production of gametes (sperm) and synthesis/secretion of hormones (primarily, testosterone) [3, 4].

Despite these clearly dissimilar functions and the apparent structural and morphological differences between human brain and testis, in the last four decades it has become increasingly evident that these tissues share several features. The similarity was further confirmed by analysis of gene expression, with evidence that human brain and testis, among all the organs of the body, share the highest number of genes [5, 6]. More recently, authors found a positive correlation between general intelligence and three key measures of semen quality: sperm concentration, sperm count and sperm motility [7]. A possible association between male sexual dysfunction and neurological disorders was also proposed by several authors [8, 9]. These findings raise some interesting questions. (i) Why do the human brain and testis share a similar gene expression profile? (ii) Have these tissues a similar cellular organization and cooperation between cell types? (iii) Are their functions related? (iv) What are the implications of the similarities between human brain and testis?

In this context, we review the similarities between human brain and testis, and between human neuron and sperm at the cellular and molecular levels. The proteomic profile of the two human tissues (brain and testis) and the two types of cells (neuron and sperm) were also compared and critically discussed.

17.2 Brain and Testis

17.2.1 Cellular and Molecular Similarities

When human brain and testis, two apparently distinct tissues with very different functions, were compared, several similarities, spanning from molecular to cellular levels of organization, became evident. The main cellular and molecular similarities between these two organs are summarized in Table 17.1.

Human brain and testis are both constituted by different cell types that work together to maintain the integrity and function of the tissue. Human brain is a complex and organized tissue formed mainly by neurons and support cells named glia. Neurons are the most important cells in the brain, responsible for the transmission of information. To maintain their function, glia cells are in close relation with neurons. There are four different types of glia in human brain: astrocytes, oligodendrocytes, microglia and ependymal cells, each of them essential to maintain brain function [10, 11]. Likewise, testis is a well-organized tissue, composed of seminiferous tubules, in which developing germ cells and Sertoli cells are in close interaction [12]. Adjacent to the seminiferous tubules and close to the blood vessels are the Leydig cells, which produce and secrete testosterone into blood vessels [13]. The cellular organization of these two tissues is summarized in Fig. 17.1 (on the following page).

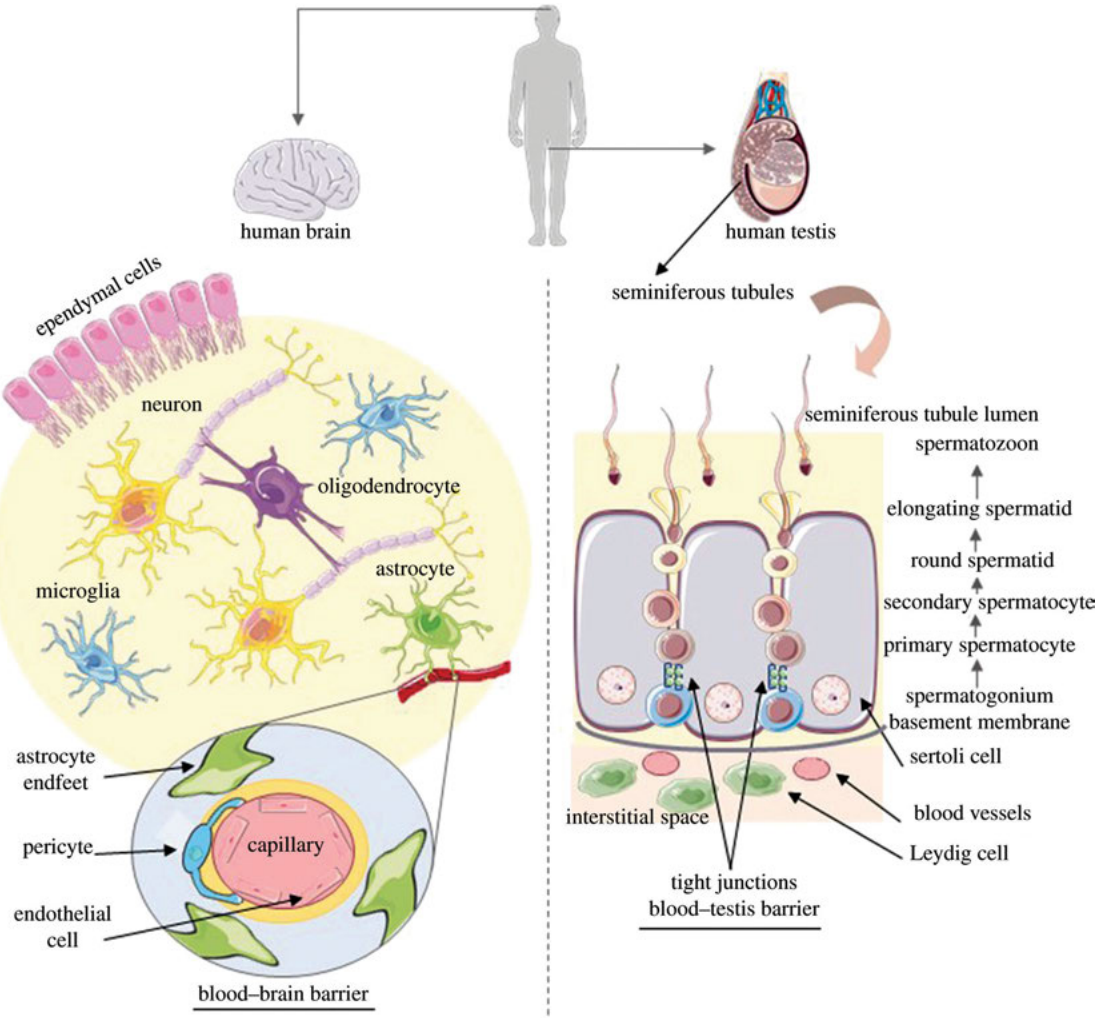

Figure 17.1 Summary of the cellular organization of human brain and testis.

Astrocytes and Sertoli cells are known as the biochemical support cells of brain and testis, respectively. Beyond their important role in the metabolism of these tissues, described below, they are responsible for the physical and nutritional support of neurons and germ cells, and essential for their development and survival [14, 15].

Human brain and testis are high-energy-demand tissues, executing energy-demanding processes such as cognitive functions and spermatogenesis, respectively [16]. To support these energy requirements, a metabolic cooperation between the different cell types is clear in both tissues [17, 18]. In the brain, astrocytes produce lactate as a glycogen-derived product, which is transported to the neurons that use it as a preferred energy source to maintain their synaptic activity. Thus, neuronal metabolic processes are highly dependent on the activity of astrocytes [17]. Similarly, a metabolic active cooperation between developing

Table 17.1 Cellular and molecular similarities between human brain and testis

Brain	Testis
Biochemical/physical support cells: astrocytes	Biochemical/physical support cells: Sertoli cells
High energy demands	
Metabolic cooperation: astrocytes produce lactate, which is used by neurons	Metabolic cooperation: Sertoli cells produce lactate, which is used by germ cells
Dependence on selenium metabolism	
High concentrations of polyunsaturated fatty acids	
Highly susceptibility to oxidative damage	
Blood–brain barrier	Blood–testis barrier
Neuroendocrine properties	
Cytoskeleton motors (kinesins and dyneins): essential role in neuronal function	Cytoskeleton motors (kinesins and dyneins): essential role in spermatogenesis

germ cells and Sertoli cells is evident. Sertoli cells convert glucose to lactate, which is transported to and used as a central energy metabolite by developing germ cells to maintain their metabolic activity [18, 19]. In addition to a similar metabolic cooperation, brain and testis both depend on selenium metabolism. A selenium-deficient diet has been associated with increased susceptibility to neurotoxicity and impaired spermatogenesis [20]. In selenium-deficient conditions, brain and testis compete for selenium utilization so that castration was associated with attenuation of neurodegeneration, mainly by increasing selenium-dependent antioxidant activity in brain [20].

Compared to other tissues, the human brain and testis are particularly susceptible to oxidative damage, due to their high energy and oxygen demand, and abundance of polyunsaturated fatty acids (PUFAs). Indeed, Kabuto et al. [21] exposed mice to bisphenol A, an oxidative stress inducer, during embryonic/fetal life and infancy, and collected several tissues, finding a particular underdevelopment of brain and testis, caused by increased oxidative injury. Furthermore, brain and testis have the lowest transcriptional levels of oxidative stress-related genes (for example, the gene that encodes catalase), compared to other tissues [16]. To counteract their high susceptibility to oxidative stress, these two tissues have specific blood–tissue barriers, called the blood–brain and the blood–testis barrier [22, 23]. An essential role of high concentrations of PUFAs in human brain and testis function and/or development has been reported [24, 25]. In the brain, the most abundant PUFA is docosahexaenoic acid (DHA), which it is mainly located at the synaptic terminals of neurons, playing a central role in neurodevelopment, function and maintenance [24]. The human germ cell line has an active lipid metabolism and displays stage-specific differences in fatty acid

pattern. The inverse correlation found between the percentage of abnormal sperm and the percentage of DHA suggests a role of PUFAs in sperm morphology and development [25].

In recent decades, the Leydig cells of the human testis have been recognized as members of the neuroendocrine system. The synthesis and release of a large number of biologically active substances that are typical for nerve and neuroendocrine cells has revealed that Leydig cells are neuroendocrine cells [26]. Indeed, several neuron-specific peptides and proteins, such as Substance-P [27], synaptophysin and neural cell-adhesion molecule, have been detected in human Leydig cells [28]. Some glial-cell-specific antigens—(glial fibrillary acidic protein (GFAP), galactocerebroside (GalC), cyclic 2′,3′-nucleotide-3′-phosphodiesterase (CNPase), A2B5-antigen and O_4-antigen, which are considered to be marker molecules of astrocytes and oligodendrocytes—were also found in Leydig cells of human testis [26]. Besides Leydig cells, Sertoli cells also express some neuron- and glial cell-specific proteins. In fact, the three isoforms of neurofilament proteins, and GFAP, GalC and CNPase were found in both Leydig and Sertoli cells of human testis [26, 29].

Cytoskeleton motors, including myosin, kinesins and dyneins, play essential roles in the brain, namely in neuron polarization, extension, shape and neurotransmission processes [30]. Motor proteins also play key roles in the formation of mature sperm [31]. Spermatogenesis includes several mitotic and meiotic divisions, for which the role of motor proteins in spindle organization, chromosome congression, chromatid separation, among others, are clear. Also in the final step of spermatogenesis, called spermiogenesis, kinesins seems to be crucial in acrosome biogenesis, nuclear shaping, tail formation, and spermatid maturation and transcription [31]. The vital role of these cytoskeleton motor proteins in brain and testis function is evident by several neurodegenerative and reproductive diseases that arise from mutations or other dysfunctions of these proteins in brain and testis, respectively [32, 33].

17.2.2 Proteomic Comparison

According to the apparent cellular and molecular similarities between human brain and testis, it has become clear that these tissues have a similar gene expression pattern. In a UniGene pilot investigation carried out by Guo et al. [5], the expression data of 760 human UniGenes in 17 tissues were retrieved and compared. Unexpectedly, among the 17 tissues compared, the highest similarity in gene expression patterns was between human brain and testis with a total of 364 shared expressed UniGenes [5]. According to this study, a large-scale analysis of the expression of 33,689 genes in 15 human tissues revealed that human brain and testis shared the greatest similarity in gene expression [6]. In addition, these authors demonstrated that the similarity of gene expression between brain and testis is not exclusive to humans and may be widely present in other mammals, including rodents [6]. Several authors have demonstrated that some genes are highly or selectively expressed in brain and testis of mice (*Tb-rbp*,

Gpr37, *Hst-1/Fgf-4*) and rat (*Ugt1a6*, *Glutx1*, *α4-b*, *Lancl1*, *Nep*) [34–41]. Moreover, Danielsson et al. [42] found that human brain and testis share the highest number of group-enriched genes. Although transcriptomic profiling has become a standard approach to understand the (dys)function of tissues, it is also important to evaluate how gene expression relates to the proteins that are actually being expressed. To that purpose, it is possible to use proteomics, which gives information about protein composition of a cell, tissue or organism [43].

Herein, we compared the brain and testis proteome with that of 31 other tissues, representing all major tissues in the human body (heart, skeletal muscle, adrenal gland, parathyroid gland, thyroid gland, lung, gastrointestinal tract, salivary gland, oesophagus, stomach, duodenum, small intestine, colon, bone marrow, lymph node, spleen, appendix, pancreas, kidney, liver, gallbladder, epididymis, seminal vesicle, prostate, breast, cervix, endometrium, ovary, placenta, adipose tissue and skin), using the Human Protein Atlas (HPA) (available at www.proteinatlas.org) and the Jveen tool (available at http://jvenn.toulouse.inra.fr). The HPA is a programme that aims to map all the proteins in cells, tissues and organs using the integration of various technologies (e.g. antibody-based imaging, mass spectrometry-based proteomics, systems biology). Consistent with the gene expression analysis mentioned in the previous section [5, 6], the highest number of common proteins was observed between brain and testis, suggesting that human brain and testis are the most similar tissues of the human body. The common proteins between these two tissues were retrieved and, to prevent redundancy, all proteins were annotated using the UniProtKB/Swiss-Prot accession number. From the total of 14,315 and 15,687 proteins that constitute the human brain and testis proteome, respectively, 13,442 are common to both tissues (Fig. 17.2; electronic supplementary material, Table S1).

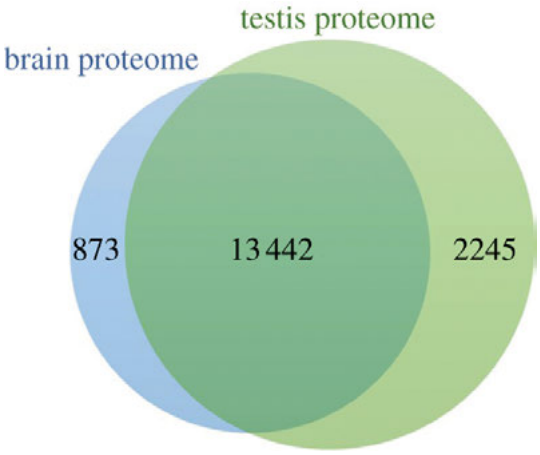

Figure 17.2 Veen diagram demonstrating the overlap between the human brain and testis proteome (based on the Jveen tool). The data of human brain and testis proteome were collected on 20 August 2019.

Table 17.2 List of proteins highly expressed only in brain and testis, along with their UnitProt ID, gene name and the main biological process(es) associated (according to UnitProt)

UnitProt ID	Gene name	Protein name	Biological processes
Q9H172	ABCG4	ATP-binding cassette sub-family G member 4	Cellular response to leukaemia inhibitory factor; cholesterol efflux; transmembrane transport
Q96M02	C1ORF90	(E2-independent) E3 ubiquitin-conjugating enzyme FATS	Protein polyubiquitination and stabilization; regulation of centriole replication
Q13536	C1ORF61	Protein CROC-4 (contingent replication of cDNA 4)	Positive regulation of transcription by RNA polymerase II
Q5T035	C9ORF129	Putative in characterized protein C9orf129	—
P08912	CHRM5	Muscarinic acetylcholine receptor M5	Chemical synaptic transport; dopamine transport; transmission of nerve impulse
Q12926	ELAV2	ELAV-like protein 2	mRNA splicing, via spliceosome; regulation of transcription
Q49AJ0	FAM135B	Protein FAM135B	Cellular lipid metabolic process
P43080	GUCA1A	Guanylyl cyclase-activating protein 1	Cellular response to calcium ion; signal transduction; visual perception
Q8NE63	HIPK4	Homeodomain-interacting protein kinase 4	Histone phosphorylation; peptidyl-serine phosphorylation; protein autophosphorylation
A6NGN9	IGLON5	IgLON family member 5	—
Q7Z553	MDGA2	MAM domain-containing glycosylphosphatidylinositol anchor protein 2	Spinal cord motor neuron differentiation
P60323	NANOS3	Nanos homolog 3	Germ cell development; multicellular organism development; regulation of cell cycle; spermatogenesis
O14594	NCAN	Neurocan core protein	Cell adhesion; CNS development; chondroitin sulfate biosynthetic process
Q9NQ35	NRIP3	Nuclear receptor-interacting protein 3	—
Q9Y5K3	PCYT1B	Choline-phosphate cytidylyltransferase B	**Spermatogenesis**; phosphatidylcholine biosynthetic process

(*Continued*)

Table 17.2 (*Continued*)

UnitProt ID	Gene name	Protein name	Biological processes
P01213	PDYN	Proenkephalin-B	**Chemical synaptic transmission**; G protein-coupled receptor signalling pathway; **neuropeptide signalling pathway**
Q96PV4	PNMA5	Paraneoplastic antigen-like protein 5	Positive regulation of apoptotic process
Q8WY54	PPM1E	Protein phosphatase 1E	Cellular response to drug; negative regulation of protein kinase activity
Q33E94	RFX4	Transcription factor RFX4	Positive regulation of transcription by RNA polymerase II; cilium assembly
Q8N6R1	SERP2	Stress-associated endoplasmic reticulum protein 2	Endoplasmic reticulum unfolded protein response; protein glycosylation; protein transport
Q6ZV89	SH2D5	SH2 domain-containing protein 5	—
Q99963	SH3GL3	Endophilin-A3	**CNS development**; endocytosis; **positive regulation of neuron differentiation**
Q8TF17	SH3TC2	SH3 domain and tetratricopeptide repeat-containing protein 2	**Peripheral nervous system myelin maintenance**; regulation of intracellular protein transport
Q8N5S1	SLC25A41	Solute carrier family 25 member 41	—
Q99726	SLC30A3	Zinc transporter 3 (ZnT-3)	Regulation of sequestering zinc ion; response to zinc ion
O00570	SOX1	Transcription factor SOX1	Cell differentiation; **CNS development;** chromatin organization; **forebrain neuron development; neuron differentiation**
Q16650	TBR1	T-box brain protein 1	**Brain development**; cell fate specification; **regulation of axon guidance**; regulation of transcription
O95409	ZIC2	Zinc finger protein ZIC 2	**Brain development**; cell differentiation; positive regulation of transcription
O96T25	ZIC5	Zinc finger protein ZIC 5	Cell differentiation; **CNS development**

Note: The biological processes associated with brain or testis function/development are bolded.
Abbreviation: CNS, central nervous system.

From the 13,442 common proteins between human brain and testis, we decided to highlight the proteins that are highly expressed in these two tissues, when compared with other human body tissues. To do that, we cross-checked the information from HPA with GeneCards (available at https://www.genecards.org/) and identified a total of 29 proteins highly expressed in brain and testis (Table 17.2). To better understand the similarities between human brain and testis, we decided to explore the biological processes in which these 29 proteins are involved, using UniProt which is summarized in Table 17.2. The analysis of protein-associated biological processes revealed specific roles of some proteins in brain and testis function and/or development. Since brain plays a key role in the control of testis function, particularly by the secretion of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the hypothalamus and pituitary, we expected more common specific proteins involved in testis function/development. Counterintuitively, 31% of the proteins are involved in brain function development, as opposed to 7% of testis function/development-related proteins.

17.2.3 Why Do Brain and Testis Appear to Have Similar Proteomes?

The increasing evidence for similarity between the human brain and testis gene expression and protein composition raises the question of the importance of these findings. It has been hypothesized that the testis could participate in human speciation along with the brain and placenta, which may contribute to the expression of the same set of genes in both tissues [44]. It has been suggested that evolutionary changes in gene expression contribute to most of the phenotypic differences between species, but how these gene expression patterns might be passed down to the offspring is a misunderstood topic [6, 45]. The involvement of testis, along with the brain and placenta, in speciation was first suggested by Wilda et al. [44] and the hypothalamus–pituitary–testis axis was proposed to be implicated in maintaining the similar gene expression between brain and testis [6]. Indeed, testis has been proposed as the hotspot for the appearance of new genes, which are the raw material for the evolution of species [46]. Sperm seem to be the motor of speciation. On one hand, sperm competition, that is the competitive process between sperm of different males to fertilize the same egg, is important in the formation of new species. On the other hand, sperm is also crucial to maintain the integrity of a species, by acting as a reproductive isolation barrier that precludes gene flow between species. In fact, male hybrids, characterized by the combination of two different species, seem to produce significantly fewer mature spermatozoa due to incompatibilities in the last stages of sperm development [47]. The high and specific expression of fragile X mental retardation 1 gene (*Fmr1*) in brain and testis suggests that speciation recruits the same set of tissue-specific genes that are active in those organs that are important for speciation [44].

More recently, 60 new protein-coding genes that originated *de novo* in the human lineage since its divergence from chimpanzee (human-specific genes) were

identified [48]. These proteins became fixed in the human population, and the high levels found in testis are also in agreement with the role of this organ in the transmission of gene expression patterns to the offspring [48]. The highest expression levels in cerebral cortex and testis suggested that these genes may contribute to phenotypic features that are exclusive of humans, such as the improved cognitive ability. Indeed, human-specific *NOTCHNL2* genes were associated with a role in cortex development and neurogenesis, and have been proposed as a driving force in the evolution of human large brains [49]. Additional evidence seems to suggest that brain and testis function-associated genes are changing unusually quickly, becoming the most divergent genes between species [50].

The similarities between the human brain and testis proteome seems to be reflected in an apparent association between the (dys)function of these tissues. Indeed, an association was observed between the degenerative process in the central nervous system and testicular degeneration, without coexisting hypophyseal lesions [51]. In addition, mutations in X-linked aristaless-related homeobox gene (*Arx*) were associated with the X-linked lissencephaly with abnormal genitalia (XLAG) syndrome, a disease characterized by simultaneous microcephaly and hypogonadism [52]. Evidence in mouse suggested that alterations in the same protein may be simultaneously responsible for brain and testis dysfunction. Inactivation of Huntington disease gene (*Hdh*) in mouse brain and testis results in a progressive degenerative neuronal phenotype, along with sterility [53], while mutations in *Arx* caused abnormal development of forebrain and testis [52]. Moreover, a negative correlation was observed between testis volume and parental behaviour and nurturing-related brain activity [54].

17.3 Neuron and Sperm

17.3.1 Cellular and Molecular Similarities

The morphology, genomic activity and function of human neuron and sperm are as different as any other two cells in the body [55]. Sperm is a very distinct cell, compared to other cells in the human body, mainly because it is a haploid cell and virtually devoid of transcription and translation [56]. However, beyond the similarities between brain and testis, several bodies of evidence of the similarities between human neuron and sperm, the fundamental units of these tissues, have been reported and are summarized in Table 17.3.

Both neuron and sperm can activate other cells, though the activation mechanisms involved are different. After the plasma membrane interaction of sperm with oocyte, the sperm activates the oocyte and triggers a signal transduction cascade that ultimately results in the conversion of the oocyte to a diploid embryo [57]. Neurons also have the capacity to activate other cells, namely other neurons or somatic effector cells, through chemical synapses or gap junctions (electrical synapses), not requiring contact between cells [57].

Table 17.3 Cellular and molecular similarities between human neuron and sperm

Neuron	Sperm
Activate other cells: neurons or somatic effectors	Activate other cells: oocyte
Exocytosis of neurotransmitters in the synaptic space (essential for neuron function)	Acrosomal exocytosis at the oocyte surface (essential for sperm function)
Synaptic vesicles	Acrosome
High concentrations of PUFAs	
Presence of 'neuronal' receptors	
Excitable cells	
presence of Ca^{2+} channels	
Ca^{2+} signalling involved in regulation of key functions	
Common signalling pathways	

Human neuron and sperm seem also to share similarities in exocytic process. Exocytosis is a central process to their individual abilities to carry out their functions. Several components of the neuronal synaptic vesicle exocytotic machinery have been found in sperm, notably including an intricate system of plasma membrane proteins, like synaptotagmins and SNARE complex [58–61]. Neurons use exocytosis for neurite outgrowth and to release neurotransmitters from synaptic vesicles, which is essential for communication between neurons [62]. The synaptic vesicles can be compared to the acrosome of sperm, which essentially contains hydrolytic enzymes and other important fertilization factors. These enzymes are released from the sperm through a specialized form of exocytosis. This process includes membrane loss and is necessary for zona pellucida breakdown and consequent sperm–oocyte fusion [55, 63]. Despite the similarities of the exocytotic process in neurons and sperm, in sperm this event only occurs once, in contrast to the continuous exocytotic activity of a neuron [55].

After the release of neurotransmitters at the synaptic gap, they interact with post-synaptic receptors ('neuronal' receptors) to induce or inhibit neurotransmission. Several types of 'neuronal' receptors, like glutamate and gamma-aminobutyric acid ($GABA_A$), glycine and nicotinic acetylcholine receptors, have been found in sperm [64–66]. Also in sperm, the 'neuronal' receptors play vital roles for its normal function, including in sperm acrosomal reaction, capacitation and motility [55, 67]. Due to the presence of various voltage-gated ion channels and several ligand-gated receptor channels, involved in rapid membrane potential changes, neurons are considered excitable cells [68]. In sperm, diverse types of high- and low-voltage-activated channels have been reported, suggesting that sperm may, like neurons, be considered an excitable cell [69, 70].

Calcium (Ca^{2+}) signalling is central to the regulation of function in both neuron and sperm cells. These distinct cell types both need to generate precisely

timed and localized $[Ca^{2+}]_I$ signals. It appears that this has resulted in some striking similarities in the ways in which their Ca^{2+} signalling toolkits are employed [55]. Though all cells express a Ca^{2+} signalling toolkit, the types, locations and combinations of channels and pumps can vary significantly between cell types, because they are adapted to the requirements of the cell and its activities. Both neuron and sperm Ca^{2+} signalling toolkits involve a diverse range of components (including Ca^{2+}-permeable channels and Ca^{2+} pumps) in both the plasma membrane and intracellular membranes, though the diversity in sperm is low in comparison to that of neurons [67, 71]. In neurons, Ca^{2+} signalling is involved in the regulation of various key functions, including transmission, processing and storage of information [72]. For instance, synaptic neurotransmitter secretion, modulation of synaptic efficacy (underlying memory formation) and excitability of the neuronal membrane (the ease with which a nerve impulse can be induced) are all dependent on or modulated by $[Ca^{2+}]_I$ [73]. In mature sperm cells, Ca^{2+} signalling is arguably at least as important as in neurons, playing central roles in the regulation of motility and capacitation (post-ejaculatory acquisition of fertilizing ability) [71]. The complex signalling pathway that leads to acrosome reaction also requires mobilization of Ca^{2+} stores within the acrosome [74]. The best-characterized Ca^{2+} channel in sperm is CatSper, which is a sperm-specific channel essential for hyperactivated motility [75, 76]. The influx of extracellular Ca^{2+} is also required for acrosome reaction, though the involvement of CatSper here is unclear [69].

Several signalling pathways are common to neuron and sperm, and seem to play essential roles in both cell types. For instance, anandamide (AEA) signalling seems to modulate human sperm motility [77]. A role as a modulator of synaptic function was also described for AEA signalling pathway [78]. In addition, Wnt signalling occurs also in both cell types where it controls both sperm maturation and neuronal differentiation [79, 80]. The mTOR signalling pathway was also associated with crucial events in both neuron and sperm. Indeed, mTOR signalling regulates sperm quality in older men and is important for normal neuronal growth [81, 82].

17.3.2 Proteomic Comparison

The sperm proteome was recently extracted, using the PubMed database, by Santiago et al. [83]. To avoid redundancy, from the total list of sperm proteins, we excluded duplicates and only reviewed proteins (annotated using the UniProtKB/Swiss-Prot accession number) were considered (electronic supplementary material, Table S2). Based on the same criteria, the neuronal cells proteome was retrieved from HPA. To avoid redundancy, duplicates and unreviewed proteins (according to UnitProt) were excluded. A list of all neuron proteins (available at 31 March 2021) were obtained (electronic supplementary material, Table S2).

A total of 13,193 and 6653 reviewed proteins constitute the neuron and sperm proteomes, respectively. A Venn diagram analysis was conducted using the Jveen tool to recover the common proteins between these two cell types. From the total proteins, 5048 are common to both human sperm and neuron (electronic supplementary material, Table S2; Fig. 17.3).

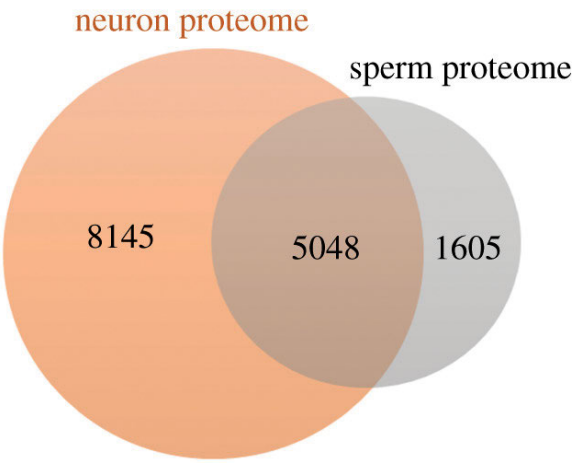

Figure 17.3 Venn diagram demonstrating the overlap between the neuron and sperm proteome (based on the Jveen tool).

From the 5048 common proteins in human neuron and sperm, a sublist was made considering the proteins with elevated expression in neuronal cells, according to HPA (www.proteinatlas.org). This analysis results in a total of 682 common proteins. Considering these common proteins, a GO analysis (using STRING: functional protein association networks) was performed and revealed a total of 328 GO terms significantly enriched, with an FDR < 0.05 (electronic supplementary material, Table S2). In Table 17.4, we summarize some of the most important biological processes in the context of the present study, together with the number of proteins associated with the GO term and the respective FDR of the annotation.

Among the common proteins between human neurons and sperm, several GO terms related to cell/tissue development were significantly enriched, suggesting that both cells play important roles in human tissue development. Also comparing neuron and sperm proteomes, it is observed that there are many common proteins involved in brain/neuron development and function. As expected by the important role of exocytosis in both sperm and neuron function as discussed above (Table 17.4), both neuron and sperm express a huge number of proteins involved in exocytic process. Cell signalling-associated biological processes were also highlighted in this analysis, corroborating the idea that sperm and neuron share several important signalling pathways (Table 17.4).

Table 17.4 Main biological processes associated with the common proteins between human sperm and neuron

GO term	Description	Count in gene set	FDR
Development			
GO:0032 502	Developmental process	268/5401	4.08×10^{-9}
GO:0048869	Cellular developmental process	175/3533	2.61×10^{-5}
GO:2000026	Regulation of multicellular organismal developmental	90/1876	2.23×10^{-2}
GO:0021700	Developmental maturation	17/216	3.35×10^{-2}
GO:0048639	Positive regulation of developmental growth	14/165	3.93×10^{-2}
Nervous system development			
GO:0007399	Nervous system development	181/2206	1.11×10^{-23}
GO:0022008	Neuron projection development	68/616	6.03×10^{-13}
GO:0048666	Neuron development	76/758	1.11×10^{-12}
GO:0061564	Axon development	47/377	1.28×10^{-10}
GO:0007417	Central nervous system development	60/861	3.14×10^{-5}
GO:0007420	Brain development	47/650	1.80×10^{-4}
GO:0021695	Cerebellar cortex development	7/49	3.63×10^{-2}
Brain/neuron-associated processes			
GO:0030182	Neuron differentiation	86/940	1.64×10^{-12}
GO:0010975	Regulation of neuron projection development	47/443	1.22×10^{-8}
GO:0007411	Axon guidance	27/220	4.38×10^{-6}
GO:009893	Axonal transport	9/43	1.50×10^{-3}
GO:0008038	Neuron recognition	8/34	1.80×10^{-3}
GO:0001764	Neuron migration	14/118	3.70×10^{-3}
GO:0007158	Neuron cell–cell adhesion	5/14	7.50×10^{-3}
GO:0019228	Neuronal action potential	6/31	2.17×10^{-2}
GO:0036514	Dopaminergic neuron axon guidance	3/5	2.87×10^{-2}
Exocytosis			
GO:0017156	Calcium ion regulated exocytosis	12/74	9.00×10^{-4}
GO:0016079	Synaptic vesicle exocytosis	11/64	0.0011
GO:0006904	Vesicle docking involved in exocytosis	6/38	0.0439
Cell signalling			
GO:0007267	Cell–cell signalling	95/1073	4.86×10^{-13}
GO:0035637	Multicellular organismal signalling	21/110	1.89×10^{-7}
GO:0023052	Signalling	232/5108	1.10×10^{-4}

GO term	Description	Count in gene set	FDR
GO:0007215	Glutamate receptor signalling pathway	9/43	1.50×10^{-3}
GO:1905114	Cell surface receptor signalling pathway	30/383	1.80×10^{-3}
GO:1990034	Calcium-mediated signalling	13/132	2.02×10^{-2}
GO:0035556	Intracellular signal transduction	76/1528	2.17×10^{-2}
GO:0016055	Wnt signalling pathway	22/303	2.41×10^{-2}

Note: A list of all the associated biological processes is found in electronic supplementary material, Table S2.

17.4 Concluding Remarks

Human brain and testis share several molecular characteristics, which are reflected in a very similar proteomic profile. Our *in silico* analysis revealed that, surprisingly, human brain and testis have the highest number of common proteins, compared with other human body tissues. The common proteins are mainly involved in the function and/or development of brain, rather than in testis-associated processes. The human neuron and sperm are very distinct cells; however, they share several molecular features, and a huge number of proteins are common to both cells, mainly those involved in exocytotic and cell signalling processes, tissue development and brain/neuron-associated processes.

The similarity between human brain and testis may be explained by a biochemical convergence and by the involvement of these two tissues in the speciation process. The high similarity of proteins between human brain and testis may have clinical relevance. Indeed, the common proteins may be associated with the simultaneously impairment of brain and testis function. The identification of these proteins, along with the analysis of their role in brain and/or testis function, could help in better understanding the pathophysiology of these conditions, as well as in the development of new therapeutic strategies for treating brain or testis diseases.

Supplementary Material

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5427713.

Disclosures and Conflict of Interest

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Chapter 18

Myomatous Erythrocytosis Syndrome: A Case Series

Glaiza S. de Guzman, MD, and Eileen M. Manalo, MD, MSc

Department of Obstetrics and Gynecology,
University of the Philippines, Philippine General Hospital, Philippines

gsdeguzman1@up.edu.ph

Keywords: bilateral salpingo-oophorectomy, body mass index (BMI), erythrocytosis, erythropoietin (EPO), exploratory laparotomy, fibroids, hematocrit, hemoglobin, hysterectomy, jak2 mutation assay, laparotomy, leiomyoma, magnetic resonance imaging (MRI), myoma, myomatous erythrocytosis syndrome, myomectomy, total hysterectomy, uterine fibroids

18.1 Introduction

Leiomyomas or uterine fibroids are the most common solid tumors in women. Women over 45 years of age have an estimated 60% lifetime risk of having fibroids, based on recent longitudinal studies. Associated risk factors include family history, race, age, menopausal status, obesity, and consumption of food additives [1, 2]. Although 40–60% of those affected remain asymptomatic, some present with abnormal uterine bleeding, pelvic pain or pressure symptoms, reproductive dysfunction, and a pelvic mass [3]. Rarely, hemoglobin and hematocrit levels are elevated, owing to ectopic production of erythropoietin.

Thomson and Marson were the first to describe this condition, in 1953. Since then, three criteria have been used to diagnose myomatous erythrocytosis syndrome: isolated erythrocytosis, myomatous uterus, and restoration of normal hematologic values after a myomectomy or hysterectomy [4]. According to a review by LevGur and Levie, fewer than 40 cases of myomatous erythrocytosis syndrome have been reported in literature over the past six decades [5].

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18.1.1 Case Series

18.1.1.1 Case 1

A 46-year-old nulliparous postmenopausal woman consulted with a one-year history of a gradually enlarging hypogastric mass associated with intermittent hypogastric pain. She had congestive heart failure (functional class I) from hypertensive heart disease. She noted occasional exertional dyspnea. Her family medical history was unremarkable. She had had her menarche at 13 years old and subsequent menses had been regular and monthly until the age of 45.

The patient was overweight, with a body mass index (BMI) of 26.3 kg/m^2. She was not plethoric, and systemic physical examination findings were normal. On pelvic examination, the cervix was flushed and deviated anteriorly, and the corpus was enlarged to 26 weeks' size. Abdominopelvic ultrasound showed an enlarged uterus with a large subserous myoma with intramural component (FIGO Grade 5) which measured 28.2 × 22.0 × 6.3 cm at the posterior corpus. Color flow of the mass showed absent vascularity. A complete blood count showed isolated erythrocytosis, with a hemoglobin level of 197 g/L and hematocrit of 0.58. Serum erythropoietin was elevated at 29.53 (normal range: 2.59–18.5). 2D echocardiography showed concentric remodeling of the left ventricle with mildly depressed systolic function and Grade I diastolic dysfunction from chronic hypertension.

The patient was referred to the hematology service to rule out any blood dyscrasia and for preoperative clearance. Jak-2 mutation assay was negative; hence, polycythemia vera was ruled out. She was started on hydroxyurea 500 mg once daily. Venesection was done twice. She was cleared for exploratory laparotomy, and total hysterectomy with bilateral salpingectomy.

On admission, repeat hemoglobin and hematocrit were 213 g/L and 0.62, respectively. Venesection was done every other day to obtain a hematocrit of 0.55. Pelvic magnetic resonance imaging (MRI) showed a well-defined, slightly enhancing mass with smooth margins measuring 20.7 × 20.4 × 12.4 cm (Fig. 18.1) and color flow of the mass showed absent vascularity. The mass exhibited good planes of differentiation from the adjacent structures. The primary consideration was a leiomyoma. The patient underwent surgery on the seventh hospital day.

Intraoperatively, the uterus measured 22.5 × 23.5 × 17.0 cm and had a smooth serosal surface. There was a well-circumscribed mass at the cervicocorporeal junction measuring 18.0 × 18.5 × 12.0 cm (Fig. 18.2). This mass displaced the uterine corpus superiorly. It had a whorled pattern on cut section, with no areas of hemorrhage or necrosis. The endometrium was 0.2 cm thick. The rest of the abdominopelvic organs were grossly normal.

The patient tolerated the procedure well and had an unremarkable postoperative course. She was discharged with normalized values of hemoglobin and hematocrit, at 144 g/L and 0.44.

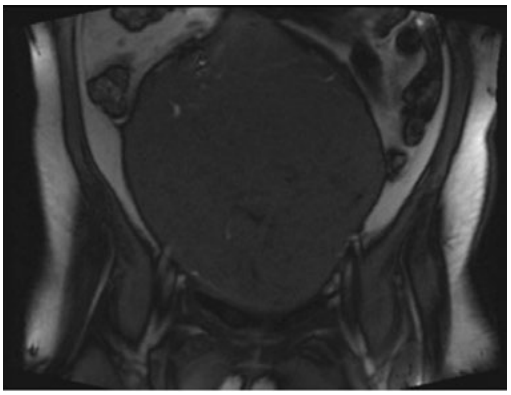

Figure 18.1 Magnetic resonance imaging shows a well-defined, slightly enhancing mass with good planes of differentiation from adjacent pelvic structures.

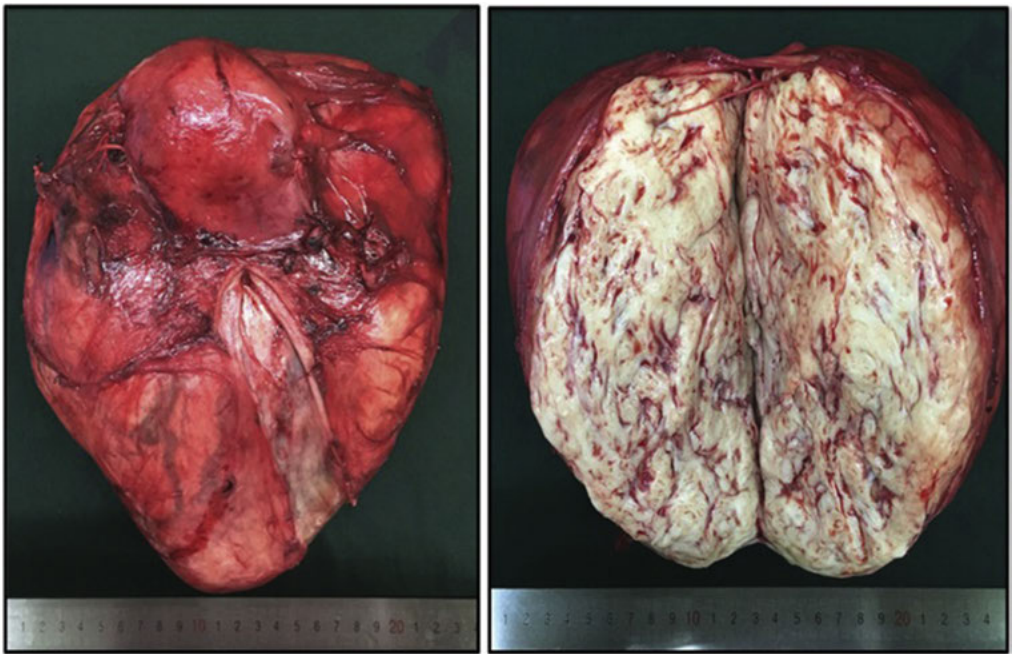

Figure 18.2 There was a well-circumscribed mass at the posterior isthmic area, displacing the corpus superiorly. Cut section showed a whorled pattern.

18.1.1.2 Case 2

A 45-year-old nulliparous postmenopausal woman had a two-year history of a gradually enlarging abdominopelvic mass associated with pelvic pain. She had a 15-year history of uninvestigated primary infertility. Family medical history was unremarkable. Menarche had been at 12 years of age, and subsequent menses were regular and monthly until the age of 44. There was no history of increase in menstrual flow or duration.

On pelvic examination, the corpus was enlarged to 24 weeks' size. The rest of the findings on systemic physical examination were essentially normal. Pelvic ultrasound showed a 22.7 × 21.3 × 20.2 cm well-circumscribed heterogeneous mass at the fundal portion of the uterus, subserous with <50% intramural component (FIGO Grade 6). Color flow mapping of the mass showed absent vascularity. Hemoglobin was shown to be elevated at 184 g/L with a hematocrit of 0.55. Serum erythropoietin was increased to 24.63 (normal range: 2.59–18.5). She was cleared for surgery by the hematology service.

The patient underwent exploratory laparotomy and total hysterectomy with bilateral salpingo-oophorectomy with an unremarkable operative course. Intraoperatively, there was a subserous, lobulated mass at the fundal area measuring 28.0 × 21.5 × 19.0 cm (Fig. 18.3a). The rest of the abdominopelvic structures were smooth and grossly normal. Cut section of the mass showed a trabecular pattern with no necrosis (Fig. 18.3b).

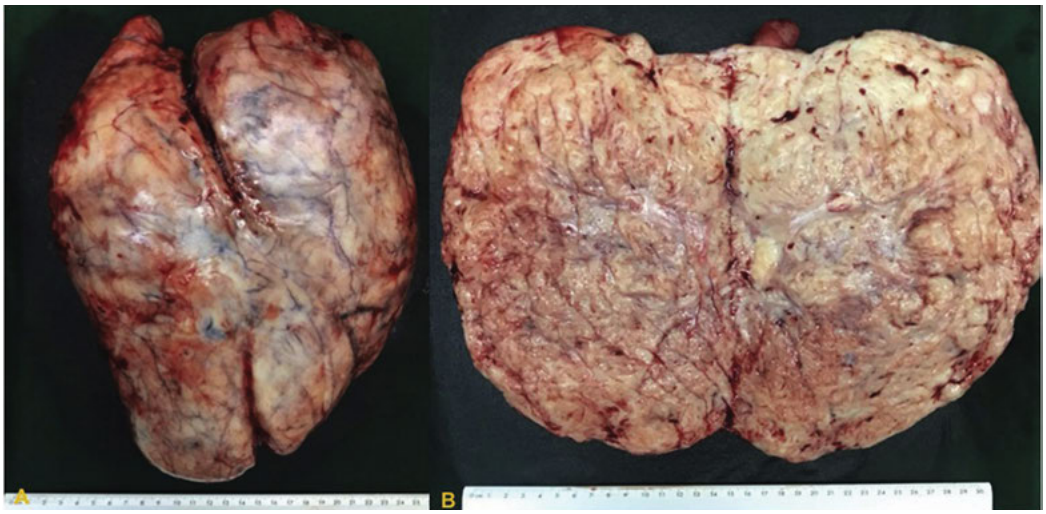

Figure 18.3 A 28.0 × 21.5 × 19.0 cm firm, well-circumscribed mass at the fundal area, subserous. Cut section showed a trabeculated surface with no areas of hemorrhage or necrosis.

18.1.1.3 Case 3

A 27-year-old nulliparous premenopausal woman presented with a three-year history of gradually enlarging abdominopelvic mass associated with pelvic pain. There were no associated menstrual disturbances or bowel movement changes.

She had a BMI of 22.1 kg/m^2. On pelvic examination, the cervix was deviated to the right and posteriorly. The corpus was enlarged to 24–26 weeks' size. Pelvic ultrasound (Fig. 18.4a) showed a well-circumscribed, lobulated heterogeneous mass measuring 34.5 × 14.9 × 8.6 cm at the left anterolateral uterine wall, intramural with subserous component (FIGO Grade 6). Baseline

hemoglobin was 174 g/L while the hematocrit was 0.53. She was cleared by the hematology service for surgery. She was given three doses of GnRH agonist monthly to induce amenorrhea while awaiting elective surgery. The patient was offered ulipristal acetate but had no funds to procure the medication.

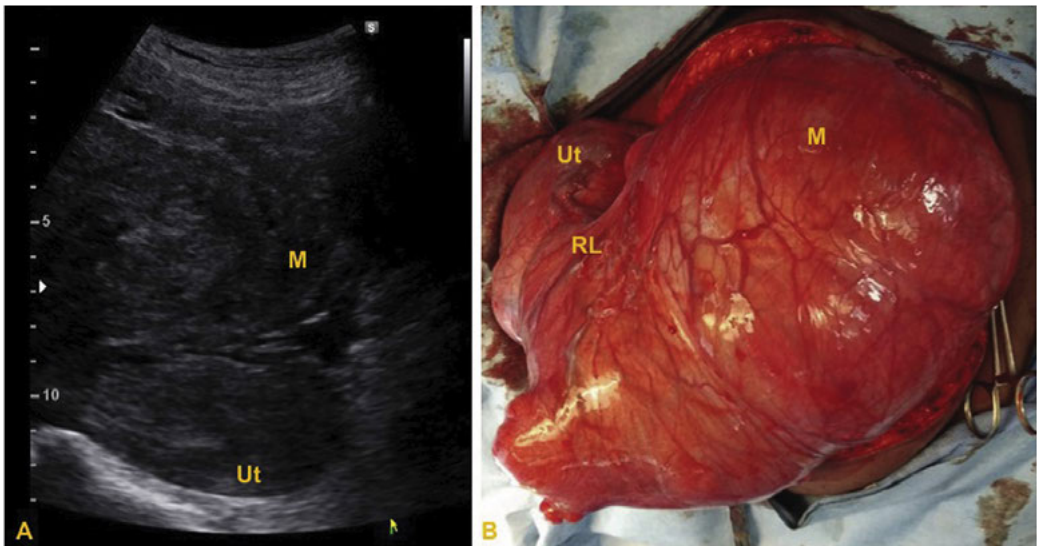

Figure 18.4 (A) Pelvic ultrasound shows a well-circumscribed, lobulated heterogeneous mass at the left anterolateral uterine wall, intramural with subserous component; B: Intraligamentary myoma stretching the round ligament at the left uterine wall. *Abbreviations*: Ut, uterus; M, myoma; RL, round ligament.

The patient underwent open myomectomy. There was a 30.0 × 25.5 × 9.0 cm well-circumscribed mass at the left lateral uterine wall, with a subserous intraligamentary component (Fig. 18.4b). No compromise to the endometrium was noted. Cut section showed a whorled pattern with no areas of necrosis or hemorrhage. Both adnexa were grossly normal. The patient tolerated the procedure well.

18.1.1.4 Informed consent

Informed consent was obtained from all three patients.

18.2 Results

All three cases had histopathologic confirmation of myoma uteri with postoperative normalization of hemoglobin and hematocrit levels. Serum erythropoietin levels returned to normal as well. These cases fulfilled the criteria for the diagnosis of myomatous erythrocytosis syndrome.

18.3 Discussion

18.3.1 Myomatous Erythrocytosis Syndrome

Isolated erythrocytosis has been shown to result from ectopic production of erythropoietin (EPO) in various malignancies, including renal cell and hepatocellular carcinoma. It has been found to occur in benign tumors such as cerebellar hemangioblastoma and adenomas. It is less commonly associated with uterine leiomyoma [4].

Various mechanisms have been proposed to explain this occurrence. One hypothesis is the autonomous production of erythropoietin by the leiomyoma, which is not subjected to any negative feedback mechanism [4]. Suzuki and colleagues documented this through radioimmunoassay studies in 2001 [6]. Epo mRNA expression in the tissue samples was confirmed through reverse transcription polymerase chain reaction. Ectopic erythropoietin production accounts for the large sizes of leiomyoma encountered in this syndrome through its mediation of angiogenesis, mitogenesis, and inhibition of apoptosis [7].

18.3.2 Preoperative Management

The etiology of isolated erythrocytosis must be identified and secondary causes identified in collaboration with hematology services. Secondary causes include chronic respiratory disease and polycythemia vera. The possibility of polycythemia vera or a myeloproliferative disorder should be confirmed through Jak2 mutation testing. Serum erythropoietin levels may likewise be checked with considerations of EPO-mediated entities [8].

Risks for thrombosis, embolizations, and other cardiovascular complications should be considered prior to surgery. Preoperative planning should be undertaken to minimize or avoid these complications and may involve venesection, as was undertaken in case 1. This may be done once or twice weekly to reduce hematocrit levels to below 0.60 [9, 10]. Hemoglobin levels returned to normal immediately after surgery and were maintained on subsequent outpatient visits.

18.4 Conclusions

All reported gynecologic tumors associated with erythrocytosis have been shown to be benign. Awareness of the association between uterine leiomyoma and erythrocytosis is important for patient counseling and appropriate preoperative planning. Various hormonal mechanisms involved in this condition must be explored to guide workup and treatment strategies. To date, these are the first reported cases of myomatous erythrocytosis syndrome in the Philippines.

Disclosures and Conflict of Interest

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Chapter 19

Vascular Involvements in Cholangiocarcinoma: Tips and Tricks

Roberta Angelico,[a] Bruno Sensi,[a] Alessandro Parente,[a,b] Leandro Siragusa,[a] Carlo Gazia,[a] Giuseppe Tisone,[a] and Tommaso Maria Manzia[a]

[a]*Hepatobiliary Surgery and Transplant Unit, Department of Surgical Sciences, University of Rome Tor Vergata, Rome, Italy*
[b]*Department of Hepatobiliary and Pancreatic Surgery, Queen Elizabeth Hospital, Birmingham, UK*

tisone@uniroma2.it

Keywords: *ante situm*, artero-portal shunt (APS), cholangiocarcinoma (CCA), Dacron® (polyethylene terephthalate), distal cholangiocarcinoma (dCCA), Doppler-confirmed intrahepatic arterial flow, *ex vivo*, Gore-Tex®, hilar/perihilar CCA (pCCA), hypothermic perfusion (HP), *in situ*, inferior right hepatic vein (IRHV), inferior vena cava (IVC), intrahepatic cholangiocarcinoma (iCCA), liver transplantation (LT), low-molecular-weight heparin (LMWH), neoadjuvant therapy (NAT), polytetrafluoroethylene (PTFE), portal vein (PV), portocaval shunt (PCS), total vascular exclusion (TVE), vascular involvement, vascular reconstruction

19.1 Introduction

Cholangiocarcinoma (CCA) is a rare cancer, yet the second most common primary liver cancer after hepatocellular carcinoma. The incidence of CCA has been increasing over the past decades, affecting 0.5–2.0 in 100,000 individuals in Western countries and approximately 100 in 100,000 individuals in Thailand, and is becoming a global health problem [1]. CCA arises from the biliary tree and is divided into three subgroups, based on its localisation: intrahepatic CCA (iCCA), hilar/perihilar CCA (pCCA) and distal CCA (dCCA). The anatomical distinction between pCCA and dCCA is represented by cystic duct insertion, whereas iCCA

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emerges from the secondary order intrahepatic bile ducts. Up to 80–90% of CCAs are extrahepatic (pCCA, dCCA), while the remaining 10–20% of lesions are iCCA [2].

Surgical treatment is the gold standard for CCA, but unfortunately, the tumour is frequently diagnosed in late stages due to its asymptomatic course, resulting in unresectable disease at diagnosis. In fact, CCA frequently involves major hepatic vessels, such as the inferior vena cava (IVC) and/or hepatic veins, portal vein (PV) and hepatic artery, which might limit the surgical strategies.

In the past decades, many efforts have been made by the surgical community worldwide to push the boundaries on surgical management of CCA [3]. The surgical indications for CCA have been constantly expanded, and aggressive approaches to CCA involving vascular structures have been pursued with satisfactory outcomes. While iCCA and pCCA share similar surgical approaches involving liver resection, dCCA requires duodeno-pancreatic surgery. Therefore, for the sake of clarity and concision the current review is limited to iCCA and pCCA, and does not cover dCCA.

The aim of this chapter is to review the main surgical techniques described for iCCA and pCCA with vascular involvement, with a particular focus on vascular resection and reconstruction.

19.2 Materials and Methods

A comprehensive review using the PubMed database with the goal to investigate the current management of vascular involvement in CCA was performed. Data were retrieved from published registries, case series and trials reporting surgical outcomes after vascular resection and reconstruction, combined with liver resection, during operations for CCA. Inclusion criteria for this review were as follows: (1) any study (original article, systematic review, case reports, case series, trials) including liver resection for intrahepatic or hilar/perihilar CCA with vascular involvement; (2) studies in which resection and/or reconstruction of PV, hepatic artery, IVC and/or hepatic veins for hilar or intrahepatic CCA were reported; and (3) peer-reviewed manuscripts written in English. The references of each of the selected articles were also evaluated in order to locate additional studies that were not included during the initial search. Reports that included or were mixed with distal CCA were excluded.

The relevant articles were extracted independently by three authors (A.P., B.S., C.G.) who evaluated and excluded duplicates. No specific search dates were used. Consensus for the relevance of an included study were carried out by two senior authors (R.A., T.M.M.) who are experts in the field. Given the heterogeneity of the selected studies and paucity of patients identified within the selection criteria, the results are reported as a narrative review.

19.3 Intrahepatic Cholangiocarcinoma with Vascular Involvement

19.3.1 Oncological Considerations

Vascular involvement in the setting of iCCA can be distinguished into three different subsets: (1) microscopic/microvascular invasion; (2) macroscopic invasion of a major vessel ipsilateral to the lesion; and (3) macroscopic invasion of a major vessel of the contralateral liver, namely the future liver remnant (FLR).

Microvascular invasion is diagnosed histologically after surgical specimen excision and is not predictable preoperatively. The presence of iCCA with microvascular invasion represents a negative prognostic factor; in a large, multivariate analysis study of 1333 patients diagnosed with iCCA, the authors found that vascular invasion was an independent predictor of poor survival (p = 0.011) [4]. The one-, three- and five-year overall survival (OS) rates in patients with or without vascular invasion were 56.8%, 16.5% and 9.4%, and 58.5%, 26.8% and 18.5%, respectively (p = 0.002). Of note, no significant difference in survival was found between patients with macroscopic (n = 106) and microscopic (n = 100) vascular invasion (p = 0.790) [4]. In addition, the same authors analysed the survival rates according to the surgical margin status, demonstrating that when complete tumour resection (R0) is achieved, results are markedly improved. In fact, one-, three- and five-year OS rates in R0 and R1 were 79.1%, 42.6% and 28.7%, and 60.5%, 20.1% and 13.9%, respectively, and fell to 0% at five years after surgery for patients with R2 resection.

Since this challenging surgery is the only option for these patients, the current guidelines of the American Hepato-Pancreato-Biliary Association (AHPBA) and the European Network for the Study of CCA recommend surgery for patients with preoperatively diagnosed macrovascular invasion [2, 3].

Macrovascular invasion of a major vessel of the theoretical FLR has historically been considered a contraindication to resection. In the past, some of these patients were considered candidates for liver transplantation (LT), but the initially reported poor outcomes have established iCCA as a contraindication for transplantation. Nowadays, with the expansion of the concept of "transplant oncology", there has been renewed interest in LT as treatment for CCA, but vascular invasion is still considered an exclusion criterion. Two trials are currently ongoing to investigate whether transplantation might be an option in select patients with local advanced iCCA that has been stable for at least six months after neoadjuvant therapy (ClinicalTrials.gov Identifier: NCT04556214 and ClinicalTrials.gov Identifier: NCT04195503) [5, 6]. Nonetheless, the International Liver Transplant Society consensus defined that LT for CCA should be performed only under strict clinical trials, remaining an option only in the "experimental" setting [7].

Other options have been developed throughout the years to extend the possibility of surgical resection for these patients. In general, these strategies aim

at complete resection of the tumorous mass through vascular resection of the affected vessel, followed by reconstruction to preserve the liver remnant.

iCCA may involve any major vessel in the liver, including "posterior/central" pattern when the tumour extends to the main hepatic veins or IVC, and "hilar" pattern when it invades the portal structures. The latter, however, is more common with pCCA. Major vascular resections can be used successfully to obtain R0 resection in up to 84% of patients [8]. In the largest study to date, morbidity (41.9% vs. 55.5%; odds ratio (OR) = 0.68, 95% confidence interval (CI) = 0.32–1.45) and mortality (7% vs. 8.2%; OR = 1.05, 95% CI = 0.32–3.47) rates have been shown to be comparable between patients undergoing vascular resection (n = 128; 21 IVC resections, 98 PV resection, 9 combined vascular resections) and those treated by standard resection (n = 1087) [9]. In the same study, the median recurrence-free rates and the OS rates were similar between the groups. Another recent study comparing hepatectomies with vascular resection, major hepatectomies and the combination of the two, did not find any differences in terms of postoperative complications among the three groups [10]. Based on these data, current guidelines consider resectability for iCCA as a possibility to completely remove the disease with curative intent (parenchymal free tumour margin, R0) while ensuring an adequate FLR, even when the tumour invades the portal or hepatic veins/IVC (V status) [2, 3]. Furthermore, in recent years, some authors have pushed the boundaries of liver surgery with the first reports of major two-staged resection combined with *ex situ* liver surgery [11, 12].

When iCCA presents with vascular invasion, any of the major involvement patterns has its own significance and treatment. Among these, IVC involvement represents a major surgical challenge. In the literature, iCCA represents an indication for liver and IVC resection in 20–33% of cases [13, 14]. In the last few decades, many studies have demonstrated the feasibility of hepatectomies combined with IVC resection, obtaining R0/V1 resection in most patients [15]. However, morbidity and mortality rates are still high when IVC resection is performed, ranging from 42% to 64% and 4.3% to 19.5%, respectively [13, 16–18]. Post-hepatectomy liver failure (PHLF) and sepsis represent the main causes of death, while complications directly related to IVC resection and reconstruction (e.g., graft infection or thrombosis, lower limb oedema, deep vein thrombosis, pulmonary embolism) were reported in approximately 2.5–5% of cases in a recent review [14]. In particular, the incidence of graft thrombosis after IVC resection/reconstruction is 2.5% [14]. Given the relatively limited data thus far reported, the long-term outcomes of patients undergoing combined liver and IVC resection for iCCA have been reported in only a few studies and range from 11.6% to 37% at five years of follow-up [14]. Although prognosis remains poor, these outcomes should probably be considered acceptable, given the absence of alternative options for cure.

Hepatic vein infiltration also necessitates resection followed by reconstruction, and outcomes result from a very small series with postoperative mortality of approximately 12%, which is comparable to outcomes obtained after IVC resection [19].

In addition, PV resection for iCCA has been specifically investigated in a few studies [9, 20]. Reames et al. reported the largest experience of PV resections (n = 98) and showed no difference compared to standard hepatic surgery [9].

Lastly, hepatic artery resection for iCCA is relatively rarely undertaken and there is a scarcity of literature on this topic.

When this extreme liver surgery is not feasible, neoadjuvant therapy (NAT) should be considered for two reasons: firstly, NAT can convert as many as 53% of patients with previously unresectable disease to secondary resectable disease [21], and secondly, NAT may serve as a selection process, with progression of patients unlikely to benefit from surgery and those with stable or regressing lesions who are viable candidates for extreme resection [22].

19.3.2 Surgical Approaches in iCCA with Vascular Involvement

19.3.2.1 Inferior vena cava involvement

Site and depth of IVC involvement are fundamental for operative planning, as different strategies have been developed for increasingly complex situations. The various approaches are described consequentially, beginning with the "simpler" cases to the most challenging ones, requiring "very complex" resection attempts. Table 19.1 summarises the main studies on the subject.

Table 19.1 Major studies on IVC resection

Study	Number of patients	Major morbidity (%)	Mortality (%)	Survival
Hemming [23]	60	NA	8.00%	35% 5 years OS
Malde [24]	35	NA	11.43%	Median OS 29 months
Nuzzo [15]	23	39.13%	4.35%	NA
Azoulay [17]	22	NA	4.55%	38.30% 5 years OS
Nardo [25]	19	NA	5.90%	Median OS 32 months
Sarmiento [26]	19	NA	5.26%	Median OS 38 months

Abbreviations: NA, not available; OS, overall survival.

Minor IVC involvement below the hepatocaval confluence

Resection of the cancer could necessitate resection of the IVC itself to ensure radicality. When the IVC is involved below the hepatocaval confluence, it is theoretically possible to clamp it by different techniques (Fig. 19.1) without affecting drainage from the liver/FLR, thus minimising hepatic ischaemia time.

If IVC involvement is tangential/minor (<60–120° of its circumference and <2–3 cm longitudinally) [24, 27], it may be possible to side clamp the vein without complete interruption of flow through the vessel (Fig. 19.1A). This situation is highly advantageous as it minimises derangement in physiologic functions, avoiding pre-load reduction, splanchnic stasis and ischaemia to vital organs.

Complete IVC isolation and liver mobilisation is mandatory at the early step of the operation. In only exceptionally large cancers when rotation of the liver from the IVC might be not feasible, a primary anterior approach could be justified and transection of the liver parenchyma may be performed initially to expose the retro-hepatic IVC [23, 28]. With the exposed IVC as the only remaining tumour site, a side-biting clamp could be placed, and thus, it is possible to resect the cancer and anterior IVC wall, completing the hepatectomy. The use of a Pringle manoeuvre to minimise bleeding is legitimate, but not mandatory; however, the ideal balance between parenchymal ischaemia and blood loss may be often difficult to achieve [29].

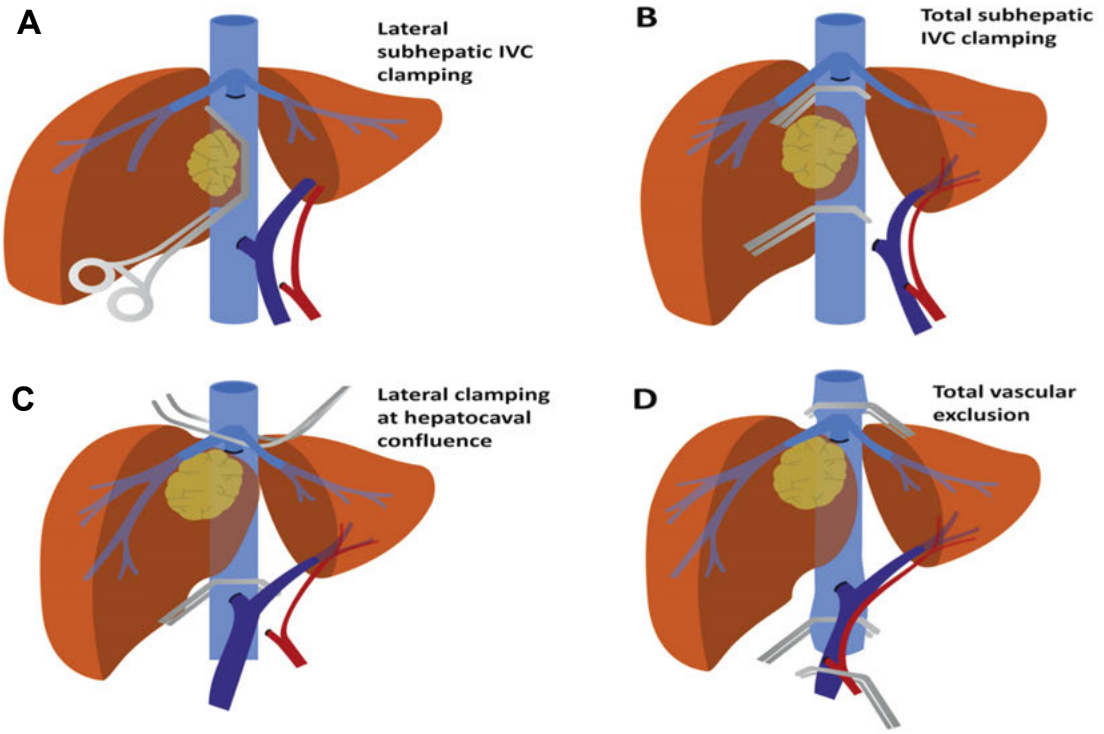

Figure 19.1 Different IVC clamping strategies depending on infiltration site. (A) Lateral IVC clamping below hepatocaval confluence: this strategy can be readily advantageous when involvement is <60–120° circumferentially and <2–3 cm longitudinally. (B) Total IVC clamping below hepatocaval confluence: this permits venous return from the liver and does not require hepatic ischaemia (C) Side clamping at hepatocaval confluence is a strategy to permit venous return from one side of the liver, despite "very high" neoplastic infiltration. (D) Total vascular exclusion necessitates complete IVC clamping above the hepatocaval confluence, concurrent Pringle manoeuvre and hepatic ischemia. *Abbreviation*: IVC, inferior vena cava.

In these cases, vascular reconstruction can be straightforward: if resection affects less than 120° of the IVC wall, it is possible to proceed with direct suture repair without risking excessive luminal narrowing (Fig. 19.2A). Direct repair

may be longitudinal in small or transverse defects to minimise risk of stenosis [27, 29]. In this regard, a recent review investigating the safety and efficacy of IVC reconstruction during liver resection demonstrated that in cases in which tangential resection of the cava was required without IVC replacement (n = 118), primary closure of the IVC defect was possible with direct suturing in up to 86 (72.9%) of the cases, whereas only 32 (27.1%) patients required the use of a polytetrafluoroethylene (PTFE) graft [14].

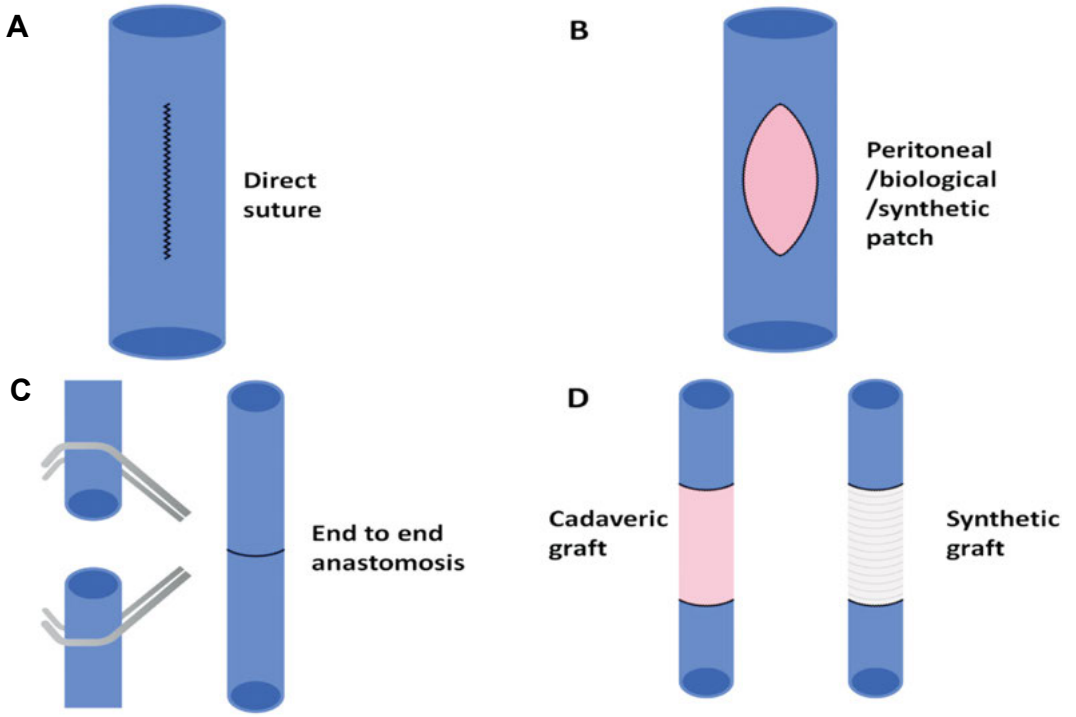

Figure 19.2 Reconstructive options after IVC resection. (A) Direct suture is possible when resection involved less than 120° of the IVC wall. Transverse repair (not depicted) may be used to lower chances of stenosis for long longitudinal defects. (B) Patch repair can be used when less than 180° of the IVC has been resected. Peritoneal, biological and synthetic options exist. (C) End-to-end anastomosis can be fashioned for circumferential IVC resection <3 cm in length. (D) Interpositional grafts are the preferred option for defects longer than 3 cm and autologous, cadaveric and synthetic grafts have been used.

Major IVC involvement below hepatocaval confluence

When IVC involvement is greater than 60–120° circumferentially and/or 2–3 cm longitudinally, side clamping could be impossible and a full IVC clamp might be mandatory. In this scenario, it is appropriate to place clamps above the renal veins and below hepatic veins whenever possible in order to minimise liver ischaemia and splanchnic stasis (Fig. 19.1B).

Although collateral circulation would partly replace IVC flow, porto-systemic shunting should be taken into consideration.

Parenchymal resection is often carried out using an anterior approach, as described in the previous paragraph. Alternatively, some authors have proposed a different technique: after dissection, ligation and division of inflow and outflow vessels of the affected hemi-liver, the IVC is clamped and resected, and after IVC reconstruction and re-established flow, the parenchymal transection might be undertaken in a very controlled setting [30].

In any case, the vascular reconstructive phase is more complex. If less than 180° of the IVC has been resected, patch repair can be fashioned (Fig. 19.2B). In a recent systematic review, patch repair was required in up to 13% of IVC resections [18]. Patch vascular repairs might be performed using: (1) autologous materials, such as the peritoneum or external iliac vein; (2) biological materials, such as bovine pericardium; and (3) synthetic grafts, including Dacron® (polyethylene terephthalate) and Gore-Tex® (PTFE).

A peritoneal patch can be retrieved from the peritoneum anterior to the renal fascia, right hypochondrium, falciform ligament or the anterior abdominal wall (possibly including the posterior rectus sheath), trimmed to fit the defect and sutured in place [31–33]. Pulitano et al. used a peritoneal patch for 21 IVC repairs, reporting no vascular complications related to the patch [31]. The advantages of this technique include very low risk of thrombosis, greater resistance to infections and absence of costs. Sano et al. published their results using an autologous external iliac vein patch, in which they report no thrombosis nor transient oedema of the lower limb as complications [34]. Autologous vein grafts are optimal patches, yet they often require an additional incision, which may lead to complications and increased operative time. Bovine pericardial patches have also been used with good results, although the cost of this modality is high [35].

In many studies, surgeons chose synthetic patches, mainly Dacron® and Gore-Tex® [13, 20, 21]. The disadvantages of synthetic grafts include higher rates of thrombosis and infection, rigidity and cost. Nonetheless, in the largest studies to date, thrombotic events seem very rare and outcomes are acceptable [23, 36]. When a synthetic patch is used, anticoagulation drugs are commonly adopted, but regimens used are highly variable. In-hospital anticoagulation with low-molecular-weight heparin (LMWH) or heparin infusion seems to be the most common peri-operative management [11, 24, 32, 37]. Long-term anticoagulation with LMWH or warfarin and the treatment duration to prevent graft thrombosis are still controversial [14]. Potential benefits may exist for patients with previous venous thromboembolism, coagulopathy due to massive intraoperative bleeding and transfusion, large tumours or undergoing re-implantation of hepatic or renal veins [14]. Currently, there is no evidence to recommend one material for repair over another.

When more than 180° of the vascular wall needs to be sacrificed, complete resection of the infiltrated segment of IVC is required, followed by IVC reconstruction, which might be performed by different techniques.

The first reconstructive option is the primary anastomosis, which has been described for small resections <3 cm in length [23] (Fig. 19.2C). The advantages of reconstruction by primary anastomosis include the rapidity of performing a single anastomosis, no further dissection in other districts (required for autologous graft), no antigenicity (occurring with cadaveric graft) and avoiding the use of synthetic grafts, which increases thrombotic and infectious risks, as well as cost effectiveness. However, primary anastomosis might be associated with the possible disadvantage of developing tension on the anastomosis, with potential narrowing.

When the direct primary anastomosis of IVC is not suitable, an interpositional graft might be required (Fig. 19.2D). Synthetic grafts have been extensively used and demonstrated to be very reliable [13, 23]. Alternatively, the resected IVC may be substituted by autologous conduits obtained by the iliac vein or peritoneum; however, although generally safe, a few reports of thrombosis with these grafts (probably due to the calibre difference between IVC and graft) have emerged in the literature [38, 39]. Papamichail et al. described IVC reconstruction using cadaveric IVC graft without post-operative vascular complications [18]. For other hepatic tumours invading the IVC, aortic grafts from deceased donors were also adopted, with the possible advantage of reducing the risk of vessel collapse due to its thickness [40]. Moreover, cadaveric grafts have the possible advantage of not requiring long-term anticoagulation therapy [41].

In some cases, although the tumour does not directly involve the hepatocaval confluence, it might be necessary to clamp the IVC above the hepatic veins. This is achieved through successive orderly clamping of the infra-hepatic IVC, hepatic hilum and supra-hepatic IVC, obtaining total vascular exclusion (TVE) (Fig. 19.1D). Given the significant influence on cardiac pre-load, in some cases, it might be necessary to perform a portocaval shunt (PCS) in order to maintain haemodynamic stability. In this setting, haemodynamic tolerance to TVE has been defined as a decrease in mean arterial pressure of at least 30% or a decrease in cardiac index >50% [17]. When TVE is predicted to be in place for long periods of time, systematic veno-venous bypass set-up should be strongly recommended (the fashioning of veno-venous bypass is described in the following paragraph). A threshold of 60 min of TVE is cited in most studies as being an indication for veno-venous bypass, reducing blood loss and respiratory complications [42]. One study on TVE found maximum diameter of the lesion, preoperative PV embolisation and planned vascular reconstruction as independent predictors for TVE > 60 min [37]. Physiological circulation is restored by de-clamping the supra-hepatic cava, followed by the infra-hepatic cava, and finally, the PV and hepatic artery.

In a systematic analysis of liver and vena cava resection with or without TVE, without the use of perfusion strategies, operative mortality was 8% (nine of 111 cases) and graft patency was 98.2%. The liver can tolerate TVE

for a limited amount of time and when complex surgery lasting more than 60–90 min is predicted, an effort to minimise ischaemic damage should be sought [43].

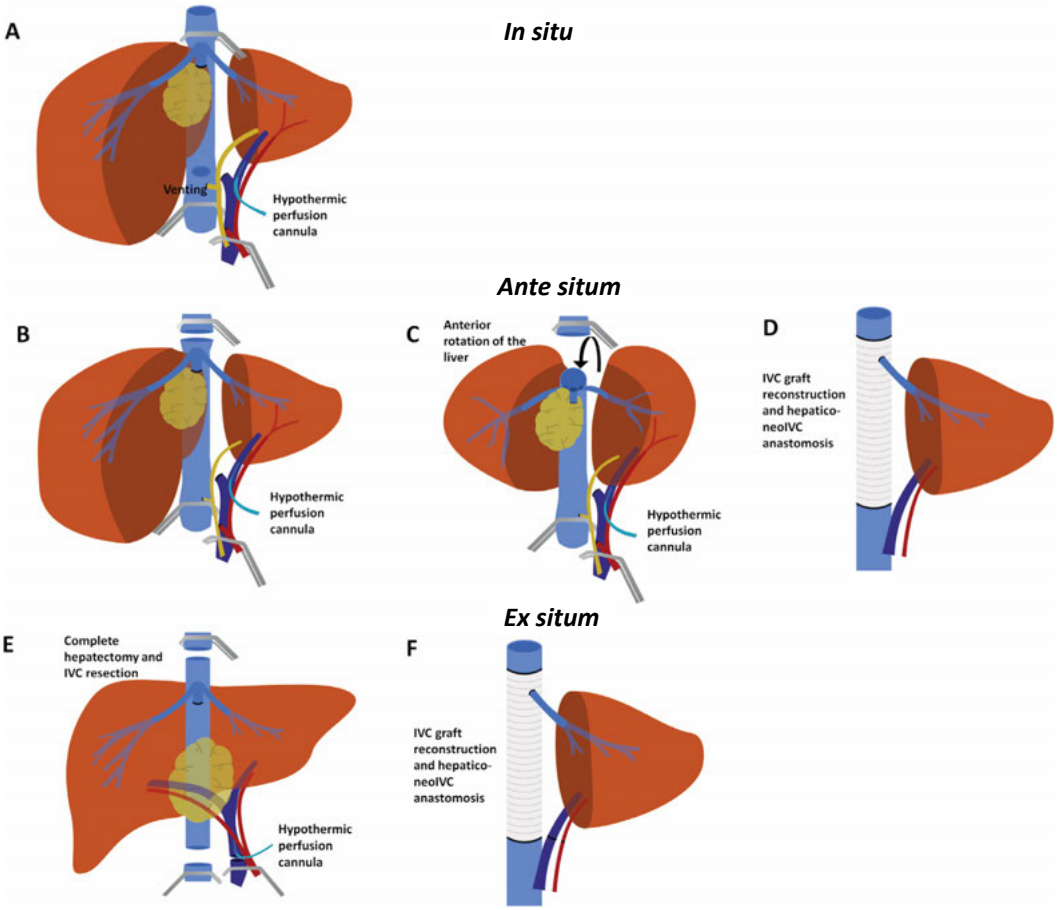

Figure 19.3 Hypothermic perfusion strategies. (A) *In situ* hypothermic perfusion: total vascular exclusion is in place and a perfusion cannula is inserted in the portal vein. A cavotomy is also performed, to be used for venting. (B) *Ante situm* technique: total vascular exclusion is in place, hypothermic perfusion is used and the vena cava has been sectioned below the superior clamp. (C) The liver can now be rotated anteriorly towards the surgical team, to perfectly expose the caval plane. (D) Resection is completed and reconstruction requires grafting of the IVC and hepatic vein re-implantation. (E) *Ex situ* technique: TVE and hypothermic perfusion are followed by PV, hepatic artery and biliary division and subsequent complete hepatectomy with IVC resection. (F) Reconstruction requires IVC grafting and portal and arterial anastomosis. Biliary anastomosis is also required (not depicted).

A "trick" to reduce TVE time and liver ischaemia when reconstructing the IVC is to "slide down" clamps below the hepatocaval confluence as soon as possible (for instance, right after the supra-hepatic anastomosis is fashioned, before construction of the inferior anastomosis [17, 30, 44]). Other

authors have described the possibility of switching from TVE to oblique clamping of the IVC after transection of the parenchyma if the reconstruction involves only part of the vena cava and can be performed with a patch, obtaining good results in minimising TVE time [45] (Fig. 19.1C).

To minimise liver ischaemic damage during TVE, in 1974, Fortner et al. described *in situ* hypothermic perfusion (HP) [46] (Fig. 19.3A).

This technique warrants TVE, followed by *in situ* perfusion of the liver with a hypothermic cytoprotective solution combined with cooling of the organ surface. Ringer's lactate solution, chilled to 4 °C and with 5 mg/L heparin, was used. HP is delivered through cannulae inserted in the portal system distal to the occluding clamp. Cannulation can occur either through the main PV, or more conveniently, through the contralateral PV, which will be resected with the specimen. The original description also included arterial HP through the gastroduodenal artery, although this is not considered strictly necessary today. The perfusate is then drained through a cavotomy, which is usually placed just superior to the inferior caval clamp. *In situ* HP permits longer ischaemia times and gives the opportunity for a meticulous and careful approach to the difficult resection. Before re-perfusion, flushing at room temperature with a potassium-poor solution is recommended. The HP procedure seems to be effective in increasing hepatic tolerance to ischaemia. In one study, TVE with HP resulted in significantly lower liver function test peaks during the postoperative period when compared to TVE without HP, and especially when TVE without HP was prolonged for more than 60 min [37]. In this same report, there were no differences in mortality, although the cohort might have been too small to highlight any effect (20 vs. 49 patients). Nonetheless, mortality and morbidity of this extreme surgery remain high (Table 19.2). In the largest series to date, Azoulay et al. describe 19.5% mortality and 27.3% major morbidity [13]. The predictors for 90-day mortality were Charlson Comorbidity Index > 3 [47], tumour size >10 cm and the 50/50 criteria on postoperative day 5 (which included prothrombin time <50% and serum bilirubin > 50 μmol/L or 2.92 mg/dL) [48]. The one-, three- and five-year OS rates were 67%, 42% and 11.6%, respectively [13].

Table 19.2 Major studies on *in situ* hypothermic perfusion technique (excluding case series with less than 5 cases or where technique-specific outcomes were not available)

Study	Number of patients	Major morbidity (%)	Mortality (%)	Survival (%)
Fortner [46]	29	NA	10.34%	59.09% at 30 months
Azoulay [13]	77	27.30%	19.48%	30.40% at 5 years
Navez [42]	27	40.10%	18.52%	NA

IVC Involvement at hepatocaval confluence

This complex situation demands TVE and challenging hepatocaval reconstruction might be required. For this reason, the use of HP is often contemplated and veno-

venous bypass should be considered. Furthermore, to obtain sufficient IVC length for clamping, resection and anastomosis, the surgeon may need to use the intrathoracic portion of the vein. The latter can be accessed either by an intraabdominal, trans-diaphragmatic approach or by median sternotomy.

A veno-venous bypass should be planned preoperatively, entailing percutaneous femoral and jugular catheter placement and intraoperative cannulation of the inferior mesenteric vein (which is ligated at procedure's end). Some groups have proposed the immediate reconstruction of the IVC with a temporary PCS and without bypass [49]. Other authors used this technique without PCS, while others did not reconstruct the IVC immediately or used bypass; only a temporary PCS was utilised [50, 51].

In these circumstances, HP and complex vascular resection/reconstruction can be performed in three ways: *in situ*, *ante situm* (*ex situ in vivo*) or *ex vivo*. The *in situ* approach is convenient when the hepatic vein of the FLR does not require resection and re-implantation in the neo-cava; the other two techniques (*ante situm* and *ex vivo*) possess specific advantages when all hepatic veins are involved, along with the IVC.

The *ante situm* operation was first described in 1991 by Hannoun [52] (Fig. 19.3B–D). This technique entails complete mobilisation of the liver and vena cava, TVE and HP through the PV, followed by sections of the hepatic veins or the vena cava itself [40]. This allows complete rotation of the liver (*ex situ*), giving full access to the posterior liver, cava and hepatocaval confluence, while preserving the hepatic pedicle (*in vivo*) and reducing the risk of vascular complication into the hilum vessels, especially to the hepatic artery. In this way, the surgeon gains operative access to a region otherwise particularly difficult to approach, representing a critical step in the operation, especially when complex resection and reconstruction are required. The *ante situm* technique seems to be a valid option, with mortality ranging from 0% to 14%, depending on the few available series [16, 52–54]. In 2018, Ye et al. reported their extensive series with this technique, reporting only one death (4.3%) out of 23 patients, stressing how poor outcomes can be minimised with experience [16].

The *ex vivo* approach, described by Pichlmayr in 1990, requires all the steps used in the *ante situm* approach, followed by careful section of the hepatic pedicle and excision of the liver from the patient's body [55] (Fig. 19.3E,F). Resection is then carried out on the "back table", permitting complex vascular resection and reconstruction in a most convenient setting. Once *ex vivo* resection is completed, autotransplantation of the remnant liver is required, generally performing vascular anastomoses in the same order as for LT (suprahepatic cava, infrahepatic cava, portal, arterial and biliary). The main advantage of the *ex vivo* procedure is maximal control over resection and reconstruction. The disadvantages include the need to re-implant, making the procedure a true autotransplantation. However, in cases in which both hilar and outflow structures are compromised, this might be the only surgical option.

The results of pioneering studies, even from highly experienced centres, have shown a prohibitively high mortality rate of 32% [49]. Nonetheless, in

a recent meta-analysis of 244 patients treated with *ex vivo* surgery, complete tumour resection was achieved in 98% and 30-day mortality was 8%, not far from the results of "ordinary" liver resection [56]. However, due to the possible complications of this complex procedure (i.e., thrombosis, pulmonary embolism, renal impairment), the 90-day mortality would probably better reflect the outcomes of this type of surgery, rather than the 30-day timepoint.

Despite this, there is no evidence to support one HP technique over another, and the choice should be selected according to the tumour size and position, vascular involvement and the surgeon's skills. Shen et al. reported the only study comparing the *in vivo* and *ex vivo* techniques in 71 patients with end-stage hepatic alveolar echinococcosis involving the IVC [27]. In their series, 26 patients in the *in vivo* group and 45 patients in the *ex vivo* group had similar postoperative morbidity (26.9% vs. 24.4%) and mortality (11.5% vs. 6.7%). In this retrospective study, patients undergoing *ex vivo* procedures had larger lesions, most frequently involving the portal structures, and underwent lengthier procedures with longer periods of ischemia, requiring more blood products. Overall results were very promising, yet this group of patients suffered from a benign disease that permitted conservation of large percentages of liver parenchyma, featuring low rates of postoperative liver failure.

Only a few cases of vena cava resection without reconstruction have been reported, possibly due to the extensive collateral circulation that forms after complete obstruction of the IVC by the mass [27].

19.3.2.2 Hepatic venous involvement

When iCCA invades the contralateral hepatic vein, its resection and reconstruction is the only option to achieve complete tumour excision. The surgical approach includes complete mobilisation of the liver from the vena cava, acquisition of inflow and outflow control (without necessarily clamping the vessels) and parenchymal transection until the involved hepatic vein is met. A clamp is then placed at the origin of the hepatic vein and resection is accomplished with completion of parenchymal transection and sectioning all hepatic veins. When the hepatic veins are involved very close to the IVC, *ante situm* or *ex situ* procedures may be necessary, with or without HP [19, 57–59]. In any case, the reconstructive phase necessitates TVE of the liver and HP (as described above), as well as porto-systemic or portocaval shunting, which can be considered depending on the time needed for reconstruction [36, 58]. Reconstruction of the hepatic vein may include a patch, or anastomosis to the IVC or an IVC prosthesis [19, 33, 58]. Options for reconstruction include direct anastomosis, synthetic grafts, cryopreserved vein grafts or autologous grafts. Direct anastomosis is the ideal method when there is enough hepatic vein length to technically perform the anastomosis, but in most cases, a graft is needed. Much of the discussion on the different kind of grafts mirrors the considerations made for IVC grafts. Two further interesting options have been reported for hepatic vein reconstruction: jugular vein procuring or use of a PV graft harvested from the resected hemi-liver [19, 59, 60]. Although

this surgical procedure may be complex and morbid, results are generally favourable, with a reported 12% mortality rate [19].

In some cases, alternatives to resection and reconstruction exist and can be pursued. For example, an inferior right hepatic vein (IRHV) can be present in up to 25% of livers [61]. In these patients, the IRHV may be enough to drain the liver, bypassing the necessity of right hepatic vein reconstruction/re-implantation after its resection with the involved IVC. Therefore, careful study of preoperative computer tomography can assist in the search for less morbid solutions. If flow through the IRHV is not convincing, embolisation of the proper right hepatic vein may allow increased transit and optimal postoperative results [62]. Other authors performed reconstruction of the middle hepatic vein when the FLR volume was <40% of total liver volume to decrease hepatic congestion and induce a volume increase [63].

19.3.2.3 Central/hilar involvement

Surgical techniques used for iCCA involving hilar structures are the same as those used for pCCA (described in the following section).

19.4 Perihilar Cholangiocarcinoma with Vascular Involvement

19.4.1 Oncologic Considerations

Due to the tight anatomical relationships, vascular infiltration from pCCA usually indicates involvement of large portal branches or the main PV trunk and/or arterial involvement. Van Vugt et al. studied the prognostic significance of vascular involvement in 674 patients with pCCA [64]. Unilateral PV involvement did not affect median survival, while main PV or bilateral portal involvement was associated with reduced survival in a univariate, but not multivariate, analysis. Hepatic arterial involvement significantly reduced survival, whether unilateral or bilateral/main, and was confirmed to be an independent prognostic risk factor by multivariate analysis.

Extent and laterality of hepatectomy are mainly determined by neoplastic biliary extension. Contralateral vascular infiltration has been considered a contraindication for surgery for many years. This paradigm has changed in the last two decades, with many investigators reporting acceptable outcomes for vascular resection and reconstruction for pCCA. Nonetheless, current guidelines do not recommend routine vascular resection due to doubts that the benefits justify the significant surgical morbidity/mortality, and counsel that these operations should be undertaken only in the most experienced hepatobiliary centres [2, 65].

Today, PV resection is performed with relative frequency in high-volume centres, where it is included in as many as 35% of operations for pCCA [66].

When PV resection is performed, mortality rates are approximately 3–5%, which does not differ from standard major liver resection, although rates as high as 12% have been reported [67, 68–70]. A recent meta-analysis considering only very large volume institutions [71] reported similar mortality rates of resected pCCA with or without PV resection. PV resection permits the achievement of R0 resection in approximately 85% of patients. This is of relevance considering that R0 resection can double and triplicate survival, compared to R1 and R2, respectively [67]. In a multi-centre study by De Jong et al., long-term survival offered by PV resection was similar to those experienced by patients who underwent standard hepatectomy [67]. The results of main studies on PV resection are summarised in Table 19.3.

Table 19.3 Major studies on portal vein resection for pCCA

Study	Number of patients	Major morbidity (%)	Mortality (%)	Survival
Ebata [70]	52	2.90%	9.60%	9.90% 5 years OS
Miyazaki [72]	34	38.00%	8.82%	16.00% 5 years OS
Song [73]	51	NA	9.80%	22.80% 5 years OS
Hemming [69]	42	NA	2.0%	NA
Tamoto [74]	36	58.33%	2.78%	Median OS 20.5 months
Neuhaus [75]	50	NA	NA	58.00% 5 years OS
Wang [76]	16	37.50%	0.00%	Median OS 20 months
Molina [77]	23	NA	22.00%	NA
Schimizzi [78]	31	47.37%	15.79%	Median OS 24 months
Mizuno [68]	157	48.00%	3.00%	Median OS 30 months
Kim [79]	35	34.30%	2.90%	37–70% 5 years = S
Kuriyama [80]	31	33.33%	3.40%	37.60% 5 years OS

In a more recent single centre study, 303 of 787 patients undergoing surgical resection underwent vascular resection (either portal or arterial). The median OS was significantly shorter in the vascular resection group, compared to the standard resection group (30 vs. 61 months; $p < 0.0001$) [68]. Nonetheless, survival was significantly longer compared to patients who did not undergo resection (30 vs. 10 months; $p < 0.001$). Similar results were presented in a meta-analysis evaluating the outcomes of PV resection for pCCA [81]. In general, PV resection may offer long-term survival to these patients without additional morbidity/mortality. Based on this, Neuhaus et al. [75] advocated for routine "in principle" *en bloc* hilar resection with PV resection associated with left hepatectomy. This procedure was also named the "no-touch" technique and its long-term results are promising, since the one-, three- and five-year OS after hilar *en bloc* resection were 87%,

70% and 58%, respectively, which was significantly higher than after conventional major hepatectomy (p = 0.021) [75]. Unfortunately, these outstanding results were not replicated elsewhere [74, 82].

Arterial resection for pCCA is still a source of debate, as the first reports that appeared in 2000 and initial results were extremely discouraging. In 2007, Miyazaki et al. [72] published their series of pCCA resections with and without vascular resection, including nine arterial resections. As the intra-operative mortality was 11% and in-hospital mortality was 33%, the authors concluded that arterial resection was not justified due to high morbidity and little (if any) benefit. In 2010, Nagino et al. published their series of 50 consecutive patients undergoing simultaneous PV and arterial resection, reporting a morbidity rate of 50% and mortality rate of 2%, a R0 rate of 66% and five-year survival of 30% [83]. A recent update included 146 arterial resections with a 4% mortality rate and a median OS of 34 months [68]. In this series, no difference in survival rate was detected between patients undergoing portal or arterial resection.

In 2018, another Japanese group [71] proposed a new approach to Bismuth type I/II pCCA. The latter, as it often invades the right hepatic artery, is usually resected with a right hepatectomy and caudate lobectomy. However, some of these patients' FLR may have been too small and not sufficiently increased with portal vein embolisation (PVE). In these cases, the authors described the feasibility of a left hepatectomy plus caudate lobectomy with concomitant resection and reconstruction of the right hepatic artery. In this series, 12 patients who underwent left hepatectomy with caudate lobectomy and right arterial resection were compared to 24 patients who underwent right hepatectomy; morbidity and mortality were similar, no reconstruction-related events occurred and long-term outcomes were comparable [71]. Hepatic artery resection is not yet a standard option, but the Nagoya experience has proved its feasibility and its benefits, including long-term survival.

Although LT is not yet an established option for iCCA, this procedure is a codified strategy with satisfactory outcomes in select patients with pCCA [84]. In particular, patients with unresectable pCCA (either due to locally advanced tumour with extensive vascular and/or biliary invasion precluding complete resection or because of poor hepatic function reserve predisposing the patient to post-hepatectomy liver failure) undergo neoadjuvant chemoradiation followed by transplantation, with a 5-year disease-free survival of 65% (although drop-out rates are still high, around 50%) [7, 84]. The transplant team should be prepared for arterial and venous jump grafts for hepatic artery and PV reconstructions [7]. In this context, PV encasement and perivascular invasion have been identified as risk factors for recurrence [85, 86].

Involvement of the hepatic veins or IVC by pCCA is quite rare. When this does happen, it may be associated with concomitant hilar vascular invasion, forcing *ex situ* and autotransplantation techniques; however, the scarcity of reported cases does not permit evaluation of outcomes.

19.4.2 Surgical Approaches in pCCA with Vascular Involvement

19.4.2.1 Inferior vena cava/hepatic vein involvement

The surgical techniques used for pCCA involving the IVC or hepatic veins are the same used for iCCA (described in the preceding section).

19.4.2.2 Portal vein involvement

As pCCA develops near the PV bifurcation, both the confluence and left (LPV) and right (RPV) branches can be involved in the disease process.

When the LPV is involved in a patient undergoing right hepatectomy, its resection and reconstruction are necessary. This reconstruction is usually straightforward as the LPV has a long extrahepatic course. Resection can be performed indifferently either before or after parenchymal transection. Most of the time, it is possible to reconstruct it by direct end-to-end anastomosis [70] (Fig. 19.4A,B). Otherwise, grafts can be used (i.e., autologous iliac/jugular veins, cadaveric iliac veins, synthetic grafts).

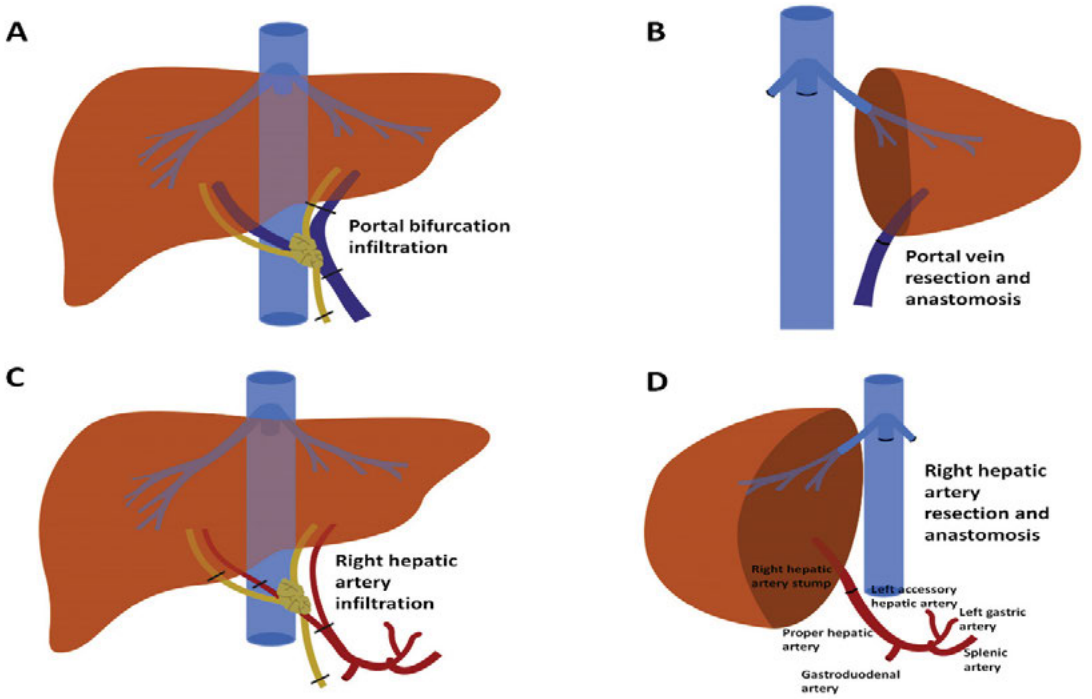

Figure 19.4 Resection and reconstruction for hilar cholangiocarcinoma involving portal vein or hepatic artery. (A) Hilar cholangiocarcinoma involving the portal bifurcation. (B) Resection has been performed, with portal end-to-end anastomosis. (C) Hilar cholangiocarcinoma involving the right hepatic artery. (D) Resection and reconstruction with end-to-end anastomosis between the right hepatic artery stump and the proper hepatic artery. Reconstruction is also possible using a rotating arterial graft with any of the named arteries in the picture. Finally, reconstruction with a radial artery graft or a saphenous vein graft is also possible (not depicted).

In the case of RPV involvement, given its shorter length, reconstruction could be technically challenging. Direct anastomosis is rarely possible and if resection is conducted up to the first-order branches, reconstruction may then involve an autologous or cadaveric iliac Y-graft. During extended left hepatectomy, the main PV and right posterior branch could feature a large size discrepancy. For these reasons, wedge resections are more commonly attempted on the right side, followed by direct suture closure or patch closure (using cadaveric vein/peritoneum) [33, 70, 87]. Ebata et al. reported direct closure to be appropriate in most cases (90%) [70].

The "no-touch" technique or "*a priori* en bloc hilar resection" proposed by Neuhaus et al. entails right hepatectomy with systematic PV resection and reconstruction [75, 88]. Hilar structures are dissected as little as possible: the right hepatic artery is divided just after its stemming from the proper hepatic artery, the main PV is sectioned away from the tumour and the LPV is divided just after its entering of the umbilical fissure. In this way, hilar dissection is largely avoided. Reconstruction is then performed, mostly with direct end-to-end anastomosis.

19.4.2.3 Hepatic artery involvement

Hepatic arterial involvement in pCCA is much more common on the right side, since the right hepatic artery is normally located just beneath the common bile duct in the hilum, whereas the left hepatic artery usually travels at a distance. Arterial resection is usually performed last, just before excising the specimen. Nonetheless, it can be performed early, as long as the main principle is respected: immediate reconstruction, minimising liver remnant ischaemia time. De Santibañes et al. claimed pre-excisional arterial resection and reconstruction to provide the major advantage of giving the surgeon an opportunity to halt the operation before transection is begun, if satisfying reconstruction cannot be achieved [89]. End-to-end direct arterial anastomosis is the most common reconstruction method [68] (Fig. 19.4C,D). In the largest single-centre series, end-to-end anastomosis was possible in 59% of cases [68]. In three of 89 cases (3.3%), end-to-end anastomosis "failed" intraoperatively due to thrombosis/insufficient blood flow and a secondary reconstruction method had to be used. Other reconstructive options entail the use of a rotating artery or an interpositional graft. Rotating grafts make use of arteries conveniently located nearby, possibly with the most adequate calibre. For this purpose, any of the following vessels are appropriate: gastroduodenal artery, left hepatic artery, left gastric artery, right gastric artery or splenic artery (Fig. 19.4D). Alternatively, a jump graft between the proximal and distal cut ends of the hepatic artery can be fashioned. Interpositional grafts are commonly retrieved from the radial artery and greater saphenous vein. Comparative results of direct anastomosis vs. rotating artery or interposition grafts are not currently available.

Hu et al. describe a case series in which reconstruction was not attempted if ischaemic demarcation was not seen intraoperatively upon clamping/resection [90]. In this study, 29 patients with arterial resection without reconstruction

were compared to 34 patients with arterial resection and reconstruction. There were no differences in major postoperative complications, mortality or long-term survival among the two groups. Peng et al. analysed results from 26 patients who underwent arterial resection without reconstruction and three who underwent standard left hepatectomy, and increased morbidity or mortality were not reported in patients undergoing arterial resection [91]. When this strategy is considered, right lobe mobilisation should be minimal to preserve collateral circulation from the diaphragmatic and retroperitoneal arteries. Furthermore, to enhance chances of success, Yasuda et al. described preoperative proper hepatic artery (or both sided) embolisation, increasing collateral flow, three weeks prior to left hepatectomy [92].

When arterial reconstruction is not deemed possible, and reconstruction is not an option (in cases of absent collateral circulation, absent Doppler-confirmed intrahepatic arterial flow or ischaemic change in colour), an artero-portal shunt (APS) could be performed as a salvage procedure. This procedure involves fashioning of an end-to-side anastomosis between the common hepatic artery and main PV [93], re-establishing sufficient hepatic oxygenation. The shunt can be radiologically obliterated later if collateral formation is demonstrated. Noji et al. investigated whether the APS procedure could serve a primary role as an alternative to hepatic arterial reconstruction [94], as the authors reported acceptable results of APS, but with significantly increased liver abscess formation compared to patients undergoing arterial anastomosis, and concluded that APS should be reserved as a salvage procedure when arterial anastomosis is not possible.

19.4.2.4 Combined portal and arterial involvement

When both the PV and artery are involved, resection and reconstruction of both can be undertaken. When both are resected simultaneously, the portal anastomosis is generally performed first [68]. When feasible, one vessel should be resected and reconstructed at a time while the other is patent, maintaining perfusion of the parenchyma and limiting ischaemic damage.

19.5 Conclusions

Radical surgical treatment remains the only curative option for CCA with vascular involvement. For CCA requiring complex vascular resection and reconstruction, the recent advantages of surgical techniques and satisfactory outcomes, in terms of complete tumour excision, justify the aggressive surgical approaches in selected patients. However, these surgical procedures might be extremely complex, yielding elevated risks, and continue to present fairly high mortality and morbidity rates. Therefore, careful evaluation by experienced hepatobiliary surgeons in a multidisciplinary setting is highly recommended in order to achieve favourable outcomes.

Abbreviations

AHPBA:	American Hepato-Pancreato-Biliary Association
APS:	artero-portal shunt
CCA:	cholangiocarcinoma
CI:	confidence interval
dCCA:	distal CCA
FLR:	future liver remnant
HP:	hypothermic perfusion
iCCA:	intrahepatic CCA
IRHV:	inferior right hepatic vein
IVC:	inferior vena cava
LMWH:	low-molecular-weight heparin
LPV:	left portal vein
LT:	liver transplantation
NAT:	neoadjuvant therapy
OR:	odds ratio
OS:	overall survival
pCCA:	hilar/perihilar CCA
PCS:	portocaval shunt
PHLF:	post-hepatectomy liver failure
PTFE:	polytetrafluoroethylene
PVE:	portal vein embolisation
PV:	portal vein
RPV:	right portal vein
TVE:	total vascular exclusion

Disclosures and Conflict of Interest

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Chapter 20

Rehabilitation for People Living with Dementia: A Practical Framework of Positive Support

Linda Clare, PhD, ScD

Centre for Research in Ageing and Cognitive Health, College of Medicine and Health, University of Exeter, Exeter, UK

l.clare@exeter.ac.uk

Keywords: Alzheimer, cognitive impairments, cognitive rehabilitation, dementia, neurodegenerative conditions, nonpharmacological intervention, psychotherapy

Awareness of the need to improve accessibility of services and opportunities for people with disabilities is growing, but people with "hidden" disabilities such as dementia can be excluded from these developments. Conceptualizing dementia in terms of social disability highlights the way in which symptoms such as memory problems—and the secondary effects of these, such as loss of confidence or negative reactions from others—affect the possibility of engaging in activities and participating in society [1]. It also suggests some practical solutions that can support participation and inclusion and promote the ability to live well [2]. Activity limitation and participation restriction can be tackled from two directions. From a community perspective, the focus is on dismantling external barriers to participation by changing public attitudes and creating accessible, dementia-friendly environments. A growing social movement led by people with dementia, Alzheimer associations, and supporters promotes acceptance, inclusion, and awareness of rights [3]. From a personal perspective, the focus is on enabling people with dementia to participate in everyday life, and in their families and communities, in a way that is meaningful to them. This is the aim of rehabilitation [4].

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20.1 Why Is Rehabilitation Relevant?

The experience of disability confers a right to rehabilitation for people living with dementia. The United Nations Convention on the Rights of Persons with Disabilities outlines the right of people with disability to be able to attain and maintain maximum independence, with the assistance of comprehensive rehabilitation services (Article 26(1)) [5]. We are used to thinking of rehabilitation in terms of physical rehabilitation following injury, but it is equally relevant for people with cognitive, rather than physical, impairments. This includes people whose impairments result from long-term, progressive neurodegenerative conditions. The rehabilitation of people with cognitive impairments is called cognitive rehabilitation [6]. In community settings, this approach may be called reablement or restorative care, and from a public health perspective, it can be considered synonymous with tertiary prevention. These concepts share similar aims [7], and for convenience, I will use the term "rehabilitation" here. Rehabilitation provides both a set of guiding principles to shape a model of service provision and a coherent practical framework for supporting people with dementia and their families [8].

20.2 How Can Cognitive Rehabilitation Benefit People with Dementia and Carers?

A rehabilitation-focused service would be organized around key principles of enabling people to function optimally in the context of their intrinsic capacity and current health state. This means ensuring that people are as independent as they wish to be, have as much control as possible over daily life, have opportunities to engage in meaningful roles and activities, and are able to integrate the changes they experience into a coherent and enduring sense of identity. The rehabilitation philosophy is genuinely person centred [9] and reflects important values underpinning good dementia care. Rehabilitation involves working with people to achieve the goals that are important to them. It is based on individual formulations and not a one-size-fits-all approach [10], acknowledging that each individual has a unique set of experiences, values, motivations, strengths, and needs.

In cognitive rehabilitation, these principles are applied to enable people with dementia to maintain or optimize functioning. The term "cognitive" is perhaps misleading, as cognitive rehabilitation does not set out to train or improve cognition but uses a goal-oriented approach to facilitate improved management of functional disability. Potential targets include everyday functioning, activities of daily living, self-care, language and communication, social interaction, and the effects of dementia-related physical disability. Cognitive rehabilitation therapists work collaboratively with each individual to formulate meaningful goals that are realistic and potentially achievable. They evaluate the person's strengths

and the resources needed for goal attainment, identify areas of mismatch, and collaboratively develop a plan to support goal attainment or address the identified need using evidence-based methods. These might involve new learning, relearning, use of compensatory strategies, or a combination of these. The person's intrinsic capacity may be augmented by additional resources such as assistive technology. Therapists also provide important psychological support as people confront the emotional impact of functional disability.

Principles of rehabilitation can be flexibly applied to address different types of need at various stages of dementia. For example, a person in the early stages of dementia may want to learn to use email to keep in contact with friends, develop strategies to feel confident enough to go out alone, or be able to cook a meal without getting distracted, while for someone with more advanced dementia, the focus may be on maintaining the ability to dress herself, managing difficulties with swallowing, or enabling participation in an enjoyable activity. Each individual might have several episodes of rehabilitation support over time as needs and goals change or as particular circumstances arise, such as a return home after hospitalization. Cognitive rehabilitation is usually carried out in the setting where the person lives or undertakes activities, to ensure direct relevance, and carers and families, when available and willing, are fully involved and appropriately supported. There is a small but growing evidence base demonstrating that cognitive rehabilitation is effective in supporting everyday functioning, reducing disability, and delaying institutionalization [11–15].

20.3 Where Do Other Nonpharmacological Interventions Fit In?

Many different types of nonpharmacological intervention for people with dementia have been described. Most of these are not cognitive rehabilitation; it is important that this term is understood correctly and is not applied to interventions that do not warrant it. Some approaches, however, are directly complementary to cognitive rehabilitation. These either address related aims (for example, self-management groups to enhance self-efficacy or therapeutic groups to promote adjustment to living with dementia) or address problems that negatively affect functioning and participation (for example, psychotherapy for depression or individualized interventions for agitation). Also directly relevant is support for family carers. Other intervention approaches focusing on providing pleasurable and meaningful activity or encouraging social contact can complement rehabilitation by creating opportunities to enjoy positive experiences and relationships. Within a rehabilitation-focused service, an intervention pathway would include these approaches where there is evidence that they offer benefits. Less likely to be recommended in the context of a rehabilitation model are nonpharmacological interventions that address symptoms in isolation or out of context so that gains, if any, are unlikely to transfer to daily life. People with dementia should also have full access to specialist physical rehabilitation

where needed following injury or illness, as well as any other appropriate medical treatment [16].

20.4 How Could Services Adopt a Rehabilitation Model?

A rehabilitation-based model of positive support could potentially be introduced in part through a redeployment of available resources. The costs of this positive approach might be offset to some extent by preventing difficulties, limiting the costs of managing distressing symptoms, and delaying institutionalisation. There is a need to develop service systems with a clear focus on optimizing functioning and supporting relationships, identity, and engagement and a need to train staff to implement rehabilitative interventions. It is essential to fully involve people with dementia and carers to ensure a thorough understanding of their perspectives.

20.5 Why Should We Acknowledge the Right to Rehabilitation?

Acknowledging the right to rehabilitation offers a tremendous opportunity to create a focused and coherent approach to positive support for people with dementia, of any age, subtype, or severity, and their families. A rehabilitation model offers a guiding framework for services and for health and social care practitioners and a practical means of providing person-centred, evidence-based interventions to maintain or enhance functioning, engagement, and participation.

Disclosures and Conflict of Interest

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Chapter 21

Are Dementia with Lewy Bodies and Parkinson's Disease Dementia the Same Disease?

Kurt A. Jellinger, MD, PhD,[a] and Amos D. Korczyn, MD, PhD[b]

[a]*Institute of Clinical Neurobiology, Vienna, Austria*
[b]*Tel-Aviv University, Sackler Faculty of Medicine, Ramat Aviv, Israel*

kurt.jellinger@univie.ac.at

Keywords: α-synuclein (αSyn), Alzheimer's disease (AD), amyloid, anti-αSyn-immunotherapy, biomarkers, cholinesterase inhibitors (ChEIs), clinical overlap, clozapine, dementia, dementia with Lewy bodies (DLB), dopamine transporter (DAT), epidemiology, fluid biomarkers, genetics, mixed dementia, morphological overlap, neuroimaging, neuropathologic criteria, neuropathology, neurophysiology, Parkinson disease-dementia (PDD), pathogenic aspects, quetiapine, REM sleep behavior disorder (RBD), synucleinopathies, tau, transcranial sonographic hyperechogenicity, white matter hyperintensities (WMH)

21.1 Background

The nosologic relationship, as defined by DSM-5 [1, 2], between dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD), both of which are major neurocognitive disorders with α-synuclein (αSyn) deposition/Lewy bodies (LB), is continuously being debated [3–22].

The clinical features of DLB and PDD are similar and include dementia, cognitive fluctuations, and (visual) hallucinations in the setting of clinical or latent parkinsonism. The cognitive domains of both disorders overlap, with progressive executive dysfunctions, visual-spatial abnormalities, and memory disorders [10]. Based on international consensus, DLB is diagnosed when cognitive impairment precedes parkinsonian motor signs or begins within 1 year from its onset [23], whereas in PDD, cognitive impairment develops in the setting of well-established Parkinson's disease (PD) [24]. DLB patients will also develop parkinsonism of increasing severity over the years, although 25% of them never develop parkinsonian

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symptoms [25]. Despite different temporal sequences of motor and cognitive deficits and several quantitative clinical differences, both disorders show largely convergent, albeit locally and quantitatively divergent neuropathological lesions, associated with increased Aβ and tau loads in DLB [9, 26–30]. The overlap of clinical and morphological features has led to the debate of whether DLB and PDD are the same disease [17], different phenotypic expressions of the same αSyn/Lewy body disease (LBD) spectrum, or distinct 'diseases' [3, 31] sharing genetic risk features with PD and Alzheimer's disease (AD) [10, 32], despite recent studies indicating a regional overlap of pathologies [33–37]. The present chapter will critically review the major current findings in DLB and PDD, their possible nosologic interrelations, and the available biological markers and therapies. Of note, this review does not include mild cognitive impairment in LBD (see [8, 38–46]).

21.1.1 Clinical Features and Diagnostic Criteria of DLB

The presenting features of DLB can be broadly placed into three categories, namely cognitive impairment, behavioral/psychiatric phenomena, and physical symptoms [47]. Essential for its diagnosis are dementia with moderate memory impairment, deficits in attention, executive dysfunction and visuoperceptual ability, fluctuating cognition (presumably related to thalamic damage and cholinergic imbalance [48]), and recurrent visual hallucinations that are well formed and detailed [2]. Hallucinations in DLB may occur spontaneously, independent of visuospatial and perceptional impairment [49], and possibly related to LBs in the temporal lobe [50], while in PDD they typically occur after dopaminergic therapy [10, 23, 51]. Nevertheless, hallucinations had been reported prior to the levodopa era [52] as well as in drug-naive PD patients even in the premotor phase [53]. Language impairment tends to be mild, with verbal and semantic fluency deficits. Spontaneous parkinsonian features, such as bradykinesia and rigidity, are common in DLB (over 85%) [31], while rest tremor is less frequent [54]. REM sleep behavior disorder (RBD), which shows a high prevalence in DLB and may precede cognitive decline by decades, is now included as a core clinical feature [55]. RBD may reflect a distinct subtype of DLB with earlier disease onset [56], associated with severe brain metabolic decreases [57]; however, as an early manifestation, it is not specific to DLB [58, 59]. The pattern of initial cognitive dysfunction differs between DLB and PDD [60], with greater deficiencies of attention, executive function, and constructive abilities, as well as significantly lower ratings in episodic verbal memory tasks, in DLB [61, 62]. Further, the rate of cognitive decline is reportedly faster in DLB than in PDD and AD [63, 64] (Table 21.1).

Supporting clinical features for the diagnosis of probable or possible DLB are repeated falls, syncopes, hyposmia, severe autonomic dysfunction, hypersomnia, hallucinations in non-visual modalities, apathy, depression, and severe sensitivity to antipsychotic agents [2, 65]. However, since these changes also occur in advanced PD, they cannot differentiate DLB from PDD, e.g., the prevalence of neuroleptic sensitivity does not differ significantly between them [66].

Table 21.1 Clinical overlap and dissimilarities between dementia with Lewy bodies (DLB) and Parkinson disease dementia (PDD)

Overlap	Dissimilarities
Rigidity, akinesia	Some cognitive dysfunctions: deficiencies of attention greater, episodic verbal memory tasks lower in DLB
Cognitive impairments	Tremor less frequent in DLB
Frontal executive dysfunction	Motor performance: slower walk and poorer balance in DLB
Visual-constructive impairment	Hallucinations (visual) more frequent in DLB Relative timing of dementia and parkinsonism (one year rule)
Mild language impairment	Onset of dementia earlier in PDD
Mood disturbances (depression, anxiety)	Orthostatic hypotension more frequent in DLB
REM sleep behavior disorder (RBD)	Frontal/temporal-associated cognitive subsets more severe in DLB, cognitive decline is faster in DLB/DLB+AD
Neuroleptic sensitivity	Delusions, visual hallucinations, and attentional fluctuation more frequent in DLB
	Visual hallucinations: spontaneous in DLB; after L-dopa therapy in PDD, but also in drug-naive cases

Abbreviation: AD, Alzheimer disease.

A diagnosis of clinically probable DLB requires (1) two or more core clinical features to be present, with or without indicative biomarkers, or (2) the presence of only one core clinical feature but with one or more indicative biomarkers [2]. Although the diagnostic specificity of these criteria is high (range 79–100%), the sensitivity can be low (12–88%), improving with additional supporting features such as biomarkers [67–70]. A recent meta-analysis reported a pooled sensitivity, specificity, and accuracy of 60.2% (95% CI 30.9–83.7%), 93.8% (83.8–97.6%), and 79.7% (62.6–90.7%), respectively, for the diagnostic [23] criteria of DLB [68]. Thus, currently, approximately 20% of DLB diagnoses are incorrect [68, 69].

21.1.2 Clinical Features and Diagnostic Guidelines of PDD

The clinical features of PDD are in many respects similar to those seen in DLB, although, by definition [23, 71], the occurrence of parkinsonism distinguishes one from the other. Rigidity and akinesia occur both in PDD and DLB [62]. Cognitive impairments in PDD are common and are similar in quality to those of DLB [8]. However, the timing, profile, and rate of cognitive decline vary widely; indeed, the average time to dementia after PD diagnosis is almost 10 years, but may be as long as 20 years [39]. Consensus criteria for PDD [24, 72, 73] require cognitive impairment across multiple domains, mood disturbances, and visual-spatial impairment similar to that seen in DLB. Attentional fluctuations, which are characteristic of DLB, are less frequent in PDD [72] but are clinically indistinguishable in the two conditions [74]. Executive functions are probably

more impaired in PDD, while language deficits are rare [71]. Visual symptoms, common in PDD [75] likely due to a reduced metabolism in both dorsal and ventral visual pathways [76], include visual hallucinations, although they are less common than in DLB [77]; yet, the phenomenology of hallucinations is similar in both disorders [78]. Other non-motor features, including autonomic dysfunctions and sleep disorders, may occur disproportionally to the severity of dementia [24, 72], while mood disturbances have a similar frequency as in DLB. The psychosis spectrum of PD has recently been reviewed [79]. RBD can evolve in PDD and DLB [80] in up to 90% of patients after >10 years [81]. Finally, clinical validation efforts for PDD have shown variable diagnostic sensitivity and specificity [82, 83] and should be considered using the Movement Disorder Society criteria for the diagnosis of PDD [84].

21.1.3 Epidemiology and Natural History of DLB and PDD

Approximately 1–2% of those aged above 65 years are diagnosed with DLB worldwide [16], affecting approximately 5% of all dementia cases in those over the age of 75 [85]. Its incidence is 0.7–1.4 new cases/100,000 person-years [16] or 3.5/100,000 person-years [86]. For PDD, the cumulative prevalence is of 75% of PD patients surviving more than 10 years [87], 83% after 20 years [88], and up to 95% by age 90 years [16], with an overall prevalence of 31.1% (95% CI 20.1–42.1) and incidence rates from 0.43 to 1.13/100,000 person-years [89], indicating that, annually, approximately 10% of a PD population will develop dementia [24]. The data concerning age at disease or dementia onset are highly variable. Whereas in the Olmsted County study [86] DLB patients were younger at symptom onset than those with PDD and had more hallucinations and cognitive fluctuations, others have reported younger age at disease onset in PDD [27, 90, 91], or no essential differences between disorders [14, 37, 92, 93].

Individuals with DLB or PDD have an increased mortality compared with the general population [94]. DLB patients with a cerebrospinal fluid (CSF) AD profile and structural MRI changes (hippocampal atrophy) have a shorter survival [95, 96]; similarly, dementia and/or neuritic AD pathology in PD are related to a significantly shorter survival [97]. PDD is associated with high mortality, advancing death by approximately 4 years [98]. For typical DLB, the average survival time from the beginning of symptoms is 5–8 years [99], while rapidly progressing cases have a mean duration of 9 months [100]. In both disorders, older age, hallucinations, and fluctuating dementia at onset are the best predictors of poor outcome [101, 102].

21.1.4 Diagnostic Tests (Table 21.2)

21.1.4.1 Neuroimaging

The neuroimaging characteristics have been reviewed in a quest for multimodal methods able to improve ante mortem diagnosis [103, 104]. Studies using ^{123}I-β-CIT (DaTScan) SPECT or ^{18}Fluorodopa PET demonstrated reduced dopamine

transport binding in caudate and posterior putamen in DLB compared to AD, but observed no differences between DLB and PDD [105, 106]. Further, lower ^{123}I–ioflupane-CIT has been observed in caudate nucleus in DLB and a greater asymmetry of uptake was seen in the posterior putamen in PDD [104, 107]. Dopamine uptake in striatum is significantly lower in PDD compared to DLB ($P < 0.04$), consistent with dopaminergic cell loss in substantia nigra pars compacta and the severity of parkinsonism [108]. The disruption of dopaminergic pathways impacts the modulation of intrinsic brain networks, resulting in poor motor and cognitive performance [109].

Table 21.2 Laboratory findings overlap and dissimilarities between dementia with Lewy bodies (DLB) and Parkinson disease-dementia (PDD)

Overlap	Dissimilarities
Decreased DAT binding in putamen	Grey matter cortical atrophy more frequent and more severe in DLB
Reduced cardiac MIBG binding	White matter hyperintensities in temporal lobe more severe and more frequent in DLB Different functional connectivity, corticostriatal disruption: *PDD*: frontal cortical disruption; *DLB*: parietal and occipital disruption
Medial temporal lobe relative preservation	Greater amyloid binding in DLB
Occipital hypoperfusion	Tau-PET imaging more severe in
Similar EEG abnormalities	DLB Several genetic differences (APOE ε4, TFAM)
Similar metabolic decrease in cerebral cortex	Decreased DAT binding in caudate related to functional impairment in DLB, not in PDD
GBA mutations	SN sonography (size, asymmetry)
	CSF AD profile more common in DLB
	CSF αSyn oligomeres increased in PDD

Abbreviations: AD, Alzheimer disease; DAT, dopamine transporter; MIBG, scintigraphy using meta-iodobenzylguanidine labeled to Iodine-123 or Iodine-131; SN, substantia nigra; CSF, cerebrospinal fluid.

SPECT imaging using ^{123}I–metaiodobenzylguanidine, a marker of postganglionic sympathetic innervation, showed reduced cardiac uptake in both DLB and PDD as compared with AD [110, 111]. The sensitivity, specificity, and accuracy for the diagnosis of probable DLB is 82.4%, 96.3%, and 92.5%, respectively [112]; yet, although specific data on PDD are not available, ^{123}I–metaiodobenzylguanidine imaging is unlikely to differentiate PDD from DLB.

Voxel-based morphometric MRI studies revealed greater grey matter loss in frontotemporal, occipital, and parietal areas in DLB compared to PDD [113–118]. Decreased grey matter volumes in association areas (left precuneus and inferior temporal lobe) are correlated with visual hallucinations in DLB [119], and atrophy of caudate, putamen, and pallidum have been observed in DLB but not in PDD [120–123]. However, since greater volume loss in various brain regions has not been statistically confirmed [124], these differences cannot be used for individual diagnoses.

White matter hyperintensities (WMH) on T2-weighted MRI have been observed in parieto-occipital areas in PDD cases with low CSF Aβ levels [125], without significant difference of progression between PDD and DLB [126], but more severe WMHs have been observed in the temporal lobe in DLB [127]. Thus, evaluation of WMH and medial temporal lobe atrophy using MRI may be a powerful diagnostic tool to investigate the progression of AD-related pathology in DLB and perhaps to distinguish DLB from PDD [126, 128]. Magnetic resonance spectroscopy studies found relatively normal N-acetylaspartate/creatinine ratios in DLB, with similar reductions being observed in PDD and AD [129].

PET, perfusion SPECT, and arterial spin labelling MRI studies showed parietal, frontal, temporal, and occipital hypoperfusion common to both entities [104, 130–135]. Further, ^{11}C PIB-PET imaging showed increased Aβ brain deposition in more than 50% of DLB cases, with more modest and less frequent Aβ accumulation in PDD [106, 136–139], while others reported increased cortical Aβ binding without dissimilarity between PDD and DLB [140]. Tau-PET imaging, along with temporal atrophy, may indicate co-existing AD pathology in DLB with variable cortical tau ^{18}F–AV-1451 uptake, which appears more common than in PDD [141, 142]. Preliminary tau-PET studies suggest a gradient of tau binding from PD/non-demented (minimal) to PDD (low), DLB (intermediate), and AD (highest) [143]. Finally, the recently described additional ^{18}F–AV-1451 binding to (neuro)melanin [144] deserves further investigation.

21.1.4.2 Electrophysiology and other studies

EEG abnormalities from posterior leads have been observed in all DLB cases and in three-quarters of those with PDD [145]. Further, a multicenter study supported the validity of quantitative EEG analysis as a tool for diagnosis of both disorders and their distinction from AD [146, 147], although some components may be reduced more in PDD than in DLB [148]. Finally, transcranial sonographic hyperechogenicity was inconclusive in differentiating DLB from PDD [149]; a comparative electro-oculographic study showed similar impairment in all tasks in both disorders [150].

21.1.5 Genetics

Both DLB and PD are primarily sporadic diseases, yet genetic factors may be involved in their causation. Recent studies have uncovered certain genetic differences between PDD and DLB, albeit none of which is diagnostic. There is a substantial genetic contribution to DLB, heritability being estimated at about 36% [151, 152], while different genetic markers within the α-Syn gene (SNCA) may be associated with PDD [153, 154], although this is not unexpected in PD (Table 21.3). Analyses of SNCA expression in PDD and DLB showed an overlap of αSyn biology, indicating that they have distinct genetic etiologies and predicting that several mechanisms may be specific [154]. Genome-wide association studies (GWAS) identified variants in the GBA, SNCA, APOE, and MAPT loci influencing the

individual risk for DLB, suggesting that it has shared genetic risk features with PD and AD [32, 155], while the APOE4 haplotype may be an indication of PDD [156]. However, to date, the genetic differences between both entities have not been studied in detail [157]; further studies will increase our understanding of the pathophysiology of these diseases [158].

Table 21.3 Potential genetic risk factors for dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD)

Gene	DLB	PDD
GBA (glucocerebrosidase)	Mutations are most prevalent risk factors for sporadic DLB [271, 272]; associated with increased levels of AD pathology [183, 273]	Mutations associated with risk of PDD and agressive cognitive decline [274–283]
MAPT (microtubule-associated protein tau) H1 haplotype	Associated with increased risk of DLB [284]	Strongly associated with dementia in PD [153, 275, 285–289]
APOE (apolipoprotein E)	*APOE ε4* is overrepresented in DLB, and it is an increased risk for DLB [35, 290]	Mixed evidence for dementia risk in PD [291–297]
SNCA (α-synuclein)	Multiplication is not a common cause of DLB [298, 299]	Rare multiplications and mutations are associated with dementia in monogenic PD [292, 300], but show phenotypic variations and clinical heterogeneity [301–306]
COMT (catechol-O-methyltransferase)	–	No evidence for dementia risk [287, 288, 307–309], but polymorphisms may contribute to cognitive deficits in PD [310]
UBQLN1 (ubiquilin-1) and *FMR1* (fragile X mental retardation protein 1)	No association with cognitive impairment [311, 312]	
LRRK2 (leucine-rich repeat serine/threonine-protein kinase 2)	Not essential for DLB [313]	No association with PDD [314–322]
C9orf72 repeat expansion	Not related with DLB [313]	
RAB39B (Ras-related protein Rab-39B) mutations	Not related with DLB [323]	

Abbreviations: AD, Alzheimer's disease; PD, Parkinson's disease.

21.1.6 Fluid Biomarkers

The development of broadly applicable CSF and other biomarkers for both DLB and PDD remains elusive, with only few biomarker candidates having been shown to specifically reflect the underlying disease process [159–161] (Table 21.2). A CSF AD profile is more common in DLB [162], while cortical atrophy in PDD is associated with increased total CSF αSyn and t-tau [159]. However, cognitive impairment in GBA-associated PD does not seem to be associated with Aβ and tau profiles in CSF [163]. The elevated tau/Aβ42 index in the order PD < PDD < DLB < AD may be related to an increased AD pathology [164]. Further, levels of αSyn oligomers in CSF are increased in PDD but not in DLB [165–167]. Although many CSF and some plasma markers have been identified in both disorders, very few studies have examined samples from both disorders simultaneously, and only a minority have been confirmed by post mortem studies [167, 168].

21.1.7 Neuropathology

The pathological substrates of DLB and PDD have been extensively investigated [9, 27, 29, 30, 35–37, 41, 169–181]. The most difficult problem in defining DLB and PDD at autopsy is their relationship with AD. DLB is, in part, conceived as a variant of AD ('Lewy body variant of AD') [182] and significant AD pathology is a consistent but not universal finding in both disorders [181]. Cerebral neurofibrillary tangle burden, along with αSyn and Aβ plaque pathology, are the strongest predictors of a shorter interval between motor and dementia symptom onset and shorter survival [183].

The pathological substrate of PDD includes (1) Lewy/αSyn pathology in cortical, limbic, and brainstem structures, (2) AD-related pathologies, and (3) a combination of these lesions that has been shown to most robustly correlate with the severity of cognitive impairment [41, 169, 173, 174]. Approximately 50% of PDD patients showed Braak LB stages 4–6 plus severe AD-type pathology [92, 174], which may act synergistically [9, 27, 35, 172–174, 184, 185], influencing clinical features including a shorter duration or a more malignant course [169, 172]. AD neuropathology seems to be a more specific correlate of dementia than cortical αSyn pathology [169, 173]. Substantia nigra cell loss is more severe in PDD than in DLB [15], consistent with more advanced parkinsonism.

Multiple neurotransmitter deficits occur in PDD [29, 172], including loss of limbic and cortically projecting dopaminergic neurons in the mesocortical limbic system [172] and involvement of the cholinergic system with loss of neurons in the nucleus basalis of Meynert leading to cortical cholinergic denervation [9, 171, 186]. Severe pathology also involves the noradrenergic locus coeruleus, causing dysfunction of the related circuitry [170]. Pedunculopontine cholinergic cell loss occurs in hallucinating PDD patients but not in DLB, which may indicate a different pattern of degeneration of cholinergic input structures [187].

DLB is featured by the co-occurrence of Lewy/αSyn pathology involving cortical and limbic areas (Braak LB stages 3–6) and AD-related pathologies. While some authors suggest that high cortical LB burden is the only independent

predictor of dementia in DLB [177], others consider AD-related pathology to be more important [188]; however, studies have shown a strong correlation between both cortical pathologies [169, 173].

The DLB clinical syndrome is positively correlated to the extent of LB pathology (LBP) and negatively to the severity of neuritic AD pathology, while Aβ load has no effect [189]. A subgroup with the clinical picture of DLB was shown to have minimal cerebral amyloid deposition [190]. The higher cortical LB load in the temporal and parietal regions, which seems to be a distinguishing feature of DLB, may account for the shorter latency to dementia and could be accelerated by the APOE ε4 allele [177]. Further, αSyn is an important predictor of disease duration both independently and synergistically with tau and Aβ load [191].

Other co-occurring pathologies (cerebrovascular lesions, cerebral amyloid angiopathy, hippocampal sclerosis, argyrophilic grain disease, and TDP-43 deposits) in PDD (19%) and DLB-AD (31.3%) brains appear to be of minor importance [35, 172, 192–194], although they may influence the development of dementia [195]. Cerebrovascular lesions in DLB are relatively mild, showing an inverse relationship with the severity of LBP [196–198]. Cerebral microbleeds are more frequent in DLB than in PDD [199], with highest densities in the occipital lobe [200], but they appear to be independent of cerebral amyloid angiopathy [201].

21.1.7.1 Morphological overlap

Both PDD and DLB may show similar neuropathological features, with a ariable mixture of αSyn/LB and AD-related pathologies (Table 21.4). A common pathophysiological factor is synaptic dysfunction due to the initial aggregation of αSyn in the presynapses causing functional disconnection [202] due to interference with axonal transport and neurotransmitter deprivation [178, 180, 203, 204].

Table 21.4 Morphological overlap and dissimilarities between dementia with Lewy bodies (DLB) and Parkinson disease-dementia (PDD)

Morphological overlap	Morphological dissimilarities
Variable mixture of cortical and subcortical LB/αSyn pathology and AD-related pathology	Higher Aβ load in cortex and striatum in DLB
Similar Braak LB stages (4-6) and neuritic stages (5 or 6)	Neuritic plaque scores higher in DLB
Relationship between pαSyn and tau aggregation to Aβ deposition in frontal and temporal cortex	Higher cortical LB load in temporal and parietal cortex in DLB
Initial αSyn aggregation in pre-synapses inducing neurodegeneration via interference with axonal transport	Increased tau loads in cortex and striatum in DLB
Postsynaptic protein downregulation	More frequent and severe αSyn load in hippocampal subareas C2(3) in DLB
	Minor deviations in lesion pattern in SNc
	Pedunculopontine cholinergic cell loss in hallucinating PDD, but not in DLB
	Higher 5-HT1A receptor binding in cerebral cortex in DLB
	More frequent cerebral microbleeds in DLB

Abbreviations: LB, Lewy body, AD, Alzheimer disease, SNc, substantia nigra pars compacta.

The relationship between phosphorylated αSyn and tau accumulation to Aβ deposition in the cerebral cortex [205, 206] suggests that there is an overlap in the pathology between AD and DLB, and that Aβ promotes the accumulation of both αSyn and tau [35–37]. Thus, cognitive decline and related symptoms are not a consequence of αSyn-induced neurodegeneration alone since Aβ and tau pathologies also contribute to the overall deficits [33, 35–37, 207].

21.1.7.2 Morphological differences

Despite many similarities, several morphological differences have been demonstrated, including higher Aβ load in striatum [34, 208], cortex, and claustrum [33, 177, 197, 209–211] and in the entorhinal cortex, amygdala, and putamen in DLB [27]. The presence of Aβ in DLB and less so in PDD, along with its great sensitivity to differentiate between the disorders, have been extensively investigated [33, 34, 177, 209], with a hierarchy PD < PDD < DLB in both Aβ and tau burden [143] (Table 21.4).

Further differences include a more severe αSyn load in hippocampal subarea C2 in DLB [29] and in amygdala in DLB compared to in PDD (78.7% vs. 36% and 92% vs. 30%, respectively) [212], whereas αSyn loads in PD are highest in the cingulate cortex [33]. Other deviations include the severity and distribution pattern of lesions in substantia nigra pars compacta (predominant neuronal loss in the ventrolateral parts in PDD versus more severe damage in the dorsolateral parts in DLB) and less marked nigral neuronal loss causing less severe postsynaptic dopaminergic upregulation [209, 213]. Additionally, significantly higher 5-HT1A receptor binding density in the cortex was seen in DLB compared to PDD [214]. The heterogeneous neurochemistry of both DLB and PDD, which depends on differences in pathology, suggests that these αSyn-related disorders and AD share a common, underlying molecular pathogenesis; however, this needs further elucidation.

21.1.8 Pathogenic Aspects

The clinicopathological features of DLB, PDD, and other synucleinopathies are highly variable and heterogeneous [9, 29, 215–217], although the spread of LBP was originally suggested to be uniformly ordered according to the Braak scheme [218, 219]. There are three current major staging systems in use for LB disorders, including one for PD [218, 219], one for DLB [23], and revised guidelines for LB disease [2, 220, 221]. Based on semiquantitative assessment of LBs in large autopsy series, a staging of the chronological spread of LBP was proposed to designate its predictable caudo-rostral sequence in the CNS, which, however, is not identical with the spreading and location of αSyn pathology [222, 223]. Cases with severe LBP (Braak 'neocortical' stages 5 and 6) that show overlap or transition between PD and DLB are frequently associated with cognitive impairment, which increases with progressing neuropathological changes [223].

The validity of the Braak staging scheme, which corresponds roughly to the classification of LB disorders as either a (1) predominantly brainstem pathology,

(2) limbic system (limbic/transitional type) pathology, or (3) diffuse neocortical pathology [224], has gained wide support as a standard for assessment of LBDs [98, 225, 226], but has also been a matter of vigorous debate [216, 227–231]. The Braak staging scheme often, but not consistently, shows acceptable correlations between morphological findings and clinical data, mainly in a subgroup with early onset and prolonged disease duration [232], whereas a new unified staging system allows the classification of all cases of LBDs, including PD, PDD, DLB, incidental LBD, and DLB-AD [220].

According to the Braak scheme, αSyn aggregates, forming the major components of LBs, and Lewy neurites appear first in the olfactory structures and enteric nervous system and then progressively spread into the brain, moving from cell to cell (neuron to neuron) and through neuronal circuits in a 'prion-like' manner, thus contributing to synaptic failure [233] due to impaired axonal transport and accounting for the progression of LBP [234–236]. More recently, it has been hypothesized that αSyn itself may be a critical factor in mediating transmission of disease pathology by such a 'prion-like' process, which appears essential for the pathogenesis of both PDD and DLB [237]. It remains to be seen if the species of aggregates of αSyn responsible for propagation and neurodegeneration are different and whether the various strains of αSyn fibrils underlie the differences in cellular and regional distribution of lesions in different synucleinopathies, as has been observed following the injection of αSyn aggregates in animal models [238, 239].

An essential problem in distinguishing between DLB and PDD is the impact of AD-related pathology and its co-occurrence with LBP, although both types of lesion have been shown to be strongly correlated with one another [169, 173]. However, recent clinicopathological studies showed that the clinical features of DLB are the consequence of multiple regional pathologies that are less pronounced in PDD [9, 27, 30, 73]. Nevertheless, the genetic and molecular mechanisms responsible for the, at least partially, different pathogenetic factors of both disorders await further elucidation.

21.1.9 Therapy

Currently, there are no disease-modifying therapies for LBDs available (however, see [240]), although robust evidence supports the use of cholinesterase inhibitors (ChEIs) to treat these disorders [241, 242], related to the reduction of cholinergic markers in both PDD and DLB [243, 244]. Meta-analyses have indicated beneficial effects of both donepezil and rivastigmine for cognitive and psychiatric symptoms in both disorders [245–248], while only one study found an effect of memantine in PDD [249]. The efficacy of memantine in DLB is thus less clear, but may have benefits either as monotherapy or as adjunctive to a ChEI [241]; further, it induced longer survival in patients with DLB and PDD [250]. Although the effects were relatively small, ChEIs gave a better response of cognitive impairment in DLB and PDD than in AD [251], and may produce reduction in apathy, visual hallucinations, and delusions [252]. The use of antipsychotics should be avoided

given the risk of serious reactions in DLB [2, 253]. When atypical antipsychotic agents are needed, quetiapine, and particularly clozapine, are less likely exacerbate parkinsonism [251]. Levodopa is generally well tolerated, but produces significantly less motor response in DLB than in PD and may be associated with an increased risk of psychosis [242, 254, 255]. Additionally, strategies to decrease the level of αSyn, to prevent cell-to-cell transmission of misfolded αSyn, and deep brain stimulation of the cholinergic nucleus basalis of Meynert have been discussed [39, 256]. Future therapeutic strategies should include disease-modifying strategies, possibly based on recent vaccination trials against αSyn, Aβ, and tau proteins [257, 258]. Preliminary results of anti-αSyn-immunotherapy in a combined model of synucleinopathy [259] may open the way to potential new treatments. A recent review of non-pharmacological interactions for DLB gave no definite results [260], while bilateral deep brain stimulation of the NBM for PDD showed potential improvement of neuropsychiatric symptoms [261].

21.2 Conclusions

DLB and PDD are major neurocognitive disorders with LBD, sharing many clinical, genetic, pathophysiological, imaging, and morphological features. Thus far, a clear and objective distinction between the two entities, other than the arbitrary timing of the appearance of cognitive and motor impairments (1-year rule), has not been established [5, 10, 15, 220], while others maintain that the two entities may merge [262] or may become the same disease [17]. The revised Movement Disorder Society clinical definition of PD, considering DLB with presence of parkinsonism a 'DLB subtype of PD' [18, 31], was criticized since it would confuse rather than clarify the distinction between both entities [3]. However, the 1-year time period may not be the optimal method for diagnostic distinction between both disorders [3] since cognitive decline has been reported to start as early as 6 years prior to PD diagnosis [263]. Yet, it appears questionable whether this and other recent clinical studies on impaired cognition years before manifestation of parkinsonism [264] may blur the distinction between PD and DLB, which has been supported by recent neuroimaging and postmortem studies indicating that, in addition to predominant LB/αSyn pathology, AD-related lesions may contribute to the timing of dementia onset relative to motor signs [177].

The clinical pictures of both phenotypes, characterized by recent diagnostic criteria (for DLB [2] and for PDD [24, 72, 84]), despite individual variability, show many overlapping and distinguishing features [3, 8, 126, 265] (Table 21.1).

Several genetic markers have been shown to be risk factors for DLB and/or PDD, with some differences among them (Table 21.3). However, it appears premature to recommend genetic testing for clinical diagnosis and differentiation between DLB and PDD. A number of indicative and supportive biomarkers may contribute to the clinical diagnosis of probable DLB and PDD (Table 21.2).

Despite considerable overlap between DLB and PDD, recent neuroimaging and postmortem studies have demonstrated differences in the quantity and distribution

pattern of LB/αSyn and AD-related pathologies between these two entities (Table 21.4). A correlation between these lesions suggests (1) a synergistic/additive or triggering effect between these protein pathologies [266], with increasing levels of AD pathology inducing an increasing burden of αSyn pathology; (2) an overlap in the pathology between DLB and AD; and (3) that the cognitive decline and related symptoms are not a consequence of αSyn-induced neurodegeneration alone, but of mixed pathologies contributing to the overall deficits [30, 35, 37, 183, 207, 266].

A possible interpretation of the available data would be that PDD and DLB are sub/phenotypes or two ends of the LBD spectrum [19], in which DLB may reside at the more severe side next to AD, while incidental LBD would be on the other (initiating) end [267]. The suggested spectrum is as follows: incidental LBD > PD/non-demented > PDD > DLB > DLB/AD nearing AD. Recent GWAS studies suggested, as another possibility, that DLB and PDD would be distinct diseases with shared genetic risk features with PD and AD [32]. Although some genetic factors that predispose to the development of dementia may differ in PDD and DLB, further extensive GWAS studies in autopsy-confirmed cohorts are warranted.

21.3 Future Perspectives

DLB and PDD are clinically similar illnesses, distinguished on the basis of the relative timing of dementia and parkinsonism (the 1-year rule). In view of the heterogeneity of the clinical course and symptomatology of both disorders that share the same pathophysiology [30], the question of whether this is a biologically valid distinction, or whether they are merely subtypes in a continuum of LBDs remains to be elucidated based on the results of combined biomarkers, new molecular imaging tracers [268, 269], and multimodal imaging [106]. Their distinction would be useful for further diagnostics and, in particular, new and disease-specific preventive and curative measurements.

At present, neuropathological (differential) diagnosis of DLB and PDD with no or insufficient clinical data would be difficult [181]. However, according to the preliminary criteria proposed in Table 21.5 (which need further validation and reproducibility), this may be possible. In view of the recent data on the clinical diagnostic criteria for DLB [68], their accuracy remains limited, while, to the best of our knowledge, no comparable studies are available for PDD. In order to support the notion that DLB and PDD are separate diseases, a unique pathogenic process should be identified for either one or the other. Therefore, at present, they cannot be strictly separated as distinct, whereas clinical, imaging, and morphological parameters can distinguish DLB from AD and frontotemporal dementia. The solution of this problem – if at all possible – warrants extensive multidisciplinary studies designed to shed further light on the relationship between PDD and DLB, including identifying genetic and environmental risk factors, and improving our understanding of the biological mechanisms responsible for their pathogenesis such that preventative or curative management can be developed [270].

Nevertheless, the wide acceptance of the term DLB is evidence of its clinical utility, which is likely to result in the maintenance of the term; it is useful in the differential diagnosis of cases presenting with cognitive decline. Whether such patients are likely to develop extrapyramidal symptoms (DLB) or not (AD, etc.) has prognostic value and indicates the type of therapy (e.g., typical or atypical neuroleptics) and is thus of clinical importance. Although we favor the concept of a continuum between DLB and PDD, it must be recognized that biological factors must exist that determine whether the synucleinopathy will present earlier with cognitive decline or with extrapyramidal features. Identifying such factors is important scientifically and may lead to the development of disease-modifying therapies.

Table 21.5 Preliminary neuropathological features of dementia with Lewy bodies (DLB) and Parkinson disease with dementia (PDD)

Type of lesion	DLB	PDD
LB/αSyn pathology	Both subtypes are characteristic by a combination of progressed LB pathology (LB Braak stage 5–6) and AD pathology of variable severity and extent	
Aβ load	More severe and extended in cortex and striatum	Less severe and less extended
Tau load	Higher tau load, particularly in medial temporal cortex	Comparatively low tau load in cortex and striatum
αSyn load (hippocampus)	CA 1/2 more severely involved	CA 2/3 more frequently involved
SN neuronal cell loss	Preferentially involving dorsolateral substantia nigra pars compacta	More severe, preferentially involving medioventral SNc
Pedunculopontine cholinergic cell loss	Negative	Positive in hallucinating PDD
5-HT1A receptor binding density in cortex	Higher	Lower
Cortical LB load	Higher in temporal & parietal cortex, hippocampus	Diffuse or focal

Abbreviations: LB, Lewy body, AD, Alzheimer disease, SN, substantia nigra.

Abbreviations

αSyn: α-synuclein
AD: Alzheimer's disease
ChEIs: cholinesterase inhibitors
CSF: cerebrospinal fluid
DAT: dopamine transporter
DLB: dementia with Lewy bodies

GWAS: genome-wide association studies
LB: Lewy bodies
LBD: Lewy body disease
LBP: LB pathology
PD: Parkinson's disease
PDD: Parkinson's disease dementia
RBD: REM sleep behavior disorder
SN: substantia nigra
SNc: substantia nigra pars compacta
WMH: white matter hyperintensities

Disclosures and Conflict of Interest

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Chapter 22

Regenerative Medicine: Could Parkinson's Be the First Neurodegenerative Disease to Be Cured?

Mariacruz L. Díaz, DVM, MSc, PhD

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Departamento de Reproducción Animal, Madrid, Spain

lopez.maria@inia.es

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The increasing prevalence of neurodegenerative diseases in developed countries is a fact recognized by the scientific community. Parkinson's disease (PD) is one of the most insidious, and deserves our attention and efforts. It is probable that more than one therapeutic approach will be needed before we unravel the primary/ultimate cause. One of the most promising treatments is based on regenerative therapy. In the 90s, the transplantation of fetal cells in patients with PD aroused great expectation, but drawbacks such as the difficulty in obtaining fetal cells, disparity of results and graft-induced dyskinesias slowed down its use. Later, Yamanaka's attempt to produce an unlimited source of stem cells was a success [1]. He applied a breakthrough procedure by introducing four transcriptional factors (Oct3/4, Sox2, Klf4 and c-Myc) with retroviruses in human fibroblasts. The fibroblasts were transformed into cells with similar potential to embryonic stem cells (ESCs); these were called induced pluripotent stem cells (iPSCs). The iPSCs are capable of self-renewal and differentiation into all three

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germ layers. Since that publication, there has been an avalanche of work in which iPSCs are induced, multiplied, differentiated into different types of nervous cells and implanted both in patients and in murine disease models. Nowadays, hPSCs can be produced from embryonic or adult cells. Significant advances have been made solving some important drawbacks of Yamanaka's strategy; for instance, derived neurons reach functional maturation preventing formation of tumors; the production efficiency has been increased by millions of cells; and transcriptional factors have been reduced, to be substituted by effective small molecules (SMs). However, to date, it is with human embryonic and fetal cells that best clinical outcomes have been achieved, but their use has also been restricted because the extraction process is really complex, and it entails important ethical objections. Here a ground-breaking approach must be mentioned that has been developed with human parthenogenetic cells. Cells are derived from human nonfertilized oocytes whose second meiosis is halted resulting in diploid cells [2]. These diploid cells are derived into parthenogenetic neural stem cells (pNSCs) with only maternal chromosomic material. Currently, DOPAminergic neurons derived from parthenogenetic pluripotent stem cells (pPSCs) are being tested in clinical trials in Australia [3]. It is too early to know what the results will be; although it could open new research avenues, we still have to learn and see the long-term parthenogenetic cell evolution [4].

For centuries, it was believed that there were no neural progenitor cells (NPCs) in the human CNS. Today, it is clear that in the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus, there are NPCs that could regenerate the nervous cells and be manipulated '*in vivo*' with regenerative purposes [5]. Moreover, it has recently been proven in mice that ependymal cells of the fourth ventricle also have neural stem activity; this supports the idea that the entire lining of the CNS probably has dormant NPCs [6]. Still, in addition to fetal and embryonic cells, NPCs are the most studied and promising cells up to date. Theoretically, NPCs have almost unlimited multiplication capacity and are multipotent; that is, they are able to differentiate into several types of nerve cells.

Although this overview will focus on the status of regenerative medicine regarding its application in PD, we should be aware that the available information supports the hypothesis that most insidious neurodegenerative diseases (Alzheimer, Parkinson, Amyotrophic Lateral Sclerosis [ALS], etc) share a common pathogenesis. In most of them, at least one protein with abnormal structure is implicated, (for instance β-amyloid in Alzheimer, α-synuclein in Parkinson and superoxide dismutase in ALS) and cell-to-cell transmission follows the paradigm accepted for prion diseases [7]. That is why they are so-called 'neurodegenerative prion-like diseases'. In some instances, more than one protein is implicated (for instance α-synuclein and tau or β-amyloid and tau). It should be noted that the spectrum is growing, including diseases such as depression. Focusing on synucleinopathies, Prusiner, in 2015, proved that multiple system atrophy (an α-synucleinopathy frequently misdiagnosed as PD) is the second prion disease in

humans [8]. Moreover, the sporadic cases account for 90% of all neurodegenerative clinical cases, emphasizing how important it is to consider additional therapeutic approaches besides the regeneration of the damaged tissues.

22.1 Multiplication and Differentiation of NSCs

Compared with other tissues, the nervous system has very limited capacity of regeneration '*in vivo*'. Therefore, many studies have been devoted to pursuing the production of NSCs '*in vitro*' with regenerative purposes. Due to the extensive scientific literature, we will focus only on relevant studies performed with human cells. Adult cell-derived hiPSCs have been utilized in the majority of research studies, in which the cells later on differentiate into NSCs and finally into mature neurons. The more frequently used cells have been the fibroblast [9], bone marrow mesenchymal stem cells (BM-MCs) [10, 11] and umbilical cord cells [9, 12]. Nevertheless, other adult cells have also been differentiated such as pericytes of the cerebral cortex, as assayed by Karow et al. in Germany [13], ecto-mesenchymal stem cells from olfactory mucosa in France by Nivet et al. [14], stem cells from carotid body in Spain by Platero-Luengo et al. [15], human urine epithelial cells in China by Cheng et al. [16], stem cells from dental pulp by Zhang et al. [17] and so on.

Since Yamanaka's procedure publication [1], many advances have been made in order to avoid tumor formation and to obtain mature neural cells. In a study published in 2015 by Bardy et al. [18], the authors developed a new culture medium superior to existing media traditionally used for growing and maturing nervous cell lineages. Fully functional glial and neuronal cells were derived from human fibroblasts directly into induced neural cells (iNCs) by using two transcriptional factors, Ascl1 (Mash1) and Ngn2 (Nurr1). The same year, Lee et al. were able to obtain directly iNSCs from human blood cells by using a single transcriptional factor (Oct4) and chemical inhibitors. The iNSCs were postderived into five types of NCs: GABAergic, DOPAminergic, astrocytes, oligodendrocyte and, for the first time, nociceptive sensory neurons [12].

The crucial step to avoid the unwanted use of retroviruses was taken earlier in 2014 by Cheng et al. They derived human epithelial cells extracted from urine into NSCs by using a cocktail of three chemicals (Valproic acid VPA, Repsox and CHIR 99021) [16]. From the functional standpoint, these SMs are inhibitors; VPA inhibits deacetylation of histones, Repsox inhibits glycogen synthase kinase 3β (GSK3β) and CHIR 99021 inhibits EGF. These cells were called chemically induced neural progenitor cells. They obtained cells with an elevated plasticity and a great potential for neuronal conversion by using these chemicals together with hypoxic conditions (mimicking the physiological conditions of oxygen partial pressure of 5% O_2 in the CNS). It deserves to be mentioned that this procedure could be applied to other cells of an epithelial nature such as uterine menstrual

cells, which have already been transformed into iPSCs using Yamanaka's procedure by Ding et al. [19]. In August 2015, the same scientific group obtained the direct conversion of human fibroblasts into mature neuronal cells with four additional SMs: Forskolin, SP600125, GO6983 and Y27632. The majority of these human chemically induced neuron cells (hciNCs) had a glutamatergic nature [20]. In October, they went another important step forward in mice; the direct conversion of astrocytes into four types of neurons: dopaminergic, GABAergic, glutamatergic and motor neurons [21]. It is important to emphasize that astrocytes are very interesting neural cells in terms of regeneration because they actively multiply after brain injury and because they are considered precursor cells of neurons in mammals.

It is clear that the type of starting cells, either fetal or adult, determines the final derived cell type. Whether we use transcriptional factors or chemical inhibitors to dedifferentiate adult cells, the epigenetic memory is specific and determinant of the final result. Regarding this issue, we must mention a suggested multicellular eraser of epigenetic marks, an inhibitor of the BET superfamily proteins (I-BET). The I-BET151 competes with BRD4 (bromodomain-containing protein 4) inhibiting its binding to the super-enhancers of core genes. The results indicate that the addition of I-BET151 in the chemical cocktail resulted in a dramatic increase of functional neurons directly from mice fibroblasts [22]. Therefore, proper chemical SMs acting first as erasers of the epigenetic marks and later as inhibitors of growth factors (EGF, TGF and BMP) or enzymes (protein kinase, GSK3β) could be used locally in such a way that theoretically the regeneration of the required cell could be achieved.

In terms of efficient production, the number of neurons obtained by Haus et al. in a developed xeno-free culture system was remarkable. They started with hESCs and isolated $CD133^{+}/CD34^{-}$ cells with magnetic sorting. In only 10 days, 300 million hNSCs could be produced and in transplanted mice no teratoma were developed [23].

22.2 Production, Transplantation and Characterization of Precursor DOPAminergic Neurons

Parkinson's was first described in 1817 by James Parkinson, and the pathognomonic location of the Lewy bodies in the DOPAminergic neurons of hippocampus, described by FH Lewy in 1910. Initially, the degenerated DOPAminergic neurons are confined into the sustantia nigra and the striatum of the hippocampus a relatively small area of the brain which could be easily substituted. However, it has been more than 24 years since the first transplants of fetal cells were performed in PD patients. To date, the best clinical results obtained in humans are still those using human fetal cells from the ventral mesencephalon [24, 25]. The most important obstacle has been the acquisition of fetal tissues together with a high variability in the recovery rates and the development of dyskinesias in treated patients (Table 22.1).

Optional sources have been sought; among the most promising ones are the precursors of DOPAminergic neurons derived from hPSCs, either from reprogrammed adult cells or from ESCs of early blastocysts. Thanks to many excellent studies, several protocols have been published by which precursor DOPAminergic neurons are produced with high efficiency and proper functionality from hESCs [26, 27].

Table 22.1 Advantages, disadvantages and results of neural precursors derived from human fetal, embryonic, reprogrammed somatic cells and parthenogenetic cells

Cell type	Advantages	Disadvantages	Results
hfventral	First clinical studies made	Ethical concerns Scarce source	Excellent long-term results
hESC	Guaranteed cell source H9WA09 GMP in place to produce cells of quality Detailed description of cell markers	Immunosuppression treatment	Functional recovery No tumorigenicity Variability
hiPSC	Absence of ethical concerns Matched HLA is possible No immunosuppression treatment Detailed description of cell markers Time-consuming and high-cost	Functional recovery No tumorigenicity Variability	
hpNSC	Less ethical objections Produce neurotrophic agents	Lack of 'bona fide' DOPAminergic markers	Unknown long-term evolution
iNC	No pluripotency stage needed	Unknown	Unknown

Abbreviations: hpNSC, Human parthenogenetic neural stem cell; hESC, Human embryonic stem cell; iNC, Directly induced neuronal cells from skin cells.

Up to now, fetal cells and hESCs have been used under strict management rules in top research centers, and only in those countries with permissive rules. The derived precursor DOPAminergic neurons from hESCs have been the most studied and the best characterized. We have seen that the line designated H9 (Scottish Centre for Regenerative Medicine, UK) or WA09 (WiCell University of Wisconsin Madison, WI, USA) has been deeply studied [26, 27]. It has already been approved by the NIH (MD, USA) and it complies with European rules (National Institute for Biological Standards and Control, UK Stem Cell Bank); even a commercial brand offers this H9WA09 line. Theoretically, the unlimited production of precursor DOPAminergic neurons could be possible using these hESCs lines. Therefore, if no more embryos are needed one could wonder what ethical objections there could be in expanding and differentiating the hPSCs into precursor DOPAminergic neurons.

Fortunately for patients with sporadic PD, the first estimations of the number of cells that need to be replaced are very low, approximately 'hundred thousand' cells would be sufficient for one patient. Obviously, these numbers are low for '*in vitro*' culture systems and few laboratories would be required to cover the needs of PD patients in each country.

Regarding DOPAminergic precursors derived from hiPSCs, the results of preclinical studies made in a monkey model for PD were published in 2017. Takahashi et al. [28] reported that the DOPAminergic cells survived for at least 2 years and formed connections with the monkey brain cells. In the treated monkeys, there was a gradual onset of motor functions and no tumor formation was reported. However, the results in terms of survival of DOPAminergic, neurons were highly variable, suggesting that there are still unknown factors in the hiPSC lines that should be characterized before clinical trials can be made.

Table 22.2 Ongoing clinical trials worldwide

Cell type	Clinical trials	Country/Leaders	Consortium/Company/University
hfC	NCT001898390 2010-2021	UK, Sweden Dr Barker RA, Dr Parmar M	TRANSEURO GFORCE
hfC	NCT03128450 2017	China Dr Chun-Feng Liu	Second Affiliated Hospital of Soochow University
hESC	NCT03119636 2017-2020	China Dr Qi Zhou	Chinese Academy of Sciences
hiPSC	R000038278 2018	Japan Dr Jun Takahashi	Center for iPS Cell Research and Application, Kyoto University
UCMSC	NCT03550183 2018	China Dr Shengjun An	Newtherapy BIo-Pharma Technology Co., Ltd Shijiazhuang, Hebei
ASC	NCT02184546 2014-2018	USA	StemGenex, San Diego, CA
BMSC	NCT01446614 2011-	China	Hospital Guanjzhou, Guanjdong
BMSC	NCT03297177 2016-	USA MD Stem Cells	The Healing Institute Margate, FL, USA Euro-Arabian Hospital Dubai, Sharjah, United Arab Emirates
BMSC	NCT02611167 2017-2019	USA Dr Mya Schiess	The University of Texas Health Science Center at Houston Houston, TX
hpNSC	NCT02452723 2016-2020	Australia, Royal Melbourne Hospital, Parkville	International Stem Cell Corporation, Carlsbad, CA

Source: NIH https://clinicaltrials.gov/.

Abbreviations: ASC, Adipose stem cell; BMSC, Bone marrow mesenchymal stem cell; hfC, Human fetal cell; hpNSC, Human parthenogenetic neural stem cell; hESC, Human embryonic stem cell; UCMSC, Umbilical cord mesenchymal stem cell.

We are carefully looking at the ongoing clinical trials worldwide (Table 22.2). In China, they have recruited PD patients for the transplantation of matched human leukocyte antigen (HLA) cells derived from hESCs. In Australia, ISCO started

clinical trials with DOPAminergic neurons derived from human parthenogenetic cells. Some drawbacks have been pointed out regarding the use of parthenogenetic cells: these cells express PAX6 factor which have not been identified 'bona fide' ventral midbrain progenitors DOPAminergic neurons [27, 28]. There are great expectations ahead, but the hurry and commercial interests need to be put in second place when the hopes of millions of patients are at stake. We hope to see PD be the first neurodegenerative disease to be cured thanks to regenerative medicine.

22.3 Rejection of Transplanted Cells

Single cell transcriptome studies have recently revealed that human fetal cells extracted at 16–18 weeks of gestation are not rejected in part due to the absence of the major histocompatibility complex MHC type I. Unfortunately, the results of this study imply that the CNS is not as immunologically inert as we thought, and that ESCs hPSCs could be very distant from adult neurons [29]. The union of the NSCs with the extracellular matrix of the recipient patient, without any doubt, must be important in the rejection or not of the donor NSCs. The extracellular matrix is in contact with the glycocalyx of NSCs and very little is known about how these components are associated in the human brain. Studies concerning glycoconjugates (glycoproteins or glycolipids) of human NPC's glycocalyx are almost absent.

The important role of the chemical structure of carbohydrates in the CNS is illustrated by experiments made in chickens with intracerebral injections of 2-deoxigalactose. The 2-deoxigalactose induces amnesia, which is reverted when coinjected with galactose [30]. As far as we know, only in rodents have the patterns of specific glycans in CNS been studied. All types of neuronal cells in SG and SV zones could be identified with labeled lectins [31]. In fact, also by using lectins, Hamanoue et al. isolated NPCs and deciphered the roles of N-acetylglucosamine and its enzymes on NPC functions, such as cellular migration and immunity [32, 33]. Taken together, all these facts stress how important the implication of glycoconjugates and their carbohydrates are in the interneuronal connections of the CNS.

Therefore, if we want to advance into successful tissue replacements more glycobiology studies will be needed to support future glyco-engineering approaches [30].

22.4 From Bench to Bed

There are translational prospects for neurodegenerative diseases, for example, from the studies made in China. The astrocytes localized in the SG and SV zones behave as neurogenic precursor cells in adult mammals [34]. This peculiar characteristic makes them ideal targets for regenerative use *in vitro* and *in vivo*. In order to convert astrocytes into neuronal cells, Cheng et al. dissected the essential chemicals used in their initial work from three (valproic acid, Repsox

and CHIR 99021) to two SMs (valproic acid and Repsox). Then, they skillfully replaced SMs by drugs already authorized, which should simplify the authorization process for the new pharmacological indications. Valproic acid, an antiepileptic drug (inhibitor of histones deacetylases), and Tranilast, an antiallergic drug (inhibitor of EGF), were used '*in vitro*' with successful results in astrocytes of adult mice [21]. The authors suggested the possibility of applying these chemicals directly in patients with PD. From a translational perspective, this could be a 'from bench to bed' study. Therefore, we would expect that the local treatments with valproic acid and Tranilast could favor the differentiation of human astrocytes into neurons whenever required.

We have to point out that the use of valproic acid in patients with PD could add other advantages apart from its important antidepressant therapeutic effect, that is, its positive effect on the expression of galectin-1 [35]. Galectin-1β (galactose-binding lectin) is known as a neuroprotective agent and promotor of the axonal regeneration [36], emphasizing again the important role that the galactose and galectin-1 could have in the functional connection between neurons and extracellular matrix. On the other hand, strong evidences support that α-synuclein is propagated by exocytosis–endocytosis from cell to cell. Importantly, the receptor implicated in the endocytosis of pathogenic α-synuclein fibrils (N-methyl-D-aspartate receptor NMDA receptor) was competitively inhibited by galectin-1 in hNSCs. In fact, it has been proven that human BM-MCs produce galectin-1 which, in turn, behaves as a competitive protein with α-synuclein in cocultured hNSCs. Moreover, in a parkinsonian mouse model, galectin-1 improved neuron survival and motor function [37]. Studies in Alzheimer's models, both *in vitro* and *in vivo* have also supported the role of the NMDA receptor in the cellular internalization of β-amyloid fibrils. In truth, both increased β-amyloid clearance and survival of hippocampal neurons were seen when BM-MCs were injected in the tail vein of mice [38], probably through the same mechanism described for α-synuclein. Therefore, in α-synucleinopathies as well as in diseases with β-amyloid fibrils formation, pharmacokinetics studies of SMs deserve to be conducted.

Chronic systemic treatments with VPA have reported some worsening of Parkinson's symptoms. These cases are reversible when the treatment ceases. Therefore, to undertake pharmacokinetic studies of local application would allow decreased doses, the overcoming of unwelcome secondary effects and, hopefully, the promotion of NSCs present in the hippocampus. In order to apply the treatments with SMs, one of the best routes could be through the nose. Anatomically, the olfactory bulb is very close to the roof of the nasal cavity and functionally, it is connected with the hippocampus. In a recent study made by Alvarez-Buylla et al., the authors discovered that in the subventricular zone of the lateral ventricle, the NSCs or type B1 cells divided symmetrically giving rise to an asymmetric population of 20% self-renewal NSCs and 80% differentiated neurons [39]. We wonder if we could reverse these percentages in PD patients. We also know, thanks to the work made by Chen et al. in 2017, that the specific inhibition of GSK3β favors NSC self-renewal while the inhibition of GSK3α

favors neuronal differentiation in mouse ESCs [40]. Therefore, if this holds true in humans, it could be possible to use nose instillations of GSK3β inhibitors or other SMs in order to increase the number of NSCs. To end this exposition on the state of the art of nervous tissue replacement, the good news is that the results of the transplantation of NSCs derived from human fetuses into Parkinson's patients have been more than encouraging. 24 years after implantation of fetal ventral cells, which were injected in the putamen, there was a complete recovery of the innervation with still functional dopaminergic neurons. Motor symptom recovery and withdrawal of the treatment with LDOPA resulted, and following deaths were not owing to the disease [41, 42].

22.5 Future Perspective

Today, human ESCs are becoming available in many countries around the world [43–45]. Agreements on robust protocols for multiplication and differentiation of ESCs and iPSCs, good practice codes and standards, disclosed specs for the starting somatic cells, and robust cryopreservation procedures of cells [45, 46] and so on, should be reached as soon as possible. This will assure a fruitful outcome for the enormous efforts that are being made in this research field around the world. In fact, a few European countries together with USA and Japan have formed a GForce-PD consortium, which has already begun working in this direction and will start very soon with human clinical trials [47].

We must not forget that α-synuclein prions exist in patients with multiple system atrophy, that α-synuclein is present in organic fluids and in other parts of the body besides the brain, and finally, that in some patients other prion-proteins could coexist. Therefore, we should take important sanitary actions because prion proteins do not respond to standard decontamination procedures. Generally, in laboratories and hospitals around the world, standard conditions used for sterilization in an autoclave are 120°C for 20 min; these are not enough to decontaminate prions. Therefore, we strongly recommend strict protocols for decontamination of prion-like protein, especially in the use of surgical material which should be new for each patient in clinical trials.

Some other clinical therapies have been suggested such as the reduction of the levels of pathological proteins with plasmapheresis [48] or the restoration of intestinal leaking with fecal transplants [49, 50]. Although ideally these treatments should be done before regenerative therapy, unfortunately little or nothing is known about their efficacy and safety. If we do not take into consideration all these precautions, transplanted cells could be negatively affected by the presence of prion-like proteins in the recipient patients, endangering their recovery or masking good results. There is a fine compromise between 'rejection' and 'teratoma formation'; the less differentiated a cell is, more probability exists that a teratoma will develop yet there is a lower probability of rejection. Regarding 'teratoma formation' many technical advances have been made since Yamanaka's first

studies. Almost all scientists already claim that their cells have not developed tumors in long-term trials. To circumvent the 'rejection' either an immunosuppressive treatment should be applied or compatible grafts should be used. Almost all clinical studies have included an immunosuppressive treatment, except in the trial made with autologous iPSC from which we still do not know the results.

Unfortunately, there is still a high variability in the recovery rate in the studies made with DOPAminergic precursors derived from ESC and hiPSC (Table 22.1). Nowadays, autologous hiPSCs derived from patients with genetic Parkinson's cannot be used. Hopefully, in the near future, different types of stem cells will be produced and used in agreement with the pathogenesis of PD.

Certainly, there is still a way to walk before nervous tissue replacement therapies are fully implemented in daily clinical practice. Meanwhile, '*in vitro*' studies should focus on the key elements for human embryonic and fetal cell transplants to be successful. In the case of PD, the joint efforts of many researchers from around the world are bringing us closer.

Abbreviations

ALS: amyotrophic lateral sclerosis
ASC: adipose stem cell
BMSC: bone marrow mesenchymal stem cell
ESCs: embryonic stem cells
GSK3β: glycogen synthase kinase 3β
hciNCs: human chemically induced neuron cells
hESC: human embryonic stem cell
hfC: human fetal cell
HLA: human leukocyte antigen
hpNSC: human parthenogenetic neural stem cell
I-BET: inhibitor of the BET superfamily proteins
iNCs: induced neural cells
iPSCs: induced pluripotent stem cells
NPCs: neural progenitor cells
PD: Parkinson's disease
pNSCs: parthenogenetic neural stem cells
pPSCs: parthenogenetic pluripotent stem cells
SMs: small molecules
UCMSC: umbilical cord mesenchymal stem cell

Disclosures and Conflict of Interest

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Chapter 23

Changes in the Functional Brain Network of Children Undergoing Repeated Epilepsy Surgery: An EEG Source Connectivity Study

Giulia Iandolo, MS,[a,b] Nitish Chourasia, MD,[c,d] Georgios Ntolkeras, MD,[a,e] Joseph R. Madsen, MD,[f] Christos Papadelis, PhD,[e,g,h] Ellen Grant, PhD,[a,e] Phillip L. Pearl, MD,[c] Fabrizio Taffoni, PhD,[b] and Eleonora Tamilia, PhD[a,e]

[a]*Fetal Neonatal Neuroimaging and Developmental Science Center, Department of Pediatrics, Boston Children's Hospital, Boston, Massachusetts, USA*
[b]*Advanced Robotics and Human-Centred Technologies-CREO Lab, School of Engineering, University Campus Bio-Medico di Roma, Rome, Italy*
[c]*Division of Epilepsy and Clinical Neurophysiology, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[d]*Health Science Center, Department of Pediatrics and Neurology, Division of Peds Neurology, Le Bonheur Children's Hospital, College of Medicine, University of Tennessee, Memphis, Tennessee, USA*
[e]*Division of Newborn Medicine, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[f]*Epilepsy Surgery Program, Department of Neurosurgery, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[g]*Jane and John Justin Neurosciences Center, Cook Children's Health Care System, Fort Worth, Texas, USA*
[h]*Department of Bioengineering, University of Texas at Arlington, Arlington, Texas, USA*

Eleonora.Tamilia@childrens.harvard.edu

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23.1 Introduction

Epilepsy is widely regarded as a brain network disorder, corroborated by an increasing body of evidence demonstrating alterations in an inter-regional anatomofunctional relationship among brain areas in the epileptic brain [1–5]. Consequently, clinical seizures are hypothesized to be secondary to involvement of large-scale synchronized brain networks [6, 7]. This understanding of seizures and epileptic networks has been often elaborated in a network-level perspective by using functional connectivity (FC), a concept that is based on the assumption that the extent to which different brain areas are functionally connected depends on their level of synchronous temporal activity [8]. FC analysis in epilepsy has been investigated via resting-state functional magnetic resonance imaging (fMRI) [9–14], intracranial electroencephalogram (iEEG) [15, 16], scalp electroencephalogram (EEG) [17–19], or magnetoencephalography (MEG) [20–22].

In patients with focal drug-resistant epilepsy (DRE), the understanding of epileptogenic network connectivity has therapeutic relevance, especially regarding epilepsy surgical evaluation. Underpinning this process is the formulation of a network hypothesis regarding identification of structures within the epileptogenic networks important in the generation of seizures [23]; however, despite elaborate pre-surgical evaluation (consisting of scalp and intracranial EEG, MRI, fMRI, PET, MEG, and neuropsychological testing), nearly one-third of patients suffer failure of first resective epilepsy surgery with continued seizures [24, 25]. Some of these patients may still benefit from repeat surgery, although defining such a re-operation plan after failed surgical resection can be challenging [26–29]. Thus, exploration of additional noninvasive techniques that can complement the pre-surgical evaluation test battery would be valued clinical tools in such complex cohorts.

There are few studies that have reviewed patients undergoing repeat epilepsy surgery in the pediatric age group [26–28], evaluating outcomes and their predictors after re-operation using clinically available pre-surgical data (such as etiology, pathology, type and site of resection, age, time between surgeries, and so on). There is a paucity of research studies investigating new analytic approaches in this population, so as to improve or complement the clinically established pre-surgical evaluation tests. To this effect, deconstructing the functional brain network and evaluating its connectivity changes after surgery is a new approach in such a cohort. It can be speculated that the quantification of brain network characteristics may aid in providing supplemental information for surgical planning in these cases.

Among all the available non-invasive modalities that can be used to quantify FC, scalp EEG stands out as an ideal tool owing to its frequent and universal usage in clinical management of patients with epilepsy. Besides its large availability, scalp EEG provides high temporal resolution (millisecond time scale typically) that allows the investigation of brain networks at different frequency bands as

opposed to fMRI. Although scalp EEG suffers from lower spatial resolution than its invasive counterpart (stereotactic EEG or electrocorticography) or fMRI, it allows the estimation of whole-brain functional networks, especially if combined with the spatial information of the individual MRI (performing what is called electric source imaging or ESI) [30–34]. Despite the contribution of aberrant brain connectivity in generation of seizures being increasingly recognized, it remains to be explored whether connectivity changes following a surgical brain resection can be quantified via routine scalp EEG and linked to seizure occurrence. In addition, even though pre-surgical aspects of the FC brain network have been extensively studied [2, 12, 35–40], there is little knowledge about how FC evolves before and after epilepsy surgery.

In this study, we study serial scalp EEGs from children with DRE who underwent repeat focal epilepsy surgery with the aim of (i) quantifying surgery-related changes in the children's functional brain network (i.e., evolution of their brain network organization) and (ii) identifying whether such brain network evolution differs depending on the surgical outcome. For this latter point, we also study FC evolution in children with DRE who underwent a single successful surgery. We perform FC analysis at the source level (between cortical regions) using scalp EEG measurements collected during sleep before and after each surgery along with the individual patient's MRI scans. We compute the FC of the brain during the interictal state using EEG epochs free of any interictal epileptiform discharges.

23.2 Materials and Methods

23.2.1 Subjects

We studied children with DRE who underwent focal epilepsy surgery at the Epilepsy Center of Boston Children's Hospital (BCH) between November 2013 and March 2019 and had a minimum duration of follow-up of 1 year since last surgery. We studied two groups of patients: (1) repeated surgery group, i.e., patients who had two focal resective surgeries (because of seizure recurrence after the first one); (2) seizure-free group, i.e., patients who had one focal resective surgery followed by good outcome (namely, who were Engel 1 after 12 months from surgery). Inclusion criteria for both groups were: (i) availability of sleep EEG data prior and after each surgery; (ii) availability of both pre-surgical and post-surgical MRIs. Exclusion criteria were: (i) hemispherectomy or corpus callosotomy (non-focal resections); (ii) absence of at least 1 min of non-REM sleep in the EEG recordings that was free of artifacts and epileptic spikes; (iii) unsatisfactory quality of the MRIs for coregistration and delineation of resection. The study protocol received approval by the BCH Institutional Review Board (IRB-P00035192, PI: Tamilia), which waived the need for written informed consent due to the study's retrospective character. Post-surgical outcome

following each surgery was evaluated by a pediatric epileptologist from the most recent follow-up visit using the Engel scale, as is clinical practice at our institution, dichotomized into good outcome (Engel 1) and poor outcome (Engel ≥ 2).

23.2.2 Scalp EEG Data

All patients underwent conventional video-EEG recording at BCH using the standard clinical EEG setup with 19 electrodes, plus two additional frontotemporal leads (FT9 and FT10) and four optional electrodes in some cases (F9, F10, P9, P10). Data were recorded using the XLTEK EMU40 system (Natus Medical Inc., Pleasanton, CA, USA) with a sampling rate in the 200–1024 Hz range. Individual leads (gold cups) were placed according to the international 10/20 system (Fig. 23.1a). Impedances were kept below 10 KΩ during the entire recording session. Reference and ground electrodes were placed in frontocentral areas (near FC1 and FC2, respectively). EEG data were filtered using a notch filter at 60 Hz (second order IIR notch filter with zero-phase lag) and a 1–70 Hz band-pass filter (Fig. 23.1b). Band-pass filtering was performed using Brainstorm software; this implements an even-order linear phase FIR filter based on a Kaiser window design, which is then made to be zero-phase and zero-delay by shifting the sequence backward in time.

We retrieved three EEGs per patient for the repeated surgery group, as shown in Fig. 23.1b: (1) before first surgery; (2) between surgeries; (3) after second surgery. Two EEGs per patient were analyzed for the seizure-free group, before and after surgery. As part of the EEG monitoring at BCH, pediatric neurophysiologists extracted multiple 5–10 min segments of interictal data from the EEG recordings (long-term monitoring or routine EEG). For the purpose of this study, a pediatric clinical neurophysiologist (N.C.), blinded to the resection and the surgical outcome, retrospectively reviewed these segments and selected for each patient 3–5 min of data from stage N1 or N2 sleep, without including major artifacts, technical disruptions, ictal events, or continuous interictal discharges. A second independent neurophysiologist (P.L.P.) reviewed the same segments, blinded to the first reading, to confirm sleep stage and absence of ictal events. Spikes were then identified through visual inspection by two independent readers (N.C. and G.N.) on a bipolar montage. In case of disagreement, a third senior epileptologist was available (P.L.P). Epochs of 2000 ms around each spike were excluded from further analysis (in order to analyze background activity). Any activity related to an excessive electrode artifact was also marked and excluded. The signal space projection (SSP) technique was used to reject external disturbances due to cardiac events detected by electrocardiogram (ECG) recordings when present. Finally, clean background EEG data (free of artefacts and epileptiform activity) were divided into 2 s epochs (see Fig. 23.1b).

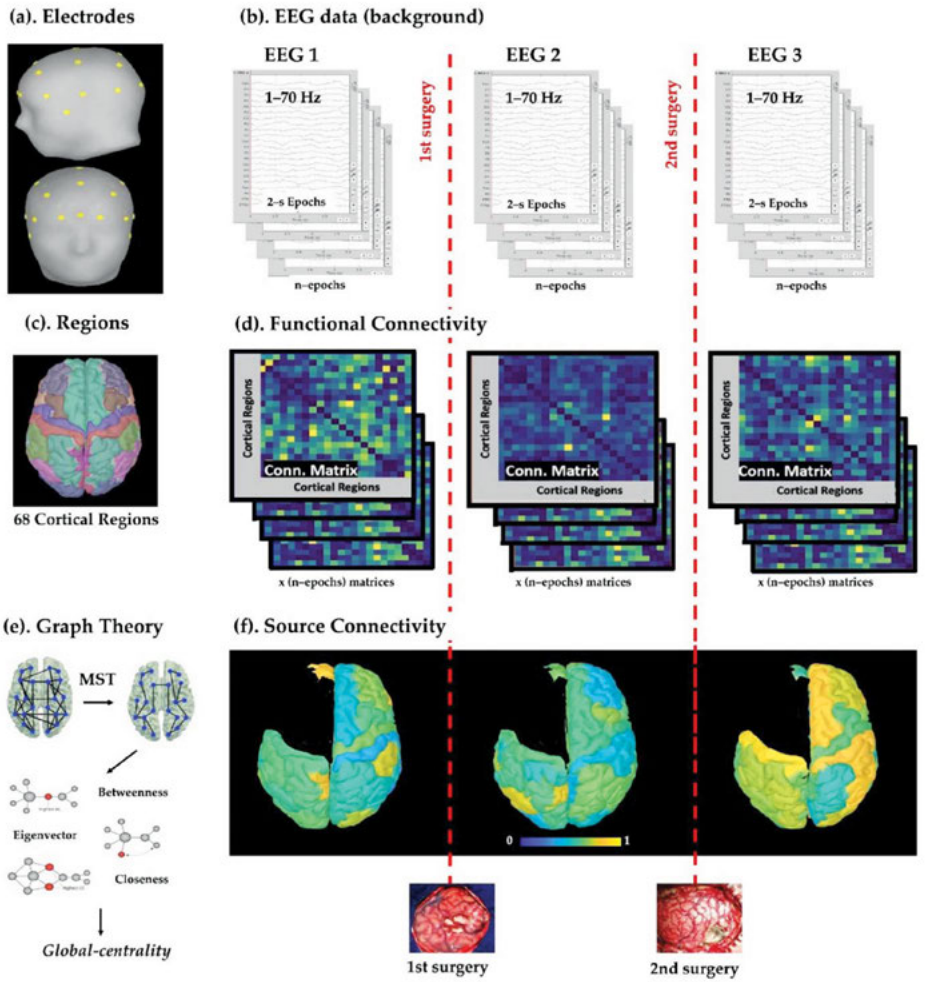

Figure 23.1 Outline of the data analysis process. (a) We retrieved conventional EEG data and the patient's MRI at the time of the EEG (to build the head model), on which electrodes were positioned according to the international 10/20 system); (b) interictal sleep EEG data without interictal discharges and artifacts were selected ("background activity"). We analyzed three EEGs per patient for the repeated surgery group (EEG1—before first surgery; EEG2—between surgeries; EEG—after second surgery) and two EEGs per patient for the seizure-free group (EEG1—before surgery; EEG2—after surgery). These EEG data were segmented into multiple 2 s epochs. (c) Each patient's cortical surface was parcellated into 68 regions of interest (ROIs) defined by the Desikan–Killiany atlas. (d) From each EEG epoch, a functional connectivity matrix was computed (amplitude envelope correlation method). (e) A graph and its minimum spanning tree (MST) were then built from each matrix, then three centrality measures (betweenness, closeness, and eigenvector) were extracted, normalized, and averaged, obtaining a unique comprehensive measure of relative centrality ("global centrality"). (f) The global centrality was estimated for each cortical ROI (average of all epochs) at each time point (before first surgery, between surgeries, after second surgery). We report here an example of global centrality across all cortical ROIs for patient #2, where yellow and blue colors indicate high and low values, respectively.

23.2.3 Cortical Parcellation and Regions of Interest

We retrieved the pre-operative and post-operative MRIs for each patient. As part of their routine pre- and post-surgical evaluation, a T1-weighted volumetric MRI was acquired, typically a magnetization-prepared rapid gradient echo (MPRAGE). Pre-operative and post-operative MRIs were pre-processed in FreeSurfer [41] (or Infant Freesurfer [42], when the first failed) to segment brain structures and extract the pial surface (cortex). As part of this process, each patient's cortical surface was mapped to the Desikan–Killiany brain atlas [43], which assigns each neocortical vertex to one of the 68 areas based on gyral morphology. Through this atlas, we automatically defined the 68 regions of interest (ROIs) for the following whole-brain connectivity analysis.

23.2.4 Estimation of Source Activity and Cortical Parcellation

ESI was performed on EEG data using Brainstorm [44], as illustrated in Fig. 23.2a. For this purpose, we constructed a realistic head model using OpenMEEG software after manually co-registering the EEG channel locations with the patient's MRI based on the 10–20 system (top of Fig. 23.2b) [31, 45, 46].

For the head model, we used a three-layer (scalp, outer skull, and inner skull) boundary element method (BEM). Source space was constrained to the cortex, which was composed of ~15,000 vertices per patient. We used a linearly constrained minimum variance (LCMV) beamformer to estimate brain activity within the source space [47]. Data covariance was computed from the EEG epochs, whereas identity matrix was used for noise covariance.

We obtained a source activation map (covering all cortical vertices) for each 2 s epoch (bottom of Fig. 23.2a). The beamformer output was used to estimate the neural activity of each ROI, defined via the Desikan–Killiany atlas, by computing their mean activation (mean across cortical vertices). For each of these regions we obtained a time series (cortical activation over time), as shown in Fig. 23.2b.

23.2.5 Classification of ROIs Based on the Resection

Resection was drawn manually in Brainstorm slice-by-slice after co-registration of the pre- and post-operative MRIs (see Fig. 23.3a). We computed the distance of each cortical ROI from the resection as the minimum Euclidean distance between all their cortical vertices. Regions that had <10% of their vertices resected were regarded as untouched during surgery (remaining cortical regions). All other ROIs were considered as overlapping with the resection and were excluded from further analysis. We excluded partially resected ROIs from our study because: (i) their automated parcellation on the post-operative cortex could be affected by errors due to the abnormal brain structure; (ii) the observed post-surgical connectivity changes could be significantly biased by the actual structural changes of these regions (due to partial resection). Given the limited sample size of our cohort, we could not control for these factors.

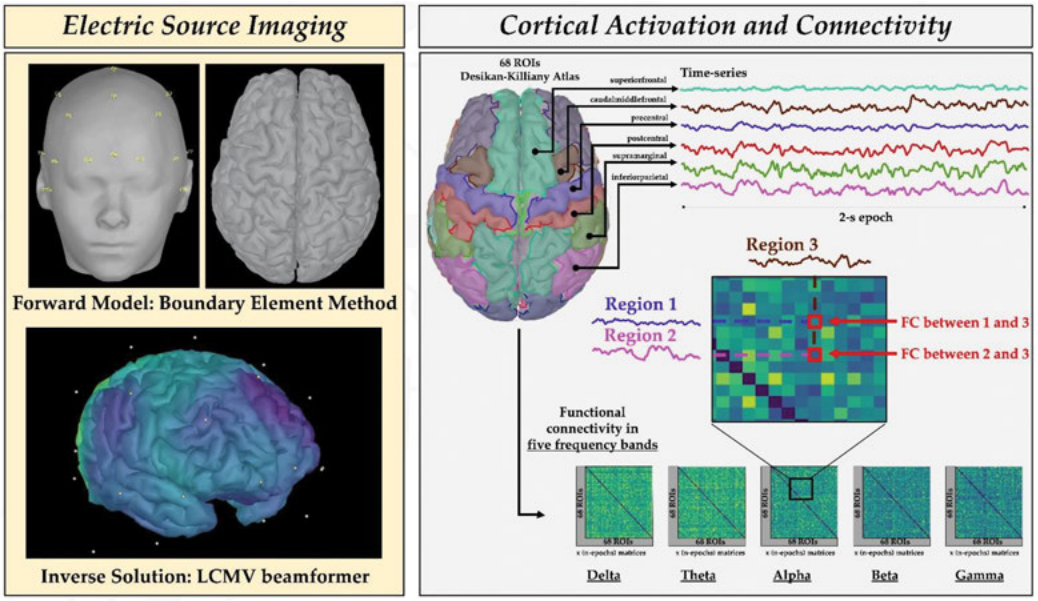

Figure 23.2 Source connectivity analysis. (a) To estimate the cortical activity from EEG data, we performed electric source imaging (ESI). A 3-layer boundary element method (BEM) was applied to solve the forward problem (i.e., compute the head model) after extracting the 3D head mask and cortical surface (top left) from each patient's MRI. Electrodes were positioned on the head based on the 10–20 system. A linearly constrained minimum variance (LCMV) beamformer was used to solve the inverse problem, which generated a cortical activation map for each 2 s epoch (bottom left). (b) We estimated the activity of each cortical region of interest (ROI), obtaining a time series for each ROI. Functional connectivity between these regions was estimated for five frequency bands (δ (2–4 Hz); θ (5–7 Hz); α (8–12 Hz); β (13–29 Hz); γ (30–70 Hz)). Each element of the connectivity matrix indicates how much an ROI is connected to another in that 2 s epoch.

The untouched regions in the same hemisphere of the resection that had a distance from the resection lower or equal to 10 mm were labeled as "adjacent"; all other remaining regions were labeled as "far" (see example in Fig. 23.3b).

23.2.6 Functional Connectivity Analysis

To estimate FC, we computed the orthogonalized amplitude envelope correlation (AEC) [48, 49] between each pair of ROIs, which consisted of the linear correlation between orthogonalized, band-limited power envelopes. Orthogonalization was used to compensate the spatial leakage and to remove all shared signals at zero lag between the network nodes. We chose this method based on the study of Colclough et al. [50], which showed that AEC is the most consistent connectivity measure for resting-state studies in source space. AEC was estimated in five frequency bands (Fig. 23.2b): (i) delta δ (1–4 Hz); (ii) theta θ (5–7 Hz); (iii) alpha α (8–12 Hz); (iv) beta β (13–29 Hz); (v) gamma γ (30–70 Hz, low gamma).

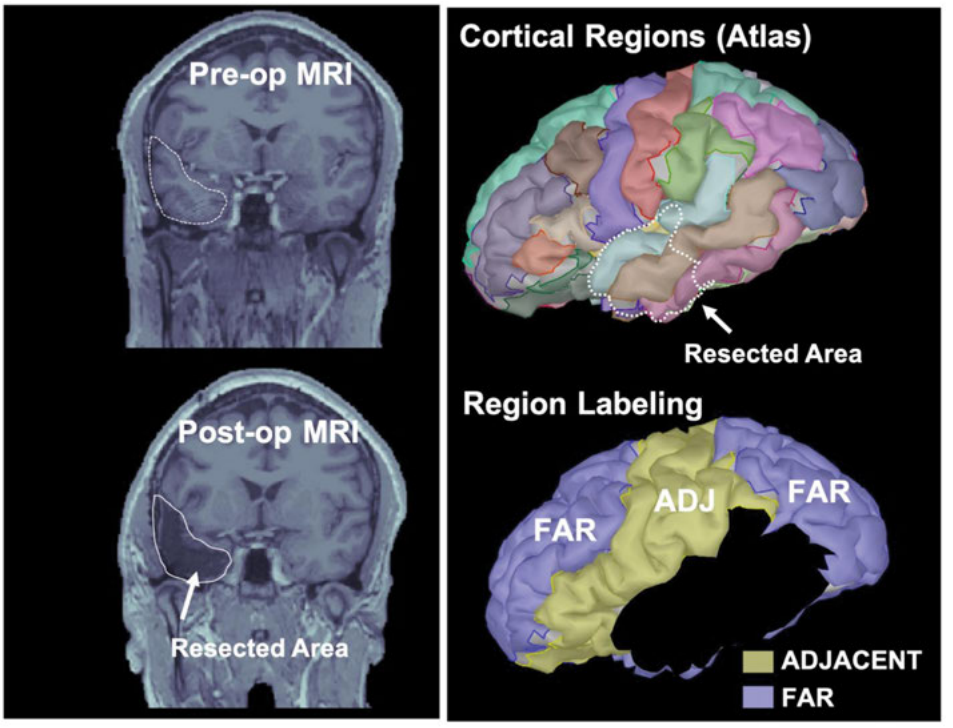

Figure 23.3 Resection and cortical region labeling. (a) Pre-operative (top) and post-operative (bottom) MRIs are displayed with the resected area (white outline). (b) Resected area (left temporal lobe in this patient) is displayed on top of the 68 cortical ROIs. The ROIs overlapping (even partially) with the resected areas were identified (black regions, bottom). All other ROIs were labelled as either adjacent to the resection (ADJ, in yellow) if within 10 mm from the closest margin of the resected area or far from the resection otherwise (in purple).

We computed the AEC for each 2 s epoch and obtained a FC matrix per epoch (Fig. 23.1d), whereby each element tells us how much each brain region is connected to another. Then, each connectivity matrix was converted into a graph consisting of nodes (i.e., cortical ROIs) and edges (i.e., AEC value between them). Each graph was then converted into a minimum spanning tree (MST) [20], as shown in Fig. 23.4, a sub-graph of the weighted graph, which captures the most important connections without forming close loops and which minimizes the functional cost. The cost of each link was calculated as the inverse of the connectivity value. From each MST, we computed three centrality measures that quantified the importance and influence of each node within the network, capturing distinct aspects of centrality in a network:

- Betweenness centrality measures how often each node appears on the shortest path between two nodes in the graph. In brain network analysis, the BC value of a brain region measures its impact on the flow of information across the brain network.

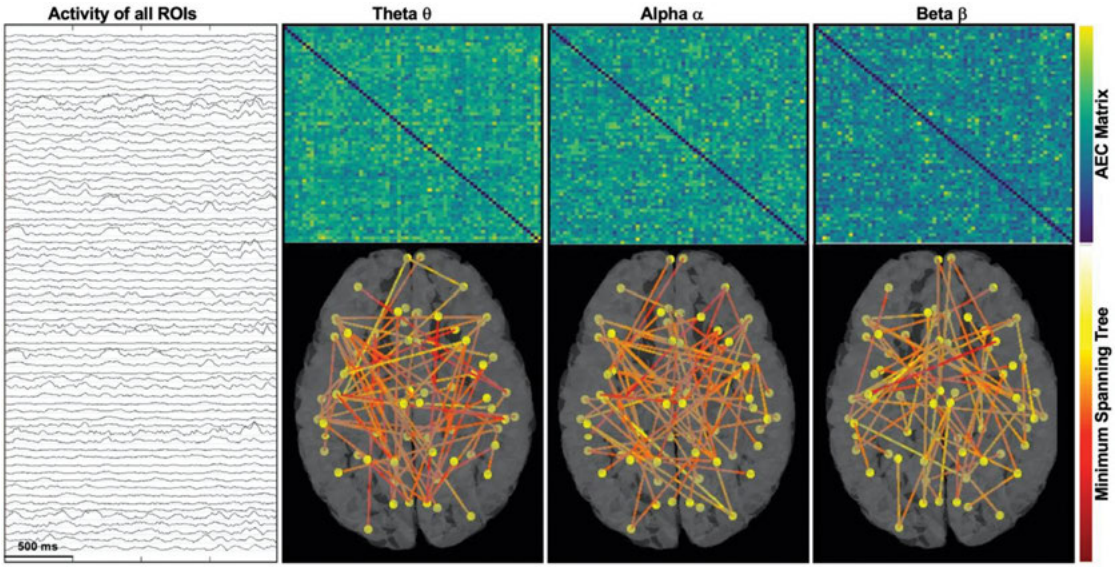

Figure 23.4 Example of source connectivity and minimum spanning tree in three frequency bands. Left panel shows one 2 s epoch of the background source activity (68 signals), whereby each signal corresponds to the reconstructed activity (via ESI) of each cortical ROI across the whole brain. From these reconstructed cortical signals, we estimated 5 connectivity matrices (via AEC method) and their minimum spanning tree (MST) for each frequency band. Here, we show matrices and graphs (MST) for theta, alpha, and beta bands (from the 2-s epoch displayed on the left), whereby each node (yellow dots) corresponds to an ROI and each edge (link or connection) is color-coded based on its cost (inverse of AEC value). Network centrality measures were estimated from each MST and then averaged across all analyzed epochs.

- Closeness centrality reflects the closeness between a node and other nodes in the brain network. In the brain network analysis, the closeness centrality of a brain region measures its indirect impact on other brain regions.
- Eigenvector centrality is a measure that considers both the quantity and quality of a node's connections. In fact, it considers both the degree of the node and the degree of its neighbors.

Each centrality measure was averaged across all epochs within a specific time point in order to obtain a single averaged value per cortical region per patient. Such values were then normalized to the patient's maximum value of relative centrality within the brain network (so that the most central region was assigned a value of 1 for each patient). Finally, we defined a new comprehensive measure of centrality, which we called "global centrality", as the average of the normalized values of the betweenness, closeness, and eigenvector (Fig. 23.1f). As a result, for each patient, we estimated the relative global centrality of each cortical region within the brain network, at each time point (1—before first surgery; 2—after first surgery; 3—after second surgery when present) and for each frequency band. Figure 23.1 provides an overview of the data analysis

pipeline for the repeated surgery group. Similar pipeline was followed for the seizure-free group, whereby only one surgery was performed and only two EEGs were analyzed.

For each ROI, we also computed their post-surgical variations in centrality (i.e., post-surgical minus pre-surgical global centrality). The normalized power spectrum density (PSD) was also computed in the five frequency bands for all cortical ROIs in Brainstorm software and their post-surgical variations in relative power were estimated.

23.2.7 Statistical Analysis

To assess whether the centrality role of the untouched cortical regions within the brain network changed after surgery, we compared their global centrality between pre- and post-surgery. The Wilcoxon signed rank test was used to test the difference between pre-surgical and post-surgical global centrality separately for all the regions that were adjacent or far from the resection and separately for each frequency band. These analyses were conducted separately for the following types of surgery: (i) first successful surgery (seizure-free group); (ii) first failed surgery; (iii) repeated (second) failed surgery; (iv) repeated (second) successful surgery. Finally, the Wilcoxon rank sum test was used to compare variables between seizure-free and repeated surgery groups. Spearman's correlation was also used to test for correlations between the patients' ages or time intervals between EEGs and the patients' post-surgical variation in centrality far from or adjacent to resection. We also tested whether the observed variations in global centrality of the cortical regions correlated with variations in relative power and whether the average relative centrality of far and adjacent regions correlated with each patient's number of AEDs (at the time of the EEG). Here, p-values < 0.05 were considered significant. MATLAB R2020b (The MathWorks Inc., Natick, MA, USA) was used for statistical analysis.

23.3 Results

23.3.1 Patient Cohort

The repeated surgery group included nine children (4 female; age at first surgery: 5.4 (3.5–12.4) years). Three of them had a second successful surgery (age at second surgery: 6.7 (5.5–16.5) years), whereas six patients had a poor outcome after the second surgery (9.7 (2.6–12.8) years). The seizure-free group (single surgery) included 12 children (4 female; age at surgery: 14.5 (10.5–17.7) years). Table 23.1 reports the clinical and demographic characteristics of all included patients. Genetic testing did not play a role in the pre-surgical workup of our cohort. The first surgery consisted of a temporal resection in five patients (55%) for the repeated surgery group and in seven patients (58%) for the seizure-free group. In the repeated surgery group, we had 2 cases out of 9 (22%, patients #2, #6) with unknown causes of epilepsy (Table 23.1) and 3 cases out of 12

Table 23.1 Clinical characteristics of our cohort

		Age (Y)					No. AED			Resection (side lobe)		
			Surgery				EEG			Surgery		
Pt/ Sex	Gr.	Epi. Ons	1st	2nd	Etiology	Pathology (histology)	1st	2nd	3rd	1st	2nd	Engel[a]
1/Fe	RS	3	5.3	6.7	FCD	FCD 2A	3	2	1	L-T	L-T	I
2/M	RS	4	16.8	19.8	Unknown	Gliosis	2	3	0	L-F	L-F	I
3/Fe	RS	0.7	1.7	2.5	Polymicrogyria	FCD 2A, Gliosis, Fused Sulci, WM heteropia	3	3	2	L-F	L-F	III
4/M	RS	1.5	5.4	7.3	FCD	FCD 2B, Gliosis	4	4	4	L-F	L-F	II
5/M	RS	10	15.5	17.4	Low grade glioma + developmental venous anomaly	Low grade glioma, dysplastic features	1	2	2	R-T	R-C	II
6/Fe	RS	0.8	11.3	12.8	Unknown	Oligodendroglial hyperplasia	2	2	2	R-T	R-T	IV
7/M	RS	10	11.3	12.0	Sphenoidal encephalocele	Dysplastic features, Gliosis	2	2	3	L-T	L-T	II
8/Fe	RS	0	2.1	2.6	TSC	Consistent w/TSC	2	3	2	R-F	R-PF	IV
9/M	RS	3	4.0	5.1	FCD	FCD 2A, MST	2	2	1	R-T	R-T	I
10/M	SF	9	17.4	n/a	Neoplasm	Ganglioma, FCD 1	3	3	na	L-T	na	I
11/Fe	SF	16	18.2	n/a	Unknown	Gliosis	2	1	na	L-T	na	I
12/M	SF	5	5.5	n/a	Lesion suggestive of DNET	Angiocentric glioma	4	0	na	L-C	na	I
13/M	SF	1.5	12.5	n/a	MTS	FCD 2A	3	2	na	L-T	na	I
14/M	SF	8	9.8	n/a	Polymicrogyria	Mild dysplastic features	2	3	na	L-P	na	I
15/M	SF	6	14.8	n/a	FCD	na[b]	3	3	na	R-F	na	I
16/M	SF	8	10.0	n/a	Posterior cerebralartery infarct (PCA)	Consistent w/PCA encephalomalacia, FCD 2D	2	2	na	R-O	na	I
17/Fe	SF	17	21	n/a	TBI	Consistent w/TBI	2	3	na	R-T	na	I
18/Fe	SF	12	18	n/a	Unknown	MTS, gliosis, oligodendroglial hyperplasia	1	0	na	L-T	na	I
19/M	SF	13	14.2	n/a	Low grade tumor	DNET	5	2	na	L-T	na	I
20/Fe	SF	0.6	16.1	n/a	MTS + FCD	MST, FCD 2A	2	1	na	L-T	na	I
21/M	SF	5	10.8	n/a	Unknown	na[b]	1	1	na	R-P	na	I

[a]For repeated surgery (RS) group, the reported Engel scale outcome is relative to their 2nd surgery.

[b]Histological exam was not performed (laser ablation cases).

Abbreviations: Pt, patient; Y, years; M, male; Fe, female; Gr, group; RS, repeated surgery; SF, seizure-free; Epi. Onset, age at epilepsy onset; Surg, surgery; n/a, not applicable; AED, anti-epileptic drugs; L, left; R, right; F, frontal; T, temporal; P, parietal; O, occipital; C, central; FCD, focal cortical dysplasia; TSC, tuberous sclerosis complex; DNET, dysembryoplastic neuroepithelial tumor; TBI, traumatic brain injury; WM, white matter; MTS, mesial temporal sclerosis.

(25%, patients #11, #18, #21) in the seizure-free group; thus, since the proportion did not differ, we can exclude that this factor influenced the observed differences between groups. Age at first surgery was lower in the repeated surgery compared to the seizure-free group (p = 0.03). The intervals between pre- and post-surgical EEG did not differ between groups (seizure-free: 1.8 (1–3.1) years; repeated surgery: 1st 1.2 (1–1.8), p = 0.50; 2nd 1.2 (0.75–2.2), p = 0.56). No differences were observed in the percentages of the cortex that was resected during surgery between the repeated surgery group (at first surgery) and seizure-free group (5% and 3%, p = 0.3). No difference was seen between the repeated surgery and seizure-free group in the number of AEDs either before (p = 0.85) or after (1st) surgery (p = 0.14).

23.3.2 Functional Connectivity Changes: Pre-Surgical vs. Post-Surgical Centrality

To explore post-surgical changes in the brain network in the different groups, we compared the global centrality of the remaining regions (far and adjacent to resection) between pre- and post-surgery. Table 23.2 shows the results that we obtained in the five frequency bands for both adjacent and far regions.

Table 23.2 Analysis of post-surgical changes in the global centrality of far and adjacent regions

		1st surgery		2nd (repeated) surgery	
Frequency	**Areas**	**Successful (SF group)**	**Failed (RS group)**	**Successful (RS group)**	**Failed (RS group)**
Delta (δ)	FAR	$p < 0.001$* (increase)	$p < 0.001$* (increase)	$p = 0.3$ (no change)	$p < 0.001$* (decrease)
	ADJACENT	$p < 0.001$* (increase)	$p = 0.04$* (decrease)	$p = 0.5$ (no change)	$p = 0.2$ (no change)
Theta (θ)	FAR	$p < 0.001$* (increase)	$p = 0.1$ (no change)	$p < 0.001$* (increase)	$p = 0.01$* (decrease)
	ADJACENT	$p < 0.001$* (increase)	$p = 0.06$ (no change)	$p = 0.02$* (increase)	$p = 0.4$ (no change)
Alpha (α)	FAR	$p < 0.001$* (increase)	$p = 0.8$ (no change)	$p = 0.01$* (increase)	$p = 0.004$* (decrease)
	ADJACENT	$p < 0.001$* (increase)	$p = 0.4$ (no change)	$p = 0.2$ (no change)	$p = 0.4$ (no change)
Beta (β)	FAR	$p < 0.001$* (increase)	$p < 0.001$* (decrease)	$p < 0.001$* (increase)	$p = 0.3$ (no change)
	ADJACENT	$p < 0.001$* (increase)	$p = 0.2$ (no change)	$p = 0.8$ (no change)	$p = 0.4$ (no change)
Gamma (γ)	FAR	$p = 0.001$* (increase)	$p < 0.001$* (decrease)	$p = 0.4$ (no change)	$p < 0.01$* (increase)
	ADJ	$p = 0.06$ (no change)	$p = 0.6$ (no change)	$p = 0.009$* (decrease)	$p = 0.9$ (no change)

*p-value statistically significant.

Abbreviations: RS, repeated surgery; SF, seizure-free; p, p-value (Wilcoxon signed rank test).

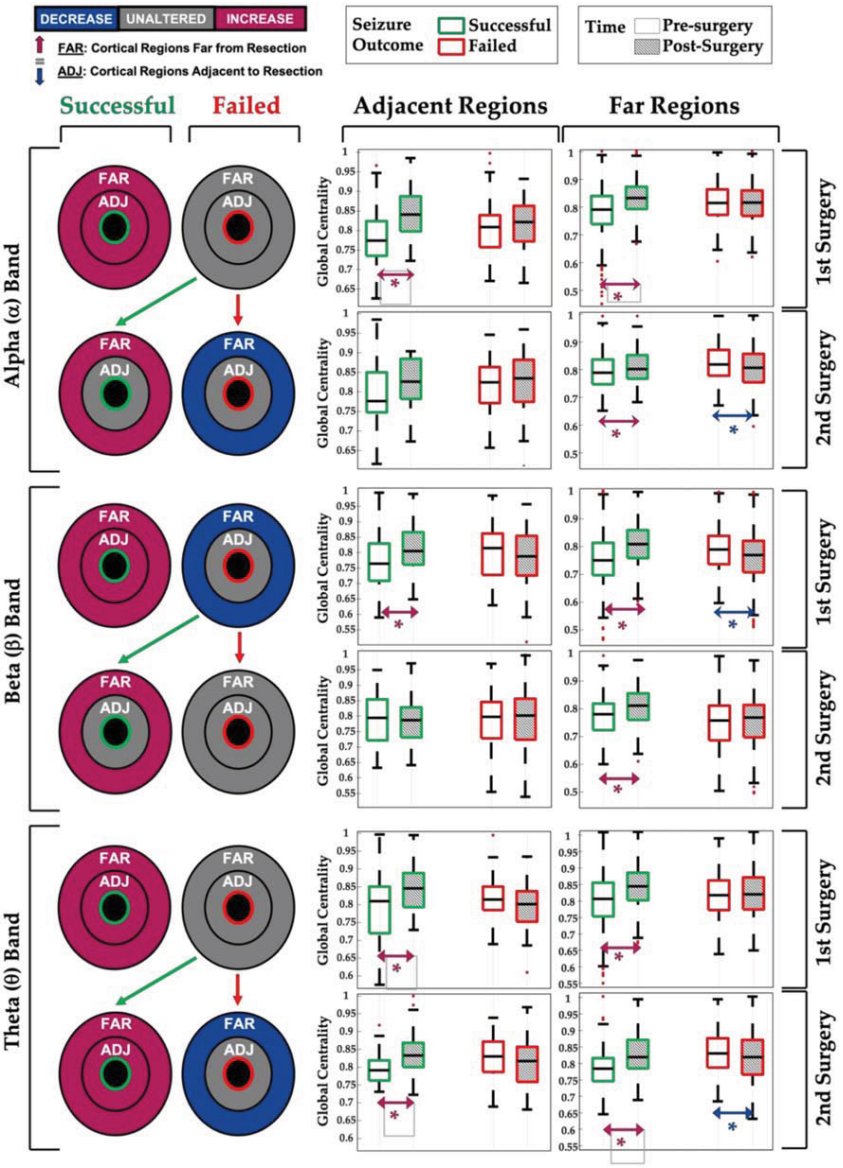

Figure 23.5 Global centrality in alpha, beta, and theta bands. For each frequency band, four circular diagrams and the corresponding boxplots are shown. For each surgery, we compared global centrality values between pre-surgery (white boxplot) and post-surgery (dashed boxplots) separately for adjacent and far regions. In the circular diagram, adjacent and far regions correspond to the two concentric sections, which are colored based on the statistical results (purple = post-surgical increase in global centrality; blue = post-surgical decrease; grey = no change). For each frequency, the top row displays the results obtained from the analysis of the first surgeries, whereby the left side shows the seizure-free group (good outcome = green), while the right side shows the repeated surgery group (poor outcome = red). The bottom row displays the results obtained from the analysis of the second surgeries (repeated surgery group only), whereby the left side shows second surgeries followed by a good outcome (green), while the right side shows second surgeries followed by a poor outcome (red). Asterisks mark significant *p*-values (<0.05).

In the seizure-free group, we found that (relative) global centrality increased after surgery in all frequency bands, both in proximity and far from resection ($p < 0.001$), with the only exception being for the adjacent regions in the gamma band (see Table 23.2). On the other hand, when looking at the repeated surgery group, after failed surgery, we found that theta, alpha, and beta networks did not present changes in centrality close to the resection (adjacent regions) or presented a decrease far from resection in some cases (as shown in Fig. 23.5). In these same frequency bands, when the 2nd (repeated) surgery was successful, we observed again an increase in the global centrality (as in the seizure-free group) far from the resection (theta: p-value < 0.001; alpha: p-value = 0.01; beta: p-value < 0.001), as well as in its proximity for the theta band only ($p = 0.02$). Figure 23.5 illustrates the results for these three frequency bands. Looking at the functional network in the delta and gamma bands instead, the same post-surgical changes were seen both after a failed surgery and a successful surgery (see Table 23.2), nullifying a possible link with seizure outcome. Figure 23.6 shows four representative examples of global centrality maps before and after successful or failed surgery (first or repeated).

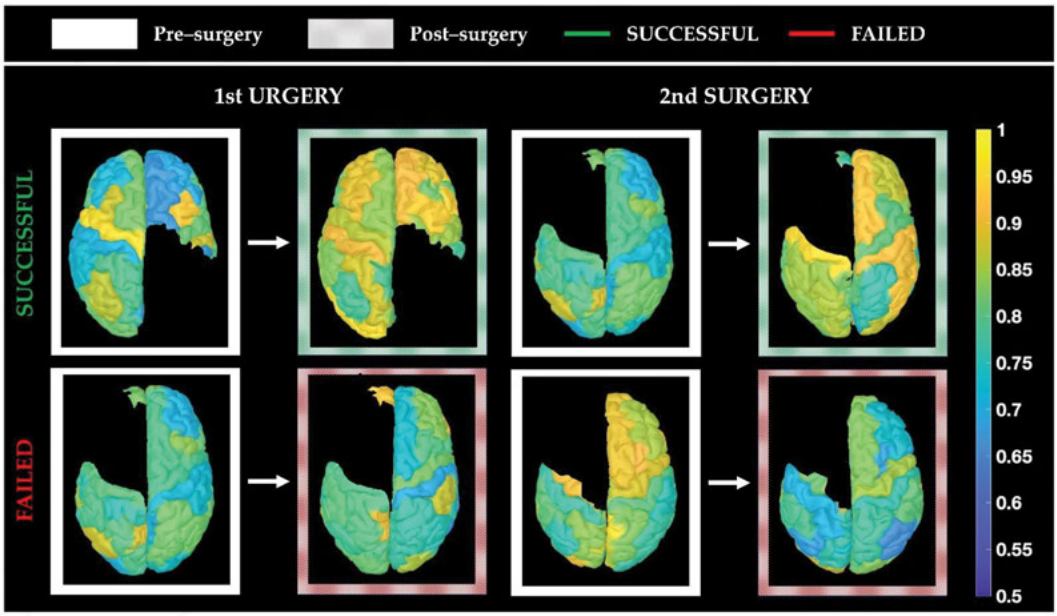

Figure 23.6 Examples of post-surgical global centrality changes in the theta band. Examples of post-surgical changes in global centrality, showing one patient image from the seizure-free group (patient 16, top left) and three from the repeated surgery group (patient 2, bottom left and top right; patient 4, bottom right). Each cortical ROI (that was not touched during surgery) is color-coded based on its global centrality value, whereby yellow indicates a high level of connectivity and blue indicates a low level of connectivity. In the successful surgery examples (top row), the color maps show an overall increase in post-surgical connectivity. On the other hand, in the examples of first and second failed surgeries, the color maps show no changes or decreases in connectivity.

23.3.3 Correlation with Other Factors (Age, Time Interval, AED, Power)

We evaluated whether the intervals between EEGs or the patient's age at the time of the pre-surgical evaluation (pre-surgical EEG) may have an influenced the observed post-surgical variation in centrality. Correlation analyses showed that: (i) averaged post-surgical variations in global centrality (for far or adjacent regions) in our cohort of patients did not depend on the time interval between EEGs ($p > 0.05$); (ii) averaged post-surgical variations in global centrality far from resection showed a negative correlation ($p < 0.05$, $R < 0$) with the patient's age before surgery, meaning that the younger the child, the more positive (higher) the post-surgical variation in global centrality.

From the PSD analysis, we also found that the post-surgical variations in global centrality of the untouched cortical regions after first surgery did not correlate with their variations in relative power for all frequency bands, with the only exception being the gamma band, where a weak negative correlation was observed for both groups ($R = -(0.10–0.16)$, $p < 0.01$)).

Finally, we did not observe any correlation in our cohort of patients between the number of AEDs (at the time of the 1st or 2nd EEG) and the averaged relative centrality of the far and adjacent regions ($p > 0.05$) in all frequency bands.

23.4 Discussion

23.4.1 Significance of the Study

Epilepsy is increasingly recognized as a neurological disorder that affects cortical network organization [36]. Several studies have shown that the brain FC and network topology can be altered through an epileptogenic focus [2, 35, 51]. By using advanced brain imaging techniques that combine ESI, FC, and graph theory, in the present study we investigated changes in the functional brain network of children with DRE following repeated surgery in comparison to a cohort of patients who had one successful surgery.

Repeat epilepsy surgery is a challenging proposition due to the inherent risks of subsequent surgery as well as the impetus for surgical success the second time around; children who have a first failed surgery can be judged to be eligible for re-operations, although the decision to initiate another pre-surgical evaluation is very difficult. Although re-operation rates vary between 3% and 15%, recent studies have suggested that about 30–40% of patients could benefit from a second surgery [29], further emphasizing the need for strong neurophysiological and epileptogenic network hypotheses. This was one of the few studies to investigate these challenging pediatric patients and to examine post-surgical changes in background EEG activity (independent of any epileptiform discharge). Our ultimate aim was to assess whether scalp EEG can reveal FC changes in a child's brain network after a surgical intervention (in the proximity of or far from the resection), which seemed to be specific to this population and to their poor post-surgical seizure outcome.

23.4.2 Main Findings: Functional Connectivity Changes in the Two Cohorts of Patients

Our study suggests that advanced scalp EEG can demonstrate brain networks changes following one or two focal surgeries in children with DRE and that these changes can be linked to the seizure outcome. Our most significant findings, summarized in Fig. 23.5, indicate that:

First successful surgeries (seizure-free group) are associated with an increase in the relative centrality of the remaining (untouched) brain regions within the alpha, beta, or theta network (see Fig. 23.5, green boxplots for 1st surgery);

Post-surgical seizure recurrence (surgery failure) on the other hand, after a first or second epilepsy surgery, is associated with a decrease or no change in the relative centrality of the remaining brain regions within the brain network (see Fig. 23.5, red boxplots);

When the repeated (2nd) surgery is successful (i.e., is followed by seizure freedom), we observed an increase in network centrality, especially for the cortical regions far from the surgical resection (see Fig. 23.5, green boxplots for 2nd surgery), similar to what is seen in patients who became seizure-free after their first surgery;

Network changes in the alpha, theta, and beta EEG bands seem to be associated with seizure outcome, as opposed to the changes observed in the delta and gamma bands, which did not correlate with the seizure outcome (see Table 23.2).

We hypothesize that the increase in relative centrality following successful surgeries may indicate a re-organization of the overall brain network towards a more uniform configuration, whereby the untouched (non-epileptogenic) regions (remaining cortex adjacent or far from resection) gain centrality within the network, likely secondary to the resection of the epileptogenic focus. Conversely, the unaltered or diminished centrality, which we observed following failed surgeries, may suggest an overall compensation effect of the untouched cortical regions aiming to isolate the persistent epileptogenic focus.

Previous fMRI [9, 11, 52], intracranial EEG [13, 15, 18], and MEG [21] studies have mostly focused on examining the connectivity of the epileptogenic focus or resected area, characterized by high local connectivity, whereby good post-surgical outcome was related to high FC of the resected brain region. In contrast, here we examined both pre-surgical and post-surgical whole-brain connectivity in children with DRE and reported how the FC of the remaining non-resected areas evolves after one or two epilepsy surgeries, depending on the outcome. This work adds to certain functional and structural connectivity studies [15, 52, 53] that have suggested that decreased distant connectivity in epilepsy results in a network that isolates the epileptic focus from the rest of the brain. In our cohort, removal of the epileptogenic focus was associated with increased relative connectivity in the rest of the network (because of the abolition of a highly central node), while the presence of a persistent epileptogenic focus after surgery led to a decreased or unaltered centrality role of the remaining cortical regions. Our scalp EEG results are also in line with previous MEG [21] and fMRI [10, 12, 14, 54]

studies in the field, which reported widespread decreases in connectivity in patients with focal epilepsy versus controls. Englot et al. [21] used MEG in adult patients to show that although focal epilepsy is often associated with increased local connectivity in the epileptogenic zone, it also leads to decreased long-range connectivity outside of it. Additionally, Lagarde et al. [15] using stereo-EEG found that in patients with DRE (mixed population of children and adults), before surgery non-epileptogenic areas showed low connectivity compared to the higher values exhibited for the epileptogenic and propagation zone.

Of note is also the fact that our findings are based on sleep EEG epochs containing "background activity", free of any epileptiform. This implies that during the interictal state, even when the brain is not "spiking", the functional brain network is reflective of a hierarchical epileptogenic organization, whereby non-epileptogenic regions do not gain a central role within the network until the epileptogenic focus is successfully removed.

23.4.3 Methodological Choices: Rationale, Pros, and Cons

We used routine scalp EEG data combined with MRI to estimate whole-brain FC. The choice of this approach was driven by its potentially high translational value since scalp EEG and MRI are the most widely used techniques to study the epileptic brain in any epilepsy center. Novel analytical methods that are EEG-based are of particular interest because scalp EEG is also potentially portable, safe, and routinely used during long-term pre-surgical investigations.

The main challenge of using scalp EEG is that the potentials recorded at the electrode level are subject to volume conduction (are attenuated and distorted before reaching the scalp electrodes). Electric source imaging (ESI) helps overcoming this problem by reconstructing sparsely sampled sensor signals on the scalp into the three-dimensional brain space. ESI basically combines EEG and MRI information of the patient to estimate the underlying brain activity that generates the recorded EEG [31, 45, 46]. As Fig. 23.2 shows, in this study we used ESI to calculate the cortical activity (we reconstructed beamformer-based time series from 68 anatomically-defined cortical ROIs) and then applied FC analysis to analyze the relationships between these cortical sources. The consistency of our findings with previous works and the ability to highlight different behaviors in our two different cohorts of patients suggest that this approach allows to monitor how the functional brain network evolves in children with DRE at the cortical level (see Figs. 23.1 and 23.2).

It is important to acknowledge that ESI-based approaches to estimate FC are inherently limited by the fact that source estimates are highly spatially correlated and that this can cause the resemblance of connectivity between regions (because of spatial leakage of the estimated sources into neighbors) [50, 55]. Many methods exist for FC estimation, and some are more sensitive than others to this spatial leakage confound. We chose the AEC (amplitude envelope correlation), as implemented in the Brainstorm open-source software, after orthogonalization correction, which removes zero-lag signal overlaps that could

be attributed to spatial leakage effects. This choice was based on: (a) the studies by Brookes et al. [49] and Hipp et al. [48], who introduced this novel measure to overcome limitations due to the limited spatial resolution of electrophysiological measures and demonstrated how it estimates functional networks that are very similar to those from fMRI; (b) the technical note from Colclough et al. [50] showing how this was the most consistent and repeatable connectivity measure for resting-state studies in the source space. It is also true that using FC methods, one can estimate a strong connection between two ROIs only because they both have high power signals. For this reason, here we also examined the relative power in the five frequency bands for each cortical ROI and tested their correlation with their FC estimate; we did not find any positive correlation, excluding the likelihood of a systematic bias in our cohort.

We must also mention that non-invasive recording techniques with a higher number of sensors [56], such as high-density EEG (>64 channels) or MEG [47, 57], allow a more precise reconstruction of the network through smaller ROIs (also called virtual sensors). Most clinics still lack the equipment or resources to perform MEG or EEG monitoring with >64 electrodes: thus, while our proposed approach can be significantly enhanced in terms of localizing value, it paves the way for an easily implementable approach in clinics. Intracranial EEG also offers a significant improvement in the spatial resolution and signal-to-noise ratio compared to routine scalp EEG, although intracranial EEG studies are inherently limited: (i) to the area of electrode implantation, not permitting whole-brain connectivity analysis; (ii) by the invasiveness of the technique, which can be used as a pre-surgical tool only for selected patients and not as a post-surgical technique to monitor the disease.

In summary, the methodological EEG-based approach that we proposed here provides an estimation of the functional brain network thatmay further the utility of traditional scalp EEG reading, which is typically limited to the interpretation of epileptiform discharges at the scalp level. Our findings must be interpreted carefully considering the above-mentioned factors that can influence the analysis (FC and ESI method, type of epilepsy, AEDs, resting-state network and graph analysis); nevertheless, they contribute to paving the way to bring source space connectivity into clinical practice.

23.4.4 Limitations and Future Directions

Our pediatric cohort was heterogeneous in terms of the type and location of resection (Table 23.1); although we could not control for this factor (due to the low sample size), results for the extent of the resected brain and type of surgery (temporal vs. extratemporal) did not differ between our seizure-free and repeated surgery group. Eventually, analysis of a large cohort of patients that allows correction for possible confounders such as pathology, seizure frequency, age at epilepsy onset, and time between resection and post-surgical EEG should be implemented to investigate these interactions.

The low sample size did not allow us to investigate the clinical value of the approach at the individual patient level and whether the proposed parameters are crucial to the surgical work-up. Larger prospective studies are recommended to test the relevance of the proposed EEG parameters in the pre-surgical work-up for re-operation. It is also important to note that our findings may depend on the choice of specific source localization and FC methods; a comparison between ESI and FC methods was out of the scope of our study but is warranted in the future.

Our seizure-free patients were older than the repeated surgery ones; although we did not control for this factor, we found an opposite correlation between a patient's age and their global centrality variation after surgery, reducing the possibility that our findings (positive variation of global centrality after surgery in the seizure-free but not repeated surgery patients) were due to such an age difference. Furthermore, to exclude the possibility that our findings were biased by the younger ages of the repeated surgery group at first surgery, we tested whether the same trends were seen when the two very young (<3 years old) patients (#3 and #8) were not included in the analysis. This confirmed the same statistical trend seen following the failed surgery, as we observed no changes ($p > 0.05$) in post-surgical connectivity in alpha, theta, and beta band both for far and adjacent areas and a decrease for the beta band in far areas ($p < 0.001$). Our data suggest that the reported findings are robust despite the age differences.

Another possible limitation is the influence of AEDs [58]. Patients in our series presented different types and number of AEDs at the time of recordings. Although we could not control for or investigate the effect of this variable on the post-surgical network changes, we observed that the number of AEDs at the time of the EEG did not correlate with the FC estimates adjacent of far from resection in any frequency, reducing the possibility of a bias introduced by this confounder on our findings.

23.5 Conclusions

This study presents the first evidence that EEG-based FC analysis at the source level can reveal epilepsy network changes following one or two epilepsy surgery in children with DRE. A decrease or no change in the centrality of the untouched brain regions within the brain network is seen for post-surgical seizure recurrence (meaning surgery failure). On the other hand, an increase in the overall centrality of the untouched brain regions is observed for successful surgeries. In particular, FC changes in the alpha, theta and beta EEG bands seem to be related to surgery outcome. We can speculate that unaltered or diminished centrality following epilepsy surgery is a consequence of an overall compensation effect, whereby the untouched cortical regions aim to isolate the persistent epileptogenic focus. An overall increase in FC after first surgery possibly indicates a re-organization of the overall brain network, whereby the untouched (non-epileptogenic) regions gain centrality within the network, since the epileptogenic focus has been successfully removed.

EEG-based FC analysis at the cortical level can provide information on the impacts of epilepsy surgery on brain networks. The low sample size and heterogenicity of our cohort did not allow us to demonstrate the value of our measures for individual patient care. Further studies are needed to investigate whether the proposed EEG measures are crucial to pre-surgical workup and can aid outcome prediction after re-operation.

Abbreviations

AEC:	amplitude envelope correlation
AED:	anti-epileptic drug
BCH:	Boston Children's Hospital
BEM:	boundary element method
DNET:	dysembryoplastic neuroepithelial tumor
DRE:	drug-resistant epilepsy
ECG:	electrocardiogram
EEG:	electroencephalogram
Epi. onset:	age at epilepsy onset
ESI:	electric source imaging
FC:	functional connectivity
FCD:	focal cortical dysplasia
fMRI:	functional magnetic resonance imaging
iEEG:	intracranial electroencephalogram
LCMV:	linearly constrained minimum variance
MEG:	magnetoencephalography
MPRAGE:	magnetization-prepared rapid gradient echo
MST:	minimum spanning tree
MTS:	mesial temporal sclerosis
PSD:	power spectrum density
ROIs:	regions of interest
RS:	repeated surgery
SF:	seizure-free
SSP:	signal space projection
TBI:	traumatic brain injury
TSC:	tuberous sclerosis complex
WM:	white matter

Disclosures and Conflict of Interest

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Chapter 24

Blood–Brain Barrier Disruption in Atrial Fibrillation: A Potential Contributor to the Increased Risk of Dementia and Worsening of Stroke Outcomes

Ritambhara Aryal[a,b] and Adjanie Patabendige, PhD[a,b,c]

[a]*Brain Barriers Group, School of Biomedical Sciences and Pharmacy*
The University of Newcastle, Callaghan, New South Wales, Australia
[b]*Brain and Mental Health Research Programme,*
Hunter Medical Research Institute, Newcastle, Australia
[c]*Institute of Infection and Global Health, University of Liverpool, Liverpool, UK*

adjanie.patabendige@newcastle.edu.au

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24.1 Introduction

Atrial fibrillation (AF) is the most common type of irregular heartbeat. Its prevalence is projected to double over the next two decades in people aged greater than 55 years [1]. AF is strongly associated with stroke, and there is an elevated risk of dementia in patients with AF who have a stroke [2, 3]. Stroke is a leading cause of global death, affecting more than 13 million people annually [4]. Around one-third of all strokes is known to be caused by AF, and these strokes are more complicated than strokes which are not related to AF [5–8]. Studies have shown that AF increases the risk of stroke by three to fivefold [5, 9–11]. In addition, around 19–30% of ischaemic stroke patients are reported to be affected by AF [12–14]. The interpretations of a 2015 meta-analysis indicate that the combination of various cardiac monitoring techniques has facilitated the detection of AF in almost one fourth of patients with stroke or transient ischaemia [13].

Importantly, both stroke and AF share the same risk factors such as age, diabetes, hypertension, heart failure, coronary heart disease and chronic kidney disease [7]. Cardioembolic stroke, which contributes greatly to mortality as well as permanent disability, is the most common subtype of stroke associated with AF [9]. Compared with 27% of non-AF-related strokes, there is a 50% probability that patients with stroke related to AF will demise within 1 year of the disease [9]. Patients with AF-related stroke have a 5-year survival rate of 39.2%, 5-year recurrence rate of 21.5% and 25.9% of these patients require nursing home care [15].

During AF, blood flow (and therefore shear stress) is altered, leading to the formation of blood clots, stroke, heart failure and death [16, 17]. AF has been shown to be associated with cognitive impairment/dementia, independently of clinical cerebrovascular events (stroke or transient ischaemic attack). A recent study in over 6000 patients showed that AF contributed to the risk of dementia, beyond the risk attributable to anticoagulant use [18]. One of the plausible mechanisms is the occurrence of AF-induced changes in critical haemodynamic events causing brain vascular dysfunction.

Dementia is the term given to a group of conditions in which there is significant impairment in memory and one or more other cognitive domains that hamper an individual's capability to carry out daily activities. In a systematic review published in 2013, Kalantarian et al. [3] reported a significant association of AF with an increased risk of cognitive decline or dementia, either in the presence or in the absence of prior stroke. This meta-analysis proposed numerous mechanisms for the link between AF and cognitive decline. Some of these were common risk factors for AF and cognitive impairment such as diabetes, hypertension [19], hypercoagulation due to increased thrombin generation [20], periventricular white matter lesions [21] and silent stroke.

A recent study found higher levels of biomarkers of cerebral injury such as microtubule-associated Tau protein and glial fibrillary acidic protein (GFAP)

in AF patients, suggesting potential blood–brain barrier (BBB, the protective physiological barrier of the brain) disruption, which could lead to cognitive decline or dementia in future [22]. Rusanen et al. [23] reported a link between AF and risk of dementia/Alzheimer's disease in late life but not in mid-life. By contrast, Bunch et al. assessed 37 025 patients from the Intermountain Heart Collaborative Study database, and reported that AF patients who were younger than 70 years old had the greatest risk of dementia. A 5-year follow-up assessment of this study found that patients with AF had several forms of dementia when compared with patients without AF [24]. Another study showed an increased risk of dementia in AF patients who were less than 67 years old [25]. Thacker et al. [26] observed a decline of cognitive scores at earlier ages in people with AF than those without AF. A Swedish study recorded dementia in 6.2% of AF patients from the year 2001 to 2007 (n = 12 057, age ≥ 45 years). In addition, this study also reported that female AF patients who had hypothyroidism and were taking levothyroxine (a medicine used to treat hypothyroidism) had a decreased risk of dementia compared with other female AF patients who did not have hypothyroidism and were not taking levothyroxine [27].

However, the underlying mechanisms that contribute to the development of dementia and stroke in AF patients remain poorly understood. Among the plausible mechanisms, the effect of altered cerebral blood flow (CBF) during AF on the BBB has been the least investigated. This is most likely due to the evident concerns relating to direct measurement of the cerebral vascular system leading to the paucity of clinical data, difficulty in studying the BBB in animals, and the complexity of replicating the cerebrovascular system in culture.

Deterioration in neuroprotective BBB function plays a major role in the pathogenesis of the disease, since the BBB dynamically responds to many events associated with flow disturbances (e.g. focal ischaemia), free radical release and cytokine generation. Any condition that affects the functional integrity of the BBB will cause secondary effects on CBF and vascular tone, exposing the brain to further damage [16–18]. Abnormal flow patterns or flow cessation can lead to changes in shear stress or pulsatility. This can deteriorate the brain endothelium and lead to barrier impairment [28, 29]. Thus, a significant role is played by biomechanical forces generated by blood flow in the induction of many BBB properties and in modulating endothelial function.

In this chapter, we summarize and analyse current evidence on the association between the development of dementia and worsening of stroke outcomes in patients with AF, and the contribution of the BBB disruption due to altered CBF in AF as a major factor in developing these pathologies.

24.2 The Structure and Function of the Blood–Brain Barrier

The BBB is the structure, which separates the central nervous system (CNS) from the systemic circulation (Fig. 24.1). It is formed by brain capillary endothelial

cells joined by complex tight junctions. The tight junction complex contains three main transmembrane proteins; namely occludin, claudins and junctional adhesion molecules (JAMs). The BBB is supported by a basement membrane, pericytes partially surrounding the endothelium and astrocyte endfeet processes that form a complex network around the capillaries [30, 31]. Recently, the term 'neurovascular unit' (NVU) has been extensively used for the collective description of these structures along with neurons, microglia and other peripheral immune cells [32–34].

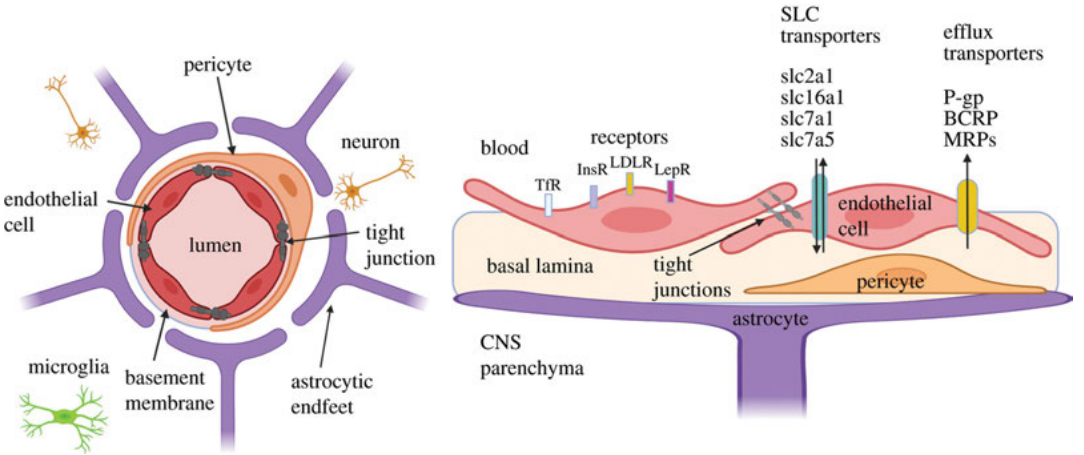

Figure 24.1 The BBB consists of brain capillary endothelial cells joined by tight junctions, basement membrane, pericytes and astrocyte endfeet processes that surround the capillaries. These structures together with neurons and microglia form the NVU. The cross-section schematic on the right demonstrates various types of transporters and receptors expressed by brain endothelial cells. Active efflux transporters transport lipophilic molecules from the CNS towards blood: e.g. ABC transporters such as P-glycoprotein (P-gp), BCRP and MRPs. The SLC transport nutrients such as glucose and amino acids into the brain and can be unidirectional or bidirectional, e.g. GLUT1/SLC2A1 (glucose), EAAT1/SLC1A3 (glutamate), SLC16A1 (lactate, pyruvate), SLC7A1 (cationic amino acids) and LAT1/SLC7A5 (neutral amino acids). Several receptors are present in the BBB to meet the brain's metabolic demand: for example, TfR, InsR, LDLR and LepR. (Created with BioRender.com). *Abbreviations*: BBB, blood–brain barrier; NVU, neurovascular unit; BCRP, breast cancer resistance protein; MRPs, multidrug resistance proteins; SLC, solute carriers; TfR, transferrin receptor; InsR, insulin receptor; LDLR, low-density lipoprotein receptor; LepR, leptin receptor.

The basement membrane is a form of extracellular matrix, which surrounds the vascular tube. The brain contains two types of basement membrane: the inner vascular basement membrane, secreted by endothelial cells and pericytes, and the outer parenchymal basement membrane, secreted by the astrocyte endfeet processes. The basement membrane predominantly contains extracellular matrix proteins such as collagen IV, laminin, nidogen and perlecan [35, 36].

Unlike highly permeable systemic capillaries, the tight junctions of the BBB seal the paracellular pathway and strictly control the passage of substances between

the brain and the circulation at the brain capillary level. Therefore, almost all molecular traffic has to use the transcellular pathway, controlled by an array of transporters, receptors and enzymes (Fig. 24.1) [37]. Such an arrangement allows the entry of important molecules like water, gases, glucose and amino acids, while preventing the entry of pathogens, potential neurotoxins and xenobiotics and protects the brain from fluctuations in plasma composition [31, 38, 39].

The CNS endothelial cells express two major types of transporters: efflux transporters and solute carrier (SLC) transporters. Efflux transporters such as ABC (ATP-binding cassette) transporter family members transport lipophilic molecules from the CNS and the endothelium towards blood. They are predominantly expressed on the luminal membrane. These active efflux pumps protect the brain by removing endogenous neurotoxins and xenobiotics. However, many potentially neuroprotective drugs may also be substrates for these efflux transporters, leading to reduced brain penetration [40, 41]. On the other hand, the SLC transporters enable the transport of various solutes and nutrients such as glucose and amino acids into the brain. SLC transporters are expressed on the luminal or abluminal membranes or both. Therefore, they are able to transport polar molecules, which cannot diffuse across the BBB, into and out of the brain, with the direction of the transport determined by the orientation of the transporter [36, 37, 42].

Contractile proteins possessed by pericytes enable the contraction of these cells to control the diameter of the capillary and regulate cerebral blood circulation [36, 43, 44]. Where the basement membrane is not present, pericytes and endothelium interlock to form peg-and-socket junctions. These junctions contain various transmembrane adhesion proteins, such as N-cadherin and connexin-43 [36, 45, 46]. Pericytes also play a crucial role in the activation of angiogenesis in the adult CNS, clearance of toxic substances, recruitment of immune cells into the brain and can act as multipotent stem cells to differentiate into various cell types of the CNS [36, 44, 47]. Hence pericytes are known to be important for the maintenance of the BBB during all stages (i.e. during the development of the BBB, adulthood and ageing [36, 48]).

To meet the metabolic requirements of neurons, astrocytes facilitate the delivery of oxygen and glucose from the vasculature to neurons [49, 50]. Astrocytes are also important for the maintenance of the BBB. By extending from the cell bodies to the basement membrane and towards the neurons, astrocyte endfeet enable bidirectional communication between the CNS vasculature and neurons [51, 52]. Astrocyte endfeet secrete vasoactive substances such as prostaglandin E2 (PGE2) and epoxyeicosatrienoic acids (EETs), hence resulting in vasodilation and increased cerebral blood circulation [53–56]. Furthermore, astrocyte endfeet processes contribute to the BBB integrity by forming a structural border called glia limitans between the CNS neural tissue and non-neural cells. This barrier restricts the entry of leucocytes and other inflammatory cells from non-neural cells into the CNS parenchyma [57–59]. Astrocytes also respond to CNS injury via a process called astrogliosis or glial scarring, in which reactive astrocytes upregulate

the expression of intermediate filament proteins such as GFAP, nestin and vimentin [60, 61]. Furthermore, reactive astrocytes not only secrete cytokines such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin-17 (IL-17), which modulate pro-inflammatory phenotypes of T-lymphocytes, but also anti-inflammatory cytokines such as interleukin-10 (IL-10) [62–64].

24.3 Mechanisms of Blood–Brain Barrier Disruption

The BBB disruption is evident in many neurological disorders, including dementia due to Alzheimer's disease [31, 65, 66], multiple sclerosis [67, 68], Parkinson's disease [69, 70], amyotrophic lateral sclerosis [71, 72], Huntington's disease [73, 74] and stroke [75–78]. In addition, emerging evidence suggest that peripheral diseases such as AF and others (e.g. heart failure, hypertension and diabetes mellitus) are also associated with BBB dysfunction [79–81].

As reviewed elsewhere, an intact BBB is essential for appropriate synaptic function, neuron connectivity and information processing [82–84]. Dysfunction of the BBB can lead to the entry of toxic substances and microbial agents into the brain and disrupt brain homeostasis. A damaged BBB can result in the activation of inflammatory and immune cells, leading to neuronal injury. Dysfunction of the BBB is associated with various factors such as oxidative damage due to reactive oxygen species (ROS) [85, 86], secretion or activation of matrix metalloproteinases (MMPs) [87, 88], angiogenic factors [89, 90], autoantibodies [91, 92] and pathogens [38, 93] (Fig. 24.2).

Studies using different experimental animal models during the 1980s demonstrated that several compounds have the ability to affect BBB permeability. The complement activation protein 5a was shown to affect the integrity of endothelial cells and astrocytes [94]. Cerebral exposure to bradykinin results in the activation of the arachidonic acid cascade, which can result in altered BBB permeability [95, 96]. Likewise, changes in Ca^{2+} influx across the BBB [97], the presence of serotonin receptors in the endothelial cells and signalling of histamine receptors H1R, H2R, H3R and H4R in neurons, astrocytes or endothelial cells can influence the BBB permeability [98, 99]. Decreased levels of tight junction proteins such as occludin, claudin-5 and zonula occludens 1 (ZO-1) are linked with increased BBB permeability in Alzheimer's disease and hypoxia/ischaemia [100].

Impaired transcytosis (a process by which large molecules enter the CNS by crossing the BBB) is one of the features of BBB disruption [101]. Brain endothelial cells of healthy adults have an increased expression of receptors and components of clathrin-coated pits to facilitate the transfer of circulatory proteins through receptor-mediated transcytosis (RMT). Degeneration of pericytes with age promotes calcification of the vasculature and a shift from ligand-specific RMT to non-specific caveolar transcytosis (Fig. 24.2). This can potentially lead to neuroinflammation due

to increased BBB permeability to neurotoxic proteins, including autoantibodies, albumin and fibrinogen [102, 103]. An increase in the number of endothelial caveolae and the rate of transcytosis at the early stages of stroke, followed by disruption in paracellular barrier mechanisms have been shown to contribute to BBB dysfunction in stroke [104]. Furthermore, the expression of molecules such as Caveolin-1 and Plasmalemma vesicle-associated protein (PLVAP), which are important in vesicular transport during transcytosis, is upregulated in the impaired BBB [36].

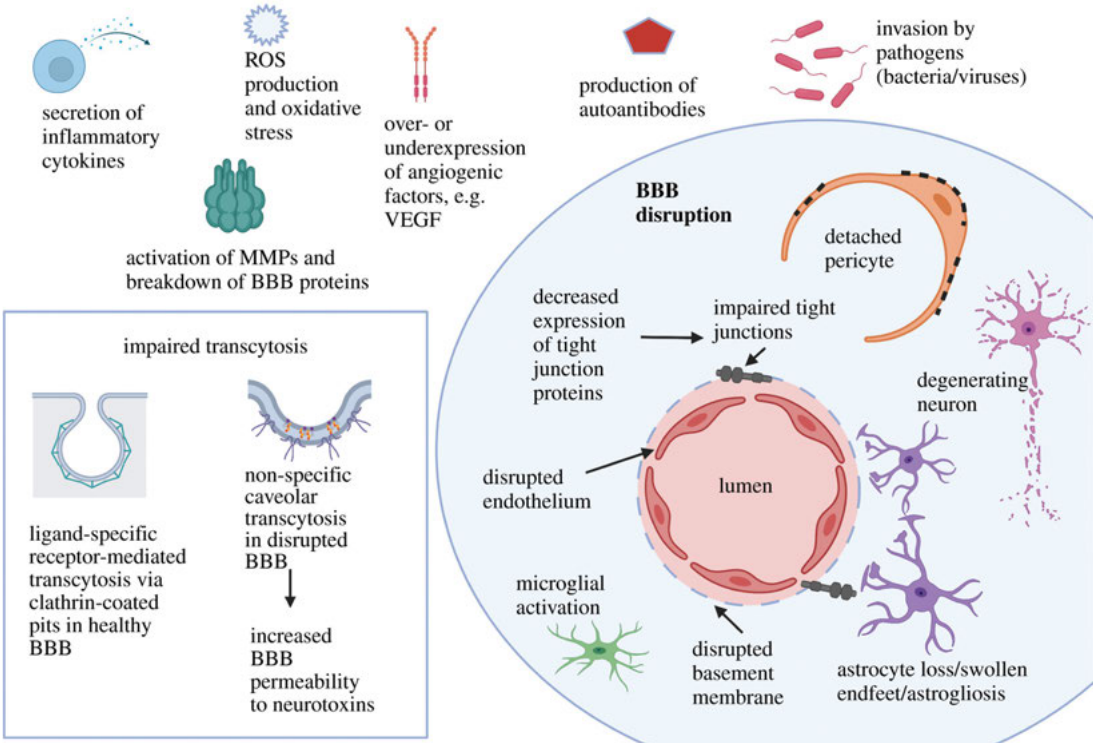

Figure 24.2 Blood–brain barrier (BBB) disruption. Several factors are associated with BBB disruption, some of which are illustrated here. Increased oxygen demand in the brain makes it susceptible to the production of ROS, subsequent oxidative stress and activation of MMPs. Likewise, over- or under-expression of angiogenic factors, production of autoantibodies, invasion by various pathogens and secretion of inflammatory cytokines are associated with impairment of BBB. Impaired transcytosis is another important factor that accompanies BBB disruption, in which the non-specific caveolar transcytosis takes over ligand-specific receptor-mediated transcytosis. Other characteristics of impaired BBB include impaired endothelium, detached pericytes, disrupted basement membrane and altered expression of tight junction proteins resulting in weakened tight junctions. Microglia, the inherent immune cells of the CNS increase their activity (microgliosis) and so do the astrocytes (astrogliosis). A compromised BBB loses its ability to restrict the entry of toxins and control brain homeostasis, and can lead to neurodegeneration, which is implicated in several neurological disorders. (Created with BioRender.com). *Abbreviations*: BBB, blood–brain barrier; MMPs, matrix metalloproteinases; ROS, reactive oxygen species.

The human apolipoprotein E (ApoE) facilitates the uptake of lipoproteins and promotes lipid metabolism. An absence of astrocyte-derived ApoE and the expression of ApoE4, a chief genetic risk factor for Alzheimer's disease leads to a compromised BBB [105, 106]. Bell and colleagues reported that ApoE activates a pro-inflammatory Cyclophilin A-nuclear factor-kB-MMP-9 pathway in pericytes, which results in BBB disruption. This is followed by the uptake of neurotoxic proteins by neurons and a decrease in CBF [107]. Likewise, other studies have proposed loss of pericytes as a precondition for BBB disruption in some chronic conditions such as diabetes [108, 109]. In human immunodeficiency virus (HIV) associated dementia, early inflammation of CNS, presence of perivascular macrophages and infiltration of monocytes are associated with BBB breakdown [110, 111].

The presence of antioxidants such as glutathione, glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase provide cerebral endothelium defence against oxidative damage [112, 113]. However, the BBB integrity is compromised in the event of oxidative stress, as observed in several pathological conditions including various neurological disorders [86, 114–116]. Increased oxygen demand for neuronal activity makes the brain susceptible to the production of ROS. High mitochondrial concentration in the endothelium also makes the brain vulnerable to increased oxidative stress. Likewise, the presence of nitric oxide (NO) in the brain can result in the generation of ROS [112]. Furthermore, the movement of the transmembrane protein occludin away from the tight junction has been shown to associate with oxidative stress-related BBB permeability [117]. Superoxide and its derivatives can cause vasodilatation by opening the potassium channels [118]. Other triggers of BBB disruption by ROS are activation of MMPs, autophagy and inflammation [119].

Shear stress or fluid shear stress is a tangential force of blood flow on the vascular endothelium. Several *in vitro* BBB models are available for studying the effects of shear stress on the BBB [120–123]. As reviewed by Wang et al. [121], shear stress under physiological conditions promotes BBB integrity by suppressing nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) activation and ROS production, upregulating the expression and assembly of tight junction proteins, e.g. via VE-cadherin/Tiam1/Rac1 pathway. However, high shear stress during pathological conditions activates the release of MMPs such as MMP-2 and MMP-9 by platelets, which leads to the depletion of extracellular matrix and tight junctions. In addition, the activation of Src/ERK1/2 signalling pathway due to increased shear stress can lead to the downregulation of tight junction markers. Janigro and colleagues [28, 124, 125] have studied the effects of shear stress on the BBB extensively using human brain endothelial cells co-cultured with human astrocytes in a dynamic *in vitro* BBB (DIV-BBB) model. They have shown that shear stress leads to tightening of the BBB, increase in the expression of multidrug resistance transporters, ion channels, several cytochrome P450 enzymes and SLC transporters, leading to differentiation of endothelial cells into a BBB phenotype while inhibiting cell proliferation [28].

Elbakary & Badhan [126] used the Quasi-Vivo 600 (QV600) interconnected chamber system to perfuse media and apply shear stress on high transendothelial electrical resistance (TEER) primary porcine brain microvascular endothelial cell (PBMEC) cultures [127]. The application of shear stress on these cells caused reorientation and improvement of tight junction formation along with an increase in TEER, in comparison with the cells which were cultured under static conditions. In another study, shear stress was applied on confluent monolayers of derived human brain microvascular endothelial cells (dhBMECs) [128]. These cells displayed a distinctive phenotype compared with cells grown under static conditions, such as the absence of elongation and alignment, significant reduction in rates of apoptosis and proliferation, and unaltered expression of important BBB markers. Garcia-Polite et al. [29] observed that the expression of tight junction markers such as ZO-1 and claudin-5 was upregulated by more than 1.7 fold by physiological shear stress, whereas their expression was reduced to basal levels by increased shear stress.

One important consideration regarding the factors/events associated with BBB disruption is that they can either precede or succeed BBB damage or both. In other words, some factors fit into the categories of both 'causes' and 'consequences' of BBB disruption. For instance, entry of pathogens causes BBB disruption leading to increased BBB permeability [129], impaired tight junctions [130] and disrupted basement membrane [93], which can facilitate further entry of other pathogens and toxic substances (consequence). Likewise, the release of inflammatory cytokines in response to CNS injury (cause) leads to BBB impairment, which can result in microglial activation [131, 132]. Activated microglia may in turn release more cytokines/chemokines (consequence) and lead to neuronal damage. In addition, activated microglia have the ability to produce ROS [133], the latter of which is also a causal factor for BBB impairment. This could also be true for other factors listed above, which are known to be contributary to BBB leakage. For example, peripheral or CNS cytokines may induce BBB leakage in neuropsychiatric lupus, hence allowing the entry of autoantibodies via disrupted BBB into the CNS [134] (see review by Obermeier et al. [38] for a discussion on how BBB disruption can cause or contribute to neurological disease).

In addition to the above mechanisms, there is a plethora of literature on other means of BBB disruption. In this chapter, we will focus specifically on how CBF changes are associated with BBB damage.

24.4 Effects of Altered Cerebral Blood Flow on the Blood–Brain Barrier

To match its metabolic requirements, the mammalian brain contains an exceptional mechanism of regional CBF regulation and neuronal activation termed

'neurovascular coupling' (NVC). Interaction of various cells and components of the BBB contributes to this process [135–137]. NVC, also known as functional hyperemia, starts with alterations in neuronal activation, which modulates vasodilation and eventually leads to an increase in CBF. Studies have shown that NVC occurs even if there is a reduction in neuronal activity, and it is facilitated by the action of excitatory as well as inhibitory neurons [135, 138, 139].

The brain also has a regulatory mechanism called cerebral autoregulation, which ensures appropriate CBF and oxygen transport over differing perfusion pressures [140, 141]. Normal CBF is maintained at around 50 ml per 100 g of brain tissue per minute, within the range of 50–150 mmHg of cerebral perfusion pressure (the difference between intracranial pressure and mean arterial pressure) [142–144]. When this pressure falls below 50 mmHg, the CBF decreases, leading to ischaemia, whereas rise of pressure above 150 mmHg results in cerebral oedema and BBB breakdown [144]. Cerebral autoregulation circumvents perfusion abnormalities and reduces the subsequent risk of haemorrhage or ischaemia.

Two types of cerebral autoregulation have been described: blood flow alterations during rapid changes in blood pressure are called dynamic cerebral autoregulation, and blood flow changes in response to continued blood pressure changes are called static cerebral autoregulation [145, 146]. An absence of autoregulatory mechanisms can lead to significant brain injuries. For instance, forced opening of cerebral vessels during acute hypertension results in a massive increase in CBF (300–400%) in a phenomenon called autoregulatory breakthrough. Reduced cerebrovascular resistance also raises hydrostatic pressure on endothelial cells, resulting in the development of oedema [142, 147, 148]. Castro et al. [144] outlined that cerebral autoregulation is disrupted focally in large vessel stroke and globally in small vessel stroke. Moreover, transcranial Doppler (TCD) studies have shown gradual degradation in cerebral autoregulation after 5 days of stroke with a recovery period of around three months [145, 149, 150]. While a normal CBF pattern safeguards proper functioning of the brain, abnormalities in CBF resulting from the disruption of cerebral autoregulation and neurovascular coupling is linked with BBB disruption in disease conditions such as ischaemic stroke, hypertension, Alzheimer's disease and AF [140, 151].

The CBF is regulated by factors such as partial pressure of CO_2 and O_2, cerebral metabolism and the autonomic nervous system. Other substances such as glutamate activate neuronal *N*-methyl-d-aspartate (NMDA) receptors, which in the presence of ample glucose and oxygen further activates NO synthase that produces NO, a vasodilator of parenchymal arterioles [135, 152]. Astrocytes respond to glutamate release from synaptic terminals by producing the vasoconstrictor, arachidonic acid and parenchymal arteriole dilators such as EET and prostaglandins [153, 154]. Moreover, the dilatation of cerebral arterioles caused by the activation of ATP-sensitive K^+ channels during low oxygen conditions (hypoxia) is associated

with an increase in CBF [142, 155]. In a study using mice pups, the transitory opening of BBB was reported in response to hypoxia–ischaemia [156]. Likewise, the lowering of CO_2 level in the blood (hypocapnia) has been known to promote vasoconstriction and a subsequent reduction of CBF. On the other hand, elevated CO_2 level in the blood (hypercapnia) causes vasodilation and successive augmentation of CBF [142, 157]. A recent study described that hypercapnia worsens BBB disruption by promoting the overproduction of interleukin-1β in blood [158].

Direct assessment of the effects of changes in CBF on brain function is challenging. However, various techniques are available for the assessment of CBF such as arterial spin labelling/pulsed arterial spin tagging and vascular-space occupancy [159]. Shen & Duong [160] have reviewed some technical developments of CBF measurement methods in animal models. One technique is inversion-recovery background suppression continuous arterial spin labelling (IR-cASL), which shows 2 times improved temporal stability and 2–2.3 times higher functional contrast-to-noise ratios for stimulation of hypercapnia in rats, in comparison with cASL without background suppression. Furthermore, this technique was used to demonstrate that whole-brain CBF values in these animals were similar across various labelling periods [161].

High spatial resolution CBF magnetic resonance imaging (MRI) is another technique that can provide valuable information on brain CBF supply at columnar and laminar levels. Using this method, Shen et al. showed that the whole-brain CBF in rat brains was around 0.89 ml g^{-1} min^{-1}. The maximum CBF values were found along the neocortex (1 ml g^{-1} min^{-1}, within the range of 0.89–1.16 ml g^{-1} min^{-1}) and the minimum CBF values were observed in the corpus callosum (0.32 ml g^{-1} min^{-1}). These observations showed a CBF ratio of 3.1 for grey matter: white matter [162]. This method was also used to study rats with stroke before and after reperfusion. It was found that brain regions of normal perfusion and hypoperfusion were diverse during middle cerebral artery occlusion (MCAO) [160].

The cardiac spin labelling (CSL) technique allows higher sensitivity CBF imaging and measurement of cerebral, retinal and choroidal blood flow in small animals such as mice [160]. A two-compartment exchange model for quantification of perfusion, described by Zhou et al. [163] allows controlled trafficking of blood/water between the microvascular compartment (containing arterioles, venules and capillaries) and the extravascular space in the parenchyma. During limited water exchange, the FAIR (flow-sensitive alternating inversion recovery) signal intensities of two compartments could be compared in magnitude but they did not overlap in time. Whereas during fast limited water exchange, flows quantified from the signal-intensity difference were underestimated. This observation was found to be more significant for bigger flows and increased magnetic field strengths. Likewise, the near-infrared spectroscopy (NIRS) indocyanine green dye dilution technique allows non-invasive bedside measurement of regional CBF and regional

cerebral blood volume [164]. Preliminary results showed that the values obtained from this technique matched with the values obtained from a standard method such as perfusion-weighted MRI.

24.5 Disrupted Peripheral and Cerebral Blood Flow in Atrial Fibrillation

As reviewed by Calenda et al. [165], histopathological features like an aggregation of glycogen granules in atrial cardiomyocytes, increased loss of sarcomeres and variable degrees of interstitial fibrosis are known to manifest as AF in the heart, along with some additional indicators [165, 166]. These changes may have been contributed by various factors such as ageing, genetic predisposition, endothelial disruption, inflammation and stretching of the atrial wall [167, 168].

Based on past and recent experimental studies, it has been widely acknowledged that AF can cause impairments in the coronary blood flow pattern in humans [169–171]. One study by Saito et al. [172] showed a significant reduction in coronary blood flow in dogs with artificially induced AF. Likewise, Kochiadakis et al. [173] reported an increased coronary circulation in patients with acute AF. While the mechanism of perturbed coronary circulation in AF remains debated, Scarsoglio et al. [174] proposed that impaired coronary blood flow was attributed to the higher ventricular rate during AF. Similarly, another study using coronary angiography showed that a fine fibrillary wave was a key factor for decreased coronary flow in AF patients, as compared to the sinus rhythm [175]. Apart from these studies on peripheral circulation, AF is also closely linked to blunted CBF as discussed below.

A recent study by Junejo et al. [140] reported reduced cerebral autoregulation and impaired neurovascular coupling response to visual stimulation in people with AF. This observation corroborates very early studies that date back to the 1980s. Lavy et al. [16] found a reduction in cerebral circulation in patients with AF. The rate of reduction in CBF was higher in patients of age group 35–50 years [17.5%] compared with the age group of 51–65 years (13.4%) and above 65 years (5.5%). By contrast, a recent study by Gardarsdottir et al. [17] reported that the elderly population with persistent AF had reduced total CBF and brain perfusion. The authors speculate that this may be an explanation to their previous finding in which AF was associated with decreased brain volumes and cognitive decline in elderly patients with AF [176].

Measurement of CBF with the intravenous xenon-133 technique by Petersen and colleagues showed a decrease in CBF in AF patients who did not have heart failure. The CBF was increased in these patients subsequent to electrical cardioversion (a technique that uses electric current to readjust heart rhythm to normal sinus rhythm). This observation led the authors to speculate that a decrease in CBF can cause recurrent cerebral abnormalities associated with

AF [177]. Another study used TCD sonography to measure CBF in patients with paroxysmal AF. They observed that the mean flow velocity significantly decreased in the middle cerebral artery but not in other arteries [178]. Neuropsychological testing and TCD procedure in patients with heart failure indicated cerebral hypoperfusion as a plausible mechanism that contributes to the worsened cognitive function in these patients [179]. Likewise, recent advances in computational algorithms have reinforced the hypothesis of altered CBF dynamics in AF, marked by hypertensive events and transitory hypoperfusions [180].

To better understand the haemodynamics during AF, some researchers have developed computational models that imitate AF. Choi and colleagues performed computational fluid dynamics (CFD) simulations to comprehend the course of blood clots in the aortic arch under normal and AF conditions. This study showed that the frequency of blood clots is significantly increased in AF when compared with the aortic flow at normal conditions. The authors suggest that this type of computational approach would be valuable in determining the effect of AF cardiac haemodynamics in the cerebral embolization of blood clots, which could result in stroke [181]. Another computational study used two coupled lumped-parameter models of cardiovascular and cerebrovascular circulations to mimic AF and sinus rhythm. There were increased cerebral haemodynamic variations (such as brief periods of decreased blood flow or increased pressure) in the distal circulation under the conditions of AF, which was proposed to be a mechanism that initiates cognitive impairment or dementia in AF patients [182]. A similar approach was used by Scarsoglio et al. [183, 184] where they noted differences in artificially built signals gained from their computational model in the direction of distal cerebral regions in AF compared with normal sinus rhythm.

Oral anticoagulants (e.g. warfarin), which are used for stroke prevention in AF patients, have also been described to be effective in dementia prevention, although this remains contentious [18, 185–187]. The CHADS2 and CHA_2DS_2VASc scores not only predict the risk of stroke in AF patients, but they also indicate the risk of dementia in these patients [18, 188, 189]. Graves et al. [18] reported that in patients with long-term warfarin treatment, the presence of AF showed an increased risk of dementia across all CHADS2 score levels compared with patients without AF. The authors speculated that dementia risk contributed by AF is not exclusively caused using anticoagulation therapy, and AF may facilitate the development of dementia towards the end-stage of the disease [18]. Friberg & Rosenqvist [190] reported that early anticoagulation treatment decreased the risk of dementia by 29% in patients with AF compared with patients without anticoagulation treatment. In another study, Jacobs et al. [191] discovered that both over-coagulation and under-coagulation treatments increased the risk of dementia, hence signifying chronic cerebral injury as a factor that links AF and dementia.

Animal models of AF can only reproduce certain but not the entire spectrum of human AF. AF induction in these animals lasts for a very short time. Animals that have been used to study AF include mouse, rat, guinea pig, rabbit, goat, dog, sheep, pig and horse [192]. Corday and colleagues measured CBF in dogs using a magnetic probe which was connected to a square wave electromagnetic flowmeter and integrator-computer. A mean reduction was observed in CBF by seven per cent in premature atrial systoles, 12% in premature ventricular systoles and 23% during AF. The authors also highlighted that cerebral angiospasm can occur during the arrythmias, which may continue afterwards [193]. Likewise, Friedman et al. [194] observed reduced CBF in the cerebellum and brain stem, along with a decrease in splanchnic and renal cortical blood flow in dogs with AF. These studies implicate that cardiac arrhythmias may cause an ample decrease in CBF which can ultimately lead to cerebral ischaemia [193].

To our knowledge, there have been very limited experimental studies on animal models of AF, other than those mentioned above, which have focused on the mechanisms of BBB disruption or impaired cerebral circulation. Future studies are urgently required to get a better understanding of the underlying mechanisms that can help develop new treatments.

24.6 What Are the Underlying Mechanisms that Increase the Risk of Stroke and Dementia in Atrial Fibrillation?

In this review, we provide a summary of our current understanding of AF, stroke and dementia associations, and demonstrate how changes in CBF due to AF could disrupt the BBB, leading to worsening or developing stroke and dementia. Old age is a major shared risk factor for dementia, stroke and AF, although these conditions are also observed in the young age group [185, 195]. While AF significantly increases the risk of stroke, it is also extremely important to highlight the fact that stroke patients have a higher risk of cognitive impairment/dementia. Development from AF to cognitive decline or dementia can occur either directly or with stroke as an intermediate [196]. Furthermore, several studies have proposed BBB disruption as a harbinger of cognitive dysfunction [197–199].

Hence either the coexistence or co-influence of these three diseases is highly likely. Very little is known about the potential mechanisms via which AF contributes to cognitive impairment/dementia and clinical stroke. We propose that changes in CBF patterns and BBB disruption during persistent AF may lead to increased risk of dementia and worsened stroke outcomes.

Our hypothesis is based on the observations of studies that showed direct or indirect impairment of BBB/CBF during AF. One such study is the work of Junejo et al. [140], which indicated that cognitive dysfunction might result from a weakened cerebrovascular regulation. Bunch et al. [200] also proposed that cerebral microvascular dysfunction may be responsible for sudden cognitive

decline in patients with AF. This concept is bolstered by the observation of Bangen et al. that cerebral atherosclerosis was the chief cerebrovascular pathology in the brain autopsies of 84 Alzheimer's disease patients. Atherosclerosis was predominantly detected in the circle of Willis, a region where several arteries join at the inferior side of the brain, to allow collateral blood flow between anterior and posterior aspects of the brain. The authors proposed that maintaining proper cerebrovascular health could result in averting or delaying dementia in people with Alzheimer's disease pathology [201].

Two other studies noted increased plasma levels of von Willebrand factor, a biomarker of endothelial dysfunction in patients with AF, which could predict stroke and cardiovascular risk [202, 203]. Freestone et al. [203] also reported that patients with chronic AF exhibit impaired brachial artery flow-mediated dilatation (FMD) response. This is suggestive of diminished endothelial NO bioavailability [140, 204, 205] and possible perturbation of CBF regulation [206]. Besides markers of endothelial dysfunction, biomarkers of oxidative stress and inflammation are known to be raised in AF as well as in Alzheimer's disease, which are also evident in BBB disruption [200, 207].

Some studies have shown that the treatments for abnormal heart rhythms can also be effective in improving cognition. Efimova et al. [208] investigated the effects of atrioventricular node ablation and pacemaker implantation in brain perfusion and cognitive function of patients with AF. Not only did the authors note improved ventricular systolic function, but also enhanced blood perfusion in temporal and frontal cortices, improved verbal and visual memory and advanced learning. Likewise, Bunch et al. [209] reported that AF patients who were treated with catheter ablation had lesser rates of dementia and stroke than the patients who were not treated with catheter ablation for AF.

A systematic review and meta-analysis of studies from 1950 to 2009 described that ten per cent of the population suffered from dementia before they had their first stroke. Furthermore, ten per cent of patients suffered from new dementia shortly after their first stroke attack, and over one-third of the population suffered from dementia succeeding recurrent stroke. In addition, pre-stroke dementia was linked with risk factors such as the family history of dementia, female gender and medial temporal lobe atrophy, whereas post-stroke dementia was associated with multiple cerebral lesions. Hence, prevention of stroke or proper care after episodes of stroke seems to be crucial in reducing the risk of dementia [210].

While many studies have supported the association between AF and dementia risk in patients with stroke [2, 211, 212], a meta-analysis by Kwok et al. [2] pointed out the ambiguity on such an association in wider population. Reports have also shown that patients with AF have an increased risk of cognitive deterioration/dementia even in the absence of medical history of previous stroke [213–215]. There is increasing evidence that silent cerebral infarcts (SCI) are commonly observed in AF patients [5, 216, 217]. A meta-analysis reported

that MRI detected SCI in 40% of patients with AF without a history of symptomatic stroke [216]. Likewise, Hahne et al. [5] reviewed that the rates of SCI were 12.3–92% in AF patients and 17–69% in non-AF patients, and the rates of silent strokes increase following AF ablation procedures.

Petersen et al. [217] observed that SCIs were mainly present in the cortex, and the AF group had a higher number of SCIs than the control group; however, there was no significant difference in the size of SCIs between the two groups. Studies show that the risks of symptomatic stroke and dementia increase by more than threefold [218] and twofold [212], respectively, in the presence of SCI. In addition, other studies have also considered SCI as a risk factor for future cognitive dysfunction and dementia [212, 219] as well as subsequent stroke [220]. Hence Kalantarian et al. [216] proposed that a high prevalence of SCI in AF patients may make these patients vulnerable to cognitive decline/dementia and future stroke. This finding is in line with our notion that altered CBF due to cerebral infarction could be one factor which links AF, stroke and dementia together.

Manifestation of other Alzheimer's disease-related pathologies in stroke or AF have also been demonstrated. For example, ApoE4, the major genetic risk factor of Alzheimer's disease has been recently shown to be involved in the escalation of dementia prior to or following transient ischaemic attack and stroke [221, 222]. Cerebral atrophy or decreased brain volume is another major characteristic of Alzheimer's disease. Reduced brain volumes are not only observed in stroke [223, 224] but also in AF patients, especially in persistent AF compared with paroxysmal AF patients [176, 225]. As vascular impairment is associated with cerebral atrophy (e.g. in vascular dementia), this reinforces our hypothesis that cerebrovascular/BBB dysfunction could be one of the regulators of cerebral atrophy in dementia, stroke and AF.

Furthermore, myelin degeneration or demyelination is another common observation in Alzheimer's disease, vascular dementia [226–228] and stroke [229–232]. Case studies have previously reported that multiple sclerosis, a demyelinating disease is associated with AF and electrocardiographic changes in some patients [233, 234]. These studies suggested that demyelination could alter cardiac conductance causing arrhythmias and result in neurogenic pulmonary oedema. In fact, demyelination is strongly linked with BBB impairment in multiple sclerosis and some other CNS diseases [235–238].

24.7 Concluding Remarks

This review highlights a critical gap in the current knowledge in understanding neurological manifestations in AF, and the need to address the missing link—a disrupted BBB (see Fig. 24.3). Protection of which can lead to improved outcomes for AF patients and stop or delay the progression of developing dementia and

stroke. However, there are important unanswered questions. There is an urgent need for further studies and the development of suitable models for assessing BBB disruption to determine the underlying mechanisms. These are outlined in Box 24.1. While several BBB models are available for assessing BBB disruption [121, 239], a lack of suitable models for investigating how different environmental stimuli such as changes in CBF or blood-borne substances can affect the BBB during AF, leading to dementia or stroke, is a major hurdle. Developing therapeutics to protect the BBB needs to be a priority to help prevent AF-related neurological complications more effectively.

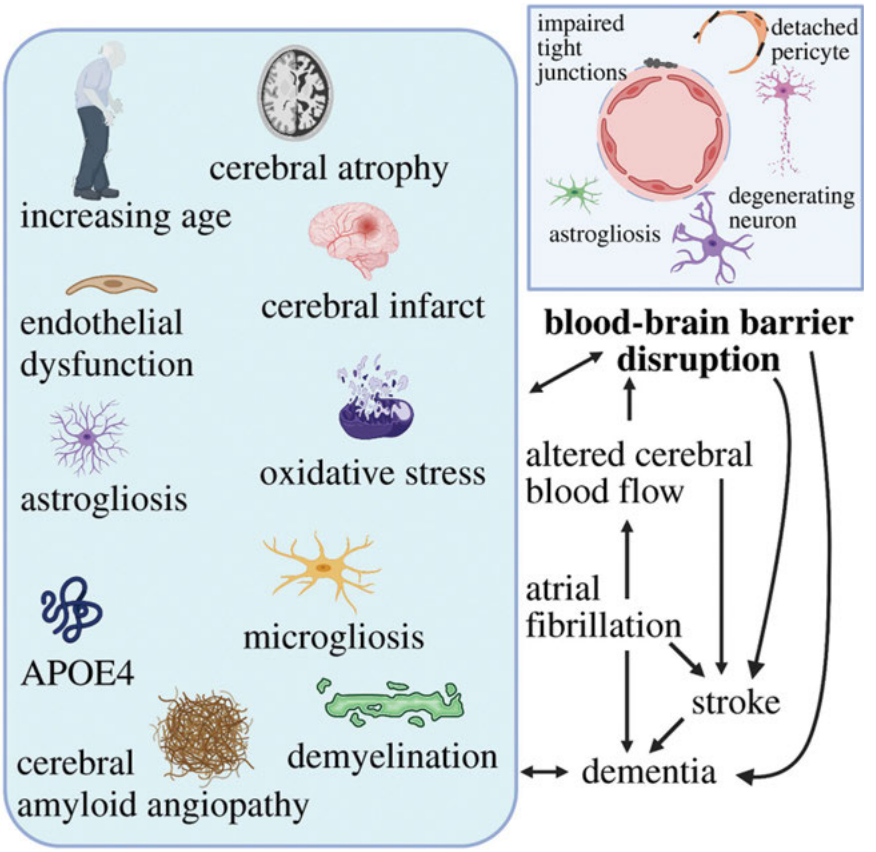

Figure 24.3 Blood–brain barrier (BBB) disruption in atrial fibrillation. The risk of atrial fibrillation (AF), stroke and cognitive impairment/dementia increases with age. Altered blood flow due to AF increases the risk of stroke, and stroke increases the risk of dementia. The progression of AF to dementia can be either direct or via transitional stroke. One of the plausible mechanisms is the occurrence of AF-induced changes in critical haemodynamic events causing BBB dysfunction. Box 24.1 lists some events associated with BBB disruption, all of which are evident in AF, stroke and dementia. Development of therapeutics that protect the BBB may help prevent or delay AF-related neurological complications more effectively. (Created with BioRender.com). *Abbreviations*: AF, atrial fibrillation; BBB, blood–brain barrier.

Box 24.1 Outlook

1. Need for further studies:
   - More studies on improved risk stratification scores and screening tools are needed for the detection of dementia/stroke in AF patients [240].
   - Need ample evidence from larger cohorts regarding the effects of oral anticoagulation/catheter ablation on cognitive function in AF/stroke; likewise, more studies are needed to determine the effects of lifestyle changes/dietary interventions on cognitive function in AF/stroke [241].
   - Efficacy of anticoagulation on SCI is not clear yet as shown by some studies [242, 243]. Future studies need to check whether anticoagulation results in reduced incidence of SCI, and if this in turn leads to decreased incidence of dementia, symptomatic stroke and mortality [216].
   - More studies are needed to determine the best biomarkers for predicting stroke and response to anticoagulants [244].
2. Challenges:
   - There is a paucity of information on the association of altered CBF dynamics and AF. As put forward by Saglietto et al. [180], this is attributed to issues regarding direct sampling of the cerebral circulation and the lack of resolving power to get information on pressure and flow signals downstream the cerebral arteries.
   - Dementia may cause reduced adherence to oral anticoagulation therapy in AF/stroke [186].
   - Improved strategies for AF detection needed as AF is undetected and untreated in many patients since they do not show symptoms, or they have paroxysmal AF [245].
3. Availability and suitability of current models and methods for assessing BBB dysfunction due to changes in CBF:
   - A range of *in vitro* BBB models has been developed over the years [246, 247]. These include *in silico* models, microfluidic systems, ECM-based models, Transwell systems, spheroidal models and isolated brain microvessels. However, not all models are suitable for elucidating the mechanisms of flow-induced changes.
   - There is no 'gold-standard' BBB system that perfectly imitates the human BBB. For example, brain endothelial cell monolayers, non-contact Transwell cocultures and monoculture microfluidic models have limited cell interactions; the BBB-on-a-chip model has limited reproducibility of cell phenotype [248]. Dynamic flow-based models or microfluidic models that incorporate brain endothelial cells as well as astrocytes, pericytes and neurons are more suitable for investigating the role of BBB during disrupted CBF in AF [121]. However, these models are more expensive and can be labour-intensive.

- MRI-based methods to assess BBB function in AF or computerized simulation of CBF to determine BBB disruption can be other options that need further validation.

4. Potential treatments focusing on protecting BBB:
    - BBB dysfunction hinders the transport of amyloid plaques, the major pathological hallmark of Alzheimer's disease, from the brain to the peripheral circulation. Hence potential treatments focusing on BBB protection could be beneficial in the treatment of Alzheimer's disease [65].
    - Manipulation of BBB could be used for targeted drug delivery for the treatment of neurodegenerative disorders [31], e.g. reversible BBB disruption by chemical or physical methods to allow entry of drugs; drug lipidization for increased permeability, development of more hydrophobic drug analogues [249].

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Chapter 25

Trauma-Induced Coagulopathy: From Pathophysiology to Outcomes—Overview of an Emerging Medical Problem

Gabriele Savioli, MD, PhD

Emergency Department, IRCCS Policlinico San Matteo, Pavia, Italy
PhD University of Pavia, Pavia, Italy

gabrielesavioli@gmail.com

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25.1 Introduction

Major trauma is one of the leading causes of mortality and morbidity worldwide and the leading cause of death under 40 years of age.

Every year, there are ~6 million deaths worldwide because of traumatic injuries.

Major trauma is also a frequent cause of hospitalization: An estimated 24 million patients are hospitalized yearly. This also results in extensive out-of-

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hospital medical care: approximately 85 million patients worldwide. Although the problem mainly affects low- and middle-income countries, it is also found in high-income countries. For example, in Europe, traumatic injuries are the third-largest cause of mortality in the general population and the leading cause of mortality in young patients. They are also one of the main causes of disability, resulting in high direct and indirect costs. Therefore, correct and quick identification of the cause of bleeding, as well as neglected coagulopathies, is of great importance for the correct management of several surgical pathologies.

Major trauma (MT) refers to an event that results in a single injury or multiple injuries of such magnitude that it constitutes a quoad vitam or quoad valetitudinem danger to the patient. Conventionally, trauma is defined as severe when the patient's injury severity score (ISS) is >15.

ISS consists of an assessment system that assigns a number based on the severity and location of the different injuries caused by the trauma. This index was chosen because it showed excellent correlation with mortality, morbidity, need for hospitalization and stay in the hospital. The value >15 was decided on the basis of a proven increase in mortality. ISS calculation is possible only after the patient has undergone diagnostic investigations mainly in a hospital setting. To overcome this limitation, it is essential that a potential MT be recognized as soon as possible, in the pre-hospital phase, and triage criteria for MT should be implemented (Table 25.1).

Post-traumatic hemorrhage is the most frequent cause of death in victims of severe trauma. This is due to two main mechanisms, but they can intertwine and be present at the same time. These are as follows:

- Bleeding caused by direct injury of blood vessels: with hemorrhage dependent on physiological or anatomical factors: These include the hemodynamic state of the patient, in particular systolic blood pressure values; the arterial or venous nature of the affected vessel; the caliber of the vessel. In cases of injury of arterial vessels of large caliber, we can witness profuse hemorrhage with shock of the patient and exitus in a very rapid time, even before the arrival of the rescue crew.
- Secondary bleeding from the development of trauma-induced coagulopathy: secondary bleeding from a widespread microvascular hemorrhage and not localized at the site of the trauma. This represents a pathological entity in its own right whose classification and pathogenesis will be discussed later.

Although as we can see, the pathogenesis and classification of approximately 30% of MT patients seem to develop trauma coagulopathy upon arrival in the emergency room (ER). Although it was once believed that trauma coagulopathy begins hours or even days after the traumatic event, it is now clear that it begins at the moment of trauma. About 40% of trauma deaths are the result of bleeding, 10% of which seem avoidable.

Table 25.1 Triage criteria for severe trauma: criteria for activating the severe trauma protocol in our trauma center; physiological, anatomical, and dynamic criteria for defining probable severe trauma (one of the following criteria is sufficient)

Physiological criteria	Anatomical criteria	Dynamic criteria
Ejection from vehicle	Penetrating head/neck/ throat/abdomen/pelvic/ armpit/groin trauma	Systolic blood pressure < 90 mmHg
Motorcycle crash with separation of rider	Amputations above the wrist or ankle	Respiratory or breathlessness rate <10 or >29 acts/min
Died in the same vehicle	Chest trauma with flap/flail chest	State of consciousness (GCS) <13
crash intrusion >30 cm at patient area	Neurological injury with paralysis of even a single limb	
Fall from height >2 m	Fractures of two or more long bones	
Pedestrian thrown or run over or hit at speed >10 km/h	Suspected unstable fracture ring of pelvis: suspected unstable fracture	
High-energy impact (speed > 65 km/h)	Open or depressed skull fracture	
Vehicle rollover	Burn >20% of body surface or airway/face	
Extrication time > 20 min		

GCS, Glasgow coma score.

25.2 Definition

Numerous definitions and terms have been proposed to identify coagulopathy resulting from trauma and to describe the specific trauma-associated coagulopathic physiology, including acute traumatic coagulopathy, early coagulopathy of trauma, acute coagulopathy of trauma-shock, trauma-induced coagulopathy, and trauma-associated coagulopathy.

Trauma-induced coagulopathy can be defined overall as a condition of endogenous hypercoagulation that is observed in the immediate post-traumatic period, that is, with onset by convention within 1 h of trauma. It is characterized by widespread microvascular hemorrhages and not only located exclusively at the site of trauma.

25.3 Pathophysiology

Hemostasis is an essential complex process for the body's defense and wound healing system. Physiologic hemostasis is a homeostatic process comprising a

balance between pro- and anti-coagulation systems as well as fibrinolysis and anti-fibrinolysis. This process takes place on the surface of endothelial cells and platelets and involves clotting factors. The endothelium is an active part of this homeostatic budget process and is not just a "passive witness." This property is maintained by several mediators, including tissue factor pathway inhibitor, endothelial protein C receptors, the endothelial glycocalyx (EGL), thrombomodulin, nitric oxide, and tissue plasminogen activator.

Despite continuing and recent advances in research into major trauma and consequent increased knowledge in the sector, the pathophysiological mechanisms that contribute to T.I.C. remain largely unknown. This is also due to the multitude of complex systems that interact with each other. A disturbance in hemostasis occurs in fact by activation/disregulation of the vascular endothelium, coagulation, natural anticoagulants, the pro-fibrinolytic system of the anti-fibrinolytic system and inflammation.

These phenomena are compounded by a number of external factors (such as hemodilution by the administration of crystalloids) and detrimental factors such as hypothermia, hydroelectrolytic imbalance and acidosis. These detrimental factors are likely to self-feed and depend on both endogenous and exogenous factors.

For years, it was considered that T.I.C. was solely due to the dilution of clotting factors due to massive fluid administration or massive transfusions, which further complicated the development of acidemia and hypothermia, which, with T.I.C., contribute to the formation of the "lethal triad" and thus further aggravate the clinical picture (Fig. 25.1).

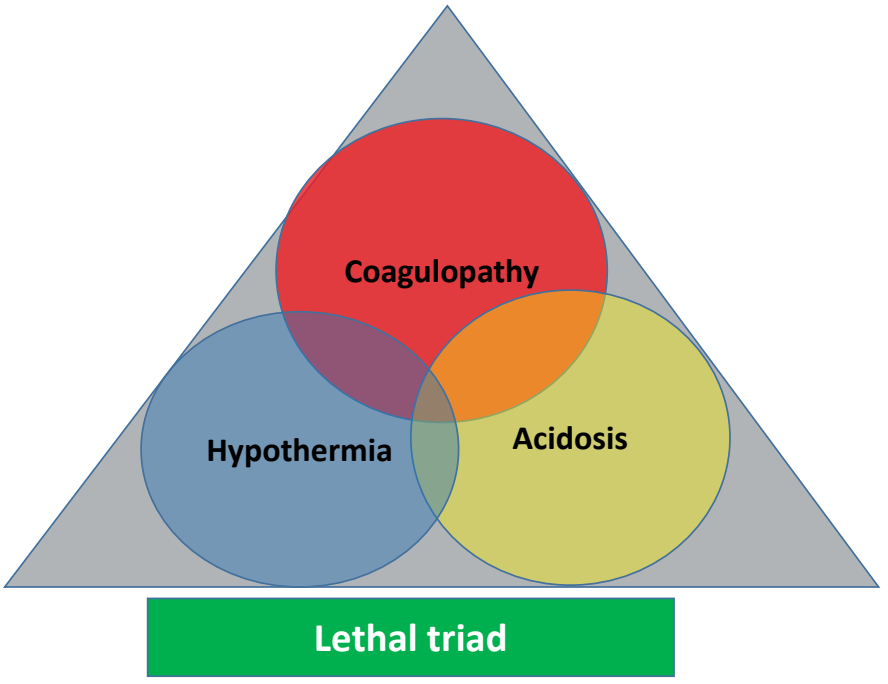

Figure 25.1 Lethal triad for major trauma.

Classically, in short, the three factors recognized as the only triggers of T.I.C. were: hemodilution, hypothermia, and acidemia. Although they are still recognized as triggers of T.I.C., it has been found that this already develops in the early stages of trauma, before any medical intervention and before the development of acidemia and hyperthermia; it is dependent on the first phase during the release of mediators by hypoperfused organs and damaged tissues.

In fact, in recent years, it has been discovered that the physiopathological mechanisms underlying trauma clot disease are much more complex than this and how fluid administration contributes to the development of intracardiac thrombus (ICT), without being the main cause; etiopathogenesis is in fact multifactorial (Fig. 25.5).

A distinction can therefore be made between acute traumatic coagulopathy and coagulopathy disease induced by resuscitating maneuvers, which can coexist, but have different mechanisms and temporal phases.

We can therefore schematically (Fig. 25.2) claim that TIC is composed of

- a pathophysiological process linked to trauma (acute traumatic coagulopathy);
- iatrogenic factors (coagulopathy induced by resuscitation maneuvers);
- detrimental factors (both iatrogenic and pathophysological).

25.3.1 Acute Traumatic Coagulopathy

Injury to the wall of a vessel as a result of trauma can expose the subendothelial collagen and the activated tissue factor that provide an adhesion platform for circulating platelets as well as for the interplay between the cellular and humoral components of the hemostatic system. This procoagulant activity is controlled by a counter-regulation system of natural anticoagulants. The net effect of these two opposing systems may be the generation of pro-coagulation at the site of endothelial injury, while preventing uncontrolled microvascular thrombosis and tissue hypoperfusion by means of endogenous anti-coagulation and fibrinolysis. Parts of the process are interconnected in a complex way.

Thrombin plays a central role in this process by activating both coagulation and anti-coagulation as well as contributing to the crosstalk with the inflammatory response.

25.3.1.1 Role of the C protein

Many theories have been put forward about the pathophysiological process that triggers T.I.C. Until recently, the activated C protein had been identified as one of the main players (Fig. 25.3).

It was thought that the activated protein C (APC) system played the most important role in the development of trauma-induced coagulopathy (TIC). APC is a physiological anti-coagulant that irreversibly inactivates the pro-coagulant factors Va and VIIIa. It is also profibrinolytic by inhibiting plasminogen activator inhibitor-1 (PAI-1). APC is also cytoprotective through anti-inflammatory and anti-apoptotic mechanisms.

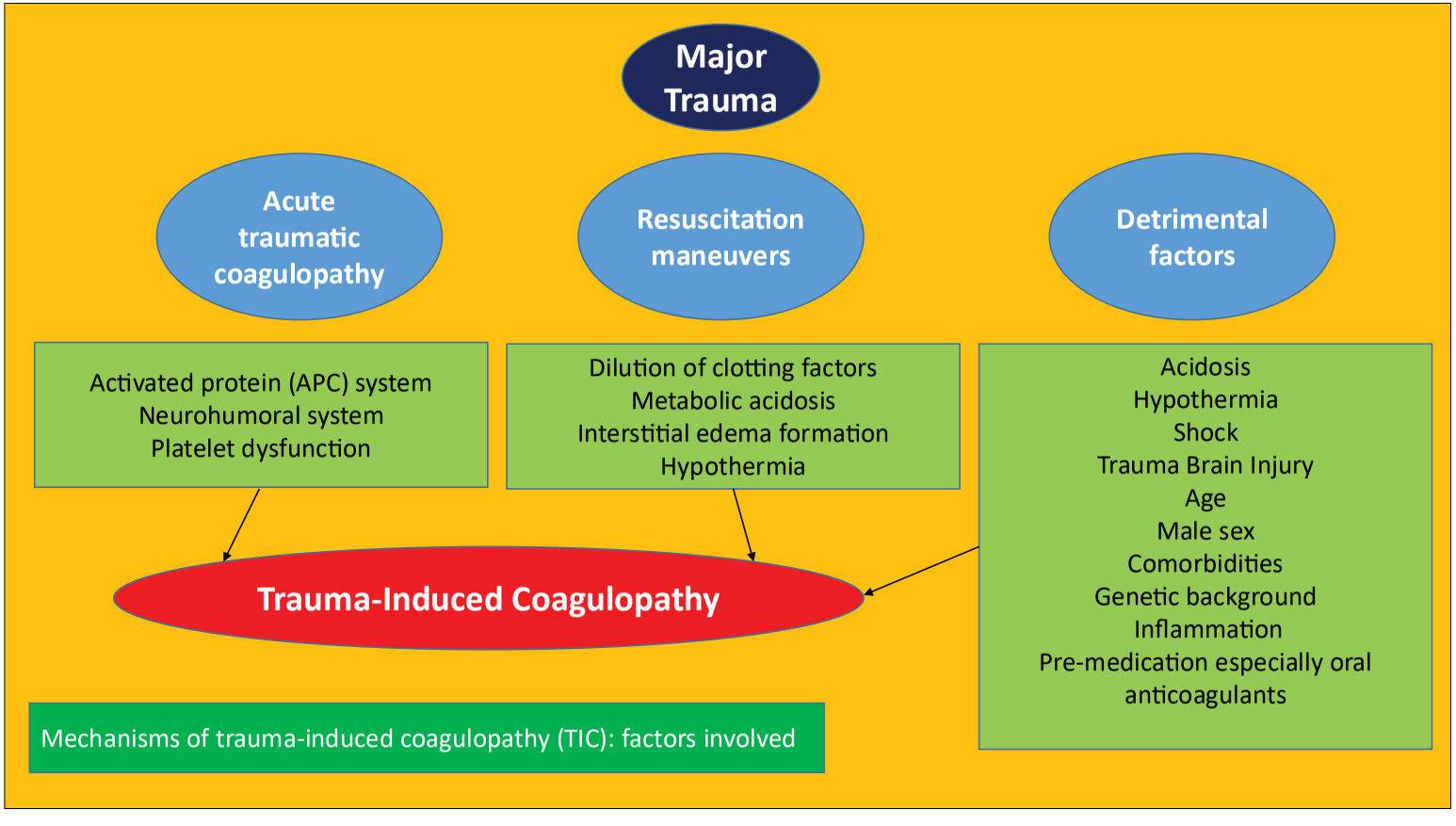

Figure 25.2 Factors involved in the development of TIC.

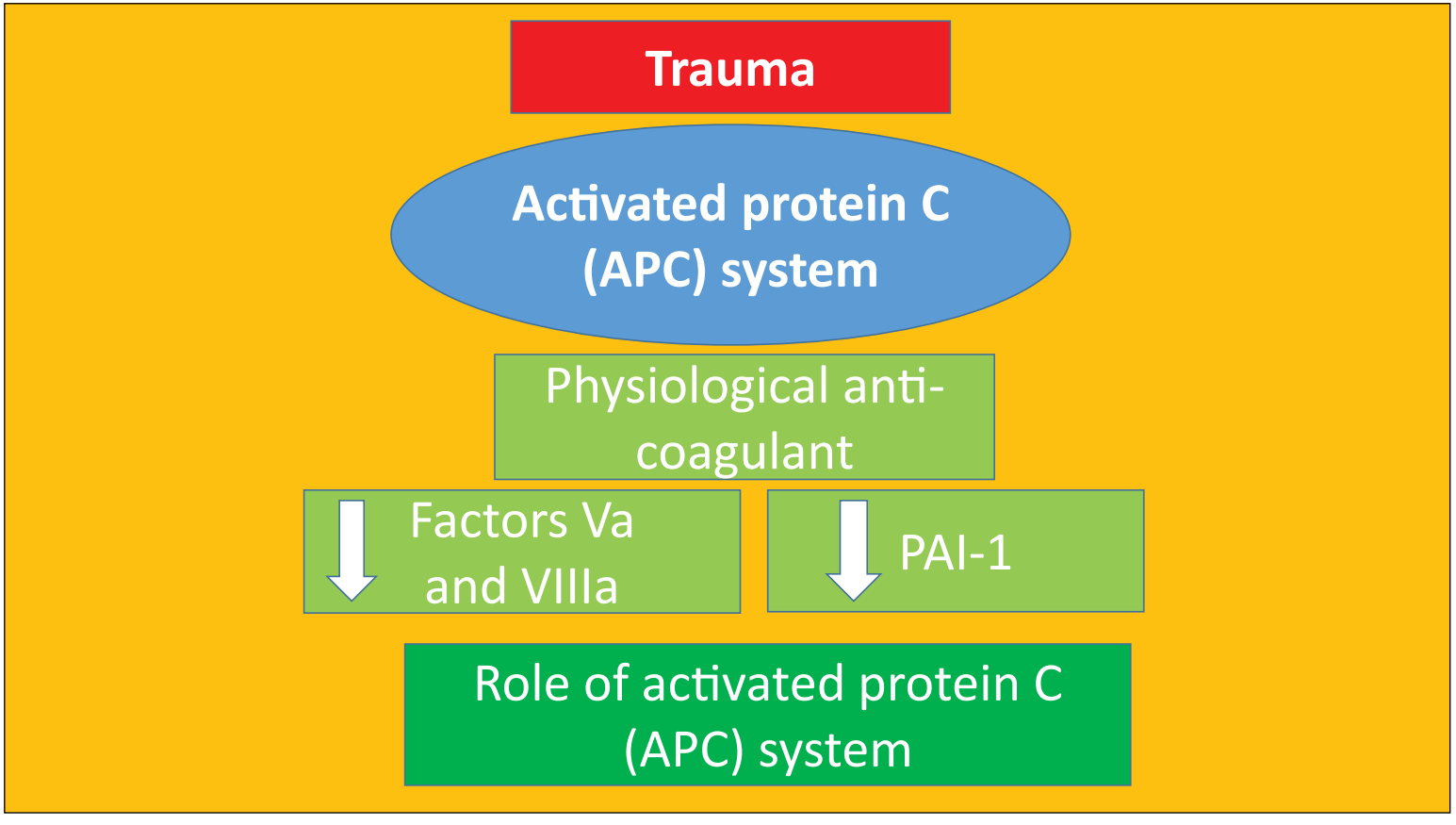

Figure 25.3 Role of Protein C.

In the PROMMTT study acute traumatic coagulopathy identified on arrival to the emergency department was associated with a depletion of the pro-coagulant factors I, II, V, VII, VIII, IX, and X and the activation of the protein C system.

Lately, however, its role has been questioned and scaled back. In fact, a recent study showed a significant early increase in tissue plasminogen activator but not PAI-1 following a severe injury. In addition, a recent systematic literature review concluded that there may not be a direct cause–effect relationship between APC and increased fibrinolysis.

This contradiction is not surprising, taking into consideration the complexity of the hemostatic system and the crosstalk with the myriad of mediators released following a trauma.

25.3.1.2 Role of the neurohumoral system

Trauma activates the neurohumoral system, which results in increasing inflammatory cytokines and hormones, such as adrenaline and vasopressin.

This increase leads to the activation of endothelial cells resulting in the release of tPA and Weibel–Palade bodies. These bind to the endothelium, release the von Willebrand factor (vWf) and encourage platelet recruitment.

In addition, the release of tPA and the high amounts of plasmin contribute to the catabolism of fibrinogen.

This increase in catecholamine levels also leads to endothelial damage and glycocalyx degradation. The process described is named endotheliopathy. Endotheliopathy may also contribute to capillary leakage following trauma.

Further, it causes degradation of the endothelium and the consequent release into circulation of proteoglycans, similar to heparin, and thus determining the phenomenon that is most properly known as self-heparinization. This process was observed in approximately 5% of trauma patients, and this was associated with a high ISS (Fig. 25.4).

High levels of adrenaline and syndecan-1 have been shown in non-survivor traumatic adult trauma patients.

The syndecan-1 is a marker of glycocalyx degradation. Some studies confirmed that high adrenaline levels and glycocalyx damage are associated with endothelial damage, hypocoagulopathy and hyperfibrinolysis. Additionally, high levels of syndecan-1 were associated with increased inflammation and endothelial damage.

Recently it has been proven that both adrenaline and syndecan-1 were independent predictors of <24 h, 7-day, and 28-day mortality, even after adjustment for ISS.

The pathophysiological mechanisms that determine severe TIC lead to hypofibrinogenemia. Hypofibrinogenemia as a consequence of TIC is frequently observed in the early phases of trauma. It has been shown that fibrinogen concentration <2 g/L was identified in about 15–20% of the patients. Low fibrinogen levels were associated with a poor outcome. Remember, however, that there may be an age-associated increase in fibrinogen levels.

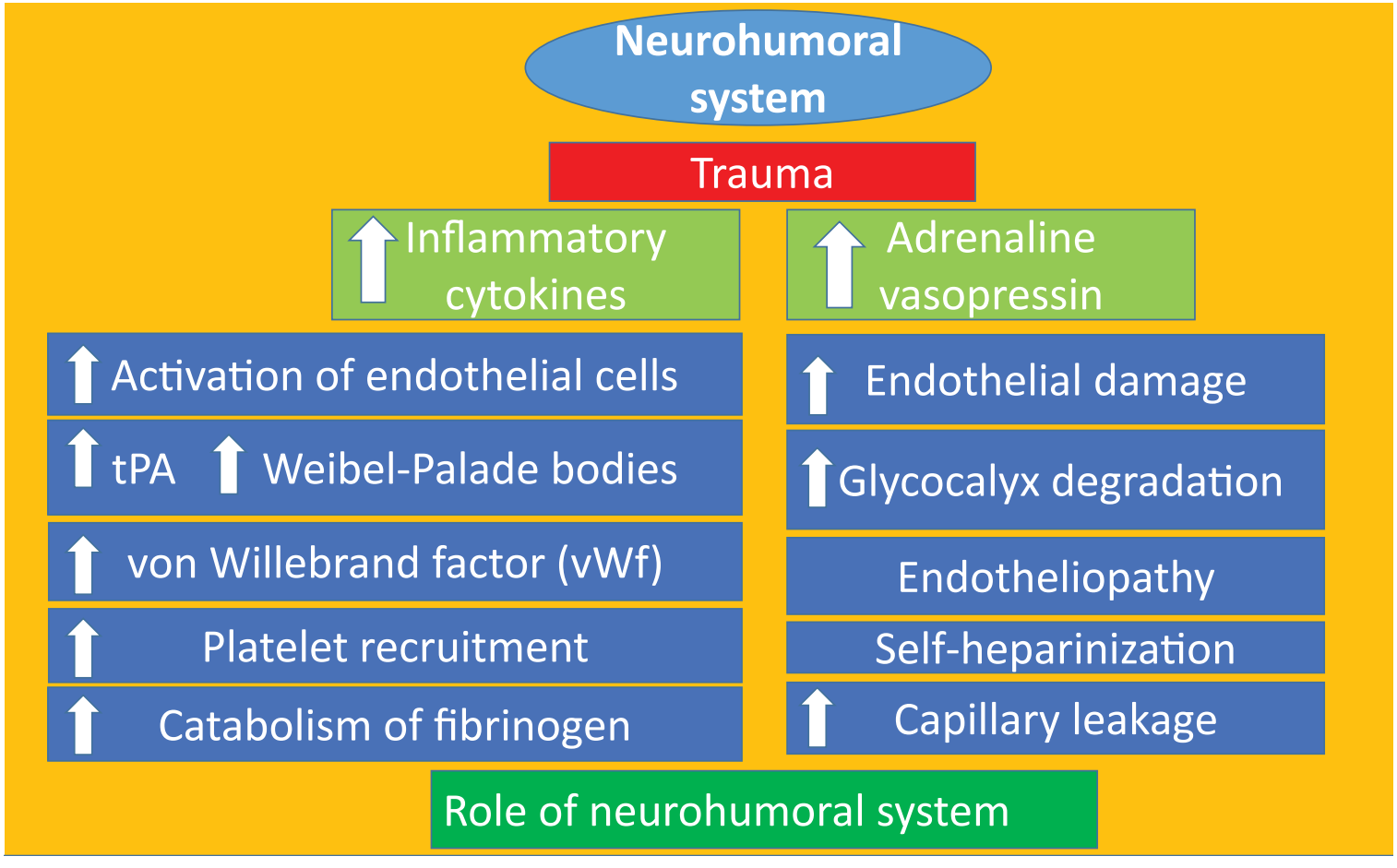

Figure 25.4 Role of the neurohumoral system.

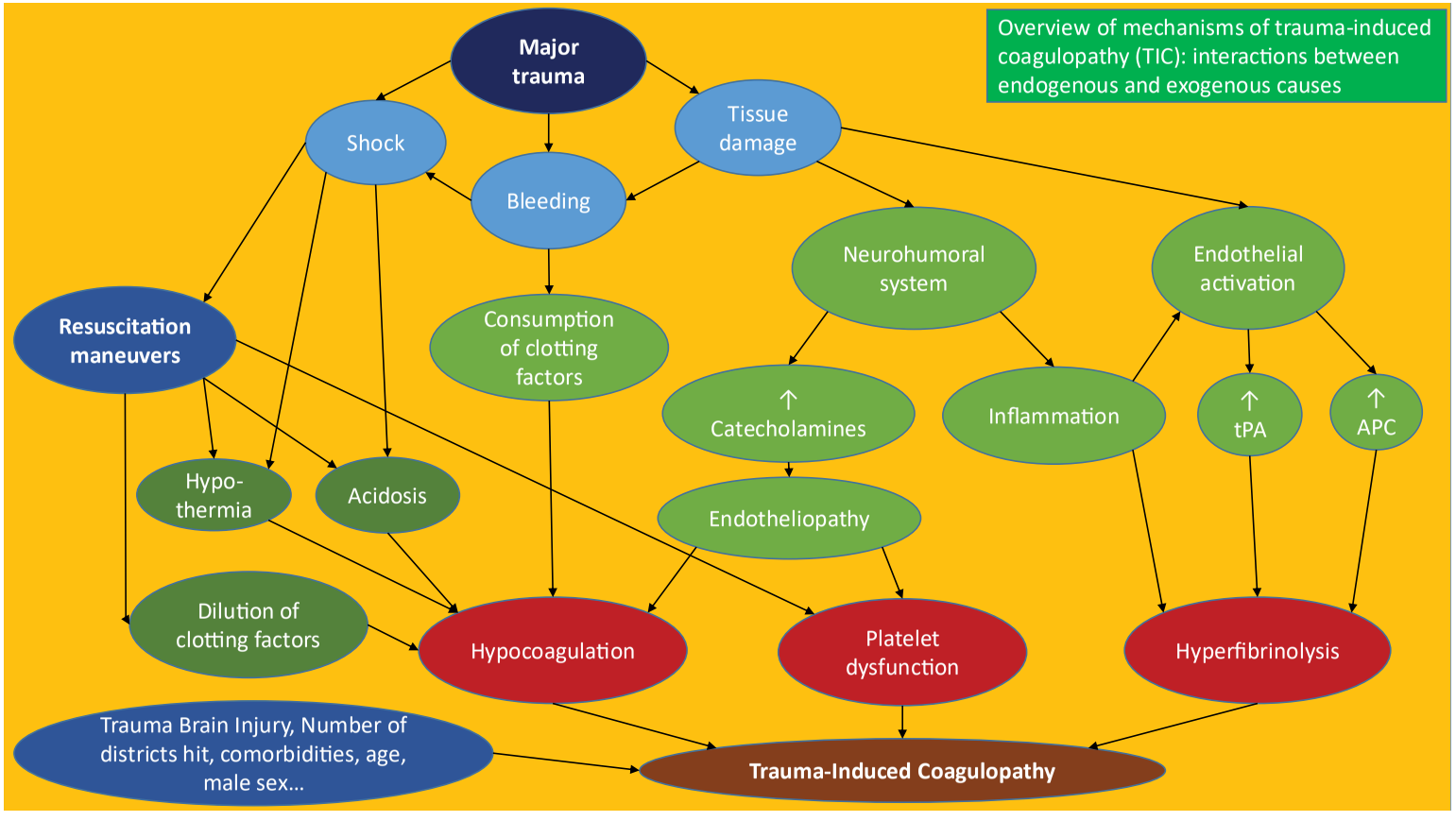

Figure 25.5 Overview of mechanisms of trauma-induced coagulopathy (TIC).

25.3.1.3 Role of platelets

Platelets contain a plethora of proteins involved in coagulation and fibrinolysis. It is not yet clear how these contradictory platelet secretions are exactly affecting TIC. Data on platelet function testing in trauma patients are scant, because platelet sample handling and availability of specific assays are complicated.

Studies have demonstrated impaired platelet aggregation in response to adenosine diphosphate (ADP), arachidonic acid, collagen and thrombin receptor activating peptide (TRAP), suggesting a prevalence of platelet dysfunction of up to 45.5% of trauma patients on admission and to 91.1% during their stay in the intensive care unit (ICU).

It has also been proposed that the thrombin receptor pathway plays an important role in trauma-induced platelet dysfunction.

However, the mechanisms and the implications of this finding are not clear. Anemia, whether it is due to hemorrhage or dilution, can also affect platelet adhesion. Considering the available evidence, endotheliopathy and anemia may be the triggers for platelet dysfunction in trauma.

Platelet count on admission may be useful as a measure for predicting the outcome as documented in some cohorts of massively transfused trauma patients, in which platelet count was inversely correlated with injury severity, morbidity, and mortality.

25.3.2 Coagulopathy Associated with Resuscitation Maneuvers

In traumatized patients aggressive resuscitation, previously recommended, with crystalloid dilutes the clotting factors and causes metabolic acidosis (hyperchloremic in the case of NaCl administration 0.9%) intertial edema formation. This also caused microcirculation impairment and impaired oxygen tissue supply. Colloids cause the movement of proteins from the blood to the interstitial space, a reduction in plasma concentration of clotting factors, particularly factor vii and von Willebrand factor, an inhibition of platelet function and a reduced interaction of factor xiii with fibrin polymers. The administration of crystalloids in traumatized patients has been shown to cause a worsening of TIC, acidemia and hypothermia that inhibit thrombin; as a result, we should try to limit the use of crystalloids to reduce dilution effects.

The effects of hypothermia, to which hemorrhage and hypoperfusion contribute, will then be discussed more extensively.

Finally, with regard to acidemia in the traumatized patient, which also happened more widely, here we remember that it depends on three factors: the use of crystalloids in resuscitation maneuvers, hypoperfusion, and the use of saline solution. In fact, hypoperfusion causes the cell to switch from an aerobic mechanismto an anerobic mechanism, resulting in the production of lactates leading to a reduction in ph. The saline solution (NaCl 0.9%) contains a higher concentration of chlorine than under physiological conditions and can therefore cause hyperchloremic metabolic acidemia.

25.3.3 Detrimental Factors and Exacerbating Trauma Coagulopathy

So we can summarize how the early acute coagulopathy associated with traumatic injury has recently been recognized as a multifactorial primary condition that results from a combination of bleeding-induced shock, tissue injury-related thrombin–thrombomodulin complex generation, and the activation of anticoagulant and fibrinolytic pathways.

25.3.3.1 Acidosis

Acidosis is a frequent and early occurrence of the traumatized patient as a result of inadequate tissue oxygenation, which then activates anaerobic metabolism.

Acidosis in itself causes dysfunction of plasma proteins and leads to rapid degradation of fibrinogen and almost all stages of clotting are compromised in this setting; in summary, for pH less than 7.4, we see

- change of shapes and structure of platelets;
- reduction in clotting factor activity;
- compromised thrombin production;
- reduction in the concentration of fibrinogen;
- increased degradation of fibrinogen (due to increased fibrinolysis and by the increase in factor XIII), while it did not affect fibrinogen production;
- increased pro-inflammatory response of platelet-mediated neutrophils.

The administration of bicarbonate to correct acidosis is not associated with a reversal of the coagulopathy.

25.3.3.2 Hypothermia

Hypothermia can ensue following a trauma as a result of heat loss, reduced heat production and the administration of fluids. Clinically significant reduction in platelet function and coagulation factor activity start at temperatures below 36°C and worsen dramatically for temperatures below 33° C.

Hypothermia influences several key stages of the coagulation process. In summary, it

- negatively affect platelet function;
- reduces the enzyme activity of clotting factors;
- induces the activation of fibrinolysis.

The effects are reversible with the normalization of body temperature, which represents a first-level goal to be achieved, both through the use of thermal blankets, or by other means of physically warming the patient, or by administering hot liquids (40°C).

Overall, the other two components of the lethal triad act on clotting in all its phases. In particular, hypothermia extensively inhibits the early stages of the process, while acidosis extensively inhibits the propagation and thrombin generation phase.

As for fibrinogen metabolism, hypothermia inhibits fibrinogen synthesis while acidosis accelerates its degradation.

With regard to the response to therapy, we can note some differences in this case as well: the effects of hypothermia are corrected when the body temperature is restored to temperatures of 36°C or more, while the effects of acidosis cannot be immediately corrected with the normalization of pH.

25.3.3.3 Shock

There is common agreement that shock is an independent risk factor for trauma coagulopathy, even though the true frequency of shock in trauma is unknown. Systolic blood pressure was frequently used in several studies as a determinant of hypoperfusion. Traumatic brain injury further hampers the use of blood pressure measurements as a sign of hypoperfusion. The systolic blood pressure goals are in fact different depending on the districts involved in major trauma: In cases of head trauma, the systolic pressure must reach 110 mmHg, while when the cranial district is not interested, the systolic blood pressure goal is 90 mmHg.

Shock and its effect on the sympathoadrenal system, the endothelium, including the glycocalyx and the hemostatic cells in the circulating blood, result in phenotypic features that characterize the clinical conditions of patients suffering from acute critical illness, despite the different types of injurious "hits" they suffer. The catecholamine-induced damage to the endothelium is responsible for endothelial breakdown resulting in glycocalyx shedding, the breakdown of tight junctions with capillary leakage, and a procoagulant microvasculature that further reduces oxygen delivery due to increased tissue pressure and microvascular thrombosis creating a vicious circle that ultimately leads to organ failure. The early genetic responses to severe trauma, burn injury, and endotoxemia are similar, indicating that the response mounted by the body to various acute critical conditions accompanied by shock, is relatively homogenous and most likely evolutionarily adapted.

25.3.3.4 Traumatic brain injury

Some studies show systemic coagulopathy often occurs within minutes of traumatic brain injury (TBI). So it has been hypothesized that it is triggered by brain-derived substances that are rapidly released systemically through the disrupted blood–brain barrier (BBB). The BBB disruption allows the vascular leakage of fluid to cause cerebral edema and releases brain-derived substances into the circulation to trigger systemic coagulopathy. Recent studies suggest that brain-derived cellular microvesicles (BDMVs) may have a role as a dissemination factor or a causal factor.

In some studies fibrinolytic product D-dimer and fibrinogen degradation products are first detected within minutes of TBI, whereas prolonged prothrombin and partial thromboplastin times are detected later, reaching their peaks ~3 to 6 h post-TBI and some authors have suggested that this time course is consistent with an early transition from a hyper- to a hypocoagulable state.

However, many steps still need to be taken to fully understand the role of head trauma in activating T.I.C., particularly with regard to its role in determining changes in fibrinolysis and platelet function. As far as changes of fibrinolysis inhibitors are concerned, there are fewer reported cases. As far as platelet function is concerned, patients with TBI seem to have moderately low counts, but often they are activated and express procoagulant activity.

25.3.3.5 Age, male sex, and comorbidities

The coagulopathy is modified by trauma-related factors such as: age, sex, and comorbidities such as diabetes and hypertension.

A significantly different sympatho-adrenal and endothelial response to the major trauma in older compared to younger patients has been reported. The patient's age also appears to significantly influence TIC, including the degree of endotheliopathy. This is in accordance with the well-described association between advanced age and progressive disruption and dysfunction of the endothelium, with the most profound endothelial disruption observed in smokers and patients with diabetes, hypertension, or atherosclerosis. In addition to age, gender also significantly influences the endogenous trauma shock response and both age and male gender are substantial and independent predictors of multiple organ failure, an outcome closely linked to endotheliopathy, following severe trauma.

Previously reported comorbidities can lead to worse outcomes probably also due to endotheliopathy.

25.3.3.6 Other factors

In addition to those previously exposed, the severity of the coagulation disorder is influenced by environmental and the resulting therapeutic factors such as genetic background, inflammation, and premedication, especially with oral anticoagulants.

As for the involvement of anatomical districts, associations were found between T.I.C. and the involvement of the abdominal district. A recent study also showed that the increased number of districts correlated with the early development of T.I.C.

25.4 Specials Clinical Forms of TIC

In addition to the forms and degrees of coaugulopathy already described (hypercoagulability, hypocoagulation, hyperfibrinolysis), here are some forms that we think are noteworthy.

25.4.1 Early Primary Hyperfibrinolysis

A limited number of patients have rapid activation during the early manifestation process of Coagulopathy and an uncontrollable pattern of the fibrinolysis process. This clinical picture is called early primary hyperfibrinolysis.

Hyperfibrinolysis is present in about 2.5–7% of all traumatized patients. Early diagnosis of this form still presents many difficulties. Viscoelastic tests (see below) highlight only a part of the cases, while occult hyperfibrinolysis seems to be much more common. This condition seems to be burdened by a much higher mortality, according to some authors even reaching levels of 60–80%.

Early administration of antifibrinolytics is required as demonstrated by the CRASH 2 study. Administration of tranexamic acid within the first 3 h in patients with active bleeding or at risk of bleeding is strongly recommended (recommendation class 1A) according to the 2016 and 2019 European guidelines.

25.4.2 Late Coagulopathy

Late coagulopathy has been seen as the hemostatic reaction following trauma, which returns to normality during recovery in patients without complications, while patients with severe injuries may suffer from the complications of massive coagulopathy.

Recovery from coagulopathy returning to normal clotting values after severe trauma may be delayed in such patients. There is a massive physiological response following trauma, which leads to a multitude of changes in the neurohumoral system, in the natural pro and anti-coagulating systems and in the other mechanisms previously reported. It remains difficult to distinguish between adaptive and maladaptive systemic inflammatory responses to injury.

From a clinical viewpoint, the feasible means to identify maladaptive systemic inflammation could be, at present, the identification of organ dysfunction. Almost 30% of severely injured patients develop a multiple organ dysfunction syndrome (MODS) and this is associated with worse outcomes and a high mortality rate. It is important to remember that late hypercoagulopathy following trauma also contributes to an increased risk of venous thromboembolism.

25.5 Diagnosis

25.5.1 Clinical Features

While blood loss can sometimes be noticeable, neither visual evaluation nor physiological parameters are effective guides to understand the degree of hemorrhage.

Trauma dynamics is an important tool to identify patients at risk for significant bleeding.

For example, the American College of Surgeons defined a threshold of 6 m (20 feet) as a “critical fall height” associated with serious injuries. Additional critical mechanisms include the high-energy deceleration effect, gunshot wounds, etc.

The dynamics of trauma combined with severity, with the patient’s clinical presentation and with the response to the first resuscitation maneuvers should

further lead to the decision to begin initial hemorrhage control as described in ATLS. An American study conducted by Mutschler et al. analyzed the adequacy of this classification and found that more than 90% of all trauma patients cannot be classified according to the Advanced Trauma Life Support (ATLS) classification of hypovolemic shock. This is composed of four classes of patients depending on the vital parameters and state of consciousness they present.

The same group analyzed the validity of the ATLS classification and concluded that this system may underestimate sensory alterations in the presence of hypovolemic shock and overestimate the degree of tachycardia associated with hypotension. A three-class scheme with three types of response to initial volemic resuscitation was proposed. The first class is made up of those who respond with stable normalization of vital parameters. The second-class, transient responders, which after initial stabilization after volemic filling present with unstable vital parameters of the state of consciousness at a later time. The third class is made up of non-responders to volemic filling. The second and third classes are candidates for immediate surgical management of bleeding.

25.5.2 Laboratory Tests

The diagnosis of TIC is still based on laboratory abnormalities that may not necessarily correspond to a distinct clinical phenotype. Despite significant advances in coagulation research, there is no adequately valid test to predict and identify a clinically relevant acquired coagulopathy. Published reports on TIC have been mostly based on the evidence of abnormal laboratory findings of prothrombin time, activated partial thromboplastin time, plasma fibrinogen concentration, platelet count, either alone or in combination.

Early identification of coagulopathy in traumatized patients is important, as this can lead to better management and overall improvement in outcomes.

The most commonly used tests are traditional clotting tests (activated partial thrombopastin time and prothrombin time), along with platelet count and fibrin monitoring.

Originally the T.I.C. has been defined as an increase in clotting plasma tests such as activated thromboplastin time, prothrombin time (PT), and international normalized ratio (INR).

Emerging evidence suggests that whole-blood viscoelastic tests, such as T.E.G. or R.O.T.E.M., may better identify coagulopathy and also identify the stage, or type, of T.I.C. and location. T.I.C. seems to be becoming more serious and has increasingly worse outcomes with the severity of the injuries and the increase of the ISS.

Three stages of T.I.C. can be proposed, corresponding to more serious clinical frameworks and worse outcomes: stage of hypercoagulation, hypocoagulation, and hyperfibrinolysis. Viscoelastic tests can provide partial results in minutes. They also have the advantage of being able to diagnose, quantify, and classify fibrinolysis, thus allowing the use of antifibrinolytic and blood-resistant drugs such as concentrated fibrinogen to be administered.

They have also been shown to be able to avoid inappropriate hemotransfusions and hemostatic infusions of blood derivatives to non-coagulopathic patients.

In addition, T.I.C. may vary with ongoing treatment, and viscoelastic tests are able to record these changes.

Current hematochemical tests (PT, aPTT, fibrinogen, platelets), though having the advantage of being available everywhere, require a long time for analysis; In addition, PT and aPTT are only useful for the analysis in the early stages of clot formation and do not give a complete view of real pro-coagulating and anti-coagulant activity, in particular on platelet and hyperfibrinolytic activity.

The use instead of viscoelastic tests, such as thromboelastography (TEG) and thromboelastometry (TEM), could remedy these problems, as they provide, in a shorter time, a more complete view of the entire clot process, giving a reflected view of the homeostatic process *in vivo*, including pertinent information regarding the analysis of platelets and fibrinogen, not available in routine hematochemical testing.

25.6 Outcomes

We summarize here synthetically (because on several occasions we have already developed the topic) that patients who develop T.I.C. have worse prognoses, regardless of the severity of the developed T.I.C. Among the worst complications they face are: a higher need for hemotransfusion, a higher rate of hospitalization, a higher rate of hospitalization in intensive care, and a higher mortality rate.

We can therefore see that not only do they have worse clinical outcomes but necessitate the use of more hospital and pre-hospital resources.

25.7 Hints for Therapy

Hypovolemic resuscitation, hypothermia prevention and early clotting support are, together with damage control surgery, the cornerstones of damage control resuscitation DCR. The convention of DCR largely arose following the discovery of the lethal triad of hypothermia, acidosis, and coagulopathy, with the goal to avoid the initiation of this "bloody vicious cycle" or to reverse its progression. CDR is the strategy by which we try to correct early conditions that promote the bleeding and compromise hemostasis and to limit the damage caused by hypoperfusion.

The international Guidelines recommend that treatment of the bleeding trauma patient is carried out in a manner that supports the concept that normalization of coagulation parameters will improve outcomes.

It is reasonable to presume that the severely injured patient is coagulopathic and "best guess" treatment should be initiated. During further resuscitation, a goal-directed approach is considered to be the most appropriate measure.

The measures to support coagulation should be initiated immediately upon hospital admission and it is essential to quickly determine the type and degree of

coagulopathy in the individual patient in order to determine the most prominent cause or causes to be treated specifically in a goal-directed manner. Early monitoring of coagulation is essential to detect trauma-induced coagulopathy and to define the main causes. Early therapeutic intervention improves coagulation tests, reduces the need for transfusion of RBC, FFP, and platelets, reduces the incidence of post-traumatic multi-organ failure, shortens the length of hospital stay, and may improve survival. The success of early intervention determines the best coagulation management in order to reduce transfusions and to improve outcomes, including reducing mortality.

In the initial management of patients with expected massive hemorrhage, it is recommended one of the two following strategies:

- Plasma (FFP or pathogen-inactivated plasma) in a plasma–RBC ratio of at least 1:2 as needed (Grade 1B)
- Fibrinogen concentrate and RBC according to Hb level (Grade 1C)

In massive bleeding, it is recommended the immediate administration of coagulation components with a 1:1:1 ratio for RBC, plasma and platelets until laboratory values are confirmed and the therapy can be adjusted.

Concentrated red blood cells must be transfused in order to achieve a hemoglobin target of 7–9 g/dl.

In patients taking oral anticoagulants, concentrated fresh plasma is recommended only in cases where the PCC is not immediately available. The use of PCC has been shown to be superior to FFP in the rapid reversal of vitamin K antagonists with evidence of a decrease in hematoma formation in those with head injuries. It is therefore the agent of choice to reverse the effects of vitamin K antagonists.

Platelets should be administered to maintain a platelet count above 50 × 109/l.

Since approximately 40% of patients arriving in the emergency room have below-normal fibrinogen values, a treatment with fibrinogen has been proposed. Treatment with concentrated fibrinogen is recommended in patients with bleeding accompanied by viscoelastic signs of fibrinogen functional deficit or with plasma levels of fibrinogen less than 1.5–2 g/L. The suggested initial dose of concentrated fibrinogen is 3–4 g or 50 mg/kg. Any repetition must be conducted by laboratory tests. Recent experimental data show that the administration of fibrinogen does not suppress endogenous fibrinogen synthesis.

Monitoring of fibrinolysis is recommended in all patients with hemorrhagic trauma. Tranexamic acid (TXA) should be administered as early as possible to the trauma patient who is bleeding or at risk of significant hemorrhage at a loading dose of 1 g infused over 10 min, followed by an i.v. infusion of 1 g over 8 h; administration should be started within 3 h after injury. TXA should not be given more than 3 h after injury. If a concentrate-based strategy is used, it may be useful to choose fibrinogen concentrate or cryoprecipitate if significant bleeding is accompanied by viscoelastic signs of a functional fibrinogen deficit or a plasma fibrinogen level of less than 1.5–2.0 g/l.

It is suggested to start an initial fibrinogen supplementation of 3–4 g. Repeat doses must be guided by viscoelastic monitoring and laboratory assessment of fibrinogen levels.

25.8 Management of Patients with Severe Trauma in an ER

Briefly we emphasize some management aspects for the ER treatment of patients with T.I.C.

It underlines the need for protocols for the management of coagulopathy in corporate diagnostics and therapeutic pathways in patients with severe trauma in line with the most up-to-date guidelines.

The usefulness of protocols for massive hemotransfusion and the need for bedside clotting analyzers, have also been demonstrated.

The European guidelines now recommend that patients be transferred directly to an appropriate trauma treatment centre and encourage the use of a restricted volume replacement strategy during initial resuscitation.

The best-practice use of blood products during further resuscitation continues to evolve and should be led by a goal-directed strategy. The identification and management of patients pre-treated with anticoagulant agents continues to pose a real challenge, despite accumulating experience and awareness.

Abbreviations

ADP: adenosine diphosphate
APC: activated protein C
ATLS: advanced trauma life support
BBB: blood–brain barrier
BDMVs: brain-derived cellular microvesicles
DCR: damage control resuscitation
EGL: endothelial glycocalyx
ER: emergency room
GCS: Glasgow coma score
ICT: intracardiac thrombus
ICU: intensive care unit
INR: international normalized ratio
ISS: injury severity score
MODS: multiple organ dysfunction syndrome
MT: major trauma
PAI-1: plasminogen activator inhibitor-1
PCC: prothrombin complex concentrate
PT: prothrombin time
TBI: traumatic brain injury
TEG: thromboelastography
TEM: thromboelastometry

TIC: trauma-induced coagulopathy
TRAP: thrombin receptor activating peptide
TXA: tranexamic acid
vWf: von Willebrand factor

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Chapter 26

Advances in Fractures and Dislocations of the Hip Joint

Thomas Pepper, MD, Philip Ahrens, MD, and M. Zahid Saeed, MD

Trauma and Orthopaedic Department, University College London Medical School, Royal Free Hospital, London, UK

z.saeed@nhs.net, mzahidsaeed@hotmail.com

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26.1 Hip Fractures

A hip fracture is defined as a fracture occurring in the region between the femoral head and 5 centimetres inferior to the lesser trochanter [1]. It is often a surrogate marker of underlying poor health, and this is reflected in a 10% mortality rate at one month and approximately 33% mortality at one year [1]. Risk factors for hip fracture include advanced age, osteoporosis and recurrent falls—the latter is the commonest mechanism of injury in the older population. Hip fractures may be divided into intracapsular and extracapsular fractures.

26.1.1 Intracapsular Fractures

26.1.1.1 Classification

These may be further defined as subcapital (immediately beneath the head), transcervical (middle region of the femoral neck) and basal (just proximal to the

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trochanters). Intracapsular fractures may be classified radiographically according to the displacement of fragments [2]:

- Stage I: an incomplete impacted fracture
- Stage II: a complete but undisplaced fracture
- Stage III: a complete fracture with displacement but some end-to-end bony contact (Fig. 26.1)
- Stage IV: a fracture with no end-to-end bony contact

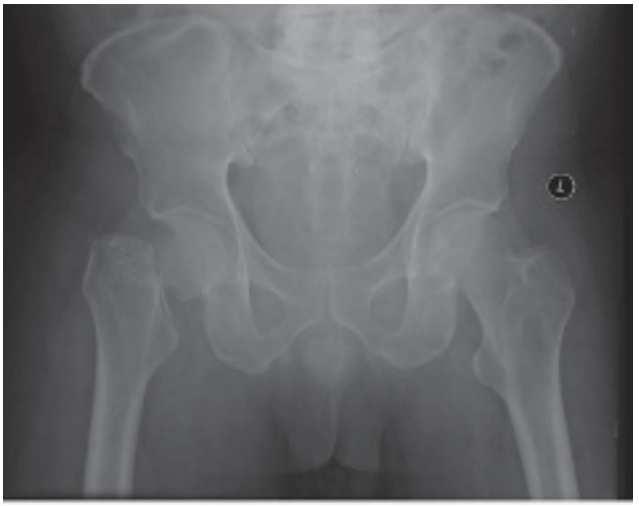

Figure 26.1 Right-sided Garden's stage III fracture.

26.1.1.2 Clinical features

A displaced fracture manifests clinically as a shortened and externally rotated leg. Patients may still be able to weight-bear on impacted fractures, and patients with dementia may not complain of pain. If there is strong clinical suspicion of a fracture but this is not demonstrated radiographically, a magnetic resonance imaging (MRI) or bone scintigraphy scan should be ordered.

26.1.1.3 Management

In stage III and IV fractures the intramedullary and retinacular vascular supply to the femoral head is interrupted, leaving the only supply from the vessels of the ligamentum teres. The latter blood supply is frequently inadequate or absent in the older population, and as a result there is a high risk of nonunion and avascular necrosis. The treatment of choice for displaced intracapsular fractures in advanced age therefore involves prosthetic replacement of the femoral head. Current National Institute for Health and Care Excellence (NICE) guidance suggests total hip replacement for patients who are able to walk independently outdoors with a stick or better, are not cognitively impaired and are medically fit for the procedure [1]. In the remainder, hemiarthroplasty is indicated. Whichever

modality is appropriate, cemented prostheses result in improved mobility and less pain [3].

Stage I and II fractures do not cause the same degree of vascular compromise to the femoral neck and may be treated reliably with closed reduction and internal fixation using cannulated screws or a dynamic hip screw. Fixation facilitates early postoperative mobilisation and minimises pulmonary complications and pressure sores, but in the bedbound or demented patients with little pain, nonoperative treatment may be acceptable. If surgery is planned it should be undertaken with urgency in order to minimise ischaemia of the femoral head, although this must be balanced with sufficient preoperative optimisation of the patient.

26.1.1.4 Complications

Complication rates accompanying hip fractures in older patients are high, but most are associated with pre-existing comorbidities, which leave them especially prone to pneumonia and pressure sores. Venous thromboembolism is a great risk in this group of patients, and this may be reduced by appropriate use of compression stockings, perioperative heparin prophylaxis and early mobilisation.

Avascular necrosis of the femoral head occurs in approximately 10% of patients with undisplaced fractures. It is only possible to diagnose this retrospectively, it being visible on bone scintigraphy several weeks postoperatively and radiographically several months postoperatively. Although the fracture may still unite, the joint suffers irreversibly damage. In patients over 45 years, total hip replacement is indicated.

Nonunion affects approximately a third of all intracapsular fractures, but the risk is higher in stage IV fractures. The aetiology may be femoral head ischaemia, suboptimal reduction or inadequate fixation, and management of the nonunion depends on addressing the underlying factor, with hemiarthroplasty or total hip replacement remaining viable options.

26.1.2 Extracapsular Fractures

26.1.2.1 Classification

Extracapsular hip fractures comprise intertrochanteric fractures, reverse oblique fractures, trochanteric avulsion fractures (Fig. 26.2) and subtrochanteric fractures. Intertrochanteric fractures (Fig. 26.3) are the most common and may be further classified according to the degree of communition and therefore instability [4].[1] Reverse oblique fractures are generally unstable as weight bearing acts to displace the fragments. Subtrochanteric fractures (Fig. 26.4) may be associated with significant blood loss; if nontraumatic the aetiology may be an osteolytic metastatic deposit.

[1]Kyle classification of intertrochanteric femoral fractures, OrthoFRACS. Available at: http://www.orthofracs.com/adult-fractures/principles/classifications/intertrochanteric-femoral-fractures/ (accessed on November 23, 2022).

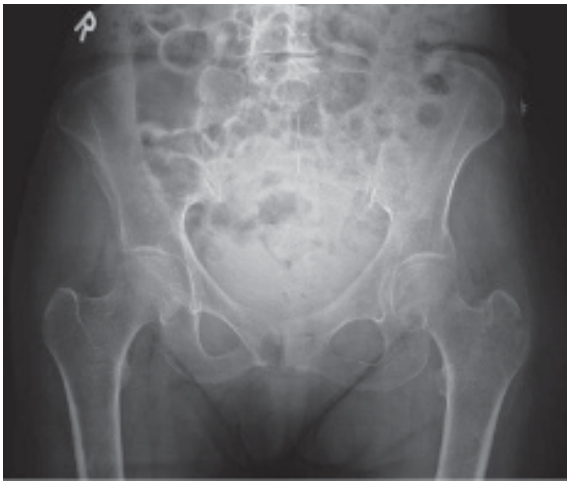

Figure 26.2 Left-sided trochanteric avulsion fracture.

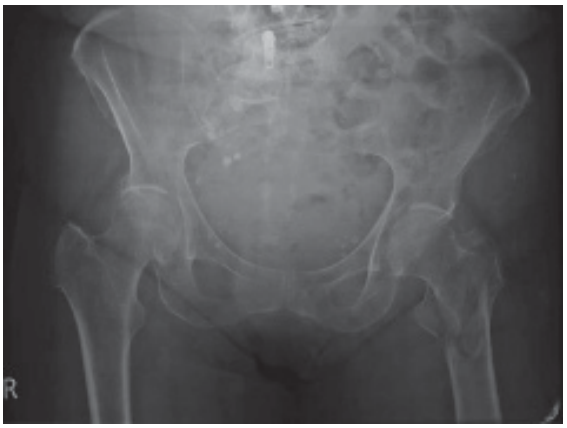

Figure 26.3 Left-sided intertrochanteric fracture.

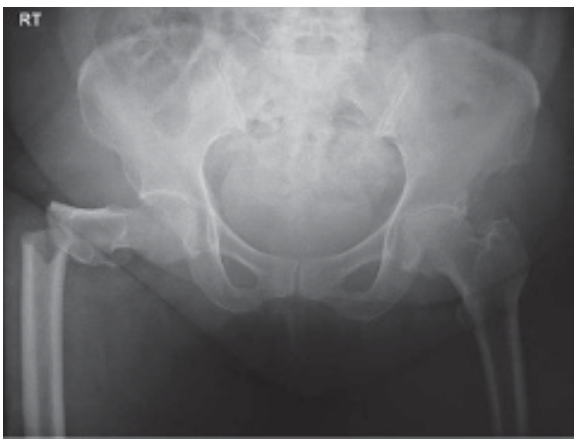

Figure 26.4 Right-sided subtrochanteric fracture.

26.1.2.2 Clinical features

Clinically, extracapsular fractures present in the same way as intracapsular fractures, although the leg may be more externally rotated as the fragments are unsupported by joint ligaments.

26.1.2.3 Management

Extracapsular fractures unite readily, and there are no significant differences in outcome between conservative and operative management [5]; there is a relatively low risk of avascular necrosis of the femoral head as the retinacular vasculature is undisrupted. These fractures are treated by internal fixation nevertheless—using a dynamic compression screw or, if subtrochanteric, an intramedullary nail—in order to facilitate early rehabilitation of the patient.

26.1.2.4 Complications

Although these fractures are less prone to avascular necrosis of the femoral head, patients are subject to the same general postoperative complications as intracapsular fractures—namely pneumonia, venous thromboembolism and pressure sores—all of which may be mitigated by early mobilisation. In addition, there is a risk of metalwork "cut out" of the osteoporotic bone, particularly if the screw has been poorly positioned or there is a suboptimal reduction. Malunion is relatively common, resulting in varus and external rotation deformities, but these are rarely clinically significant. Nonunion is uncommon, but if the fracture has not united after six months, revision is indicated.

26.2 Traumatic Dislocations of the Hip Joint

The hip joint is very stable, and as a result dislocations are relatively rare, requiring extreme force to occur. A dislocated (nonprosthetic) hip is an orthopaedic emergency and should be reduced as quickly as possible under general anaesthesia with a muscle relaxant. It is imperative to test and document neurovascular status prior to attempting reduction. Hip dislocation may be classified according to the direction of movement of the femoral head—posterior, anterior or central.

26.2.1 Posterior Dislocation

This type forms the vast majority of hip dislocations and is frequently associated with a road traffic accident involving a head- on force that causes the knee to strike against the dashboard of the vehicle, forcing the femur backwards. Acetabular injury depends on the position at the time of impact. If the hip is abducted at the time of posterior dislocation, a simultaneous acetabular fracture will occur. However, if the hip is adducted it is unsupported posteriorly and acetabular fracture may be avoided. In isolated posterior hip dislocation, clinically the leg appears shortened and the hip will be held adducted and internally

rotated. This picture may be complicated if there is a concomitant femoral shaft fracture. Plain radiographs will show the femoral head to be displaced from and superior to the acetabulum (Fig. 26.5). A computed tomography (CT) scan will better demonstrate any acetabular fracture.

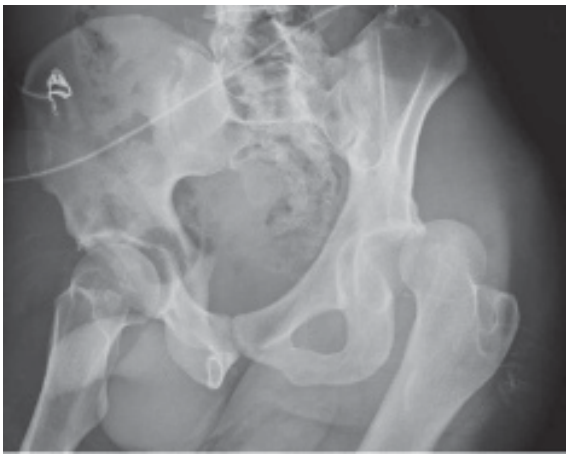

Figure 26.5 Left-sided posterior hip dislocation.

To restore joint congruity the hip and knee are flexed to 90° while traction is applied in the line of the femur and the pelvis is stabilised. A plethora of methods to achieve this have been described (Bigelow [6], Allis [7], Lefkowitz [8] and East Baltimore [9] to name a few). Following reduction the hip should be tested for instability and, if it is present, the patient should be considered for posterior acetabular repair. After reduction the patient should be put into traction for several weeks, with rehabilitation commencing as soon as pain allows. If closed reduction is unsuccessful or postreduction imaging shows intra-articular loose fragments, an operative intervention is necessary.

The sciatic nerve is closely related to the posterior surface of the femoral head, and consequently nerve injury accompanies approximately 10% of posterior hip dislocations [10]. Permanent injury is uncommon, but while nerve recovery occurs, the insensate areas of the limb must be protected from further injury and an ankle dorsiflexion splint should be worn to aid walking. A new sciatic nerve injury following reduction of the hip necessitates exploration of the joint in order to exclude entrapment of the nerve in the joint space.

A concomitant femoral shaft fracture is rare but often leads to late diagnosis of the hip dislocation, as the typical clinical picture is obfuscated. A thorough clinical and radiographic examination will mitigate this.

Avascular necrosis of the femoral head is a late-occurring complication of dislocation, and its incidence rises in proportion to the duration of time dislocated. Ischaemia is thought to result from vessel compression or spasm [11], which is relieved by prompt reduction. If the hip is reduced within 12 hours, the risk of avascular necrosis is approximately 2%–4%; if reduction is delayed past

12 hours, this risk increases by 5.6 times [12]. Osteonecrosis is appreciated earliest on MRI or at 6–12 weeks on plain radiographs. Treatment is operative and often best achieved through total joint replacement.

Secondary osteoarthritis is a potential sequela following hip dislocation and may be a result of avascular necrosis, loose bone fragments in the joint and/or trauma to the articular surface at the time of dislocation.

26.2.2 Anterior Dislocation

Anterior dislocation is much rarer than posterior but in the same way it usually accompanies major vehicular trauma. Clinically, the hip is held abducted—sometimes extremely so—and externally rotated. The leg length is maintained as the femoral head is prevented from moving proximally by the origin of the rectus femoris. The contour of the dislocated head will be visible and palpable in the groin (anteroinferior dislocation) or immediately anterior to the joint (anterosuperior dislocation).

Reduction of an anterior dislocation is achieved in much the same way as a posterior dislocation, except that the hip should be adducted while traction is applied.

Careful examination and documentation of the femoral nerve and vessel function is necessary in anterior dislocations, as these structures are closely related to the anterior hip and are at risk. Avascular necrosis of the femoral head is a possibility but occurs with less frequency than in posterior dislocations.

26.2.3 Central Dislocation

Central dislocation occurs when the femoral head is driven into the pelvis through the floor of the acetabulum. This may be caused by a fall onto the side from a height or in association with another major trauma. Clinical features are variable depending on the aetiology, but the leg is shortened. A CT scan is the most useful imaging modality, but two oblique plain radiographic views should also be sought in addition to anteroposterior and lateral views in order to conduct sufficient initial examination of the acetabulum.

Following resuscitation of the patient and reduction of the dislocation, the hip injury remaining is a complex acetabular fracture. Operative management is indicated for all unstable hips and all displaced acetabular fractures. If there are medical contraindications to surgery or the acetabular fracture is undisplaced and the hip stable, conservative management involving longitudinal traction for six weeks followed by minimal weight bearing for a further six weeks may be appropriate.

26.2.4 Dislocated Hip Prosthesis

This is a relatively common presentation and, similar to a native hip, usually requires reduction under general anaesthetic. However, this need not be

undertaken with such urgency as there is no risk of avascular necrosis of the femoral head. Physiotherapy and adherence to hip precautions are the mainstay of prosthetic dislocation prevention, but refractory hips may be improved by replacement with a jumbo prosthesis [13] or by the use of retaining bands.

Disclosures and Conflict of Interest

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Chapter 27

Predicting Scoliosis Progression: A Challenge for Researchers and Clinicians

Sabrina Donzelli, MD,[a] Fabio Zaina, MD,[a] and Stefano Negrini, PhD[b,c]

[a]*ISICO – Italian Scientific Spine Institute, Milan, Italy*
[b]*Department of Biomedical, Surgical and Dental Sciences, University of Milan "La Statale", Milan, Italy*
[c]*IRCCS Istituto Ortopedico Galeazzi, Milan, Italy*

sabrina.donzelli@isico.it

Keywords: adolescent idiopathic scoliosis, Cobb degrees, early onset scoliosis, growth disorders, idiopathic scoliosis (IS), orthosis, physiotherapy, risk of progression, scoliosis, scoliosis specific exercises PSSE, spine deformities, vertebral deformity

Idiopathic scoliosis (IS) is a three-dimensional deformity of the spine with a prevalence ranging between 1% and 4% [1, 2]. IS treatment during growth is secondary prevention with the primary aim to reduce the trunk deformity and avoid progression over 30° Cobb; the secondary aim is to avoid surgery whose threshold is above 45–50° [3]. It has been shown that ending growth below 30° allows preventing progression, disability and pain in adulthood [4].

IS has a multifactorial aetiology [4] showing a wide range of different forms: anatomical (single or multiple curves and different localization), aesthetical (milder curves with visible changes and severe hiding perfectly), and prognostical (from highly to non-progressive).

One of the major challenges faced by clinicians is related to IS prognosis and to making decisions on which would be the best treatment for every single patient [4, 5]. In this context, experts use some known clinical risk factors, the most important being residual growth: the more it is, the more the risk [6]. Other factors include the deformity in sagittal and transversal planes (rotation and flat back), familiarity and joint laxity [4]. Genetics investigations have recently highlighted the heterogeneity of IS and the major role of non-genetic factors [7].

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Considering the involvement of a multifactorial pathomechanism, Zhang and colleagues developed a clinically applicable composite model using quantitative factors including circulating markers to predict the probability of progression to 40° [8]. The test of the accuracy of the model showed 80% of specificity and 92% of sensitivity, thus meaning that the model is good in discriminating patients at high risk for progression to 40°. According to the model, there is a 20% risk of overtreating patients with less aggressive IS. Is this enough? It depends on the treatment used to avoid progression. The SOSORT Guidelines recommend that "for each patient, it is mandatory to choose the correct step of treatment, where the most efficacious is also the most demanding" [4]. Expert clinicians should always choose the option they think is the most likely to reach the goals agreed with the patient but also the less invasive in the attempt to balance between undertreatment (that leads to little or no efficacy) and overtreatment (too much burden on the patient, without further benefit). Moreover, goals of treatment may vary according to patients' perspectives, with aesthetics being one of the most important goals for patients, sometimes underestimated by researchers [5]. That means that we cannot define over- and undertreatment only according to the Cobb angle. Surgery remains the last treatment option; it exposes to higher risks, and it is the most invasive treatment [9].

The introduction of a composite model, including genetic factors, is the novelty of this study, but some clinical questions remain open. The type and quality of treatment applied, the compliance to treatment and the dosage of brace-wear have not been included in the model, although they are recognized as determinants of final results [10]. The chosen threshold of 40° is questionable, though justified by the authors. Surgery is indicated for curves exceeding 50° [2]. The 30° degrees threshold is the most important for patients' future [2]. From a clinical point of view, the 40° threshold is too low for surgery indication and too high for the best achievable result from patients' perspective.

A prognostic model should help clinicians in their choices after risks estimation, but according to the Evidence Base Practice principles, in clinical decision-making patients' attitudes towards the treatment option should always be considered [4]. The currently developed composite prediction model for progression over 40° showed that the major predictor is Cobb degrees at start. In the logistic regression equation, only weight reaches significance level, while the other factors seem to work more as confounders than covariates: delayed menarche, lower body weight, Risser sign and genetic factors play a marginal role, as shown in the comparison of the predictive power. The relatively small sample of subjects used to develop the model exposes to some risk of overfitting. The authors managed this limitation by reducing the alpha level to 0.01 and validated the model in a real sample, thus increasing the external validity of their results.

The fact that Cobb at start is the major predictor confirms the key role played by screening and conservative care: exercises and bracing to prevent progression should be started at early stages of the deformity when it is early

diagnosed. Composite models, including genetic factors, showed to offer promising improvement to the prediction of IS progression, but need to be validated in larger samples and with more complex validation techniques.

Disclosures and Conflict of Interest

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Chapter 28

Prosthetic and Mechanical Parameters of the Facial Bone under the Load of Different Dental Implant Shapes: A Parametric Study

Marco Cicciù, DDS, MSc, PhD,[a] Gabriele Cervino, DDS, MSc, PhD,[a] Antonella Terranova, DDS, MSc,[a] Giacomo Risitano, PhD,[b] Marcello Raffaele, PhD,[b] Filippo Cucinotta, PhD,[b] Dario Santonocito, PhD,[b] and Luca Fiorillo, DDS, MSc, PhD[a,c]

[a]*Department of Biomedical and Dental Sciences, Morphological and Functional Images, University of Messina, Policlinico G. Martino, Messina, Italy*
[b]*Department of Engineering, Messina University, Messina, Italy*
[c]*Multidisciplinary Department of Medical-Surgical and Dental Specialties, Second University of Naples, Naples, Italy*

lfiorillo@unime.it

Keywords: adaptation zone, anisotropy, AnyOne® External, AnyOne® OneStage, biomechanical phenomena, dental implant, dental occlusion, dental prosthesis design, elastic tangential modulus, endosseous implant, finite element analysis, functional adaptation, inhomogeneity, linear elastic isotropic model, masticatory loads, maximum masticatory load, osseointegrated implants, osseointegration, overload zone, parametrical analysis, pathologic overload zone, pathologic unload zone, peri-implantitis, Poisson's ratio, proprioceptive structures, prosthesis, stereolithography scan, subjective specificity, viscoelasticity, Young's modulus

28.1 Introduction

The dental implant (also known as endosseous implant) is a surgical-type medical device used to functionally and aesthetically rehabilitate the loss or congenital deficiency of one or more teeth, allowing the support of a prosthetic substitute through direct bone support thanks to a biological process known as osseointegration. The long-term prognosis of dental implants can be considered reliable and predictable, as it can now be based on more than forty years of worldwide clinical experience. The data reported in the literature report variable

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failure rates, depending on the operating techniques and types used. The type of surface treatment also appears to involve significant differences in the implant survival data. The failures are divided according to the causes, biological, biomechanical, and aesthetic. Biological failures are divided into early and late, depending on the period in which they occur. Early failure is typically linked to a deficient initial osseointegration process following the surgical procedure, more rarely to operational errors in the procedure itself, while late failures are due to progressive infectious processes affecting the peri-implant tissues and therefore the supporting bone that surrounds the implant (peri-implantitis) [1–6]. Biomechanical failures derive from problems due to overload and functional trauma, which can occur with structural failure at both the implants and the supported prosthetic structures. The direct implant-bone connection linked to the osseointegration process leads to a greater functional load both on the prosthetic elements of the implants and on the antagonist elements that come into contact with the implant prosthetic elements. The lack of the physiological periodontal ligament also implies the absence of the proprioceptive structures that contribute to limiting trauma, through some opportune reflex mechanisms. This explains the tendency to increase mechanical problems over time. Some systems have been proposed to limit these problems by inserting elastic elements in the structure of the plants. There is talk of aesthetic failure when in areas of high aesthetic relevance there are exposures of metal parts, bone dehiscence and gums with retraction of the interdental papillae and the creation of dark triangles below the contact points of the teeth. The success or failure of the implants depends both on the health status of the person receiving it, on any medications taken which have a possible impact with osseointegration, and the condition of the tissues of the mouth [7, 8]. The mechanical stress that the implant would encounter during its life must be carefully evaluated. The correct planning of the position and number of implants is fundamental for the long-term preservation of the prosthesis, as the biomechanical forces acting during chewing can be significant. The position of the implant is determined by the position and angle of the adjacent teeth, by laboratory simulations, or by the use of computerized tomography with CAD/CAM simulations and surgical guides [9].

The objective of this study is to evaluate how the geometric and therefore biomechanical characteristics of a dental implant, in addition to having repercussions on the components of the implant, can influence the response of the patient's oral tissues [10–13].

28.2 Results

The equivalent Von Mises stress distribution at the maximum load during the masticatory cycle has been evaluated. A vertical compressive load was applied on the three different prostheses and after the simulation was performed the obtained data were post-processed. The results are presented adopting a unified color scale for each kind of prosthesis component, ranging from a minimum value in megapascal (MPa), represented by blue color, and a maximum value, represented

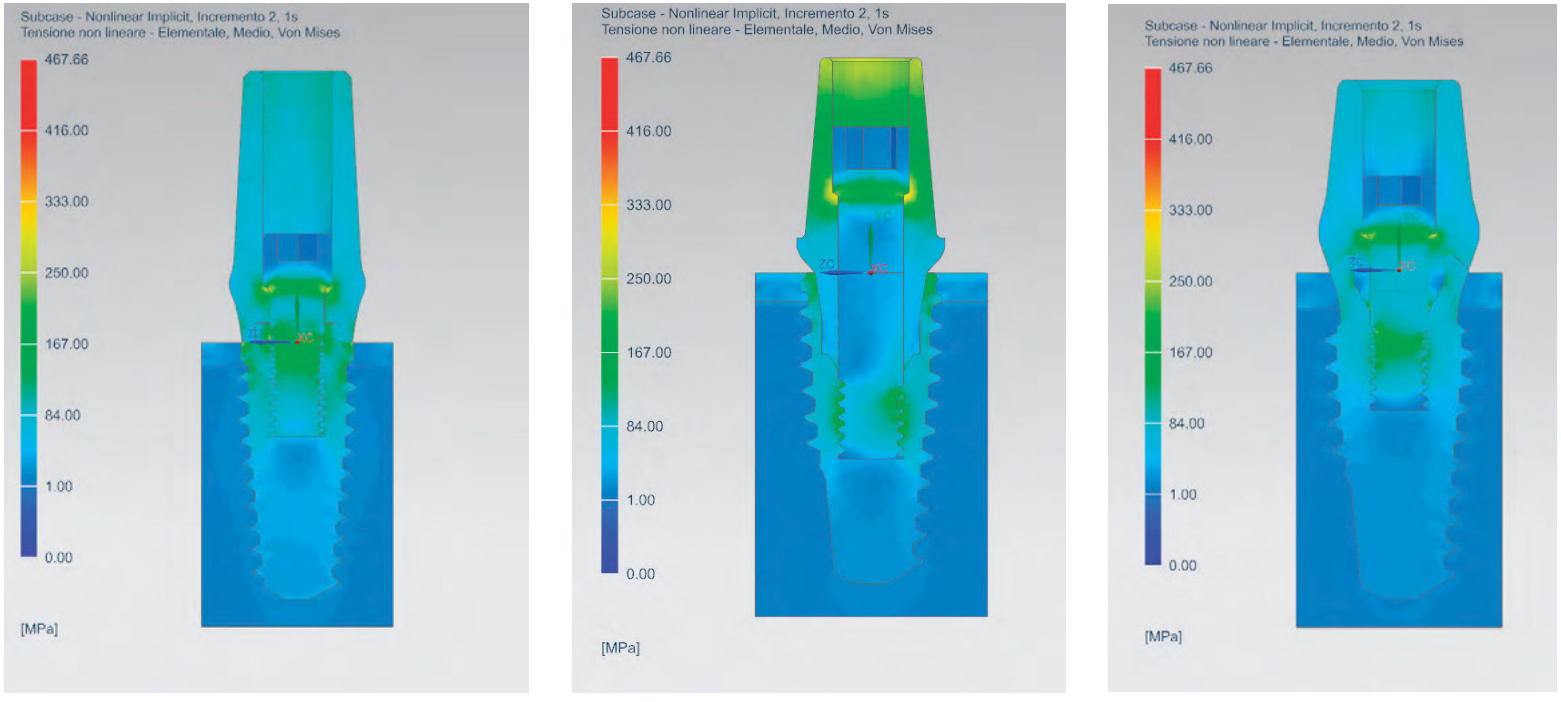

Figure 28.1 Equivalent Von Mises stress results for the complex bone fixture and prosthodontic attachments: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

by red color. In order to show the internal stress arising in the different parts of the finite element models, the stress has been evaluated in section views.

As a first result, it is possible to see how all of the prosthesis components reach a maximum stress value lower than the yielding stress of the titanium (1020 MPa), therefore plasticization and static rupture of the prosthesis are avoided (Fig. 28.1).

The AnyOne® Internal presents the most stressed area in the abutment, in the abutment-implant connection interface and in the thread of the internal screw (Fig. 28.2b). On the other hand, AnyOne® External and AnyOne® OneStage present the area of maximum stress in the internal screw thread and in the internal contact area between the screw and the abutment (Fig. 28.2a–c).

The internal screw, thanks to the applied preload, guarantees the correct mechanical joining between the implant and the abutment. For all three internal screws, regardless of the prosthesis geometry, the most stressed areas are located at the contact interface between the head of the screw itself and the abutment hole and also in the first threads of the threading (Fig. 28.3).

The implant is the joining part responsible for the load transfer between the prosthesis and the bone and it has to be perfectly osseointegrated in order to allow this load transfer. The AnyOne® External implant presents higher stress in the proximity of the first thread of the internal nut and on the first thread of the external threading (Fig. 28.4a). The AnyOne® Internal implant shows the most stressed area in the internal contact interface with the abutment and in the thread of the internal nut (Fig. 28.4b), while the AnyOne® OneStage implant has lower stress values compared to the other two implants, but located in the same areas (Fig. 28.4c).

The abutment must withstand the time-varying forces coming from the masticatory cycle. The AnyOne® External abutment presents the highest stress in the internal contact area with the retaining screw (Fig. 28.5). The AnyOne® Internal abutment has the highest stress in the same contact area of the previous one, but it exhibits also a large stress area in its upper part, where the load is applied (Fig. 28.5b). The AnyOne® OneStage, in adjunction to the internal screw contact area, presents higher stress also in the lower contacting part with the relative implant (Fig. 28.5c).

From the analysis of the stress distribution on the bone tissues (Figs. 28.6 and 28.7), it is possible to see how the AnyOne® External implant stresses a larger part of the cancellous bone, especially the first threads. On the other hand, the AnyOne® Internal presents a well-defined stressed area around the thread of the implant. The AnyOne® OneStage presents, as the External device, a wide stress area around the threading of the implant with lower stress values for the first threads compared to the other two devices.

As regards cortical tissues, a more homogeneous distribution of the stress for the AnyOne® External and AnyOne® OneStage devices is registered, while greater peaks of stress are exhibited by the AnyOne® Internal prosthesis (Fig. 28.7).

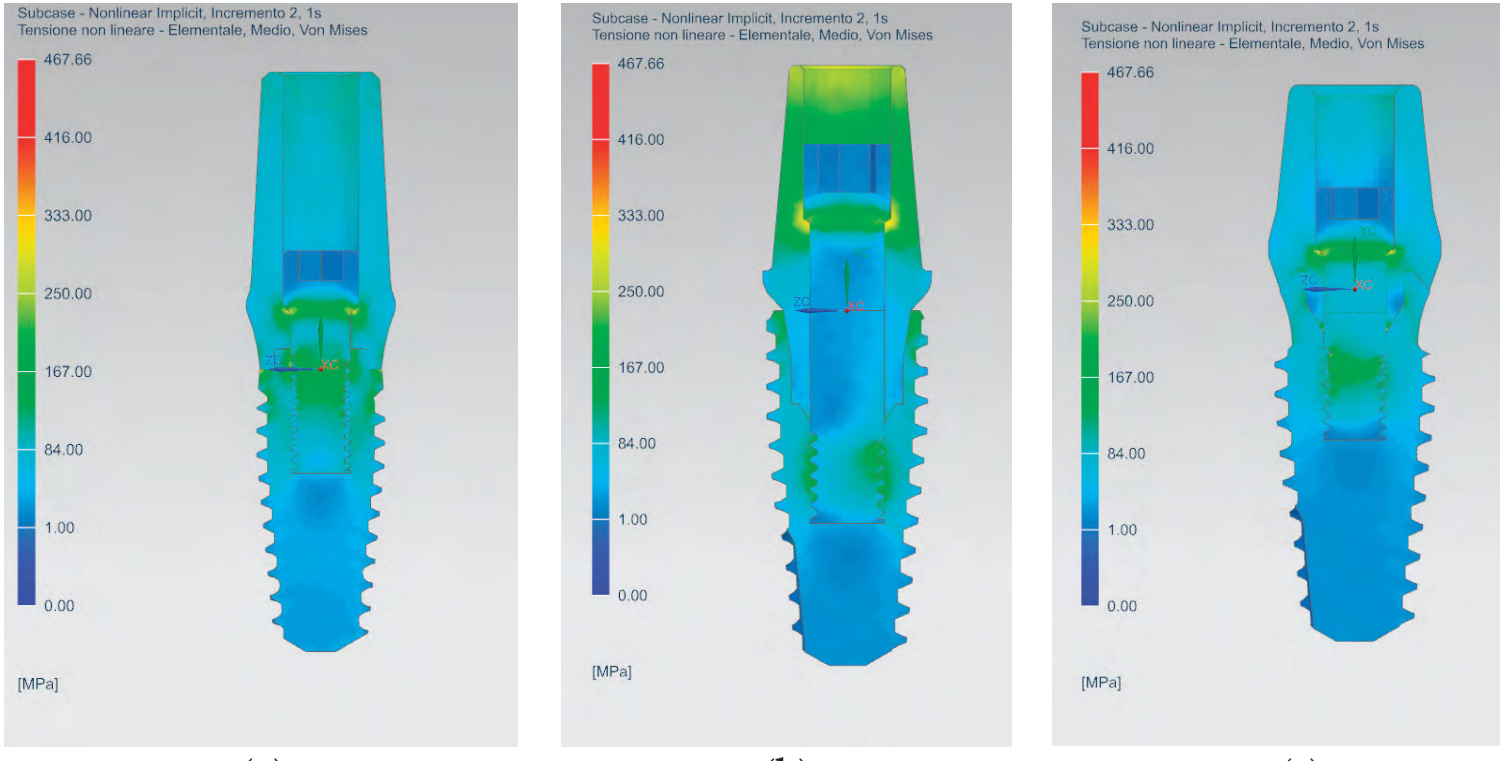

Figure 28.2 Equivalent Von Mises Stress results on the three prosthesis: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

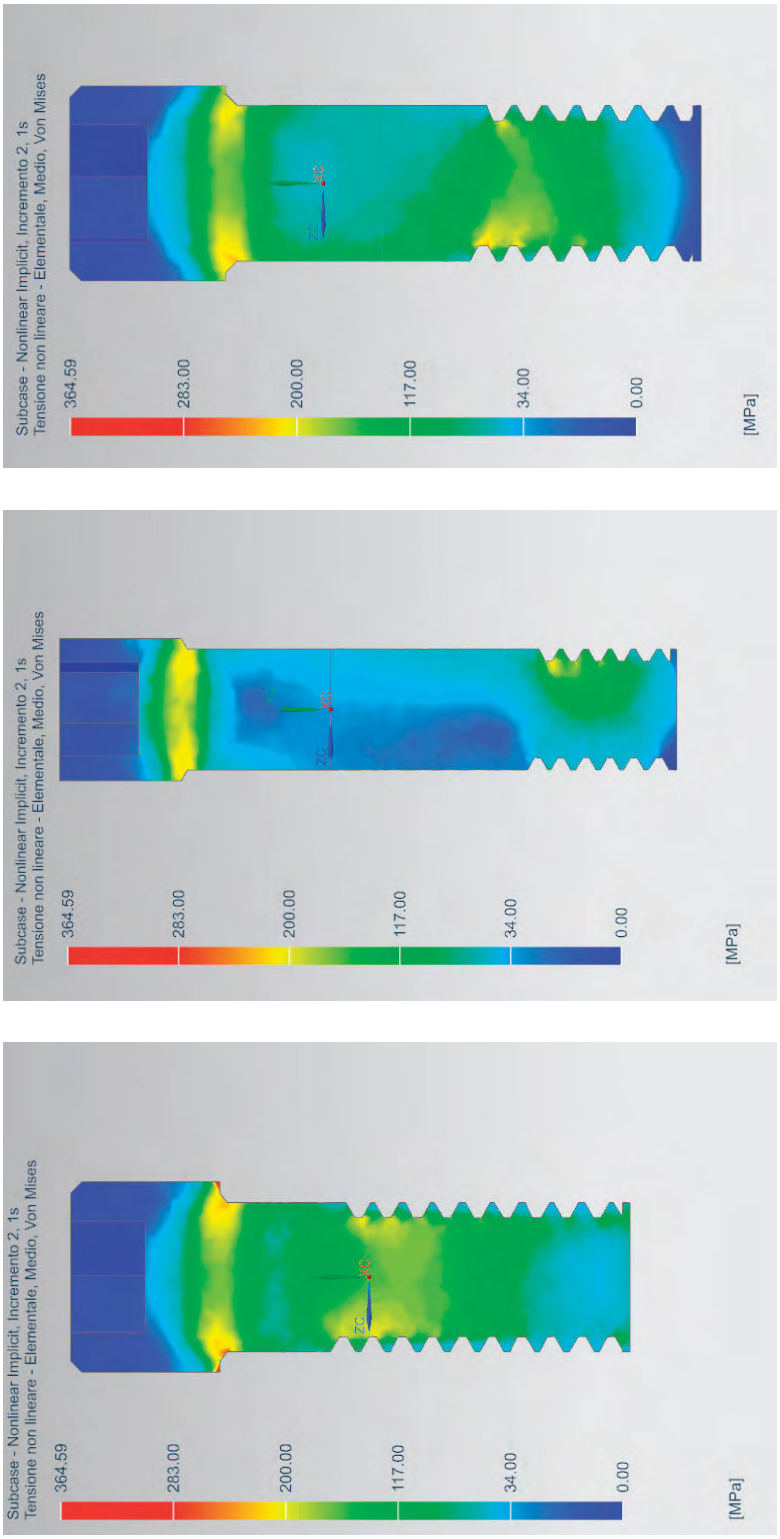

Figure 28.3 Equivalent Von Mises stress results on the internal screws: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

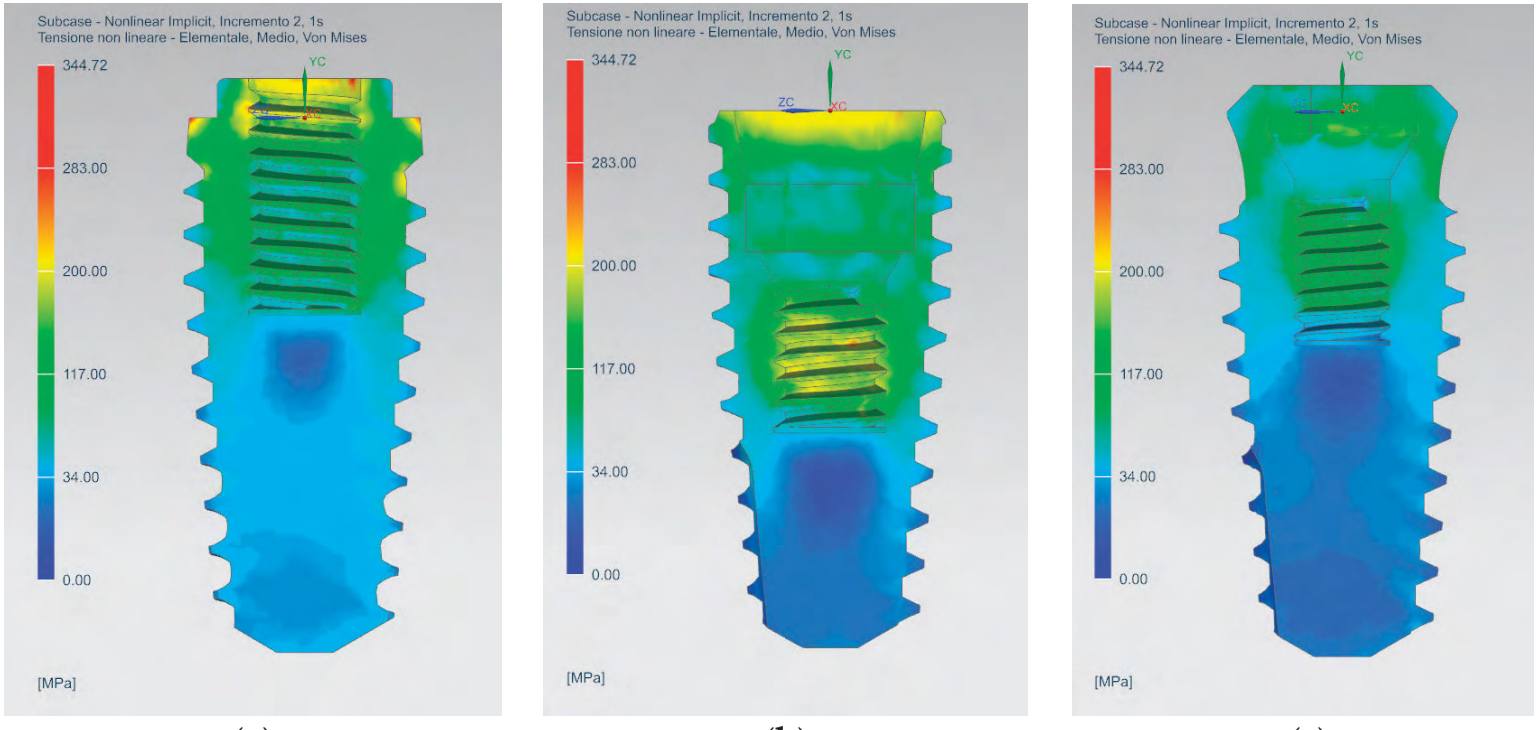

Figure 28.4 Equivalent Von Mises stress results on the implants: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

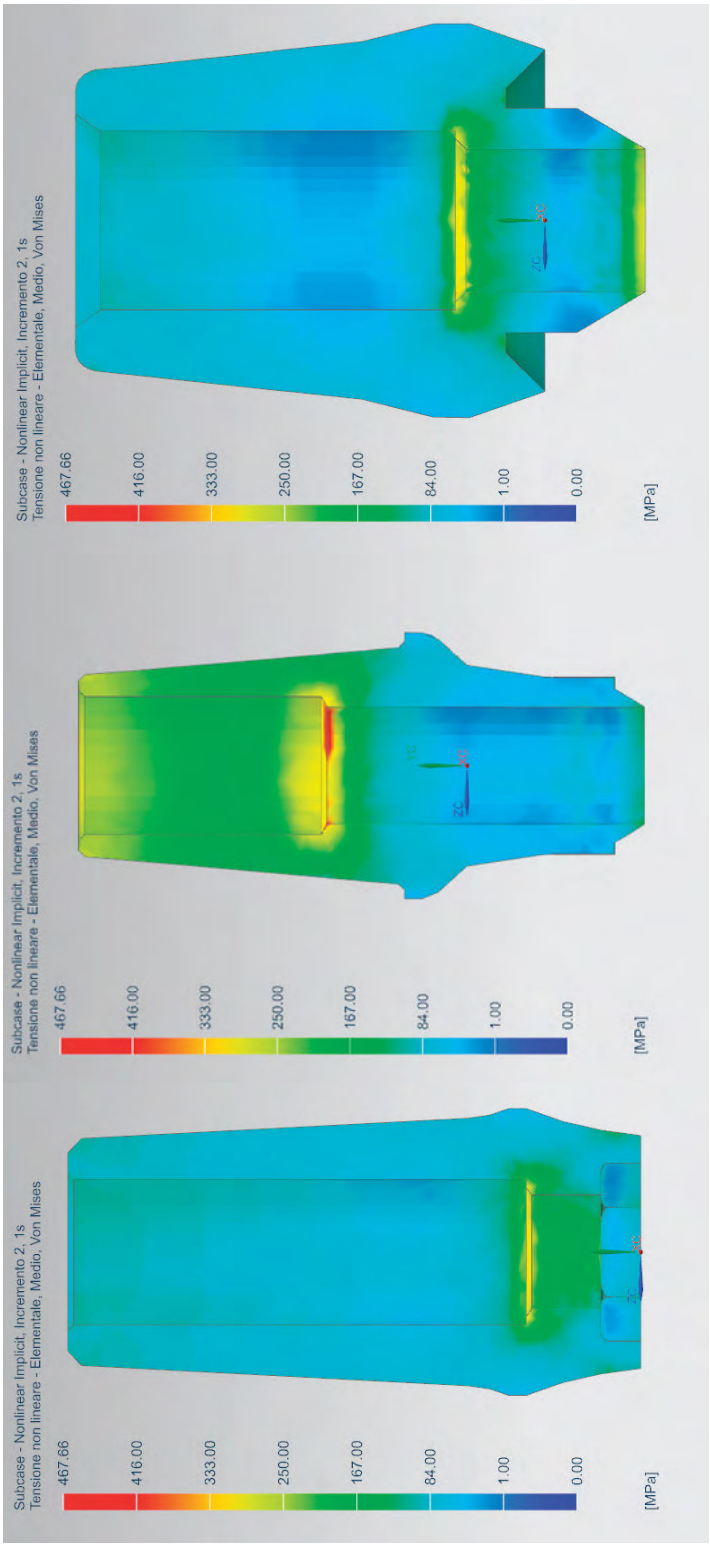

Figure 28.5 Equivalent Von Mises stress results on the abutments: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

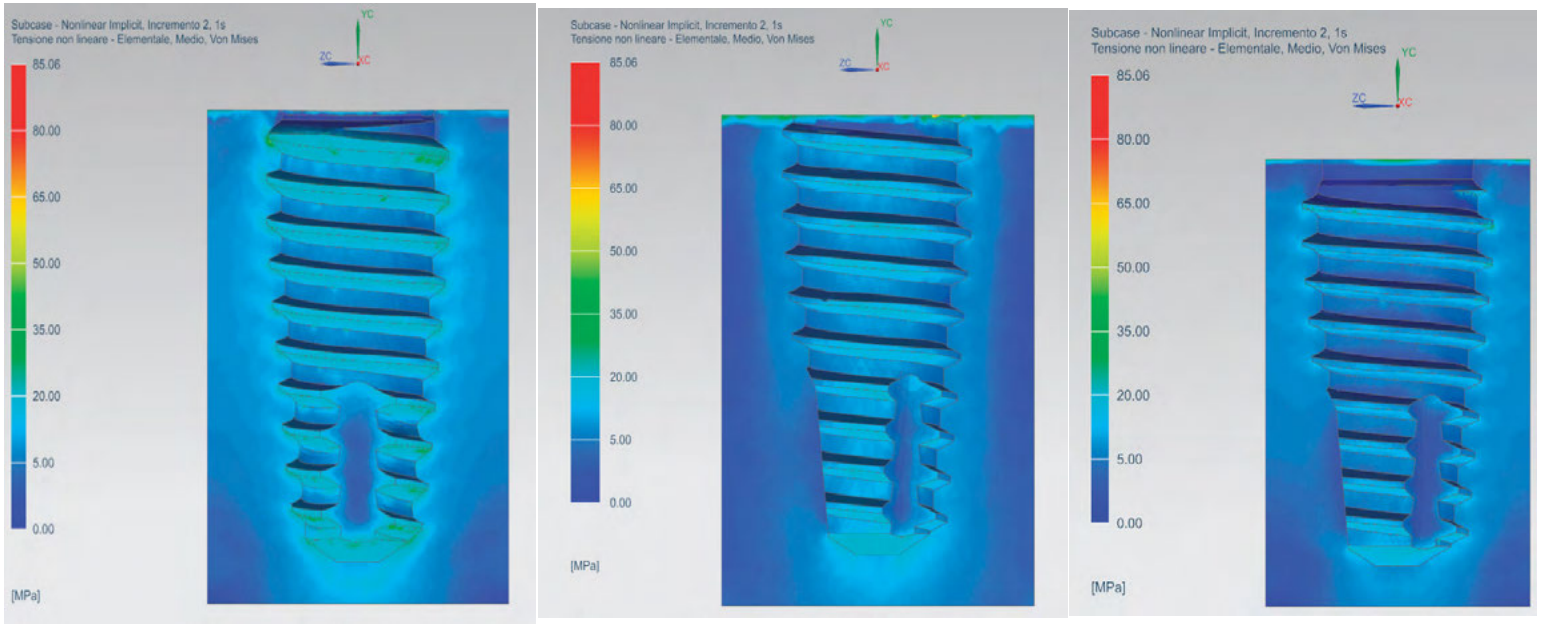

Figure 28.6 Stress distribution on the cancellous bone for: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

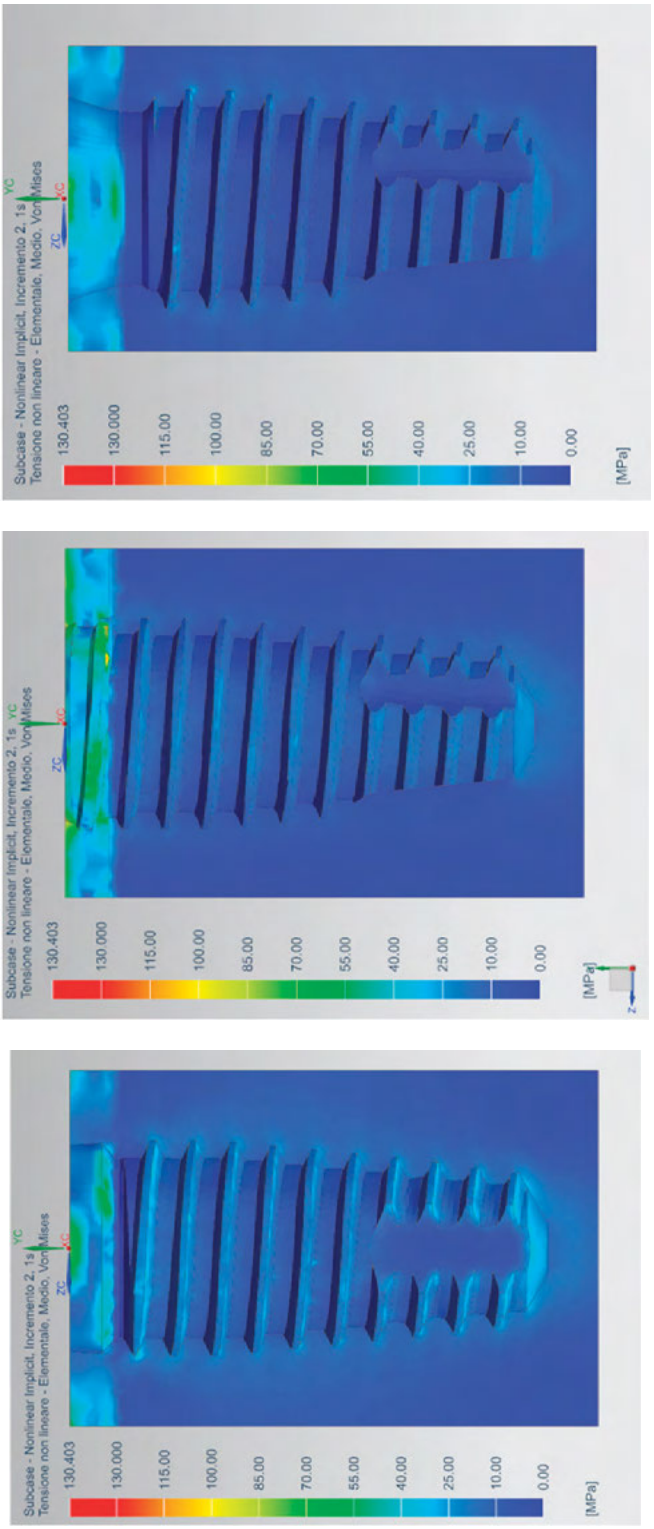

Figure 28.7 Stress distribution on the bone tissues (cancellous and cortical) for: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

28.3 Discussion

The finite element analysis is a useful aid for the assessment of stress rising in the bone due to the presence of prosthetic devices. It represents an easy way to investigate complex biomechanical systems instead of experimental techniques that are difficult to apply [14, 15]. To perform a reliable simulation, several fundamental parameters have to be taken into account, such as the bone tissues material model, the state of osseointegration of the implant, and the preload of the internal screw. Also, the reverse engineering procedure assumes a fundamental importance in order to establish the correct geometry and properly models the interaction between the different prosthesis components [16].

The mechanical behavior of the bone tissues is not easy to model due to the marked anisotropy and peculiar aspects depending on the individual biotype. Several authors [17–19] adopted, as a simplification, the linear elastic isotropic model in which the bone exhibits the same mechanical behavior regardless of the direction in which the load is applied. In that case, only a value of Young's modulus and Poisson's ratio is needed, and they can be easily retrieved from the literature or by simple mechanical tests. Other authors [20–22], in order to better represent the real mechanical behavior of the bone, adopted a linear elastic orthotropic model. In the present study, for the cancellous bone a linear elastic orthotropic model has been adopted while a transverse orthotropic model has been adopted for the cortical bone, i.e., in-plane properties (x- and y directions) are the same while the third direction differs from the other two.

The perfect osseointegration has been considered as a reasonable hypothesis [19, 23, 24]. Different biological parameters can affect the osseointegration, leading to a failure of the prosthesis [10]: Medical status of the patient, smoking, bone quality, bone age, operator experience, degree of surgical trauma, and bacterial contamination.

Several authors [16, 25, 26] have highlighted how the internal screw preload is of fundamental importance in order to prevent the loosening of the functional contact between the abutment and the implant, especially under repeated loads. Therefore, the fatigue behavior of the internal screw connection is still an open issue [27, 28]. In this study, only the maximum static load acting during the chewing cycle has been considered, hence further investigation under repeated and inclined load should be performed.

The geometry of the prosthesis is of fundamental importance and it can affect the way in which the prosthesis transfers the load to the bone [29]. The neck area and the first threads of the internal screw have the maximum stress, but the AnyOne® Internal retaining screw presents smaller and well confined stress areas compared to the other two geometries. This is due to the fact that the screw has a longer shank with a reduced threaded surface respect to the AnyOne® External and the AnyOne® OneStage internal screws, which conversely present a much-extended threaded surface.

The three dental implants shape adopt different configurations for the connection area with the abutment. The AnyOne® External has an external

hexagonal head, while the AnyOne® Internal and OneStage present respectively a hybrid conical-hexa and octa-conical internal connection. The external configuration has the highest stress value compared to the other two internal configurations. As reported by Ceruso et al. [30], the external hexagonal connection presents micro-movement, especially under lateral load, with consequent micro-gap at the abutment-implant interface that can lead to micro-leakage and bacterial infiltration. In addition, the external solution is not able to allow a good redistribution of the stress on the implant. On the other hand, the internal connections are able to withstand in a better way to the load, redistributing the stress homogeneously on the implant and reducing the micro-gap, especially under inclined load [31, 32].

The abutments are the most stressed components of the prosthetic device [33]. In this kind of component, the geometry and the shape have a great influence on the stress distribution. The AnyOne® Internal abutment presents the highest stress values followed by the AnyOne® External and AnyOne® OneStage. This behavior could be addressed to the presence of the screw seat near the loaded area of the abutment, that acts as a stress raiser due to the geometrical discontinuity. The AnyOne® External and AnyOne® OneStage are able to better distribute the load due to their unnotched shape. Several studies [34–36] have focused on the influence of the implant-abutment connection on the stress distribution in the peri-implant bone. In the present study, as observed by different authors [17, 37–39], the most stressed bone tissue is the cortical one. A possible reason could be the difference in the Young's modulus for the cortical and cancellous bones. The first has a value of about two order of magnitude greater than the latter, hence it is able to bear a greater stress. The implant design, the thread profile and the pitch distance have a remarkable effect on the contact area, hence on the stress distribution in peri-implant bone [38]. These properties are fundamental in order to guarantee the perfect osseointegration, transferring the correct amount of stress to avoid the bone reabsorption. The AnyOne® Internal prosthesis stresses in a minor way the bone given the fact that the most stressed region is confined near the threading. As noted by Lee et al. [40], the finer pitch allow an increase of the contact area and a reduction of the stress peak in the cancellous bone. The highest peak stress has been registered in the first thread of the cortical and cancellous bone [17, 18, 39, 41]. On the other hand, the AnyOne® External and AnyOne® OneStage prosthesis tend to stress a greater portion of the cancellous tissues. It is difficult to predict how forces are transmitted to the bone-implant interface, what happens to the implant and how the bone reacts by reshaping. First, the transmission of masticatory loads to osseointegrated implants is characterized by significant biomechanical differences with respect to natural teeth. The natural tooth is connected to the bone by the collagen fibers of the periodontal ligament which allow its intrusion up to 50–100 μm; instead the dental implant is in direct contact with the bone and the elasticity of the system depends on the elasticity of the bone. Secondly, we need to consider the biomechanical properties of bone tissue.

The bone tissue is characterized by:

- Anisotropy: The properties vary with the direction of the stress;
- Inhomogeneity: The properties vary from point to point within the fabric;
- Subjective specificity: Property values is different from one subject to another;
- Viscoelasticity: Mechanical properties depend on time; the deformation is increasing over time even at constant load;
- Functional adaptation: The biomechanical properties change in response to stresses. The functional adaptation of bone is characterized by the ability of bone cells to produce or reabsorb the mineral component of the bone matrix.

According to this theory of Frost [42], four levels of increasing bone tissue are distinguished:

1. Pathologic unload zone: If no force is applied to the bone, its mineralization is gradually lost and consequently its resistance.
2. Adaptation zone: If the bone is correctly stimulated, the right physiological remodeling is created which allows the maintenance of the bone itself.
3. Overload zones: If the applied force exceeds the area of adaptation, the bone tissue reacts by opposing the external stimulus with osteoblast activation and bone apposition.
4. Pathologic overload zone: If the load exceeds the physiological range the function of the osteoblasts can be inhibited, and therefore the osteoclastic function prevails. Consequently, the bone becomes weaker and in the case of dental implants the osseointegration is lost. Finally, when the elastic limit and the resistance of the tissue are exceeded, there is a bone fracture.

28.4 Materials and Methods

Finite elements analysis is a valid and important aid to assess the mechanical behavior of the prosthodontic devices. In particular, it is easy to predict possible bone overloads and failures that can occur on the prosthesis due to fractures. Despite finite elements analysis being a powerful tool, some fundamental parameters have to be taken into account to properly model the implants and deduce the correct results. Parameters such as model geometry, material properties, the loads, and the constrains, can severely affect the accuracy of the results. Not least, the model discretization operated by means of finite elements assumes a fundamental role in the precision of the results, therefore convergence test must always be performed.

In this comparative study, three commercial prosthesis devices from the same manufacturer (MEGAGEN) with different geometric characteristic were adopted: AnyOne® External, AnyOne® Internal and AnyOne® OneStage.

The simulation process undergoes through two main phases: The former involves the reverse engineering of the prosthesis, in which the stereolithography scan (STL file format) was converted into a three-dimensional CAD model and

the jaw bone tissue conditions were modelled; the latter the definition of the material properties, the discretization of the model, i.e., the creation of the mesh, and the application of the loads and of the constraints on the prosthesis and bone.

After the finite elements analysis has been performed, it is possible to post-process the data obtaining the equivalent Von Mises stress distribution on the entire model composed by the prosthesis and the surrounding bone.

28.4.1 Reverse Engineering

The reverse engineering of the three prostheses was performed in order to obtain a CAD file starting from the STL source file provided by the manufacturer. The STL file format is able to represent only the surfaces of the model, no information about the volume can be retrieved from it. As is possible to note in Fig. 28.1a, some important details are missing, like the external and internal threads, and some components are not completely represented. Therefore, it is necessary to model from scratch the missing parts, such as the internal screws, and retrieve the missing measurements from the real prosthesis. First, the STL files were automatically converted into a solid model adopting the 3D software SpaceClaim®, then the obtained model was modified in order to add the missing features. The measurements were acquired from the real prostheses adopting an electronic microscope with a resolution of 640 × 480 pixel and 5 × zoom (Fig. 28.8).

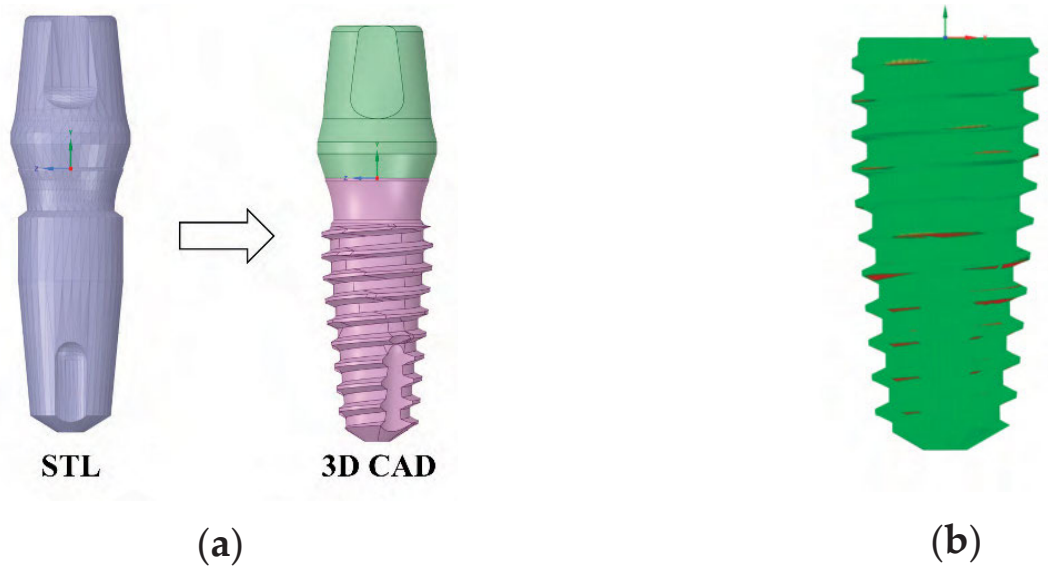

Figure 28.8 (a) STL to 3D CAD reverse engineering process; (b) in red, the deviation between the reconstructed geometry and the original STL file equals to 0.03 mm.

After the reverse engineering had been performed, it was necessary to verify the deviation of the reconstructed geometry compared to the original STL file.

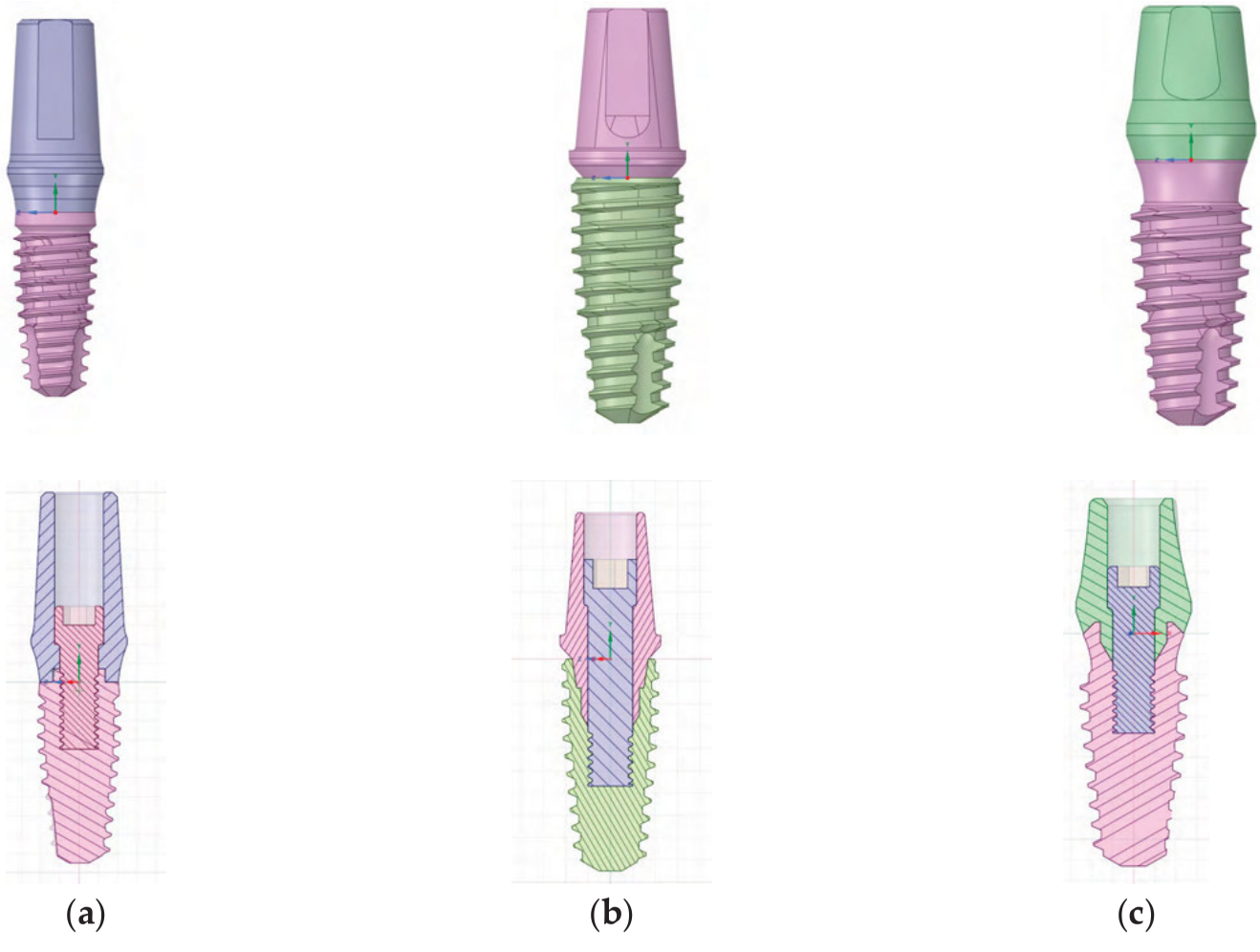

Figure 28.9 CAD reconstruction of: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

The reverse engineering procedure maintained a maximum deviation respect to the STL geometry file in the order of 1/100 of a millimeter (Fig. 28.8b). The reconstructed geometry and a sagittal section of the three prosthetic devices are reported in Fig. 28.9, in which it is possible to appreciate the different components of the prosthesis (implant, abutment and internal screw) and how they are paired.

28.4.2 FEM Analysis

In order to assess the stresses on the three different prosthetic devices, a series of 3D elastic finite elements analysis was carried out using Siemens NX Nastran® 1859. All the prosthetic devices were modelled with titanium alloy (Ti6Al4V), considered as a homogeneous isotropic material whose properties are reported in Table 28.1. The interaction between the implants and the bone tissues of the jaw were taken into account and modelled considering a small hexahedral volume of bone with cortical and cancellous bone tissues (Fig. 28.10).

Table 28.1 Material properties and E module sources accordingly to the literature data [1, 16, 27, 43]

Properties	Cortical bone	Cancellous bone	Ti6Al4V
Density [g/cm^3]	1.8	1.2	4.51
E_{xx} [GPa]	9.6	0.144	
E_{yy} [GPa]	9.6	0.099	105
E_{zz} [GPa]	17.8	0.344	
ν_{xx}	0.55	0.23	
ν_{yy}	0.30	0.11	0.37
ν_{zz}	0.30	0.13	
G_{xx} [GPa]	3.10	0.053	
G_{yy} [GPa]	3.51	0.063	38.32
G_{zz} [GPa]	3.51	0.045	

The cortical and cancellous bones exhibit a linear elastic orthotropic behavior, with the necessity of defining the Young's modulus, Poisson's ratio and Elastic tangential modulus in the three orthogonal directions (Table 28.1).

The solid geometries were meshed with solid 4-node CTETRA4 tetrahedral elements while the contact zones were modelled with BSURFS element type. This kind of finite element defines a contact region which may act as a source or target. In order to obtain reliable stress values maintaining concurrently a reasonable calculation time, a convergence test was performed and an element size of 0.2 mm was chosen with an acceptable error below 5% compared to the 0.1 mm element size (Table 28.2). The final mesh configuration (Fig. 28.11), in terms of number of nodes and elements, for the three different adopted geometries after the convergence test, are reported in Table 28.3.

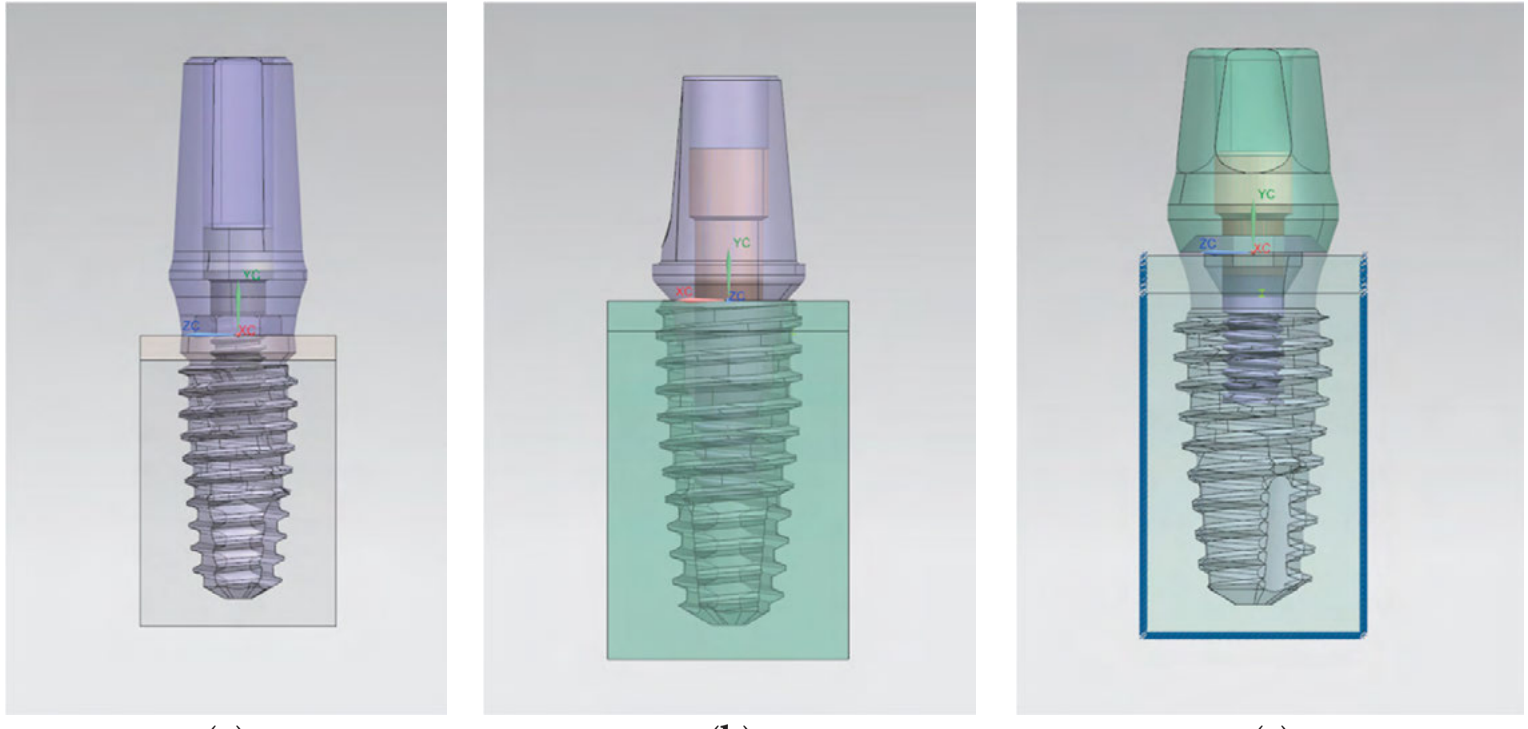

Figure 28.10 Reconstructed geometry and bone tissues (cortical and cancellous) for: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

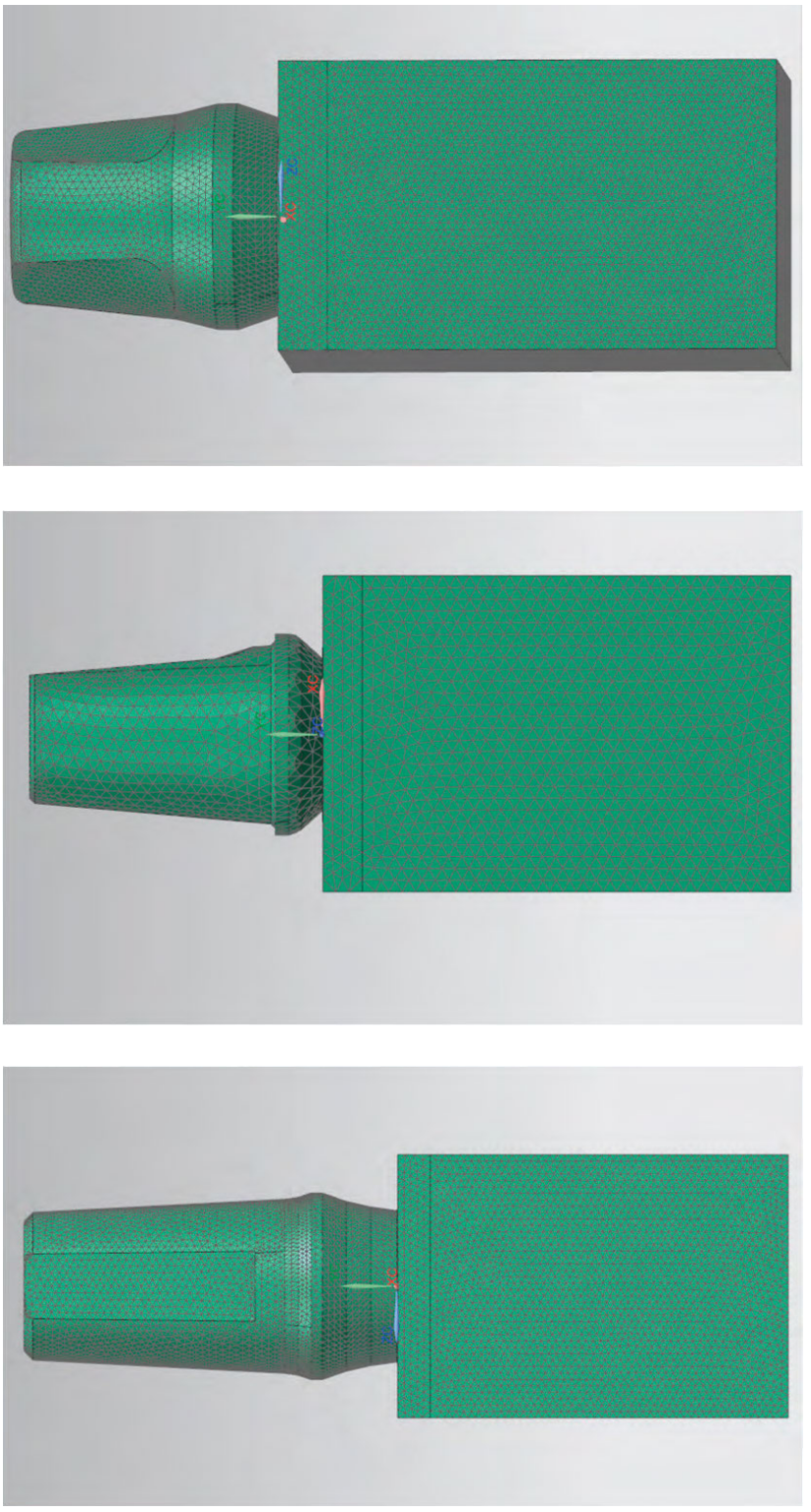

Figure 28.11 Mesh for the three prosthetic devices: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

Table 28.2 Results of the convergence test. Element size of 0.1 mm as taken as the reference

Element size [mm]	Maximum stress [MPa]	Error [%]
0.1	276.69	—
0.2	268.56	2.94
0.3	194.06	29.86
0.4	181.93	34.25
0.5	176.84	36.09

Table 28.3 Dimension of the models with 0.2 mm element size

	AnyOne® External	AnyOne® Internal	AnyOne® OneStage
Elements	498,819	443,946	508,105
Nodes	105,804	96,583	108,369

The hexahedral volumes of bone tissues were fixed at their lateral and lower faces and the bone-implant and bone-bone interfaces were modelled as bonded contacts in order to simulate the perfect osseointegration of the implant. The contact between the metal surfaces of the prostheses was modelled as frictional contact with a value of the frictional coefficient equals to 0.3.

The prosthodontic component surfaces were loaded with a distributed compressive axial force of 800 N along the Y direction in order to simulate the effects of the maximum masticatory load [43] (Fig. 28.12).

The internal screw was preloaded with a force of 875 N in order to simulate the tightening torque of 35 Ncm as suggested by the manufacturer. The value of the preload was estimated with the formula:

$$M = K \cdot D \cdot P,$$

where M is the tightening torque (expressed in Nmm), K is a global coefficient that takes into account the friction coefficient on the thread (in this case equal to 0.2), D is the diameter of the screw (expressed in mm) and P is the axial preload to apply to the screw (expressed in N).

28.5 Conclusions

Having clarified a whole series of biomechanical notions related to dental implants, and especially the influence of these on the peri-implant tissues, can lead to an improvement of the implant surfaces and even of their shape. Knowing the behavior of dental implants under masticatory load, and evaluating their effects with different angles, or prosthetic components, can lead to the realization of personalized rehabilitations for each patient and for every clinical need.

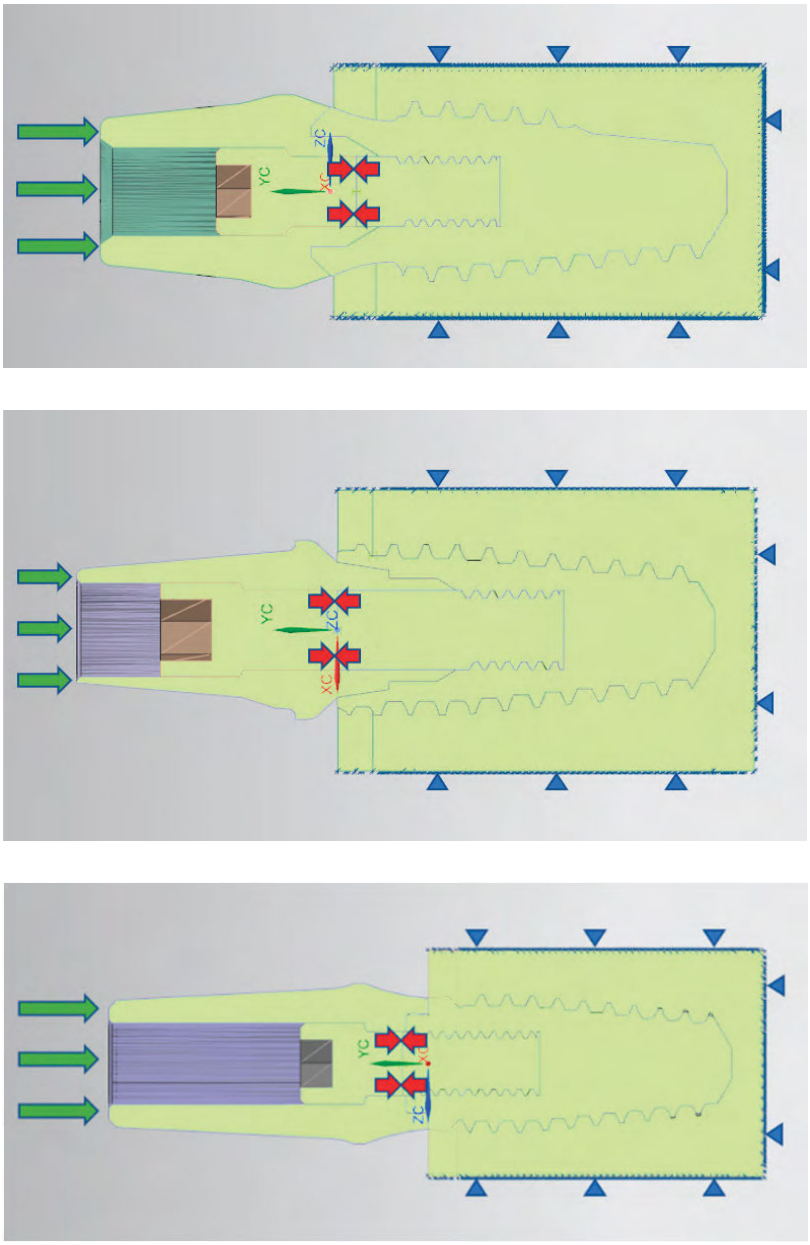

Figure 28.12 Loads and boundary condition on the prosthesis. The green arrows indicate the masticatory load, the red arrows indicate the internal screw preload, while the blue triangles indicate the constraints: (a) AnyOne® External; (b) AnyOne® Internal; (c) AnyOne® OneStage.

Disclosures and Conflict of Interest

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Chapter 29

Circulating Arsenic Is Associated with Long-Term Risk of Graft Failure in Kidney Transplant Recipients: A Prospective Cohort Study

Camilo G. Sotomayor, MD,[a] Dion Groothof,[a] Joppe J. Vodegel, MD,[a] Tomás A. Gacitúa, MD,[a] António W. Gomes-Neto, MD,[a] Maryse C. J. Osté,[a] Robert A. Pol, MD, PhD,[b] Catterina Ferreccio, PhD,[c] Stefan P. Berger, MD, PhD,[a] Guillermo Chong, PhD,[d] Riemer H. J. A. Slart, MD, PhD,[e] Ramón Rodrigo, PhD,[f] Gerjan J. Navis, MD, PhD,[a] Daan J. Touw, PhD,[g] and Stephan J. L. Bakker, MD, PhD[a]

[a]*Department of Internal Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands*
[b]*Division of Transplantation Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands*
[c]*Advanced Center for Chronic Diseases, Pontificia Universidad Católica de Chile, Santiago, Chile*
[d]*Department of Radiology, Clínica Alemana de Santiago, Universidad del Desarrollo, Santiago, Chile*
[e]*Department of Nuclear and Molecular Imaging, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands*
[f]*Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile*
[g]*Department of Pharmacy and Clinical Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands*

c.g.sotomayor.campos@umcg.nl

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29.1 Introduction

Arsenic is toxic to many organ systems, the kidney being the most sensitive target organ [1, 2]. Free radical mediated-oxidative damage is the cornerstone of arsenic-

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induced pathology [3]. Arsenic induces morphological alterations of mitochondria that lead to uncontrolled formation of free radicals [4], whilst it inhibits the production of glutathione that protects cells from oxidative damage, ultimately yielding irreversible cell damage [5, 6]. The kidney, being a major player in removal of arsenic from the system, is very much exposed to arsenic and therefore susceptible to arsenic-induced toxicity [7–10].

A large variety of arsenic compounds are known, divided into the elemental metal, inorganic, and organic compounds with a large variety of toxicity [1, 2, 11, 12]. While an extraordinary cause for arsenic intake has been described as hydroarsenicism—contamination of drinking water with arsenic in the US, Chile, and Taiwan—arsenic in food is an increasingly recognized pathway of environmental exposure. Thus, upon background regional differences, arsenic exposure substantially derives from rice consumption, as well as vegetables, fruits, and herbal tea [13–19]. Of note, however, seafood is thought to be a major route for arsenic intake, followed by alcohol consumption, with the latter mainly due to contaminated wine, therewith representing an evident public health threat [20, 21].

Basic and clinical evidence has linked arsenic exposure to nephrotoxicity, tubular necrosis, diffuse interstitial fibrosis, decline of kidney function, incident chronic kidney disease, and progress of native chronic kidney disease, among several other conditions such as hypercalciuria, albuminuria, and nephrocalcinosis [22–29]. Kidney transplant recipients (KTRs) are particularly vulnerability to the harmful effects of nephrotoxic agents. However, no study has been devoted to evaluating whether arsenic may be an otherwise overlooked modifiable risk factor in the post-kidney transplantation setting. The current study, therefore, aimed to identify independent environmental and system determinants of plasma arsenic levels and to evaluate the potential association of plasma arsenic levels with long-term risk of graft failure in a large cohort of well-characterized KTRs.

29.2 Methods

29.2.1 Design and Study Population

In this prospective cohort study, outpatient adult KTRs with a functioning graft ≥1 year, no alcohol or drug addiction, and without known systemic illnesses (i.e., malignancies, opportunistic infections) were invited to participate. The recruitment of patients took place at the University Medical Center Groningen between November 2008 and March 2011. In total, 817 KTRs were invited for the study, of whom 707 (87%) provided written informed consent to participate. All patients with missing plasma arsenic levels were excluded, resulting in 665 KTRs eligible for statistical analyses. Multiple imputations (n = 5) were used to account for missingness of data among variables other than data on circulating arsenic. The present study was approved by the Institutional Review

Board (METc 2008/186) and was conducted in accordance with the Declaration of Helsinki.

The primary outcome of this study was death-censored graft failure, defined as end-stage kidney disease requiring dialysis or re-transplantation. The continuous surveillance system of the outpatient clinic of our university hospital, in which patients visit the outpatient clinic with declining frequency in accordance with the American Transplantation Society Guidelines, ensured updated information on patient status [30]. General practitioners or referring nephrologists were contacted in case the status of a patient was unknown. Endpoints were recorded until September 2015. No patients were lost to follow-up.

All KTRs were transplanted at the University Medical Center Groningen following the establishment of standard antihypertensive and immunosuppressive therapies. Relevant characteristics including recipient age, gender, cardiovascular history, and transplant-related information were extracted from patient records. Dietary intake, clinical parameters, and laboratory measurements were extensively assessed at baseline.

29.2.2 Assessment of Dietary Intake

Dietary intake was assessed using a validated semi-quantitative food frequency questionnaire (FFQ) developed and updated at Wageningen University [31]. The questionnaire consisted of 177 food items to record intake during the last month, taking seasonal variations into account. For each item, the frequency was expressed in times per day, week, or month. The number of servings was recorded in natural units (e.g., slice of bread or apple) or household measures (e.g., cup or spoon). The FFQ was self-administered and then checked by a trained researcher on the day of visit to the outpatient clinic. Inconsistent answers were verified with the patients. The results of the FFQ were converted into total energy and nutrient intake per day by using the Dutch Food Composition Table of 2006 [32].

29.2.3 Clinical Parameters and Definitions

All measurements were performed during a morning visit to the outpatient clinic. Blood pressure was determined with a semi-automatic device (Dinamap 1846, Critikon, Tampa, FL, USA), measuring every minute for 15 min. The last three measurements were averaged, following a strict protocol as described previously [33]. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2), and body surface area (BSA) was estimated in meters squared (m^2) by using the universally adopted formula of DuBois and DuBois [34]. Diabetes was defined as use of antidiabetic medication, fasting plasma glucose ≥7.0 mmol/L, and/or HbA_{1C} higher than 6.5% [35]. Kidney function was assessed by means of estimated glomerular filtration rate (eGFR) according to the Chronic Kidney Disease Epidemiology Collaboration equation [36].

29.2.4 Laboratory Methods and Arsenic Measurement

Blood was drawn after a fasting period of 8–12 h, which included no medication intake. Serum high-sensitivity C-reactive protein (hs-CRP), HbA1C, triglycerides, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and total cholesterol were measured using routine laboratory methods. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). Serum cystatin C was determined using Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a modular analyzer (Roche Diagnostics, Mannheim, Germany). Class I and class II human leukocyte antigens (HLA) antibodies were assessed by ELISA (LATM20×5, One Lambda, Canoga Park, CA, USA) as described elsewhere [37]. According to a strict protocol, all participants were instructed to collect a 24 h urine sample the day before to their visit to the outpatient clinic. Total urinary protein concentration was determined using the Biuret reaction (MEGA AU 150, Merck Diagnostica, Darmstadt, Germany).

Arsenic plasma concentrations were assessed from EDTA plasma samples that were stored frozen at −80°C. Arsenic plasma concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS; Varian, Palo Alto, CA, USA) with a modified method for the measurement of low concentrations of heavy metals in plasma using a standard addition method. Standards were made by addition to blanc plasma known amounts of arsenic to obtain added concentrations of 0.500, 1.00, 2.00, 3.00, 4.00, and 5.00 μg/L. Control samples were made by spiking blanc plasma with known amounts of arsenic to obtain added concentrations of, respectively, 0.75 (low), 2.5 (medium), and 4.5 μg/L (high). Sample preparation consisted of diluting 100 μL sample with 1.0 mL dilution reagent. The dilution reagent contained 0.005% Triton X100, 0.005% EDTA, and 0.1 mg/L Yttrium as internal standard. Characteristics of this method are summarized in Table 29.1.

Table 29.1 Bias and precision of arsenic measurements

Arsenic concentration	*n*	μg/L	Bias (%)	Inter-assay coefficient	
				SD (μg/L)	CV (%)
Low	36	0.75	−13	0.26	40
Medium	36	2.5	−9.2	0.38	17
High	37	4.5	−6	0.48	11

Note: *n*, number of control samples; SD, standard differentiation; CV, coefficient of variation.

29.2.5 Follow-Up of Plasma Arsenic Levels in a Sample Population of the TransplantLines Cohort and Biobank Study

Additionally, to investigate plasma arsenic levels over time, we requested follow-up plasma samples (3 months, 6 months, 1 year, and 2 years post-kidney

transplantation) from 46 consecutive KTRs enrolled between February 2016 and May 2017 in the ongoing TransplantLines Prospective Cohort and Biobank Study [38]. Arsenic plasma concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, Varian 820-MS; Varian, Palo Alto, CA, USA) with a modified method for the measurement of low concentrations of heavy metals in plasma using a standard addition method, as described hereby in the preceding section.

29.2.6 Statistical Analyses

Data analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria). Continuous variables were summarized using mean (SD) for normally distributed data, whereas skewed distributed variables are given as median (IQR). Categorical variables were summarized as numbers (percentage). In all analyses, a two-sided $p < 0.05$ was considered significant. Linear regression analyses were performed to evaluate the association of baseline characteristics with arsenic concentrations, adjusted for (i) age and sex, and additionally (ii) eGFR. The assumption of homoscedasticity and normality of residual variance were verified, and a natural log-transformation was applied when appropriate. Std. β coefficients represent the difference (in SD) in arsenic per 1-SD increment in continuous characteristics or for categorical characteristics the difference (in SD) in arsenic compared to the implied reference group. In order to study, in an integrated manner, which baseline characteristics were independently associated with and were determinants of plasma arsenic, we performed forward selection of baseline characteristics according to preceding multivariable linear regression analyses (p for inclusion < 0.2), followed by stepwise backwards multivariable linear regression analyses (p for exclusion 0.05). Finally, we also performed a stepwise backwards multivariable linear regression with exclusion of eGFR in the initial model in order to isolate environmental determinants of plasma arsenic levels.

The prospective association of plasma arsenic with risk of graft failure during follow-up was examined incorporating time to event and accounting for death-censoring, by means of univariable and multivariable Cox proportional-hazards regression analyses with time-dependent covariates to calculate hazard ratios (HR) and 95% confidence intervals (CI). Schoenfeld residuals were calculated to assess whether proportionality assumptions were satisfied. Associations are shown with plasma arsenic as a continuous variable and according to tertiles of the plasma arsenic distribution. Following univariable analyses (model 1), we first performed multivariable adjustment for the most important environmental determinants of arsenic levels according to the results of our backwards linear regression analyses (model 2). To avoid overfitting, further models were performed with additive adjustments to model 2, defined as the primary multivariable model [39]. Thus, we performed additional adjustments for intake of fruits, vegetables, potato, rice, bread, and total energy intake (model 3); transplant characteristics (donor and recipient age, donor type, HLA mismatches, circulating

anti-HLA class I antibodies, circulating anti-HLA class II antibodies, transplant vintage, and immunosuppressive therapy; model 4); risk factors of graft failure (eGFR, hs-CRP, systolic blood pressure, total cholesterol, and triglycerides concentration; model 5); and primary renal disease and proteinuria in model 6.

The intra-individual coefficient of variation (CV) for plasma arsenic levels in KTRs of the TransplantLines Cohort and Biobank Study was calculated using the formula CV = (SD/mean) × 100, in which SD is the standard deviation and mean is the mean value for plasma arsenic concentrations as measured in follow-up samples taken at 3 months, 6 months, 1 year, and 2 years post transplantation. Next, box plots were used to illustrate medians (interquartile range) of plasma arsenic levels during follow-up visits. Finally, significance of potential change during follow-up visits was tested using the Kruskal Wallis test.

29.3 Results

29.3.1 Baseline Characteristics and Cross-Sectional Analyses

Mean (SD) age of the 665 KTRs was 53 (13) years, of whom 383 (58%) were male. Median (IQR) plasma arsenic concentration was 1.26 (1.04–2.04) μg/L. The baseline characteristics of the study participants along with the results of age- and sex- as well as eGFR-adjusted linear regression analyses are shown in Table 29.2. In stepwise backward multivariable linear regression analysis, fish consumption (β = 0.26; p < 0.001), eGFR (β = −0.11; p = 0.02), and proteinuria (std β = 0.18; p < 0.001) were identified as independent determinants of plasma arsenic concentrations (Table 29.2). If analyses were performed with eGFR excluded from the initial model, fish consumption (β = 0.27; p < 0.001) was identified as the only independent determinant of arsenic (Table 29.2).

29.3.2 Prospective Analyses

During a follow-up of 5 years, 72 (11%) patients developed graft failure. Chronic allograft dysfunction was the major cause of graft failure accountable for 50 (69%) of all graft failures. Other causes for graft failure included return of primary kidney disease (11%), infection (4%), acute rejection (4%), BK nephropathy (4%), vascular complications (3%), and others (4%). From low to high tertiles of the plasma arsenic distribution, 18, 25, and 29 patients developed graft failure, respectively. Prospective analyses of the association of plasma arsenic with death-censored graft failure are shown in Table 29.3. Multivariable-adjusted Cox proportional hazards models showed that plasma arsenic was directly associated with graft failure (HR 1.80; 95% CI 1.28–2.53, p = 0.001), independent of major environmental determinants of arsenic concentration, i.e., alcohol and fish consumption. In analyses with further adjustment for potential confounders, the association remained materially unchanged (Table 29.3). We did not find signs of a non-linear association between plasma arsenic levels and risk of death-censored graft failure (Supplementary Table 29.S1).

Table 29.2 Baseline characteristics of 665 kidney transplant recipients (KTRs) and their association with plasma arsenic

Baseline characteristics	Overall KTRs *n* = 665	†Plasma arsenic (ln), µg/L	‡Plasma arsenic (ln), µg/L	Backwards linear regression	§Backwards linear regression
		Std. β	Std. β	Std. β	Std. β
Plasma arsenic, µg/L, median (IQR)	1.26 (1.04–2.04)	—	—	—	—
Demographics and body composition					
Age, years, mean (SD)	53 (13)	—	—		
Sex (male), *n* (%)	383 (58)	—	—		
Diabetes mellitus, *n* (%)	160 (24)	−0.07 *	−0.07 *	~	~
Body surface area, m^2, mean (SD)	1.94 (0.22)	−0.02	−0.05		
Body mass index, kg/m^2, median (IQR)	26.0 (23.3–29.4)	−0.003	−0.02		
Waist circumference, cm, mean (SD)	99 (14)	0.003	−0.02		
Cardiovascular history and lifestyle					
History of cardiovascular disease, *n* (%)	325 (49)	−0.01	−0.01		
Heart rate, beats per minute, mean (SD)	69 (12)	0.01	0.02		
Systolic blood pressure, mmHg, mean (SD)	136 (17)	−0.04	−0.06 *	~	~
Use of antihypertensives, *n* (%)	586 (88)	0.001	−0.04		
Current or former smoker, *n* (%)	382 (57)	0.04	0.03		
Alcohol consumption > 10 g/d, *n* (%)	169 (25)	0.14 ***	0.14 ***	~	

(*Continued*)

Table 29.2 (*Continued*)

Baseline characteristics	Overall KTRs *n* = 665	†Plasma arsenic (ln), μg/L	‡Plasma arsenic (ln), μg/L	Backwards linear regression	§Backwards linear regression
		Std. β	Std. β	Std. β	Std. β
Dietary intake					
Bread, g/day, mean (SD)	133 (59)	−0.09 **	−0.08 *	~	~
Vegetables, g/day, median (IQR)	90 (50–118)	−0.03	−0.03		
Fruit, g/day, median (IQR)	123 (61–232)	−0.04	−0.04	~	~
Potato, g/day, median (IQR)	119 (72–161)	−0.11 ***	−0.11 **	~	~
Rice, g/day, median (IQR)	15 (4–32)	0.07 *	0.06 *	~	~
Fish, g/day, median (IQR)	11 (4–21)	0.32 ***	0.31 ***	0.26 ***	0.27 ***
Coffee, mg/day, median (IQR)	500 (250–625)	−0.001	0.01		
Tea, mg/day, median (IQR)	250 (54–375)	0.03	0.01 *	~	~
Laboratory measurements					
Albumin, g/L, mean (SD)	43 (3)	−0.05	−0.03		
Calcium, mmol/L, mean (SD)	2.40 (0.15)	−0.06 *	−0.04		
Phosphate, mmol/L, mean (SD)	0.97 (0.21)	0.09 **	0.03		
eGFR, mL/min/1.73 m^2, mean (SD)	53 (20)	−0.18 ***	–	−0.11 **	—
Proteinuria, *n* (%)	150 (23)	0.12 ***	0.09 **	0.18 ***	
Alkaline phosphatase, U/L, median (IQR)	67 (54–84)	0.02	0.02		

Baseline characteristics	Overall KTRs *n* = 665	†Plasma arsenic (ln), µg/L	‡Plasma arsenic (ln), µg/L	Backwards linear regression	§Backwards linear regression
		Std. β	Std. β	Std. β	Std. β
ASAT, U/L, median (IQR)	22 (18–27)	0.06 *	0.07 *	~	~
ALAT, U/L, median (IQR)	19 (14–25)	0.01	0.04		
Gamma-GT, U/L, median (IQR)	26 (18–41)	0.05 *	0.05		
Lipids					
Total cholesterol, mmol/L, mean (SD)	5.1 (1.1)	0.03	0.02		
HDL cholesterol, mmol/L, median (IQR)	1.3 (1.1–1.6)	0.04	0.08 *	~	~
LDL cholesterol, mmol/L, mean (SD)	3.0 (0.9)	0.02	0.01		
Triglycerides, mmol/L, median (IQR)	1.7 (1.2–2.3)	−0.01	−0.04		
Inflammation and oxidative stress					
Leukocyte count, per 10^9/L, mean (SD)	8.1 (2.6)	0.01	0.01		
hs-CRP, mg/L, median (IQR)	1.6 (0.7–4.5)	−0.01	−0.02		
Malondialdehyde, µmol/L, median (IQR)	2.5 (1.9–3.7)	−0.02	−0.01		
Primary kidney disease and kidney transplantation					
Primary kidney disease					
Glomerulosclerosis, *n* (%)	190 (29)	0.02	0.01		
Glomerulonephritis, *n* (%)	51 (8)	0.01	−0.01		

(*Continued*)

Table 29.2 (*Continued*)

Baseline characteristics	Overall KTRs *n* = 665	†Plasma arsenic (ln), μg/L	‡Plasma arsenic (ln), μg/L	Backwards linear regression	§Backwards linear regression
		Std. β	Std. β	Std. β	Std. β
Tubulointerstitial nephritis, *n* (%)	76 (11)	0.05	0.06		
Polycystic kidney disease, *n* (%)	136 (21)	−0.09	−0.07		
Kidney hypo/dysplasia, *n* (%)	29 (4)	0.02	0.02		
Renovascular disease, *n* (%)	38 (6)	−0.05	−0.04		
Diabetes, *n* (%)	32 (5)	0.04	0.04		
Other/miscellaneous, *n* (%)	113 (17)	0.02	0.02		
Donor type, living *n* (%)	229 (34)	−0.05	−0.04		
Donor age, years, median (IQR)	46 (31–54)	−0.01	−0.06 *	~	~
Transplant vintage, years, median (IQR)	5.5 (2.0–11.9)	−0.03	−0.01		
Immunosuppressive therapy					
Prednisolone dose, grams, median (IQR)	10.0 (7.5–10.0)	0.01	0.02		
Use of calcineurin inhibitor, *n* (%)	381 (57)	0.05	0.003		
Use of proliferation inhibitor, *n* (%)	553 (83)	−0.001	0.02		
Acute rejection treatment, *n* (%)	176 (26)	0.04	0.03		

*$p < 0.2$; **$p < 0.05$; ***$p < 0.01$. †Linear regression analysis; adjusted for age, sex, ‡and eGFR. Std β coefficients represent the difference (in SD) in arsenic per SD increment in continuous characteristics or for categorical characteristics the difference (in SD) in arsenic compared to the implied reference group. For inclusion and exclusion in stepwise backwards linear regression analyses *p* values were set at 0.2 and 0.05, respectively. §eGFR was removed from the initial model. ~ Excluded from the final models. *Abbreviations*: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HLA, human leukocyte antigens; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

Figure 29.1 illustrates the association between plasma arsenic concentration and risk of death-censored graft failure using Cox regression analyses with mean concentration of plasma arsenic as reference, adjusted for age, sex, fish intake and alcohol consumption, and in relation to the histogram of plasma arsenic distribution.

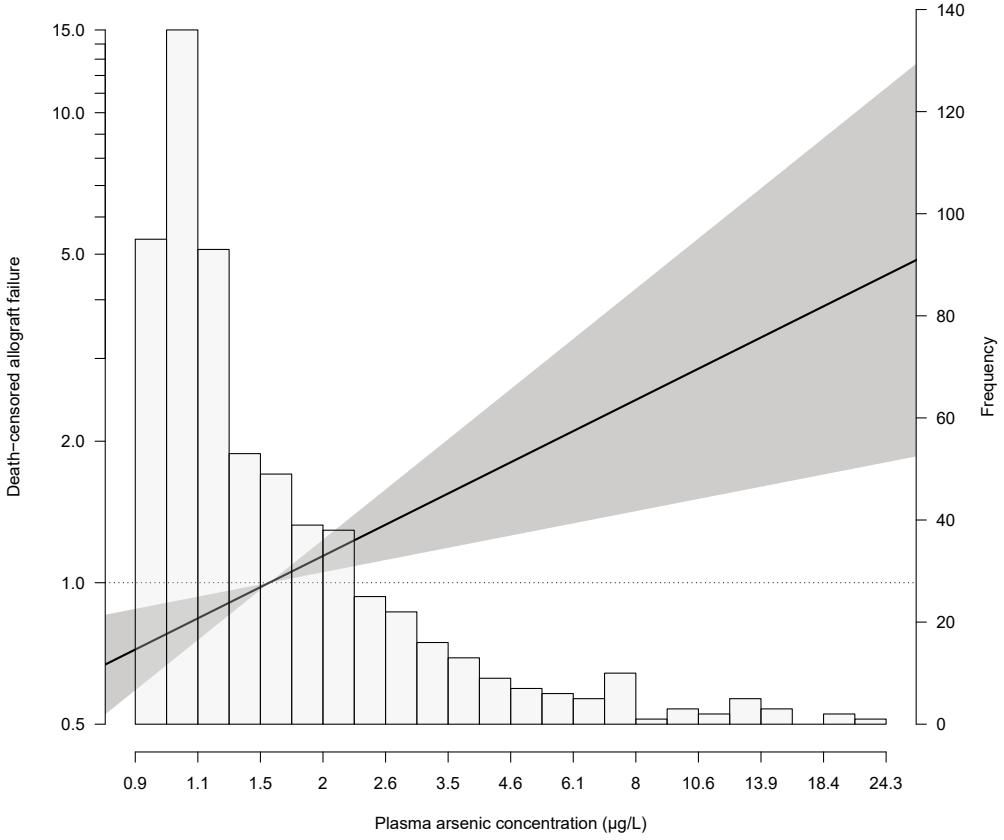

Figure 29.1 Association between plasma arsenic concentration and risk of death-censored graft failure using Cox regression analyses with mean concentration of plasma arsenic as reference, adjusted for age, sex, fish intake, and alcohol consumption, and in relation to the histogram of plasma arsenic distribution.

29.3.3 Follow-Up of Plasma Arsenic Levels in a Sample Population of the TransplantLines Cohort and Biobank Study

In supplementary Fig. 29.S1, we show box plots with medians (IQR) of plasma arsenic concentration of 46 KTRs (mean age 52±14 years old, eGFR 43±28 mL/min/1.72 m^2) from the TransplantLines Prospective Cohort and Biobank Study, at different follow-up visits post-kidney transplantation. Median (interquartile range) plasma arsenic concentrations were 1.61 (1.51–1.99), 1.64 (1.52–2.05), 1.64 (1.43–1.94), and 1.59 (1.46–2.26) µg/L at 3 months, 6 months,

1 year, and 2 years post-kidney transplantation, respectively. Median (interquartile range) intra-individual coefficient of variation was 12.2% (6.7–28.7%), and we did not find signs of a significant change in plasma arsenic levels over time ($p = 0.64$).

Table 29.3 Prospective analyses of the association of plasma arsenic with death-censored graft failure in 665 kidney transplant recipients

	Plasma arsenic				
	Tertile 1	Tertile 2	Tertile 3	Continuous (ln)	
	Ref.	HR (95% CI)	HR (95% CI)	HR (95% CI)	*p*
n events	18	25	29	72	
Model 1	1.00	1.41 (0.77–2.59)	1.69 (0.94–3.04)	1.47 (1.08–2.01)	0.02
Model 2	1.00	1.58 (0.86–2.92)	2.12 (1.14–3.95)	1.80 (1.28–2.53)	0.001
Model 3	1.00	1.55 (0.84–2.87)	2.05 (1.10–3.82)	1.74 (1.24–2.45)	0.001
Model 4	1.00	1.40 (0.75–2.61)	2.00 (1.06–3.77)	1.90 (1.32–2.73)	0.001
Model 5	1.00	1.32 (0.71–2.45)	1.76 (0.93–3.32)	1.56 (1.10–2.23)	0.01
Model 6	1.00	1.29 (0.70–2.40)	1.84 (0.99–3.42)	1.53 (1.09–2.14)	0.01

Cox proportional-hazards regression analyses were performed to assess the association of plasma arsenic with risk of death-censored graft failure (number of events = 72). Associations are shown with plasma arsenic concentration as a continuous variable and according to tertiles of the plasma arsenic distribution (tertile 1: ≤1.1 μg/L; tertile 2: 1.1-1.67 μg/L; tertile 3: ≥1.67 μg/L). Model 1 is univariable. Multivariable model 2 was adjusted for fish intake and alcohol consumption. Subsequently, additive adjustment was performed for intake of fruits, vegetables, potato, rice, bread, and total energy intake (model 3); donor and recipient age, donor type, human leukocyte antigen mismatches (HLA), circu, lating anti-HLA class I antibodies, circulating anti-HLA class II antibodies, transplant vintage, and immunosuppressive therapy (model 4); eGFR, high-sensitivity C-reactive protein, systolic blood pressure, total cholesterol, and triglyceride concentration (model 5); primary kidney disease and proteinuria (model 6).

29.4 Discussion

In these analyses of 665 well-characterized individuals from a Dutch cohort of KTRs, we identified fish consumption as the major environmental determinant of plasma arsenic levels. Prospective analyses showed that higher plasma arsenic levels are associated with increased long-term risk of graft failure, independent of donor and recipient characteristics, immunosuppressive therapy, eGFR, and proteinuria. These data pose arsenic as a potentially modifiable risk factor for late graft failure in KTRs, emphasizing the need for specific recommendations regarding arsenic exposure, as well as patient monitoring and management of arsenic-induced kidney injury, particularly in populations highly susceptible to nephrotoxic agents such as KTRs.

Being the major organ involved in arsenic clearance, the kidney is highly susceptible and the most sensitive target organ to arsenic exposure [1, 2, 9, 10].

Arsenic-induced oxidative stress has been suggested to be the cornerstone of pathological mechanisms leading to kidney injury and development of chronic kidney disease [3, 40]. On the one hand, decreased antioxidant capacity has been shown in individuals exposed to arsenic [41], wherein depletion of glutathione has been consistently described [5, 42, 43]. Of note, by protecting cells from oxidative damage, inhibition of glutathione production and subsequent glutathione depletion ultimately reverberates into increased vulnerability of cells to arsenic damage. On the other hand, it has been shown that arsenic induces morphological alterations of mitochondrial integrity that lead to uncontrolled free radical formation [4], which further feeds the circle of oxidative challenge and tissue injury. Indeed, basic and clinical evidence has linked arsenic exposure to nephrotoxicity, tubular necrosis, diffuse interstitial fibrosis, decline of kidney function, incident chronic kidney disease, and progress of native chronic kidney disease, amongst other conditions such as hypercalciuria, albuminuria, and nephrocalcinosis [22–29]. Subsequently, diminished kidney clearance of arsenic and enhanced production of reactive oxygen species longitudinally contribute to perpetuate tissue insult and progression of chronic kidney disease [22, 23]. Previous studies have also shown an association between arsenic and hypertension and type 2 diabetes mellitus, both suggesting additional mechanisms for secondary kidney damage [44, 45]. Ecological studies from the United States, Chile, and Taiwan have shown that arsenic exposure is associated with increased mortality from kidney disease [13–15, 22, 26, 28, 46–49]. KTRs are particularly vulnerable to harmful effects of nephrotoxic agents. End-stage kidney disease and maintenance immunosuppressive therapy are constant sources of oxidative challenge for the graft tissue, which shortens the capacity of oxidative stress defenses against additional environmental hazards. To our knowledge, the current study is the first to provide evidence of an independent prospective association between circulating arsenic levels and risk of late kidney graft failure.

Further supportive evidence for the key role of oxidative stress in arsenic-induced pathogenic mechanisms—and suggestive of potential management alternatives—was provided by the observation that co-administration of ascorbic acid and α-tocopherol to arsenic-exposed rats led to a reduction in the levels of lipid peroxidation, protein carbonyls, and hydrogen peroxide along with increased levels of reduced glutathione, ascorbic acid, and α-tocopherol. Investigation aimed to evaluating whether ascorbic acid and α-tocopherol supplementation may improve arsenic-induced altered microsomal functions in the kidney is warranted [50].

An increasing body of evidence supports that the kidney is a primary site of arsenic uptake and accumulation. Recently, X-ray fluorescence spectrometry allowed detection of arsenic accumulation, specifically at level of the kidney cortex [51]. X-ray fluorescence spectrometry may provide comprehensive information of bioaccumulation for biomedical and toxicological research by allowing direct measurement of the distribution of arsenic at tissue, cellular, and subcellular level. Next, X-ray absorption spectroscopy has been shown to allow *in vivo*

assessment of whole-body distribution, which is key information for the development of chelation therapies [52]. Future studies using these analytical methods may provide essential research data to understand the sequence of specific mechanisms of nephrotoxicity and deepen the understanding of the association between long-term arsenic exposure and kidney damage [51].

The current study is etiological in nature, which needs to be separated from prediction research [53]. Whereas the latter is a distinct field of epidemiologic research aimed at predicting the risk of an outcome according to a model of statistically significant predictors, which not necessarily represents causal associations, etiological studies aim to understand a certain pathway of a disease in an attempt to prevent its onset or progression [53]. Taken together, our findings and the aforementioned studies may support an etiological role of arsenic in pathways of disease that contribute to increased risk of death-censored graft failure.

Data on the average diet-derived arsenic exposure in The Netherlands are scarce. One study reported an estimated median (range) exposure of 37.8 (20.6–70.1) μg/day [54]. This was corroborated by a more recent study of Hoogenboom et al. stating that the average diet-derived arsenic exposure is <50 μg/day. In agreement with our findings, higher intake of arsenic most frequently originates from higher fish consumption [55]. A monitoring program from the Dutch Agriculture Advisory Committee (LAC), conducted in the 1980s, demonstrated that levels of arsenic in fish landed in The Netherlands varied between 0.8 and 6.8 mg/kg wet weight, showing a slight decreasing trend over time. Likewise, the arsenic levels in shrimps decreased from 4.3 to 1.3 mg/kg wet weight during that period (LAC program, 1991, in reference [41]). However, more recent data regarding arsenic-contaminated fish landed in The Netherlands are lacking and needed to evaluate strategies aiming to reduce the dietary consumption of arsenic by the population. Next, although in The Netherlands, naturally occurring arsenic concentrations in drinking water are usually below the concentrations required by the European drinking water standard (<10 μg/L in all countries, except Denmark, where it is <5 μg/L), health risks cannot be excluded at this level, and it has been recommended to optimize water supply to arsenic levels <1 μg/L [56, 57].

The current study was performed in a large cohort of extensively phenotyped KTRs, allowing us to control our main findings for several potential confounders, including donor and recipient characteristics, immunosuppressive therapy, proteinuria, and eGFR. Moreover, patients were monitored for an extensive period and patient status was updated without losses to follow-up, allowing the study of the long-term association of arsenic with graft failure. Despite considerable improvement of short-term graft survival during last decades, improvement of long-term outcomes continues to lag behind, emphasizing that future advances in the field of kidney transplantation are expected from the amelioration of long-term graft attrition [58]. Systematic description of modifiable risk factors is key to promote preventive strategies particularly addressed for this population of solid organ patients.

Our study derived from a single university center from the northern part of The Netherlands, which calls for prudence to extrapolate our results to different populations regarding potential environmental arsenic contamination and exposure. Additionally, the observational design of the current study does not allow hard conclusions on causality, nor could the potentiality of reversed causation or residual confounding be eliminated, despite the substantial number of potential confounders for which we adjusted. Furthermore, the technique used in the current study does not allow different species of arsenic to be distinguished, while arsenic species have major varieties in toxicity [1–4, 11, 12]. Elemental arsenic is nontoxic as the metal is insoluble in bodily fluids, and inorganic species of arsenic, e.g., arsenite and arsenate, are especially toxic to humans. Organic species vary in toxicity; the most common species, monomethylarsonic acid and dimethylarsinic acid, are less toxic compared to inorganic species, and arsenobetaine and arsenosugars have a very low toxicity [1, 5, 9, 11, 59–61]. Further studies utilizing techniques with the ability to distinguish between the different species of arsenic, e.g., high-performance liquid chromatography–inductively coupled plasma-mass spectrometry, could provide more information on the impact of the different species on graft failure in KTRs. A further limitation is that adjustment for immunological factors as potential confounders of the association was limited to adjustment for HLA matching, circulating anti-HLA class I antibodies, and circulating anti-HLA class II antibodies, since we had no data on donor-specific anti-HLA antibodies and biopsy findings. Finally, it should be acknowledged that graft failure can be the consequence of multiple, heterogenous causes. Unfortunately, in our study the numbers of cause-specific cases of death-censored graft failure was too small to allow for meaningful separate analyses [62]. Larger studies are warranted to comprehensively evaluate the association of plasma arsenic with different causes of death-censored graft failure. It should be noticed, however, that this study is the first to indicate a prospective association of arsenic with the hard endpoint graft failure, thus holding a plea for future studies which to only investigate arsenic plasma concentrations, but also take into account concentrations of arsenic in drinking water, and not only in KTRs to investigate associations with death-censored graft failure, but also in other populations, such as patients with diabetes and the general population.

29.5 Conclusions

In conclusion, the current study shows for the first time that circulating arsenic levels are independently associated with higher risk of late kidney graft failure, emphasizing the need for specific recommendations regarding arsenic exposure, as well as patient monitoring and management of chronic arsenic-induced kidney damage. Our findings point towards arsenic as an otherwise overlooked modifiable risk factor for adverse long-term kidney outcomes, especially in populations of vulnerability to oxidative stress challenge, e.g., KTRs. Further

studies are warranted to confirm our results and investigate the longitudinal association between arsenic exposure and graft failure in KTRs from populations with different dietary and environmental exposure.

Abbreviations

ALAT: alanine aminotransferase
ASAT: aspartate aminotransferase
BMI: body mass index
BSA: body surface area
CV: coefficient of variation
eGFR: estimated glomerular filtration rate
FFQ: food frequency questionnaire
HDL: high-density lipoprotein
HLA: human leukocyte antigens
hs-CRP: high-sensitivity C-reactive protein
ICP-MS: inductively coupled plasma mass spectrometry
KTRs: kidney transplant recipients
LDL: low-density lipoprotein
SD: standard differentiation

Supplementary Materials

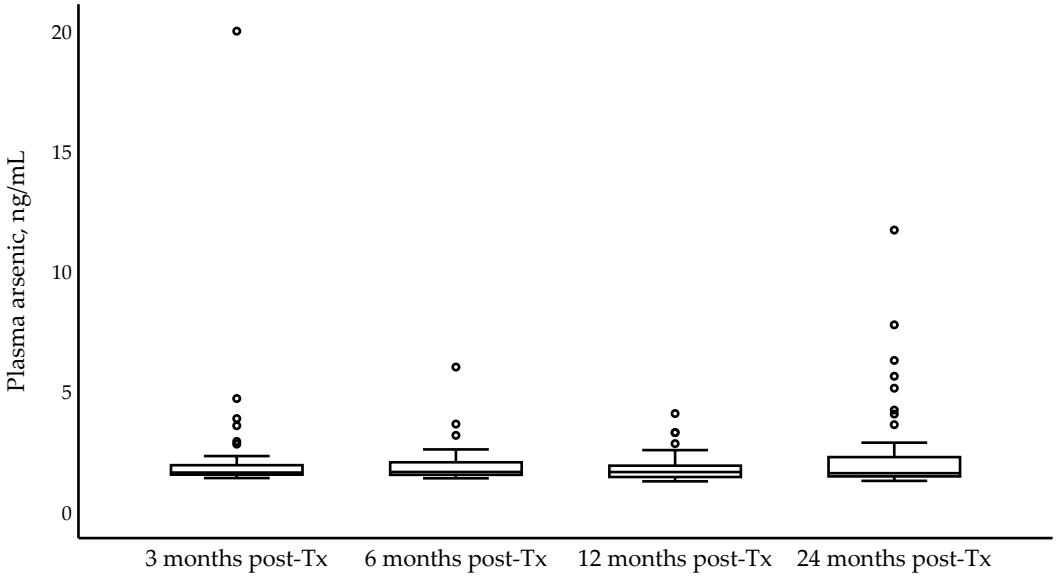

Figure 29.S1 Plasma arsenic concentration of 46 kidney transplant recipients from the TransplantLines Prospective Cohort and Biobank Study [38], at different follow-up visits after transplantation. Box plots show medians (interquartile range). Significance of potential change during follow-up visits was tested using the Kruskal Wallis test, which indicated no significant change over time ($p = 0.64$).

Table 29.S1 Verification of linearity of the association between plasma arsenic and risk of death-censored graft failure.

	HR (95% CI)	*p*	BIC	$p_{comparison}$*
Model 1a				
Log_2 arsenic	1.31 (1.05–1.62)	0.015	914.03	
Model 1b				
Log_2 arsenic	1.46 (0.82–2.61)	0.20	918.14	Model 1b vs. model 1a: 0.69
Log_2 arsenic^2	0.97 (0.82–1.14)	0.69		
Model 1c				
Log_2 arsenic	1.18 (0.42–3.36)	0.76	922.20	Model 1c vs. model 1a: 0.83
Log_2 arsenic^2	1.15 (0.55–2.41)	0.71		
Log_2 arsenic^3	0.97 (0.85–1.11)	0.64		
Model 2a				
Log_2 arsenic	1.36 (1.10–1.69)	0.0053	915.82	
Model 2b				
Log_2 arsenic	1.56 (0.86–2.81)	0.14	919.87	Model 2b vs. model 2a: 0.63
Log_2 arsenic^2	0.96 (0.81–1.14)	0.64		
Model 2c				
Log_2 arsenic	1.29 (0.45–3.76)	0.64	923.98	Model 2c vs. model 2a: 0.82
Log_2 arsenic^2	1.12 (0.53–2.37)	0.77		
Log_2 arsenic^3	0.97 (0.85–1.11)	0.69		
Model 3a				
Log_2 arsenic	1.52 (1.20–1.92)	0.0005	916.98	
Model 3b				
Log_2 arsenic	1.86 (1.00–3.48)	0.050	920.76	Model 3b vs. model 3a: 0.48
Log_2 arsenic^2	0.94 (0.79–1.12)	0.50		
Model 3c				
Log_2 arsenic	1.83 (0.61–5.46)	0.28	925.04	Model 3c vs. model 3a: 0.78
Log_2 arsenic^2	0.96 (0.46–2.00)	0.90		
Log_2 arsenic^3	1.00 (0.88–1.14)	0.97		

*p values are for the comparison with the referent model based on a likelihood ratio test. Model 1: crude model. Model 2: adjusted for age and sex. Model 3: model 2 + fish consumption and alcohol consumption. BIC, Bayesian information criterion. HR, hazard ratio.

Disclosures and Conflict of Interest

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Chapter 30

Successful Aging and Chronic Osteoarthritis

Ray Marks, EdD

Department of Health and Behavior Studies, Columbia University, Teachers College, New York, USA

rm226@columbia.edu

Keywords: Alzheimer's disease, chronic osteoarthritis, diabetes, elimination, influenza, intervention, mastectomy, Mediterranean diet, osteoarthritis, palliation, pneumonia, quality of life, rehabilitation, remediation, self-efficacy, self-management, social support, successful aging

30.1 Introduction

In the 16th century, it was reported that the explorer Ponce de Leon marched in search of the fountain of youth, only to discover death. Intruding into Indian territory located in Florida, he was killed by an arrow at the age of 47.

However, due to declines in infant mortality rates and better health care in general, the population of older Americans, as well as those in most other nations, is rapidly increasing, even though no fountain of youth has been uncovered. Indeed, the longer life sought by de Leon is being experienced by millions of older persons in the United States (US) and worldwide. In this regard, current data in the US show many older adults not only living into their second 50 years, but that some have entered their 'third' 50-year life stage. By 2050, the US census bureau predicts the number of centenarians in this country alone could exceed four million [1], even if longevity is clearly being impacted negatively by obesity, violence, and opioid epidemics, among others.

While the aging process seems inevitable though regardless of longevity improvements, the question arises as to whether this programmed phase of

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growth to maturity, must inevitably lead at some point to a functional decline. This present overview focuses on the concept of 'successful aging' as an idea that can be attained, if one can potentially impact or slow this genetically programmed process of death and debility, and if this is indeed a desirable process. It explores what the term means precisely, if this can be achieved readily, and how clinicians may help aging adults to adapt to age-associated changes and to achieve less severe or fewer age-associated deficits or levels of disability than would otherwise occur without intervention, in general, and in the context of the most prevalent joint disease among older people, known as osteoarthritis. Since the concept of optimum health for all is an acceptable current public health goal, the idea that people with health conditions can still age 'successfully' must be considered. Moreover, if the term only applies to those who are disease free, very few elders will qualify, and it will be hard to explain how some disabled elders would classify their lives as successful or highly successful, regardless of age, which is not uncommon, and vice versa. In this respect, the key behaviors individual's themselves can participate in or avoid in order to obtain a more successful, rather than a less 'successful aging' process and outcome, regardless of health status, such as the participation in appropriate levels of daily physical activity, are also highlighted. Taken as a whole, this present overview, while clearly not a meta analysis, may still be of potential relevance to older adults, as well as their families, and caregivers, or anyone working or living among the increasing numbers of older adults, especially those who have osteoarthritis of one or more joints, and chronic pain, presently a leading public health problem of epidemic proportions, and one where almost no report on the topic exists.

30.2 Methods

30.2.1 Data Sources

Related understandings and research as well as relevant materials were sought and downloaded from the PubMed® and Academic Search Complete databases using the key terms, successful aging, healthy aging, and osteoarthritis, alone or in combination. An array of relevant papers were flagged, and after reading these, only those that focused on the current topics of interest specifically were eligible. All forms of research were deemed acceptable however, and those articles deemed of high relevance were further scrutinized for salient facts, ideas, and implications for advancing 'successful aging'.

30.2.2 Procedures

Articles retrieved were categorized according to themes detailing the key concepts and findings of interest. This research encompassed articles that focused on 'successful aging' in general, the nature of osteoarthritis pathology, and the treatment and prevention of this disorder in relation to 'successful aging'. Only a narrative review of the diverse oftentimes confusing material was undertaken,

and no other disease specific entity other than osteoarthritis was included, although obvious overlaps among comorbid chronic conditions prevail. However, this work strove to solely examine this most salient costly disease disabler among older adults, regardless of where these populations reside, and the nature of osteoarthritis, in the context of aging 'successfully' with the view of possibly enabling a more inclusive conversation and body of research on this topic, along with possible encouragement to many aging adults and their caregivers.

After reviewing the widely heterogeneous data, which basically defied summative analysis, it was decided to focus the discussion on identifying how the original key concepts of 'successful aging' could be extended to encompass aging adults with osteoarthritis to compensate proactively to minimize the impact of their pathology, and heighten their subjective perceptions of being successful 'agers', despite this disability. For a very thorough review of the concept of 'successful aging' and its many varied interpretations, the concept paper by Flood [2] and the review paper by Martin et al. [3] are strongly recommended.

30.3 Results

30.3.1 Overall Findings

The literature search and the material extracted from that search, while not necessarily representing all publications in the field, showed that in general, there are many research reports that encompass one or more aspects of the theme of 'successful aging', but there is no comprehensive universally accepted model of the concept, and the concept is defined by a myriad of differing definitions, such as having achieved successful adaptations to an aging body [4], the absence of disease and disability [5], resilience/adaptation [6], and selective optimization with compensation [7]. As well, an array of research approaches, such as in-depth interviews conducted face to face or by phone [4, 5, 8], focus groups [6]; questionnaires [7], life story interviews [9], concept analysis [2, 10], literature reviews [3, 11–14] prevail. In addition, most studies examined small convenience samples of healthy cohorts of varying ages, and very few reports could be found that were devoted to the application of the concept of 'successful aging' to the osteoarthritis population. Moreover, very few studies discussed or compared interventions across time to assess the value of one or more identified potentially modifiable 'successful aging' determinants in the process of either healthy aging or aging in selected disadvantaged populations. Another feature was that very few studies examined the attribute and meaning of 'successful aging' from a holistic perspective, or from a more positive inclusive balanced perspective, rather than solely from a negative perspective.

30.3.2 Selected Aging Perspectives

Although data reveal that approximately 80% of older adults or those 65 years of age or older have at least one chronic health condition, and about 50% have at least

two or more of these conditions, as well as being at risk for infectious diseases and injuries such as falls, contrary to the stereotyped myth that aging is synonymous with an overall health decline, most adults are found to be healthy and can function at a high level. As well, research shows neither muscle function, nor memory loss is inevitable, and that physical and cognitive power does not need to decline over time, as previously believed.

Rather, while acknowledging relevant age-associated health changes do occur, positive aspects of aging, and how to prevent or deal with these inherent physiological changes, as well as any health problems that are incurred over time, provides an alternative perspective known as 'successful aging', which is seen as a process, as well as an active state of being. Indeed, although many myths and misconceptions about aging as a state of decline clearly prevail, this idea of 'successful aging' is not merely an academic one, because if one examines the leading causes of death among adults ages 65 and older, which are heart disease, cancer, stroke, chronic lower respiratory diseases, Alzheimer's disease, diabetes, influenza and pneumonia, and unintentional injury, none of these health conditions are simply inevitable consequences of aging [15]. Indeed, while it is true most chronic illnesses, once diagnosed in older adults are generally incurable, and can worsen over time, the key causes of most of these chronic illnesses and their morbidity rates as demonstrated in most recent nationwide and global public health research sites and data, are lifestyles and behaviors, changeable environmental agents, modifiable factors such as poverty, and other social factors, rather than genetic factors-once attributed an even role in determining longevity (e.g., smoking behaviors, eating behaviors). Additionally, most underlying causes or risk factors are amenable to prevention, intervention, remediation, elimination, or palliation, even if they are genetic (e.g., mastectomy for those with certain breast cancer genes, trauma prevention in those predisposed to genetically determined forms of osteoarthritis). Richardson et al. [16] also note that it is common for people with chronic conditions to report their health as good, even though models of healthy aging do not account for this.

Consequently, although many examples of debilitated older adults can be found, data show the majority of the older population to be neither debilitated, nor disabled. They may clearly be in their eighties but remain actively employed, they may be serving at the community level in volunteer endeavors, or involved in creative tasks, politics, education and other endeavors. Other data show that much of the innovation, beauty, science, and laws that prevail today are due to the efforts of talented or motivated people, who pushed themselves and developed their potentials, even though they were already deemed to be 'senior' citizens.

It can further be shown that many older adults, not only exhibit a complete 'lack' of any identifiable or seriously disabling disease or exposure to trauma, but that they may be more fit and functional than those in their 30s as a result of adopting a sound lifestyle. Even though bodily systems do still change over time, additional research shows a steady rate of decline from age 30 to 90, rather than any steep or rapid decline. These changes may also not occur simultaneously in all body systems, and even if they do change and influence the onset or risk of

disease and dysfunction, these changes may still be reversible, e.g., abnormal glucose levels can be impacted favorably in many cases of pre-diabetes.

On the contrary, since change is a constant in life, regardless of the stage of development or growth, a philosophy that enables the individual to adapt to any special challenges may yet foster a meaningful existence, rather than an inevitable and painful declining, and depressive existence, even though there is much more research on aging that deals with patients, and their hospitalization or experience in the nursing home than their longevity and health attributes. Unfortunately, gerontology, or the study of aging, largely focuses on the negative aspects of aging, while geriatrics is concerned with treating problems related to aging, rather than preventing these, as well as on disability or senescence.

However, in 1984 John D. and Catherine T. MacArthur gathered a group of scholars to develop a new set of insights regarding aging. Their mission was to stress the positive aspects of aging and to examine factors permitting certain individuals to function effectively, both physically and mentally in old age. More recently, Thompson et al. [17] found that even though polynomial regression of cross-sectional data obtained from 1546 individuals aged 21–100 years suggested a possible age-associated accelerated deterioration in physical and cognitive functioning, averaging 1.5–2 standard deviations over the adult lifespan, there appeared to be a linear improvement of about 1 standard deviation in various mental health attributes over the same life period.

30.3.3 Successful Aging

As reported in an initial publication in 1987 by the MacArthur group detailing the concept of 'successful aging' that followed their research efforts to better understand factors influencing positive health outcomes, despite aging, the idea put forth was that one did not have to age in a negative sense, but one could grow old, while maintaining their health, strength, and vitality, and could hence be deemed to be a successful 'ager' [18]. Since that time many hundreds if not thousands of articles related to this 1987 publication, and its dominant theme, along with its theoretical correlates have discussed this concept of 'successful aging', also known as healthy, active, productive, optimal, vital, or positive aging [3, 19, 20], among other definitions. This ever growing body of research continues to enlarge upon and expand on the early concept of 'successful aging', a viewpoint of aging built on the idea of harnessing the untapped resources of middle-aged adults and older individuals to enhance their well-being as they aged. It was a view encompassing attempts to maximize the potential of the aging adult to adapt to change, despite any physiological decline, and in so doing, to boost their chances of increasing their life satisfaction, mastery, growth, and longevity, as key components of 'successful aging', rather than succumbing to disablement and depression.

One overriding tenet of this idea was that aging in the negative sense was not inevitable, but was more likely than not to depend at least partly, on individual choices and behaviors, as well as effort. A second premise was that a broad focus of health issues, as well as more efficacious health policies, basic education that

promotes health literacy, quality housing, medical care, and appropriate employment opportunities, where desirable, might substantially reduce impediments to a meaningful and high quality experience in later life, thus promoting the idea of 'successful aging' or aging well through proactive adaptation processes [3] as an achievable goal.

However, Cosco et al. [11] noted that even though almost half a century had elapsed since the inception of the term 'successful aging', no clear definition and hence no unified understanding of the underlying framework and concepts underpinning 'successful aging' could be readily identified. After conducting a systematic review using MedLine, PsycInfo, CINAHL, EMBASE, and ISI Web of Knowledge and a search that focused on quantitative operational definitions of 'successful aging', 105 such definitions, across 84 studies, using unique models were observed. A further analysis showed 92.4% or 97 studies included physiological constructs (e.g., physical functioning), 49.5% or 52, engagement constructs (e.g., involvement in voluntary work), 48.6% or 51 well-being constructs (e.g., life satisfaction), 25.7% or 27 personal resources (e.g., resilience), and 5.7% or 6 focused on extrinsic factors (e.g., finances). Thirty-four definitions consisted of a single construct, 28 of two constructs, 27 of three constructs, 13 of four constructs, and two of five constructs. The operational definitions utilized in the included studies identified between <1% and >90% of study participants as 'successfully aging'. Based on these results, it appeared that the concept of 'successful aging' was clearly not a universal concept, and possibly one that was not always attainable, but its definition depended on what was being studied, who was being studied, or how the research was operationalized, among other factors. The idea that 'successful aging' is a multidimensional one, could consequently not be validated, and with no consistent research approach, no consensual definition of 'successful aging' or a framework for practice could be identified.

Earlier, Depp et al. [21] who similarly examined definitions and predictors of 'successful aging' using a large set of quantitative studies found 28 studies with 29 different definitions that met the researchers criteria. Even though most investigations used large samples of community-dwelling older adults, the mean reported proportion of successful agers was 35.8%, but this figure varied widely, and may have depended on the nature of the study, and the multiple components of 'successful aging' definitions identified. The most frequent favorable correlates of the various definitions of 'successful aging' were age (young-old), nonsmoking, and absence of disability, arthritis, and diabetes. Moderate support was also found for a positive role of greater physical activity, more social contacts, better self-rated health, an absence of depression and cognitive impairment, and fewer medical conditions in fostering 'successful aging'. These aforementioned data results were mostly based on examining older adults without disability who still portrayed quite a low response to the extent of perceiving they had aged successfully.

However, regardless of definition and clarification of what constitutes 'successful aging', Tesch-Romer and Wahl [22] have argued that even if changing environmental settings, societal policies, and fostering individual life styles will significantly extend the number of healthy life years, recent epidemiological research confirms the dilemma that the ongoing extension of life expectancy

prolongs not only the years in good health, but also those in poor health. This group thus argued that Rowe and Kahn's 'successful aging' model 2 is still not able to cover the emerging linkage between increasing life expectation and aging with disability and care needs, and presents a clear limitation in this regard. Instead, this group suggested a set of propositions be forged towards a more comprehensive model of 'successful aging', and one that would capture desirable living situations including those that encompass aging adults with disabilities and specific care needs. They consequently went on to describe a number of individual, environmental, and care related strategies and resources for promoting autonomy and life quality in the face of disabilities and care needs in late life, putting emphasis on inter-individual differences and social inequalities, and that was designed to expand upon the traditional concept of successful aging, but to do this in light of current aging science and aging population needs.

Although Gopinath et al. [23] found physical activity helped to foster 'successful aging', this group who attempted to determine the features of 'successful aging' through interviewer-administered questionnaire, still classified this concept as the absence of: depressive symptoms, disability, cognitive impairment, respiratory symptoms and systemic conditions (e.g., cancer, coronary artery disease), thus excluding almost all aging persons from achieving this state.

Yet, other determinants of 'successful aging' that have emerged from the research may or may not be helpful. These include, the adoption of proactive lifestyles that enhance health, actions that correct for health-associated behavioral risks, making healthy choices the only choice, and participating in stimulating cognitive activities. Other theories imply that eliminating factors that increase the risk for disability, or in fact increase its rate of progress—if present—such as stress, substance abuse, and infections, while fostering high quality social support, community based programs, financial security, and in the case of the individual having them adopt a positive, rather than a negative self-concept [4] are likely to be very influential in efforts designed to foster more successful rather than less successful aging outcomes. The availability of appropriate information and other resources, as well as uplifting opportunities to engage with others, may similarly enable more 'successful aging' across the lifespan [4], regardless of health status, than would otherwise be experienced.

The concept of 'successful aging' itself, however, is currently ill defined and may mean different things to different persons, for example those from different cultures may not emphasize the same subjective or objectively defined attributes that are perceived as elements of 'successful aging' [4, 5]. As well, a high degree of diversity in the perception of what this concept would mean personally, and whether this can be attained, if desirable may prevail, regardless of culture, health, and extrinsic factors. The studied attributes of 'successful aging' are also highly heterogeneous and range from 'having good health', to 'having a sense of purpose', and autonomy, among others [4], such as the absence of disease and disability [5], and the ability to maintain physical and cognitive functioning [5]. Adding further confusion is research showing these attributes and others can readily fluctuate depending on age-wherein 'successful' agers tended to be younger and report more frequent engagement in lifestyle activities than older subjects [7], gender

[5], living circumstances [9], and the extent of any disability and pain [9], among other factors.

In addition, another term related to the concept of 'successful aging', namely, 'healthy aging', similarly hosts a number of definitions and components of well-being that differ depending on the stance of the researchers, and their specialty. This latter concept also differs somewhat from that of 'successful aging', and is suggested to denote the ability of the individual to modify, reassess and redefine oneself-but does not preclude disease states. Resilience, adaptation and compensation are other important characteristics of healthy aging that appear valid, but again these may vary dependent on the values held by different societies, as well as societal and individual expectations, and the role of family, social engagement, among other factors. Another view is that if disease is seen as a normal part of aging-rather than modeling 'successful aging' on the basis of freedom from disease, Amin [4] implies it may be fruitless to identify whether any universal set of 'successful aging' constructs prevail. Rather, the individual's perception and how they define 'successful aging' or 'aging well' must be uncovered through careful interactions and communication efforts.

In short, the concept of 'successful' or healthy aging, or that of a 'good old age' is depicted as multidimensional. Its scope and meaning may depend however on what premises researchers decide to test, the type of research questions posed, the sample numbers and characteristics. Results may also depend on the individual and her perceptions, as well as how practitioners and researchers view this concept [3]. However, despite a myriad of views, and discrepancies among the diverse biomedical, psychological, and lay perspectives [21], it appears that to promote 'successful aging', something more than personal attributes and understandings can determine this life outcome. For example, an empathetic provider-patient relationship and access to quality care and living conditions and taking cultural preferences and views into account can help even those who are disabled to be more successful than not. As well, a focus on the socio-emotional aspects of health, as well as the physical aspects of health will clearly be more helpful than not, regardless of level of ability or disability.

Elements of 'successful aging' may also include sound nutrition practices, an active lifestyle, having a degree of financial security, and sound sleep hygiene strategies, and in our view are just as relevant to those with health conditions as those who have no health disability. Efforts to reduce or minimize anxiety, depression, and low self-concept are additional strategies that can be applied, regardless of health status. According to findings by Richardson et al. [16] who examined how resilience relates to how people consider themselves to be well, regardless of their adverse health condition, the experience of adversity is influenced by context and meaning, findings which support a broader version of resilience that incorporates vulnerabilities, and should not undermine an older person's sense of a resilient self.

Zanjari et al. [14] who examined 76 topical articles eligible for inclusion in an integrative review, found 14 subcategories and 5 main categories of 'successful aging' to prevail, including social well-being, psychological wellbeing, physical health, spirituality and transcendence, and the environment and economic security. Another study by Parslow et al. [24] found factors measuring mental, physical

and social health can all contribute significantly and independently to 'successful aging' and that the presence of chronic health conditions does not necessarily preclude high levels of well-being in older individuals. Another view is that disease and disability might be viewed as a normal part of aging, and thus freedom from disease or longevity is not necessarily of high import to the same degree in all cultures [4].

In the next section, we argue that contrary to early beliefs that saw 'successful aging' as a state where the individual was disease free, attaining this state is in the eyes of the individual, and its attainment should not be neglected in the presence of an irreversible disease.

30.3.4 Osteoarthritis

Osteoarthritis is a painful disabling joint disease that predominantly affects more than half of those adults who are 65 years of age or which older. An incurable disease that often affects more than one joint, the condition can be extremely debilitating and disabling is one where few risk free treatment strategies exist. Moreover, medications applied to alleviate disease symptoms are often contra-indicated or induce unwarranted side-effects, and surgery is not always effective or warranted. Given that many with this physical condition also show signs of depression [25], a diminished work capacity, restrictions of social activities [26], and most have one or more comorbid chronic diseases, and/or are overweight or obese, and all these components of the illness interact to often increase the severity of the condition [26], the idea of 'successful aging' is clearly one that is highly contrary when conceived as an ideal for those with this intractable condition.

So what can be done to assist the aging adult with osteoarthritis to maximize their overall well-being, while minimizing their disability in an effort to still attain a 'successful aging' state of being? Clearly, the disease, a multi-dimensional one that includes psychological as well as physical symptoms is not reversible, and can readily spread from one joint to another, while progressively degenerating processes proceed unhindered. To favorably alter the potential downward spiral, and ensure the goal of 'successful aging', or 'healthy aging', research shows this is feasible in light of what we do know, even though this requires very careful assessments, and interventions that are not only carefully construed, but are carefully tailored and titrated.

Fortunately, as with healthy adults, an extensive array of related research testifies to the enormous benefits of exercise adoption and muscle conditioning in this context, which is consistent with the idea that the health condition is more likely to occur in the presence of weak muscles or muscle pathology than not. As well, the finding that those who exercise, are more likely to experience fewer weight issues, and are better off than those with high body weights speaks to an important role for both exercise and weight control in efforts to attain a successful health outcome, despite the disease. Other salient strategies related to joint protection efforts, such as the avoidance of undue joint stressors, vigorous exercises, and excess use of opioid narcotics, among other approaches will help foster the ability to pursue health goals more readily than not. As well, the use of heat

and cold applications-as required, along with selected oral and topical medications, stress reduction efforts, ergonomic adaptations of the home and workplace, adaptive devices, and social support are found to help assist the patient with this condition to substantively raise their life quality, and to possible attenuate the disease symptoms, as well the risk of injury and comorbid health conditions such as diabetes. As with all attempts to foster 'successful aging', early intervention in childhood and adolescence is indicated, even though osteoarthritis is often considered an age-associated disease.

In sum, disabling and debilitating osteoarthritis, the most common joint disease affecting older populations, may not be inevitable, in all cases, and even when present, its severity and extent can be attenuated and may be amenable to amelioration if careful evaluations followed by carefully construed and timely treatment strategies are forthcoming and sustained where necessary. Some of the non-operative approaches for helping the osteoarthritis sufferer to maximize their health outcomes, may yet promote feelings of 'success' and control over their condition in the face of a very demoralizing disease state.

It is also reasonable to suggest that providers who adopt realistic outcome goals for effective rheumatic pain management for their patients, and help them to moderate any erroneous constraint beliefs [25], and to convey this clearly to the patient will help them to avoid unrealistic expectations, as well as excess pessimism, for example, believing joint surgery will be restorative or painfree, or that opioids that relieve pain are safe, or believing their condition is not amenable to intervention. In addition, even though pain-the symptom of most concern to patients is unlikely to be completely abolished, regardless of intervention, helping patients to understand the key determinants of pain, and what to avoid, or act on is very key to success. As well, to foster adherence behaviors, barriers to effective pain management from both the patient and the healthcare professional perspectives should be minimized. In this respect, acceptance therapy, where the patient is encouraged to accept their situation, but not to lose sight of their goals, appears very worthy of consideration and application. Joining a support group or acting as an advocate for others or journaling have been shown to advance well-being despite the presence of disability, and regardless of age.

In short, as per Fig. 30.1 shown below, it is highly possible for an older adult with osteoarthritis, to age 'successfully' in our view, especially if they try to proactively contribute to their own wellbeing on a consistent basis, in conjunction with their provider's recommendations, despite an associated array of cognitive and physical challenges. This ability to manage their health condition is not a given though, and may depend not only on the degree of presiding impairment, but on other intrinsic factors such as health beliefs, motivation and ability to comply with their providers' recommendations, and extrinsically on the degree to which providers are knowledgeable and accessible, program attributes are tailored and clearly imparted, including the degree of societal or family support. More direct interventions include the use of supplements, topical gels, maintaining a healthy weight and blood pressure, plus home improvements and worksite adaptations to minimize joint and overall stress. In the meantime, an acceptance of a broader definition of 'successful aging' is fundamental in this regard in this author's view,

along with the understanding that the disease affects the whole body, not just a single joint, including the neurological, structural, and cardiovascular components of the body, and their relationship to physical, mental, emotional, and social wellbeing, and that it is not inevitable or subject solely to deterioration.

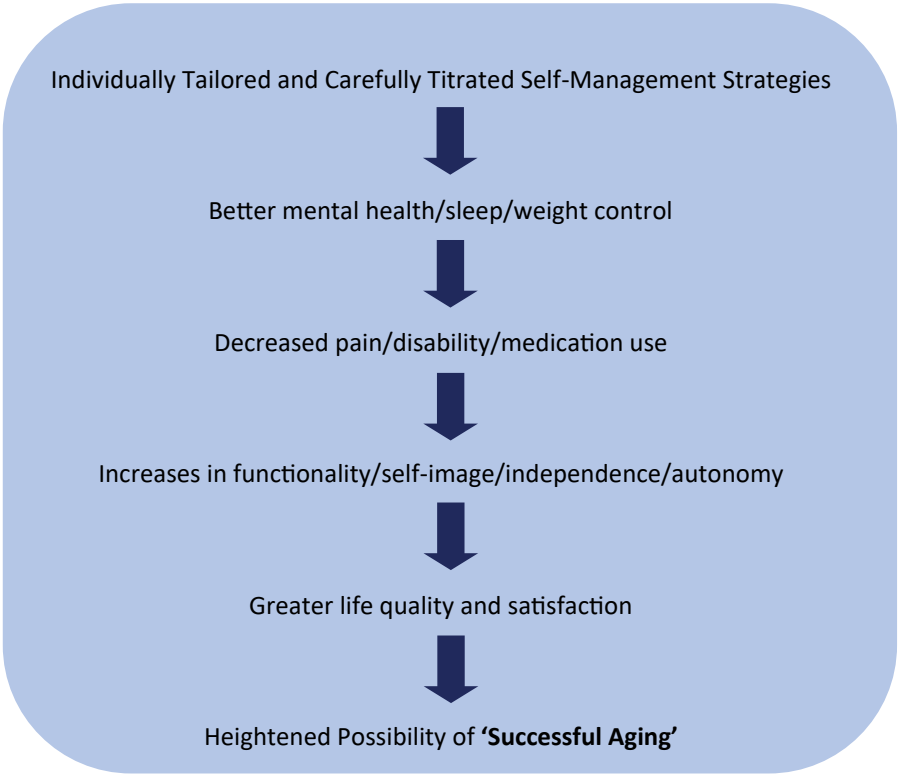

Figure 30.1 Conceptual model of anticipated outcomes of carefully tailored and titrated management.

30.4 Discussion

By 2030, the numbers of older adults in the United States will more than double to about 71 million [1]. However, not all will age successfully, even if they have no distinctive co-morbid health condition. On the other hand, this rapidly increasing number of older Americans, as well as those in other areas of the globe, will probably have high rates of chronic diseases, which may be barriers to the achievement of a fully functional life, unless a broader vision of 'successful aging' is considered, along with heightened early preventive health promotion efforts in this author's view.

Since this aging nation's efforts to ensure the achievement of 'successful aging' for all have clearly yielded suboptimal results, along with associated potentially modifiable economic and social costs, a deeper consideration of the 'successful aging' theme and opportunities in the research, academic spheres, psychology

and policy arenas to advance this concept appears imperative. However, though initially deemed an essential component of 'successful aging', namely, keeping older adults healthy and disease free, this outcome, while clearly desirable, is generally not readily observed at all today, despite modern medicine advances, and positive longevity gains, overall. Indeed, a pure aging process that has not been influenced by illness, trauma, or genetic determinants, is clearly very unlikely to prevail for most, especially if an older person has developed one or more chronic health challenges, such as osteoarthritis in their early teens or adult years.

Yet, if the goal of 'successful aging' is to enable optimal states of well-being across the life span including the later stages of the life trajectory, the perception that all individuals can 'age' optimally, may permit a more inclusive definition of this concept that embraces the potential of all citizens and does not discriminate between the presence or absence of prevailing health problems and is very likely to be more advantageous than not in this author's view. To this end, this review has described some aspects of 'successful aging' also termed 'healthy aging' in an effort to garner some insight into fostering this concept for people with chronic osteoarthritis. Based on an in depth review of all available literature, it is clear no finite definition or accepted set of constructs prevails in this regard, despite nearly 30 years of research. There is almost no literature on this concept that speaks to osteoarthritis sufferers even though more than half of aging adults will have this condition.

Since the attributes of resilience, autonomy, acceptance, the ability to cope to achieve personally valued goals, and independence are valued attributes of 'successful aging' [4, 6, 10] these traits alone, among others, such as perceived self-efficacy for coping with life in general, which can be strengthened, can clearly pertain to all aging adults, regardless of physical health status, and their attainment might potentially yield more success than not across all stages of the older adults' life trajectory. On the other hand, more health education, and availability of health resources and services, rather than seeking genetic solutions, or magic anti-aging bullets, would go a long way to helping both those adults who are unimpaired as well as those who are impaired to age gracefully-or successfully. Placing emphasis on what success means to the individual at the outset though-can surely help practitioners to guide citizens to achieve personal values that would denote success to them, rather than attempting to follow prescriptive or preconceived models. In the case of the osteoarthritis sufferer, one who proactively tries to maintain a full and independent life by means of their own efforts to accommodate certain physical changes, to prevent harm, and to promote regenerative or reparative states, the individual may feel very satisfied and thus successful-all things considered as outlined in Figure 1. On the other hand, even if their osteoarthritis condition is manageable, but is viewed in a negative way by the society, social, political, and medical systems, their level of depression might be heightened-and their self-worth lowered with dire 'aging' consequences. As well, if nothing is done to translate and apply evidence supporting primary prevention strategies, implemented early on in life, and an ecological viewpoint of health outcomes and determinants that fosters optimal safe and enriching living environments, offsetting the prevalence as well as the severity of this

condition through self-care activities alone seems unlikely. These strategies may include, but are not limited to the avoidance of injury, drinking and driving, along with better public health protection laws, fighting against criminal victimization of older people, avoidance of over- or under-eating, and sedentary activities, improved undergraduate and graduate education about aging, and community-based health promotion programs. More marketing by Aging and Arthritis Foundations of what can be done to protect against osteoarthritis, and the importance of seeking help earlier rather than later is also recommended. As well, refuting the myth that nothing can be done for their health condition, that aging is inevitable declining process and others must be actively countered with science based data. As well, more efforts by physicians and rheumatologists on efforts to combat or minimize depression and anxiety and improve coping skills in this group, as in healthy elders, will undoubtedly be very helpful in advancing their perception of 'success' across the lifespan of their later years.

30.5 Conclusions

In the author's opinion, ample research revealing the idea that aging is an inevitably declining state does not appear to be a valid one. Rather, when cases of individuals who do not appear to 'decline' objectively to any degree over time are examined, certain commonalities related to life style and behaviors, as well as socioeconomic opportunities appear more relevant than genetics. As well, equal numbers of those who are centenarians are found to have one or more health conditions, while others have few detectable problems. Not all centenarians however, are satisfied with their attainment, even if longevity is desirable, while others clearly take action overtly to prevent longevity. Thus, the idea that there is a one size fits all-or single unified formula for achieving 'successful aging' clearly seems elusive at present, as borne out by the research. However, even though no consensus on what should be studied in this realm is evident, and the varied samples and range of attributes studied to date may account for the lack of uniformity in this sphere, the idea that 'successful aging' is closely related to autonomy and goals such as physical and psychological functioning [21], suggests that this goal can be sought by most aging adults-or in fact promoted by states or countries-regardless of health status. Yet, very few related studies have actually examined aging individuals deemed to be impaired, and that take into account the fact that the mainstream cultural values of autonomy, and success in the context of aging are not likely to be uniform. The process of trying to examine only a selected number of possible 'successful aging' related determinants such as financial status, physical activity, body mass index, depression, participation in social activities with friends and family, number of yearly excursions, number of cardiovascular disease risk factors and adherence to the Mediterranean diet, may further limit understandings, and explain why these data when combined only yielded an index of 'successful aging' showing a 1/10-unit increase in the index was associated with 0.8 less annual visits to healthcare centers over 6 years

(95% CI −1.3 to −0.2) [27]. Indeed, as outlined by Kim [28], as well as Brown and Bond [8], the findings suggest that the increased longevity of centenarians and adults over the age of 65, is affected by multiple social factors, such as support services for the elderly, and social well-being programs and policies, as well as the definition of 'successful aging' applied, and these factors warrant further study.

However, based on what we do know, it appears that all aging adults can improve their health outcomes through a variety of self-directed proactive strategies if they are mentally able, as well as motivated towards achieving the promised outcomes. Moreover, despite their challenges, older adults with osteoarthritis who consistently practice positive healthy behaviors, and have access to and can take advantage of clinical and community-based services are quite likely to be able to engage in and pursue a personally meaningful life, and to achieve an acceptable level of independence, if desired, and one they personally will deem to be 'successful', despite their challenges.

Yet, despite much research, the literature in support of this concept of 'successful aging' not only shows no consensus on its attributes, and few studies that considered 'successful aging' from a holistic perspective or that examined actual ability to function physically, mentally, economically, socially, and emotionally, either in the case of those deemed physically healthy or impaired. Instead, several researchers explored the dimensions of 'successful aging' through the lens of a small array of pre-determined themes, and even if personal themes of success were identified, very little work follow up work was done to test or validate these perspectives and what these denote in persons with disabilities, as well as what the concept does not denote, or why only very few older individuals who did not need home care services in the past 59 days were not categorized as successful agers [29]. As well, although having a spouse, good mental health status, and life satisfaction were assessed and found to be salient predictors of successful aging, the fact that only disease free individuals were examined precludes this finding from being generalizable. This series of prevailing 'successful aging' research approaches in general, has also been criticized by Thompson and Forbes [17] who felt 'disease free' older persons do not represent intrinsic or true aging attributes. As well, Stowe et al. [13] who examined 'successful aging' through a life-course lens urged caution in using the model in its current formulation owing to its emphasis on personal control over one's later-life outcomes, and neglect of historical and cultural context, social relationships, and structural forces in influencing later-life functioning. Additionally, very few studies have examined the importance or role of educational attainment, health literacy, motivation for successful aging, the housing environment, food quality and availability, the possible influence of nutrigenomics [30], role loss, dependence, loneliness, medication related issues, comorbid and sleep issues in the context of moderating or mediating 'successful aging' in different age groups and genders [31]. Many if not all these factors may thus represent overlooked explanatory factors or 'successful aging' antecedents, as well as intervention points of specific import in efforts to optimize the health quality and independence of older people across the lifespan. Others points of consideration are highlighted in Table 30.1.

Table 30.1 Selected successful aging studies and conclusions

Researchers	Key conclusions
Bowling et al. [32]	A model of successful aging needs to be multi-dimensional, incorporate lay perspectives, and use a continuum for success.
Hamid et al. [33]	Showed age, educational attainment, household income, and ethnicity were significantly associated with successful aging.
Iwamasa et al. [34]	Participants perceived successful aging as optimal functioning in: physical and psychological health, cognitive functioning, socialization, spirituality, and financial security. The content of each dimension represents both culture-specific and culturally-universal elements.
Parslow et al. [24]	Chronic illness is not necessarily a barrier to successful aging.
Katz et al. [35]	Inattention to intersecting issues of social inequality, health disparities, and age relations, and possible role of social exclusion in successful aging processes remain.
Lowry et al. [12]	Successful aging is a multidimensional construct that could be viewed as a continuum of achievement. Based on the disability model proposed by the WHO International Classification of Functioning, Disability and Health, successful aging includes not only the presence or absence of disease, but also aspects of mobility and social participation.
Ng et al. [36]	Although aging well socially (engagement with life) is as important as aging well personally (illness avoidance and functioning) (Rowe and Kahn, 1997 [18]), results supported the differentiation of Rowe and Kahn's engagement with life component into caring and productive engagements.
Jeste et al. [37]	Resilience and depression had significant associations with self-rated successful aging, with effects comparable in size to that for physical health.
Pietrzak et al. [38]	Interventions and policy initiatives designed to mitigate physical health difficulties and psychological distress and to enhance protective psychosocial characteristics such as resilience, gratitude, and purpose in life may help promote successful aging in these populations.
Vahia et al. [39]	Self-rated successful aging emerged as the primary downstream factor and exhibited significant partial correlations with psychosocial protective factors, physical/general status and mental/emotional status but not with cognitive ability.

30.6 Future Research and Practical Challenges

Although this report itself is limited, and does not attempt any meta-analytic consolidation of the topic, it seems that much more effort to tease out and examine the concept of 'successful aging' alongside various aging, biological, psychological and sociological theories, technological influences, living circumstances, socially

validated work opportunities, health care access, and culture among other extrinsic factors is needed to arrive at some meaningful individual, and if possible, national strategy or orientation towards fostering 'successful aging' for all as suggested by Martin et al. [3] and Martinson et al. [40]. To better define 'successful aging', more large scale interdisciplinary efforts, more study of young healthy and impaired adolescents and adults across the range of the aging continuum [12] in well-designed prospective and intervention studies that take lay views into account [31], along with efforts to acquire a better understanding the role of stress, depression, spirituality, the epigenetic and the social determinants of health are likely to prove helpful. As well, studying those with and without specific health issues including, but not limited to osteoarthritis, using an appropriate combination of biochemical, cognitive, and functional outcome variables to define 'successful aging' among diverse samples can possibly help to indicate potential areas for maximizing successful long term health outcomes in the future. More efforts too to discern which predictors of 'successful aging' are of greatest import, such as socioeconomic, socioenvironmental, educational factors, chronic exercise, nutritional, genetic, and health status factors, among others will undoubtedly be of additional help to both policy makers and clinicians in any future effort to advance this field beyond the theoretical level. The role of smart technology, stem cell research, regenerative medicine, inflammation control, nutrients and behaviors, early life health education, and robotics in expediting 'successful aging', mostly uncharted topic areas, but increasingly relevant, also warrants attention. However, even if all this is forthcoming, moving mainstream medicine to conceptually foster the idea of 'successful aging' for all across the lifespan, as an active endeavor, regardless of age, genetics, disease, injury, functional or cognitive status, is potentially the most critically needed focal point for advancing health and optimally successful longevity in all populations.

Additional challenges that might be considered in deriving an agreed upon understanding of 'successful aging' and one that can be examined systematically are:

The failure to view the concept holistically across varying age and cultural groups, marital status, occupational prestige, years of formal education, race, annual income, and a variety of specific satisfaction with life measures related to successful aging, as well as spirituality [41, 42].

The diverse methods and measures used to examine 'successful aging'.

The widespread use of surveys to derive insights about 'successful aging' rather than functional measures.

Subjective and objective criteria of 'successful aging' may differ [43].

Availability, accessibility, role of appropriate services, and needs are poorly explored correlates.

Negative stereotypes and beliefs, control beliefs, about aging are unexplored factors.

The commonly used operational definition of successful aging (high cognitive and physical function, low probability of disease, and active engagement with life)

does not necessarily reflect the values of other cultures or those of older persons in the United States [16].

Model was primarily designed for high-income populations and may not be transferable to populations in low- and middle-income countries [44].

Model has not considered role of environmental contaminants or hazards [44].

Disclosures and Conflict of Interest

This chapter was originally published as: Marks, R. (2018). Successful aging and chronic osteoarthritis. *Medicines*, **5**(3), 105, https://doi.org/10.3390/medicines5030105, under the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/). It appears here, with edits and updates, by kind permission of the copyright holder. This research received no external funding. The author declares no conflict of interest.

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Chapter 31

Radiographic Analysis on the Distortion of the Anatomy of First Metatarsal Head in Dorsoplantar Projection

Jessica Grande-del-Arco, PhD,[a] Ricardo Becerro-de-Bengoa-Vallejo, MD, PhD,[a] Eva María Martínez-Jiménez, PhD,[a] Israel Casado-Hernández, PhD,[a] Daniel López-López, DP, PhD,[b] and Marta Losa-Iglesias, RN, PhD[c]

[a]*Facultad de Enfermería, Fisioterapia y Podología, Universidad Complutense de Madrid, Madrid, Spain*
[b]*Research, Health and Podiatry Group, Department of Health Sciences, Faculty of Nursing and Podiatry, Universidade da Coruña, Ferrol, Spain*
[c]*Faculty of Health Sciences, Universidad Rey Juan Carlos, Alcorcón, Spain*

daniel.lopez.lopez@udc.es

Keywords: dorsoplantar radiograph, first metatarsal head, foot deformity, hallux rigidus, hallux valgus (HV), intermetatarsal angle, metatarsal bones, metatarsophalangeal joint (MTPJ), orthopedics, osteoarthritis (OA), radiography, radiological health

31.1 Introduction

Hallux valgus (HV) is a highly prevalent foot deformity estimated to affect 23% of adults and 35.7% of elderly individuals (1). It presents a significant individual and public health burden, due to the high occurrence of related orthopedic foot surgery [1], and its association with foot pain [2, 3], osteoarthritis (OA) at the first metatarsophalangeal joint (MTPJ), impaired gait patterns [4], poorly coordinated stability and an increased risk of falls in older adults [5, 6].

While the development of HV is believed to be multifactorial, the exact etiology remains unclear [7]. Previous studies have suggested that several structural factors might be characteristic of HV, including various radiographic angles, first

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MTPJ congruency, metatarsal length, metatarsal head shape, sesamoid position, first metatarsocuneiform joint flexibility, and pes planus [8, 9].

First metatarsal head shape has been routinely assessed by orthopedic surgeons radiographically, and has been addressed by as many as 24 authors, as well as in systematic reviews [7], to claim that the shape is significant in the development of HV, and it has been classified as three types: round, square and crest [8, 10–28], with the crest type being the most stable to prevent the development of HV and the round shape contributing to the development of HV, and it is one of the factors in recurrence after hallux valgus surgery [11, 14, 15, 17–19, 21–23, 28–32].

Several authors have reported a relationship between a round-shaped metatarsal head and hallux valgus but have not detected a strong correlation due to a lack of substantial data between them. Therefore, it is unknown whether a metatarsal head shape predisposes one to the development of hallux valgus.

In patients with HV, radiographs are obtained as part of a clinical evaluation. On these radiographs, angular measurement is used to determine the severity of deformation. A 1951 study [33] analyzed the sources of error in the production and measurement of radiographs of the foot. This publication illustrated the need for the standardization of the radiograph of the dorsoplantar view of the foot, which has been widely advocated [33, 34].

Despite this, various authors who described “their” standard technique of the dorsoplantar radiograph use a craniocaudal tube angulation of 5° [35], 15° [36–38], or 20° [39], but the American Orthopaedic Foot and Ankle Society recommended a tube angulation of 15° [40].

One study has been performed with a tube angulation of 20° in patients with HV and reports a relatively small reduction in the distortion of the intermetatarsal angles, but it did not evaluate other anatomical structures.

To our knowledge, a systematic analysis of the relationship between tube angulation and the distortion due to the projection of the actual anatomy on the radiographs has not been performed beyond 20°.

The goal of this study was to analyze the effects and distortion that occur in the shape of the first metatarsal head when performing a dorsoplantar X-ray with the angled X-ray tube from 0° to 30° in anatomical specimens, and subsequently performing its dissection, to determine if the anatomic and radiographic findings correlate.

31.2 Material and Methods

From December 2016 to June 2019, 173 feet from embalmed cadavers were included in the study from Donation Center of the Bodies and Dissection Rooms of the Complutense, The University of Madrid. The institutional review board of the Rey Juan Carlos University approved the study data on February 14, 2017, under number 27122011600917.

Those samples that included the complete foot with the distal third of the tibia and those samples that clinically showed no signs of surgical intervention were included in the selection of anatomical pieces.

The inclusion criteria followed in the radiographic evaluation were adult feet with radiographic images, in which all the growth cartilages of the foot and the distal third of the tibia and fibula were completely closed. It was required that the radiographic images show the entire foot. Radiographs that showed traumatic or degenerative changes of the sesamoids or the surface of the first MTP joint, the presence of hallux valgus, or an intermetatarsal angle greater than 12° were excluded, as established in the article by Durrant et al. [41].

Each specimen was clinically examined to determine if it presented any deformity and those anatomical pieces that presented deformities in the foot, such as hallux valgus, hallux rigidus, osteoarthritis in the first MTF joint, fractures in the first metatarsal or presence of implants, patients with obviously abnormal shapes of the first metatarsal, due to fracture, invasion of the tumor, or congenital disease, were excluded.

Because of this, only 103 samples complying with the inclusion criteria were used in the study.

The variables to be studied on the radiographs were the shape of the head of the 1st metatarsal, establishing the following categories: Round, square and "with crest", as reported in the literature [8, 10–28].

31.2.1 Radiographic Protocol

The optimal tube angulation was defined as the angulation that was associated with the smallest average distortion. Besides the varying tube angulation, the geometry of this projection was identical to the standard technique of a dorsoplantar radiograph.

An Optima Xr200amx portable radiology equipment from GE Healthcare, 30 kW (GE HEALTHCARE, Madrid, Spain, https://www.gehealthcare.com) was used with a 24 × 30 cm chassis and FireCR Spark Medical digital reader, 4DMedical, Valencia, Madrid.

The anatomical feet were placed on the radiographic plate in a neutral position, taking into account the methodology and protocol proposed by the studies by Venning and Hardy (1951) and Tanaka, Takakura, Kumai, Samotoy Tamai (1995) [33, 42].

The standard dorsal, plantar radiographic projection proposed by several researchers was used: The X-ray beam tilts at 15° at a distance of 100 cm, to ensure the accuracy of these records, which were obtained from various articles [10, 33, 42].

31.2.2 Radiographic Representation

The samples underwent several images of the first metatarsal at different degrees of the beam projector. We performed a radiographic analysis with different degrees of projection.

We used a variable craniocaudal tube angulation in a sagittal plane 0°, 5°, 10°, 15°, 20°, 25°, and 30°, and the beam direction was set parallel to the axis of the foot and centered on the second metatarsal tarsus [42].

During X-ray imaging, the X-ray beam is perpendicular to the image intensifier, and the foot is positioned parallel to the image intensifier (Fig. 31.1).

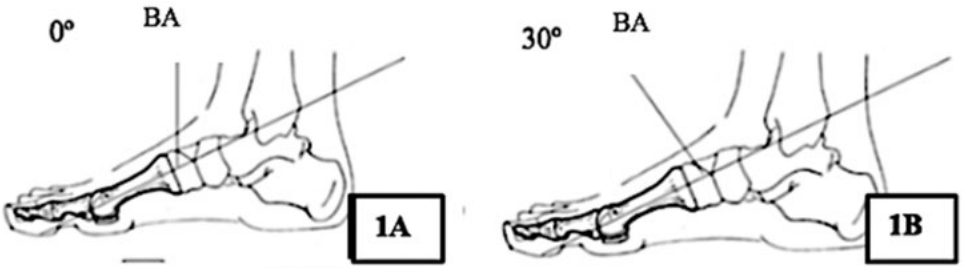

Figure 31.1 Position of the X-ray beam at a distance of 100 cm to obtain precision in the images. *Abbreviation*: BA: Beam angle. (A) relationship between the angulations with an X-ray beam projection at 0°; (B) Relationship between the angulations with an X-ray beam projection at 30°.

A neutral position, with 0° of inclination and rotation, avoiding pronation or supination of feet and beam direction focused on the second wedge joint as an exponent [43].

31.2.3 X-Ray Observation

The shape of the head of the first metatarsal was classified into three types, according to several authors: round, flat, and with crest [8, 10–28]. The observation consisted of the two assessors measuring relevant measures of 103 randomly chosen feet radiographs, and then 1 week later re-measuring all radiographs without reference to previous results.

After observing the radiographs, the samples were dissected to assess the shape of the first metatarsal head by the same two observers who assessed the radiographs.

31.2.4 Statistical Analysis

Kappa statistics and generalized McNemar tests were used to assess and test for agreement. The shape of the first metatarsal head was polycotomized into three groups; “round”, “flat”, and “crest”. As suggested by Landis and Koch, we interpreted the kappa values as follows: <0.20 indicates poor agreement, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 good, and >0.80 excellent [44].

The McNemar Bowker test describes whether the marginal distributions of two measures are similar, as one would expect if the measures agree.

Data were analyzed using IBM SPSS Statistics, version 22 statistical software (SPSS Inc, Chicago, IL, USA). Statistical significance was set at $p < 0.05$, and Confidence Interval (IC) to 95%.

31.3 Results

The interobserver agreement by Kappa analysis (Table 31.1) showed a moderate agreement at 15°, good agreement at 0°, 5°, 10°, 20° and 25° and excellent agreement at 30°.

To calculate intraobserver agreement, results were compared against angle beams. Table 31.2 shows the intraobserver A agreement when the first metatarsal head gets distorted and appears crested. Results indicate that this occurs when the angle of the X-ray beam is at 20° of inclination. These results are similar for intraobserver B (Table 31.3), where the distortion of the same head occurs at 20° relative to 15° ($p < 0.001$).

Finally, after dissecting the 103 anatomical specimens, we found that all the first metatarsal heads had a round shape and none with a square shape or a crested head, showing perfect intra- and interobserver agreement.

31.4 Discussion

The purpose of this study was to determine the presence of a distortion effect in the first metatarsal shape, due to the angulation of the X-ray beam.

Most articles on the measurement of dorsoplantar radiographs report a 15° [33, 42] or 20° craniocaudal tube angulation. The American Orthopaedic Foot and Ankle Society has recommended a tube angulation of 15° [40].

We used observations of radiographs in this study. This technique used a craniocaudal tube angulation in a sagittal plane 0°, 5°, 10°, 15°, 20°, 25° and 30° to evaluate the shape of the first metatarsal and included dissection of 103 feet embalmed cadaver by both observers.

We focused on the distorting effects of the tube angulation in the shape of the first metatarsal. We found that the distortion of the shape of the first metatarsal was minimal when the radiograph was made without angulation, or the beam angle was less than 20°.

Both observers agree that the shape of the metatarsal head is distorted in projections in which the X-ray beam with angulations is equal to or greater than 20° (Fig. 31.2).

In this study, an association between a flat- or crested-shaped head of the first metatarsal with pathologies, such as hallux rigidus or hallux limitus, cannot be supported, because these shapes are the result of distortion caused by tube angulation.

Another reason for the distortion of the first metatarsal head with the X-ray beam correctly positions at 15° is that the normal first metatarsal declination angle is 21° angle between the axis of the first metatarsal and a horizontal linear [45], and in the flat foot, the first metatarsal declination angle is lower.

So, we postulated that when tube angulation in a sagittal plane is 15° [33, 42] in a normal foot with a first metatarsal angle declination of 21°, the possibility of deformation or distortion of the first metatarsal head is minimized.

Table 31.1 Interobserver agreement about shape first metatarsal head in beam angle 0–30°

			Reader B				
	Shape metatarsal head	**Beam angle**	**Round F (%)**	**Flat F (%)**	**Crest F (%)**	**Total F (%)**	**Kappa (*p*)**
Reader A	0°	Round	69 (67.0%)	10 (9.7%)	0 (0.0%)	79 (76.7%)	0.679 (<0.001)
		Flat	3 (29%)	21 (20.4%)	0 (0.0%)	24 (23.3%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	72 (69.9%)	32 (30.1%)	0 (0.0%)	103 (100%)	
	5°	Round	72 (69.9%)	9 (8.7%)	0 (0.0%)	81 (78.6%)	0.715 (<0.001)
		Flat	2 (1.9%)	20 (19.4%)	0 (0.0%)	22 (21.4%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	74 (71.8%)	29 (28.2%)	0 (0.0%)	103 (100%)	
	10°	Round	73 (70.9%)	12 (11.7%)	0 (0.0%)	85 (82.5%)	0.613 (<0.001)
		Flat	2 (1.9%)	16 (15.5%)	0 (0.0%)	18 (17.5%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	75 (72.8%)	28 (27.2%)	0 (0.0%)	103 (100%)	
	15°	Round	70 (68.0%)	10 (9.7%)	0 (0.0%)	80 (77.7%)	0.548 (<0.001)
		Flat	6 (5.8%)	16 (15.5%)	0 (0.0%)	22 (21.4%)	
		Crest	1 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.0%)	
		Total F (%)	75 (72.8%)	26 (27.2%)	0 (0.0%)	103 (100%)	

Reader B							
	Shape metatarsal head	Beam angle	Round F (%)	Flat F (%)	Crest F (%)	Total F (%)	Kappa (*p*)
	20°	Round	38 (36.9%)	7 (6.8%)	2 (1.9%)	47 (45.6%)	0.726 (<0.001)
		Flat	1 (1.0%)	12 (11.7%)	0 (0.0%)	13 (12.6%)	
		Crest	2 (1.9%)	6 (5.8%)	35 (34.0%)	43 (42.7%)	
		Total F (%)	41 (39.8%)	25 (24.3%)	37 (35.9%)	103 (100%)	
	25°	Round	6 (5.8%)	1 (1.0%)	1 (1.0%)	8 (7.8%)	0.616 (<0.001)
		Flat	0 (0.0%)	3 (2.9%)	0 (0.0%)	3 (2.9%)	
		Crest	4 (3.9%)	4 (3.9%)	84 (81.6%)	92 (89.3%)	
		Total F (%)	10 (9.7%)	8 (7.8%)	85 (82.5%)	103 (100%)	
	30°	Round	2 (1.9%)	0 (0.0%)	0 (0.0%)	2 (1.9%)	0.853 (<0.001)
		Flat	0 (0.0%)	1 (1.0%)	0 (0.0%)	1 (1.0%)	
		Crest	0 (0.0%)	1 (1.0%)	99 (96.1%)	100 (97.1%)	
		Total F (%)	2 (1.9%)	2 (1.9%)	99 (96.1%)	103 (100%)	

Abbreviation: F, frequency.

Table 31.2 Intraobserver A agreement about shape first metatarsal head in beam angle: 0° vs. 5°, 5° vs. 10°, 10° vs. 15, 15 vs. 20°, 20 vs. 25°, 25° vs. 30°

Reader A							
	Shape metatarsal head	**Beam angle**	**Round F (%)**	**Flat F (%)**	**Crest F (%)**	**Total F (%)**	**Mcnemar (*p* *)**
Reader A	0° vs. 5°	Round	79 (76.7)	0 (0.0%)	0 (0.0%)	79 (76.7%)	2 (0.157)
		Flat	2 (1.9%)	22 (21.4%)	0 (0.0%)	24 (23.3%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	81 (78.6%)	22 (21.4%)	0 (0.0%)	103 (100%)	
	5° vs. 10°	Round	80 (77.7%)	1 (1.0%)	0 (0.0%)	81 (78.6%)	2.667 (0.102)
		Flat	5 (4.9%)	17 (16.5%)	0 (0.0%)	22 (21.4%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	85 (82.5%)	18 (17.5%)	0 (0.0%)	103 (100%)	
	10° vs. 15°	Round	79 (76.7%)	5 (4.9%)	1 (0.0%)	85 (82.5%)	3.667 (0.160)
		Flat	1 (1.0%)	17 (16.5%)	0 (0.0%)	18 (17.5%)	
		Crest	1 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	80 (77.7%)	22 (21.4%)	1 (1.0%)	103 (100%)	
	15° vs. 20°	Round	47 (45.6%)	1 (1.0%)	32 (31.1%)	80 (77.7%)	43 (<0.001)
		Flat	0 (0.0%)	12 (11.7%)	10 (9.7%)	22 (21.4%)	
		Crest	0 (0.0%)	0 (0.0%)	1 (1.0%)	1 (1.0%)	
		Total F (%)	47 (45.6%)	13 (12.6%)	43 (41.7%)	103 (100%)	

Reader A						
	Shape metatarsal head	Beam angle	Round F (%)	Flat F (%)	Crest F (%)	Total F (%)	Mcnemar (*p* *)
	20° vs. 25°	Round	7 (6.8%)	0 (0.0%)	40 (38.8%)	47 (45.6%)	50 (<0.001)
		Flat	1 (1.0%)	3 (2.9%)	9 (8.7%)	13 (12.6%)	
		Crest	0 (0.0%)	0 (0.0%)	43 (41.7%)	43 (41.7%)	
		Total F (%)	8 (7.8%)	3 (2.9%)	92 (89.3%)	103 (100%)	
	25° vs. 30°	Round	2 (1.9%)	0 (0.0%)	6 (5.8%)	8 (7.8%)	7 (0.030)
		Flat	0 (0.0%)	0 (0.0%)	3 (2.9%)	3 (2.9%)	
		Crest	0 (0.0%)	1 (1.0%)	91 (88.3%)	92 (89.3%)	
		Total F (%)	2 (1.9%)	1 (1.0%)	100 (97.5%)	103 (100%)	

Abbreviation: F, frequency.

*The McNemar Bowker test was used to compare the relative prevalence of the different grades and is given as *p*-values.

Table 31.3 Intraobserver B agreement about shape first metatarsal head in beam angle: 0° vs. 5°, 5° vs. 10°, 10° vs. 15°, 15° vs. 20°, 20° vs. 25°, 25° vs. 30°

Reader B							
	Shape metatarsal head	Beam angle	Round F (%)	Flat F (%)	Crest F (%)	Total F (%)	Mcnemar (p^*)
Reader B	0° vs. 5°	Round	72 (69.9%)	0 (0.0%)	0 (0.0%)	72 (69.9%)	2 (0.157)
		Flat	2 (1.9%)	29 (28.2%)	0 (0.0%)	31 (30.1%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	74 (71.8%)	29 (28.2%)	0 (0.0%)	103 (100%)	
	5° vs. 10°	Round	74 (71.8%)	0 (0.0%)	0 (0.0%)	74 (71.8%)	1 (0.317)
		Flat	1 (1.0%)	28 (27.2%)	0 (0.0%)	29 (28.2%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
		Total F (%)	75 (72.8%)	28 (27.2%)	0 (0.0%)	103 (100%)	
	10° vs. 15°	Round	73 (70.9%)	2 (1.9%)	0 (0.0%)	75 (72.8%)	0.667 (0.414)
		Flat	4 (3.9%)	24 (23.3%)	0 (0.0%)	28 (27.2%)	
		Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.0%)	
		Total F (%)	77 (74.8%)	26 (25.2%)	0 (0.0%)	103 (100%)	

Reader B						
Shape metatarsal head	Beam angle	Round F (%)	Flat F (%)	Crest F (%)	Total F (%)	Mcnemar (p*)
15° vs. 20°	Round	41 (39.8%)	3 (2.9%)	33 (32.0%)	77 (74.8%)	39 (<0.001)
	Flat	0 (0.0%)	22 (21.4%)	4 (3.9%)	26 (25.2%)	
	Crest	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
	Total F (%)	41 (39.8%)	25 (24.3%)	37 (35.9%)	103 (100%)	
20° vs. 25°	Round	10 (9.7%)	4 (3.9%)	27 (26.2%)	41 (39.8%)	52 (<0.001)
	Flat	0 (0.0%)	4 (3.9%)	21 (20.4%)	25 (24.3%)	
	Crest	0 (0.0%)	0 (0.0%)	37 (35.9%)	37 (35.9%)	
	Total F (%)	10 (9.7%)	8 (7.8%)	85 (82.5%)	103 (100%)	
25° vs. 30°	Round	2 (1.9%)	0 (0.0%)	8 (7.8%)	10 (9.7%)	14 (<0.001)
	Flat	0 (0.0%)	2 (1.9%)	6 (5.8%)	8 (7.8%)	
	Crest	0 (0.0%)	0 (0.0%)	85 (82.5%)	85 (82.5%)	
	Total F (%)	2 (1.9%)	2 (1.9%)	99 (96.1%)	103 (100%)	

Abbreviation: F, frequency.

*The McNemar Bowker test was used to compare the relative prevalence of the different grades and is given as p-values.

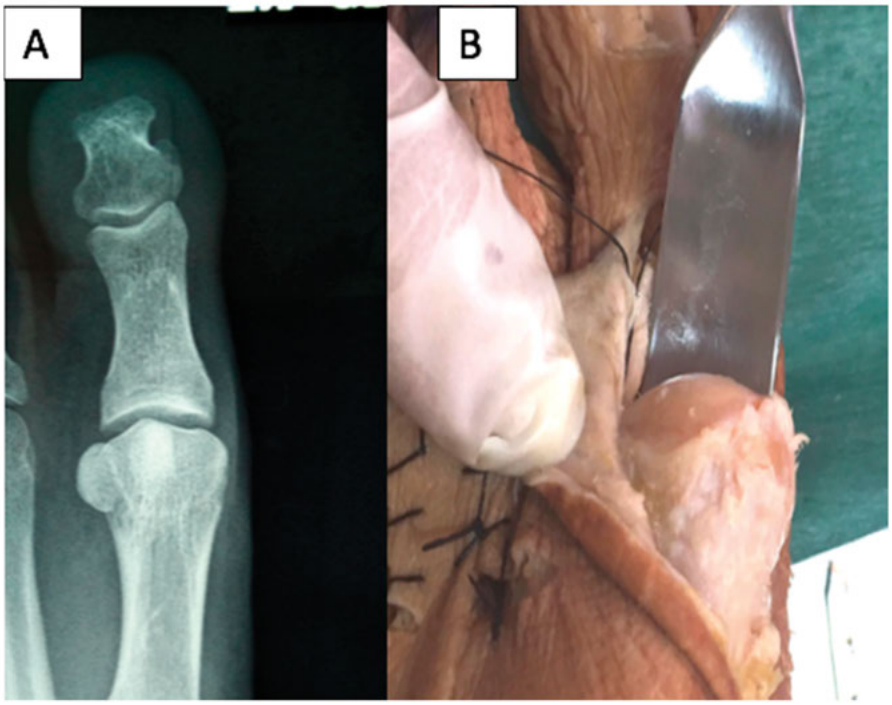

Figure 31.2 Views of a first metatarsal head showing distortion to appear crest shaped in a radiographic image performed to 30° (A) and after dissection revealing a round shape (B).

Instead, if the first metatarsal bone is dorsiflexed as a flat foot, the first metatarsal angle declination is lower, and the angle between the X-ray beam and the axis of the first metatarsal bone is a higher, thus maximizing the distortion of the first metatarsal head.

In light of these findings, it seems necessary to control the beam angulation to 5–10° in dorsoplantar X-rays of the flat loading foot, to avoid the presence of the crested or flat shape, which are artifacts produced by the angulation of the tube.

31.5 Conclusions

All of the articles that we identified state that there are three types of shapes of the first metatarsal head, and all authors relate each type of head to the diagnosis of a foot pathology, such as hallux valgus or hallux rigidus. This study demonstrates that there is only a round shape, and not three types of metatarsal head shape, and therefore, no diagnoses related to the shape of the first metatarsal head can be made.

A clinician should be aware that in patients with flat feet, dorsoplantar radiograph with weight projection should be taken at a beam angle of 5° to 10°.

Disclosures and Conflict of Interest

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Chapter 32

Clinical Aspects and Current Therapeutic Approaches for Fibrodysplasia Ossificans Progressiva

Hiroshi Kitoh, MD, PhD

Department of Orthopaedic Surgery, Aichi Children's Health and Medical Center, Obu, Aichi, Japan
Department of Comprehensive Pediatric Medicine, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan

hkitoh420@gmail.com, hiroshi_kitou@sk00106.achmc.pref.aichi.jp

Keywords: activin A receptor type I (*ACVR1*), activin-like kinase 2 (*ALK2*), aggressive juvenile fibromatosis, bone morphogenetic protein (BMP), bone morphogenetic protein receptors (BMPR), calcaneal ossifications, chondrogenesis, cyclooxygenase-2 (COX2), fibrodysplasia ossificans progressiva (FOP), fibromatosis, filamin B gene (*FLNB*), first metacarpal bone, gain-of-function mutations, garetosmab, heparan sulfate proteoglycans (HSPGs), heterotopic ossification, hypertrophic spinous processes, imatinib, mesenchymal condensations, non-steroidal anti-inflammatory drugs (NSAIDs), osseous fusion, parathyroid hormone related peptide (PTHrP), parovarotene, rapamycin, saracatinib, sarcoma

32.1 Introduction

Fibrodysplasia ossificans progressiva (FOP) is a severely disabling heritable disorder of connective tissues characterized by progressive heterotopic ossification in the skeletal muscles, ligaments, tendons, fascia, and aponeuroses, and malformations of the great toes. Painful recurrent episodes of soft tissue swelling (flare-ups) precede to heterotopic ossification. Flare-ups usually begin in the first decade of life, and several patients with FOP are misdiagnosed as having soft tissue tumors or aggressive fibromatosis before the appearance of heterotopic ossification [1]. They sometimes undergo dangerous and unnecessary diagnostic procedures that provoke heterotopic ossification formation leading to permanent harm and lifelong disability [2]. Early clinical diagnosis and confirmatory genetic testing of FOP are extremely important to prevent additional iatrogenic harm or trauma [3].

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Heterotopic ossification throughout the body is progressive, and patient's disabilities are cumulative [4]. Currently, there are no definitive treatments for FOP; however, there has been substantial recent interest in clinical trials for novel treatments for this specific disease. In this review, we specifically describe various skeletal manifestations suggestive of FOP that can usually be seen before the appearance of heterotopic ossification, to make clinicians aware of these early signs and symptoms of FOP. We also discuss current therapeutic approaches for FOP based on molecular mechanisms of this disease, especially focusing on pharmacological drugs that are currently on-going clinical trials to evaluate their efficacy in FOP patients. Patients' data presentation including photographs were approved by the ethical committee from the author's institution.

32.2 Epidemiology

The worldwide prevalence of FOP is reported to be approximately one in 2 million individuals, with no ethnic, racial, or geographical predisposition [5]. Autosomal dominant transmission with complete penetrance is established, but most cases arise as a result of a spontaneous new mutation [6]. Both genetic and environmental factors affect the phenotype of FOP. A study of monozygotic twins demonstrated that congenital toe malformations were similar within the siblings, but progression of heterotopic ossification varied greatly, suggesting that genetic factors seem to correlate to prenatal development while environmental factors strongly influence postnatal progression of heterotopic ossification [7].

32.3 Pathophysiology

FOP is caused by gain-of-function mutations in the gene encoding activin A receptor type I (*ACVR1*)/activin-like kinase 2 (*ALK2*), a bone morphogenetic protein (BMP) type I receptor [8]. Approximately 97% of individuals with FOP carry the recurrent activating mutation (617G>A, R206H) in the *ACVR1*/*ALK2* gene, causing the substitution of a conserved residue in the GS domain of the protein. There are a limited number of patients with other rare mutations in the same gene that may show the unusual clinical features for FOP (FOP variants), most notably greater or lesser severity of the great toe malformations [9, 10]. BMPs induce heterotopic bone formation in skeletal muscle *in vivo* and initiate the differentiation pathway through which myoblasts convert to osteoblastic cells *in vitro* [11]. BMP receptors (BMPR) belongs to the TGF-β superfamily, and the BMP signaling is initiated with an heteromeric receptor complex consisting of type I (BMPR-I) and type II receptors (BMPR-II). The BMPR-II activates the BMPR-I by transphosphorylating their GS domain, leading to intracellular signaling pathway through phosphorylated SMADs proteins. The mutated *ACVR1* receptor may be constitutive active leading to aberrant signaling through the kinase receptor and overactivation of the downstream SMAD1/5/8 signaling pathway. In addition, mutations appear to change the signaling specificity of the *ACVR1* receptor.

The mutated receptor is hyper-responsive to BMP ligands as well as responsive to non-osteogenic ligand, Activin A. Activin A can bind to the mutant *ACVR1* receptor and activate signaling through the SMAD1/5/8 pathway, although it does not activate SMAD signaling when it binds to wild type *ACVR1* receptors [12, 13]. Dysregulation of BMP signaling pathway is thought to trigger the formation of the ectopic chondrogenesis, osteogenesis and joint fusion of FOP [14]. To date, all *ACVR1* mutations evaluated for enhanced BMP signaling are gain-of-function mutations [9, 15].

32.4 Natural Clinical Course

Heterotopic ossification in FOP typically begins to form during the first decade of life, with sporadic episodes of flare-ups in the axial skeleton, which are sometimes misdiagnosed as having soft-tissue sarcoma or aggressive juvenile fibromatosis (Fig. 32.1). Flare-ups may occur following a localized invasion mechanism such as trauma, intramuscular injections that lead to bruising, and are occasionally accompanied by sensations of warmth and pain. Traumatic injury and surgical intervention induce explosive new bone formation in FOP. Flare-ups also occur without any causative factor and may even be provoked by systemic inflammation from viral infections such as influenza. Heterotopic ossification progresses in characteristic anatomic and emporal patterns, typically first occurring in the axial, cranial, and proximal regions of the body and later in the appendicular, caudal, and distal regions [16].

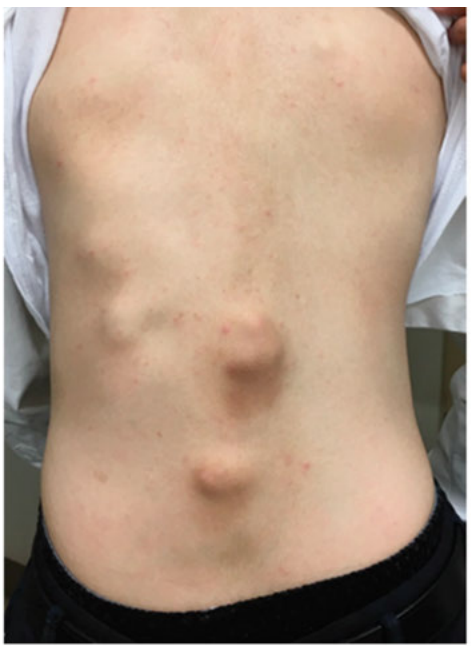

Figure 32.1 Sixteen-year-old male with fibrodysplasia ossificans progressiva (FOP) demonstrating numerous soft tissue indurations in his back.

Progressive heterotopic ossification throughout the body leads to deformities in the trunk and joint contractures in the extremities (Fig. 32.2). Oddly, the disease seems to spare some anatomical locations, including the ocular muscle, tongue, cardiac muscle, and diaphragm. Arm function reflects early decreases in the activity of daily living [17]. The process of heterotopic ossification is highly individualized. Systemic ankyloses result in difficulty in walking and respiratory dysfunction as the disease progresses in some patients. The patient's age is correlated with functional disability evaluated by patient reports, as well as the volume of heterotopic ossifications [18]. Most patients are confined to a wheelchair by the third decade of life, and require lifelong assistance in performing activities of daily living [19]. Heterotopic ossification in the temporomandibular joint and surrounding areas often results in trismus which interferes with eating and leads to severe weight loss. Heterotopic ossification of the spine and thoracic cage may cause rigid fixation of the chest wall and respiratory dysfunction. The median age at death is approximately 40 years, but the median estimated life expectancy is 56 years [20]. Death often results from complications of thoracic insufficiency syndrome or pneumonia [21]. The overall prognosis for this disease is considered poor.

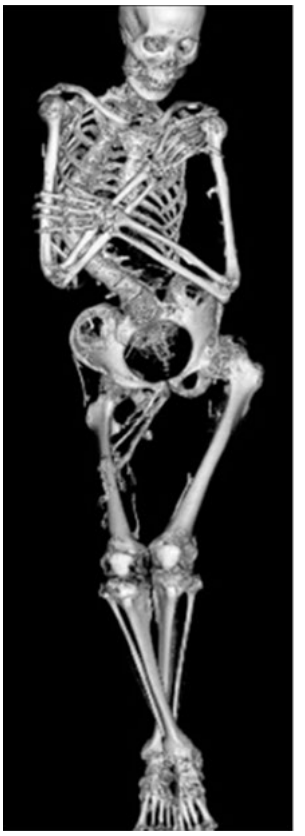

Figure 32.2 Whole body computed tomography imaging of 26-year-old male with FOP showing severe rigid scoliosis and bilateral hip joint contractures due to heterotopic ossifications across the joints.

32.5 Skeletal Malformations

Individuals with FOP appear normal at birth, but there are a variety of congenital skeletal malformations. Deformities of the great toes are well-known and are the most prevalent indicators of this disorder. A shortened great toe and hallux valgus are characteristically found before the appearance of heterotopic ossification. The tip of the great toe usually locates proximal to the distal interphalangeal joint of the second toe. The degree of hallux valgus and shortening of the great toe varies among the feet in gross findings (Fig. 32.3). Radiologically, the proximal phalanx is consistently shortened and sometimes shows triangular shape. The metatarsal bone is also shortened and sharpened to the medial side, deviating the proximal phalanx laterally from the metatarsal axis [22]. Fusion between the proximal and distal phalanx is observed with advancing age (Fig. 32.4). Although the common *ACVR1* mutation (R206H) shows a homogeneous phenotype of the great toes, several atypical mutations have been identified in patients who showed normal-appearing great toes or severe truncation deformities of digits [9, 23, 24]. BMPs exert an anti-chondrogenic effect on early limb bud mesenchymal cells [25]. The differences in genotype of the *ACVR1* may be related to the strength of the anti-chondrogenic effect on condensing mesenchymal cells via BMP signaling, leading to variety of phenotype in great toes.

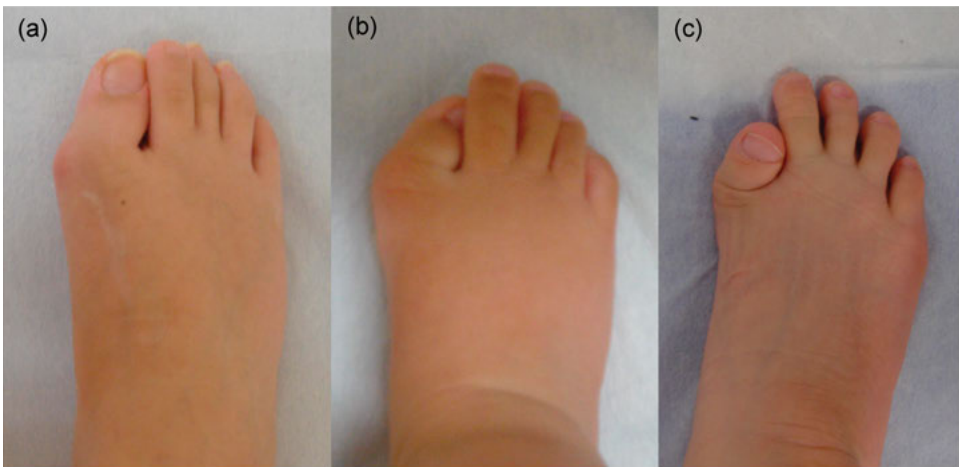

Figure 32.3 Gross appearance of the right foot in FOP patients from 18-year-old female (a), 10-month-old male (b), and 7-year-old male (c). The degree of hallux valgus and shortening of the great toes are variable.

Stiffness of the neck is an important early clinical sign of FOP in infants and it can precede the appearance of heterotopic ossification at that site. Crawling is often disturbed due to limited neck extension. Radiologically, enlarged posterior elements of the cervical spine, including pedicles, laminae, and spinous processes, are characteristic. Vertebral bodies are tall and narrow [26, 27]. The cervical spine often becomes ankylosed resulting from fusion of the facet joints early in life (Fig. 32.5). Using the chick embryos, genetically-engineered overexpression

of BMP2/4 both dorsally and laterally to the neural tube manifested combined phenotypes of hypertrophic spinous processes and large deletion of the lateral and ventral parts of vertebral bodies [28]. Mesenchymal condensations at the paraxial mesoderm in FOP, where BMP signaling is aberrantly activating, could be responsible for both enlarged spinous processes and relatively tall vertebral bodies.

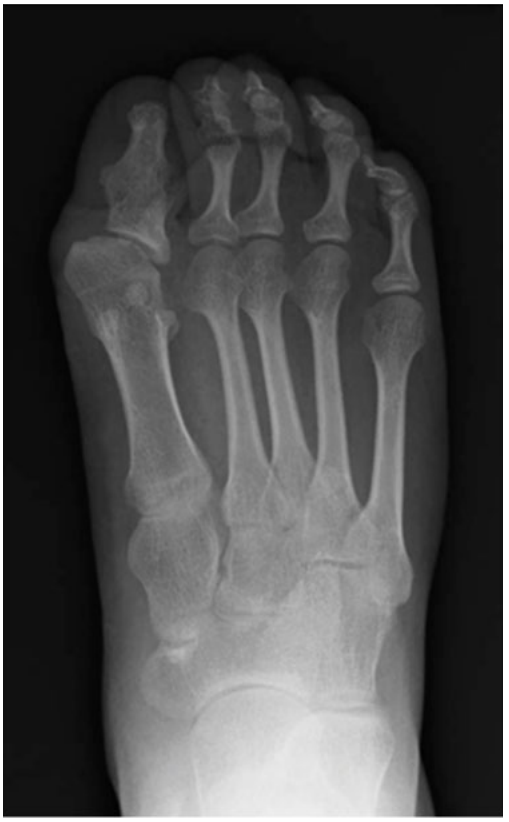

Figure 32.4 Anteroposterior radiograph of the right foot of 20-year-old female with FOP demonstrating medially deviated metatarsal bone and fused proximal and distal phalanges.

Short thumb is another clinical feature of FOP. It is mainly due to shortening of the first metacarpal bones. Quantitative radiological examinations demonstrated shortened distal phalanx relative to the second metacarpal bone and disproportionate shortening of the first metacarpal bone [29] (Fig. 32.6). The thumb is the last digit in the autopod to form, and it is different from other digits in terms of its relative position, shape, size, and number of phalanges. These unique thumb identities may be attributed to the expression profile of *HoxD* genes, which are pivotal transcriptional factors regulating limb patterning and growth [30]. *HOXD10* to *D13* genes are expressed in the future digit II-V area in the autopod during the hand plate formation, whereas the sole expression of the *HOXD13* gene in the presumptive digit I area is of great significance [31]. Interestingly, BMP

signaling-dependent SMAD1/4 proteins prevented *HoxD10* and *HoxD13* from binding to DNA targets [32]. Mesenchymal condensation and chondrocyte proliferation of the presumptive thumb area could be regulated by direct interactions between BMP-induced *SMADs* and *HoxD13*. Dysregulated BMP signal transduction during embryogenesis may cause the relative shortening of the first metacarpals and distal phalanges of the thumb in FOP.

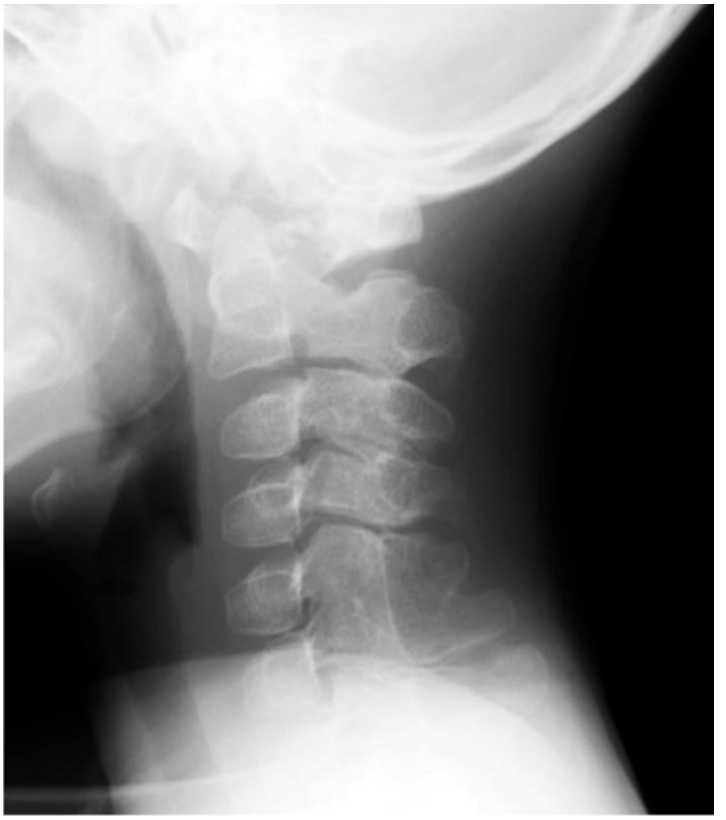

Figure 32.5 Lateral radiograph of the cervical spine of a FOP boy at the age of 7 years showing hypertrophy of the laminae and spinous processes and complete osseous fusions in facet joints and spinous processes between C5 and C6.

We have demonstrated distinctive multiple ossification centers and plantar spurs in the calcaneus in some FOP infants [33]. These findings were bilateral and symmetrical. Multiple (or punctate) calcaneal ossifications are seen in early infancy, which evolved into double ossifications and finally completely coalesced with age (Fig. 32.7). Similar duplicate calcaneus is observed in an infant with Larsen syndrome, which is caused by heterozygous mutations in the filamin B gene (*FLNB*). *FLNB* mutant mice display ectopic mineralization in various cartilaginous elements, but those on a *Runx2* haploinsufficiency background show a completely or partially rescued phenotype, indicating mutated *FLNB* interacts with Runx2-TGFβ-SMADs pathway [34]. Molecular interactions between *FLNB* and *SMADs*

signaling in skeletal morphogenesis may lead to similar phenotypes of ossifications in the calcaneal region in Larsen syndrome and FOP. Calcaneal spurs are pedunculated and projected posteriorly in early infancy, and they become sessile and finally smaller in size with age. The normal calcaneal spur is morphologically indistinguishable from the late manifestation of the calcaneal spur in FOP, but the early pedunculated appearance in FOP is not seen in the normal spur.

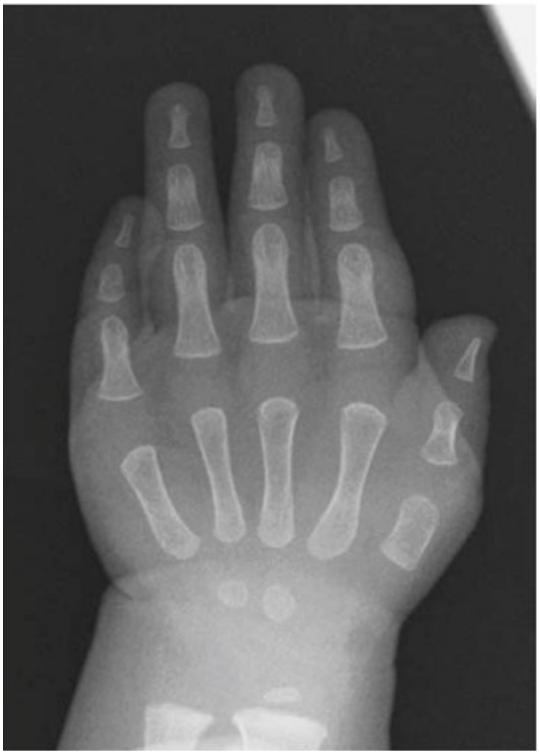

Figure 32.6 Anteroposterior radiograph of the left hand of a FOP boy at the age of 11 months demonstrating marked shortening of the first metacarpal bone.

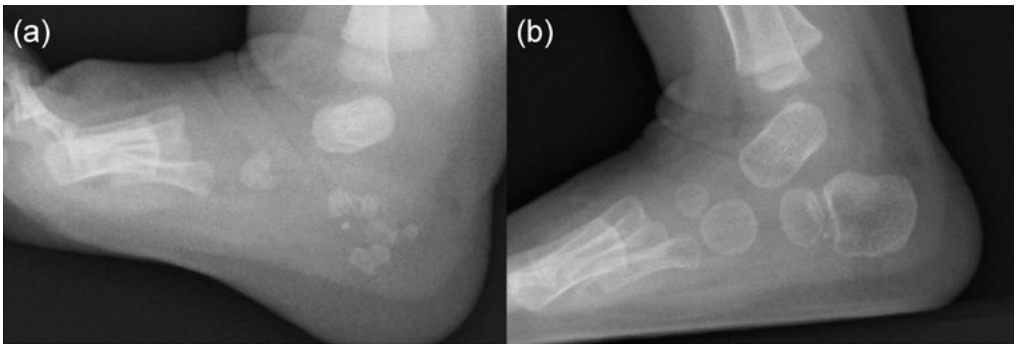

Figure 32.7 Lateral foot radiographs of a FOP boy at 3 months of age (a) and 11 months of age (b). Punctate multiple ossifications in early infancy gradually changed to double ossification centers.

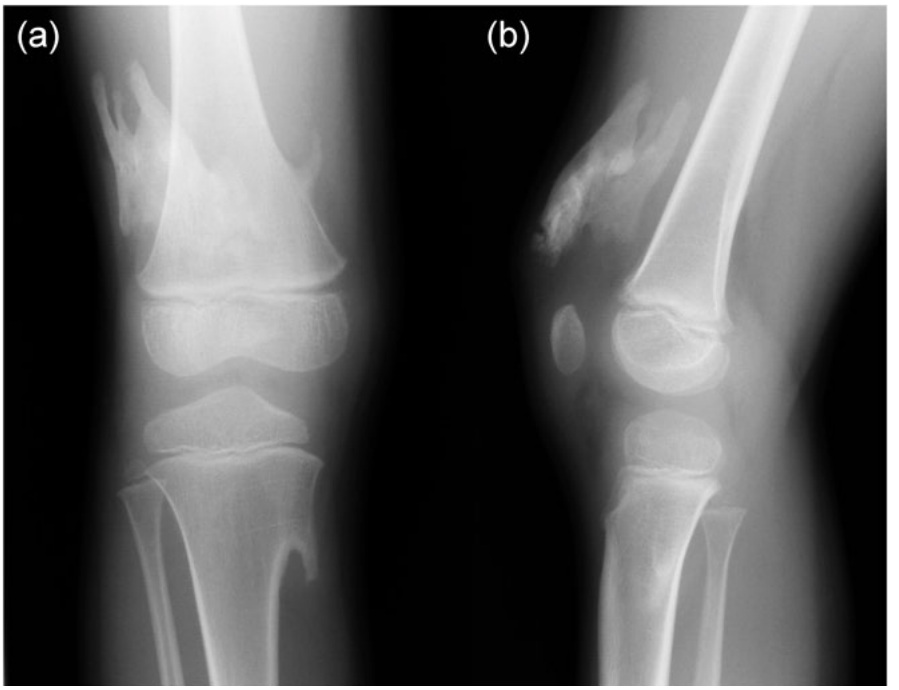

Figure 32.8 Anteroposterior (a) and lateral (b) knee radiographs of the FOP boy at the age of 5 years showing heterotopic ossification at the distal thigh and osteochondroma-like lesions of the distal femur and proximal tibia.

Broad femoral necks with metaphyseal widening and osteochondroma-like lesions in the metaphysis of the of the long bones are also early findings in FOP infants. In addition to osteochondroma-like lesions, heterotopic ossification around the knee should not be misdiagnosed as soft tissue tumor (Fig. 32.8). Osteochondroma-like lesions are commonly observed in multiple hereditary exostoses, which is caused by mutations in *EXT1*, *EXT2*, or *EXT3* genes, encoding tumor suppressors and glycosyltransferases involved in the biosynthesis of heparan sulfate proteoglycans (HSPGs) [35]. HSPGs bind to and modulate the activity of Indian hedgehog (Ihh), which is expressed in prehypertrophic chondrocytes and regulates chondrocyte maturation, and the abnormal modulation of the tightly regulated Ihh/parathyroid hormone related peptide (PTHrP)-negative feedback loop has been proposed as a molecular model of osteochondroma formation in multiple hereditary exostoses [36]. Constitutive active *ACVR1* R206H mutation resulted in dramatic upregulation of Ihh at the perichondrium and a delay in chondrocyte differentiation in a chicken limb bud model [37]. Thus, osteochondroma formation in FOP could be mediated by disruption of the BMP/Ihh/PTHrP-negative feedback loop at the perichondrium. These skeletal abnormalities suspicious of FOP in infants can lead to early clinical diagnosis, confirmatory diagnostic genetic testing, and the avoidance of iatrogenic harm or trauma.

32.6 Managements and Treatments

There is presently no definitive medical treatment to prevent, stop or reverse heterotopic ossification in FOP. Avoidance of trauma and prevention of injury remain the mainstays of therapy. Surgical removal of heterotopic ossification often leads to significant recurrence and expansion of ossification. Bracing for spinal deformity is ineffective [38]. Restriction of activity may be helpful to reduce trauma, but compromise of independence may be unacceptable to patients as well as their parents. Physical rehabilitation to maintain joint mobility may be harmful by provoking or exacerbating lesions and it should be focused on enhancing activity of daily living through approaches that avoid a passive range of motion exercises. Occupational therapy and vocational education consultations may be useful. Overstretching of the jaw and intramuscular injections of local anesthesia should not be attempted in dental care. A locked jaw sometimes necessitates surgery to avoid life threatening complications. Since conductive hearing loss is common, children should have audiology evaluations regularly. The management of FOP requires education of patients and caregivers, the use of medications to settle inflammation and flare-ups, instructions to ensure proper oral care, and other compensatory approaches that aid in rehabilitation [39].

The use of short-term high-dose corticosteroids is based on its potent anti-inflammatory effects [40]. It may help reduce the intense inflammation and tissue edema when they are used in an early stage of flare-ups. They can relieve but not completely resolve symptoms of flare-ups [41]. Corticosteroids are most effective if used within the first 24 h of a new flare-up. The dose of corticosteroids is dependent on body weight, and a recommended dose of prednisone for acute flare-ups is 2 mg/kg/day, administered as a single daily dose for no more than 4 days. Corticosteroids should be used for treatment of flare-ups that affect major joints, the jaw, or the submandibular area, and should not be used for flare-ups that involve the back, neck, or trunk due to the long duration and recurring nature of these flare-ups. Corticosteroids should not be used for long-term, and when prednisone is discontinued, non-steroidal anti-inflammatory drugs (NSAIDs) or selective cyclooxygenase-2 (COX2) inhibitors may be used for the duration of flare-ups, although there is no evidence that chronic treatment with these drugs prevent flare-ups in FOP. Bisphosphonates have been used for the symptomatic management of flare-ups in FOP, although concrete clinical data for these treatments are sparse [42]. Mast cells could provide an important role for the pathology of heterotopic ossification in FOP [43]. Imatinib, a tyrosine kinase inhibitor initially developed for chronic myeloid leukemia, has anti-proliferative and immunomodulatory effects in mast cells. The administration of imatinib demonstrated positive effects on decrease in the intensity of flare-ups in seven FOP patients who did not respond the standard medications such as corticosteroids, NSAIDs, or intravenous bisphosphonates [44].

32.7 On-Going Clinical Trials for FOP (Phase 2 or Phase 3)

Several researches to develop therapeutic drugs have focused on target inhibition of the *ACVR1* receptor, *ACVR1* ligand, BMP intracellular signaling, and inflammatory triggers of disease activity. Exciting advances in new therapeutic approaches for FOP have developed recently [45, 46, 47]. We highlight novel treatment drugs that are currently on-going phase 2 or phase 3 clinical trials (Fig. 32.9).

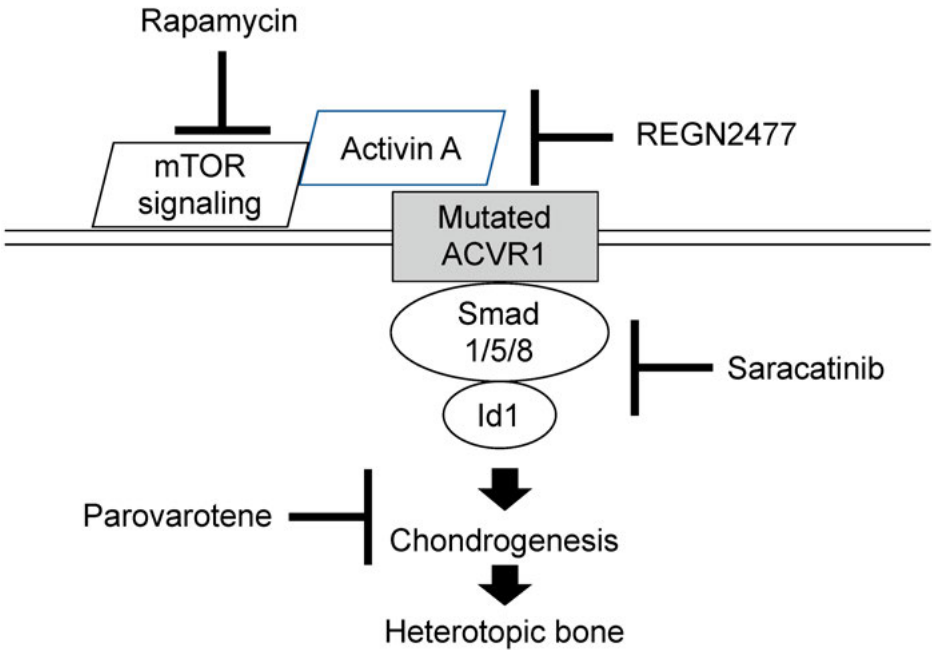

Figure 32.9 Molecular targeting of therapeutic drugs on on-going phase 2 or 3 clinical trials.

Retinoid signaling is normally attenuated during chondrogenesis and exogenous retinoid agonists can block chondrogenesis effectively and rapidly [48]. Agonists for retinoic acid receptors (PARα or RARγ) experimentally inhibited chondrogenesis of heterotopic ossification in transgenic mice model of FOP, and the RARγ agonists were far more effective [49]. One of the RARγ class drugs is palovarotene, a highly specific RARγ agonist that has already been evaluated in another clinical trial for α-1-antitrypsin-induced emphysema, and its safety profile has been well-characterized. Palovarotene inhibits heterotopic ossification and maintains limb mobility and growth in mice model of FOP [50]. Palovarotene is also evaluated in another phase 2 trials for treatment of hereditary multiple exostoses to suppress the formation of osteochondromas. Phase 2 clinical trials were initiated in 2014 by Clementia Pharmaceuticals to evaluate the safety and efficacy of palovarotene for treatment of FOP (Clinicaltrials.gov registration NCT02190747). The primary outcome was to compare the volume of heterotopic

ossification formation between treated patients and untreated patients. Palovarotene decreased the percentage of FOP patients who develop heterotopic ossification, the time to flare-ups resolution, and patient-reported pain. Phase 3 trial is currently in progress (Clinicaltrials.gov registration NCT03312634). Palovarotene is a known teratogen that causes limb malformations in the developing fetus and may decline growth in children [51]. Other potential risks of palovarotene include pancreatitis, hearing and vision impairment, mouth ulcer, sensitivity to sunlight, and dry skin. These adverse events are being monitored closely during the trials.

The R206H mutation causes the *ACVR1* receptor to misinterpret activin A and generate a signal as if BMP ligands are present [12]. The *ACVR1* mutant mice developed more heterotopic ossification throughout the skeleton when activin A was injected, and those treated with a blocking antibody of activin A did not develop heterotopic ossification [48]. Activin A is, thus, an obligatory secreted factor that is required for the initiation of heterotopic ossification in FOP, and the blocking of activin A could prevent the formation of heterotopic bone. As a result of preclinical studies, REGN 2477 (garetosmab)—an antibody that binds to activin A and blocks its activity—is now in a clinical trial to examine safety, tolerability, and efficacy on abnormal bone formation in adult patients with FOP (Clinicaltrials.gov registration NCT03188666).

Activin A enhances the chondrogenesis of induced mesenchymal stromal cells derived from FOP patients-derived induced pluripotent stem cells (FOP-iPSCs) via the aberrant activation of BMP signaling *in vitro*, and induced endochondral ossification of FOP-iPSCs *in vivo* [52]. By using a high-throughput screening system of small molecules to suppress activin A induced chondrogenesis, Hino et al. demonstrated that mTOR signaling is a critical pathway for the aberrant chondrogenesis of mesenchymal stromal cells derived from FOP-iPSCs and inhibited the heterotopic ossification of multiple model mice, including FOP-ACVR1 transgenic mice and a heterotopic ossification model utilizing FOP-iPSCs [53]. Rapamycin is a commonly-used immunosuppressant that exerts its biological effect by inhibiting mTOR1 kinase activity. Heterotopic ossification was decreased after treatment with rapamycin in mice model of FOP as well as FOP-iPSC-based heterotopic ossification model mice [54]. A phase 2 clinical trial for a 6-month randomized placebo-controlled study and subsequent open label extension study is now opening in Japan (UMIN000028429). Primary endpoint for evaluating the efficacy of rapamycin is based on objective physical function assessment using the Japanese version of Health Assessment Questionnaire or Childhood Health Assessment Questionnaire.

Saracatinib, also known as AZD0530, is an investigational drug that was initially developed as a potential treatment for patients with cancer. Saracatinib inhibits the serum activation of Id1, which is a transcriptional factor mediated by Smad 1/5/8 phosphorylation, by direct inhibition of BMPR-I kinase activity [55]. Other research also demonstrated that Saracatinib was effective at suppressing the enhanced chondrogenesis of FOP-iPSCs and suppressed the heterotopic

ossification or bone formation in multiple FOP animal models [49]. A phase 2A proof of concept study including a 6-month randomized placebo-controlled study and 12-month open label extension study using historical data is proposed in the Netherlands, the United Kingdom, and Germany (Clinicaltrials.gov registration NCT04307953).

32.8 Conclusions

Clinicians should become aware of early detectable skeletal malformations, including great toe deformities, shortened thumb, neck stiffness associated with hypertrophy of the posterior elements of the cervical spine, multiple ossification centers in the calcaneus, and osteochondroma-like lesions of the long bones, to make an early diagnosis and prevent iatrogenic harm or trauma. Although there is presently no definitive medical treatment to prevent, stop or reverse heterotopic ossification in FOP, exciting advances in novel therapeutic approaches using pharmacological drugs, including palovarotene, REGN 2477, rapamycin, and saracatinib, have been developed and are currently in clinical trials.

Abbreviations

ACVR1: activin A receptor type I
ALK2: activin-like kinase 2
BMP: bone morphogenetic protein
BMPR: BMP receptors
COX2: cyclooxygenase-2
FLNB: filamin B gene
FOP: fibrodysplasia ossificans progressiva
FOP-iPSCs: FOP patients-derived induced pluripotent stem cells
HSPGs: heparan sulfate proteoglycans
Ihh: Indian hedgehog
NSAIDs: non-steroidal anti-inflammatory drugs
PTHrP: parathyroid hormone related peptide

Disclosures and Conflict of Interest

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Chapter 33

Comparison and Lessons Learned from Neglected Tropical Diseases and Tuberculosis

Alice Wang, PhD, Adam MacNeil, MPH, PhD, and Susan Maloney, MD, MHSc

Division of Global HIV & Tuberculosis, Center for Global Health,
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

ilm1@cdc.gov

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33.1 Introduction

The World Health Organization (WHO) has identified 20 core diseases as neglected tropical diseases (NTDs), comprising primarily viral, protozoan, helminthic, and bacterial infections, as well as zoonotic or vector-borne diseases [1]. There are common features that define this group of diverse diseases [2–5]. Among the common features are that the NTDs are a proxy for poverty, often affect populations with low visibility, cause stigma and discrimination, and can be controlled, prevented, and possibly eliminated using known solutions [6]. NTDs share a heavy underlying burden of disease—both in the number of cases and the impact on the population affected, including significant economic costs for households and communities [1, 5]. Often NTDs arise in impoverished settings with poor hygiene and sanitation [3]. NTDs impair physical and cognitive development, limiting productivity and making it difficult to earn a living, thereby

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perpetuating a cycle of poverty and disease [3, 7]. Finally, NTDs are defined by neglect. Multiple interventions, when applied, have shown successful outcomes to decrease morbidity and mortality rates, yet funding gaps, competing priorities, and lack of political will allow NTDs to persist [2, 3].

Although tuberculosis (TB) incidence has dramatically decreased since the 18th and 19th centuries, the rise of the HIV/AIDS epidemic and drug-resistant strains of TB led the WHO to declare TB a global health emergency in 1993 [8]. Globally, an estimated 10 million people had TB disease in 2019; however, only 71% of these cases were notified to WHO, resulting in approximately 2.9 million missed TB cases (either undiagnosed or not reported) [9]. Without adequate diagnosis and effective treatment, the mortality rate from TB is high [10]. In 2019, TB resulted in 1.2 million deaths among HIV-negative people and an additional 208,000 deaths among HIV-positive people [9]. Despite being curable and preventable, TB is currently the leading cause of death from a single infectious agent and accounts for over one-third of all HIV-related deaths. The goal of this chapter is to elevate awareness of TB within the framework of NTDs and gain insights from successes in addressing NTDs and how these lessons can be applied to help global health programs change the trajectory of the TB epidemic.

33.2 Underlying Burden of Disease

Like NTDs, TB results not only in high morbidity but also in associated economic cost, side effects of treatment, and personal stigma and isolation. Approximately 1.7 billion individuals have latent TB infection (25% of the global population) [11]. About 5–10% of those with latent infection develop active TB during their lifetime, with differences in risk based on population. For example, the risk of TB development is much higher for children exposed to TB compared to adults, additionally for people living with untreated HIV, the annual risk of active TB disease is approximately 3–16% [12]. Globally in 2019, 1.2 million people died of TB [9]. In addition to the lungs, TB can affect many other organ systems causing morbidity. Globally in 2018, the case fatality ratio of TB was 15%; however, if left untreated, in a 10-year time horizon, TB has a mortality rate of about 70% of patients with acid-fast bacillus (AFB), smear-positive results and 20% for those with AFB smear-negative results [13, 14].

TB and HIV co-infection can act synergistically to increase morbidity and mortality rates [15]. Patients with HIV-associated TB have an increased recurrence rate, sometimes resulting from relapse but more often from re-infection [16]. TB can be difficult to diagnose in HIV-positive patients because they may have atypical radiography, may not be able to produce a diagnostic sputum sample, often have paucibacillary disease, and are more likely to have extrapulmonary TB, which can require invasive specimen collection to confirm. Globally, around 15% of the TB disease burden is due to extrapulmonary TB [17]. However, the burden of extrapulmonary TB among HIV-positive individuals with TB is

40–50% [17]. Additionally persons with TB and HIV co-infection must adhere to complex drug regimens that may interact with each other and potentially have overlapping toxicities [15].

A systematic review found that the total costs for TB patients in low- and middle-income countries ranged from $55 to $8,198 USD, with a median 20% (range, 0–62%) of the total cost for direct medical costs, 20% (range, 0–84%) to direct non-medical costs, and 60% (range, 16–94%) to income loss; half of the total cost is incurred before TB treatment initiation [18]. This systematic review also found that on average, the cost incurred was equivalent to 39% of reported household income, exceeding the threshold of 20% of the household annual income that defines catastrophic total costs due to TB [18, 19]. Diagnosis and treatment costs for multidrug-resistant (MDR) TB (resistant to isoniazid and rifampicin) and extensively drug-resistant (XDR) TB (MDR TB with additional resistance to fluoroquinolones and aminoglycosides) are considerably higher than treatment cost for drug-susceptible TB. Clearly, there is a heavy economic burden on TB patients and their families. The End TB Strategy, the WHO's global strategy with associated targets for ending TB globally, set a milestone for zero affected families facing catastrophic costs due to TB by 2020; this milestone was not met [20].

In addition to the cost burden of TB disease, the treatment itself can be a burden. The recommended regimen for drug-sensitive TB of isoniazid and rifampicin for 6 months together with pyrazinamide and ethambutol for the first 2 months, is generally well tolerated, although the reported incidence of drug-induced liver injury, which can be caused by rifampicin, isoniazid, or pyrazinamide, varies from 5% to 33% [21, 22]. Globally, 3.3% of new TB cases and 17.7% of previously treated cases had MDR or rifampin-resistant TB [9]. Compared to drug-susceptible TB, MDR TB and XDR TB require longer and more toxic treatments. Adverse events include renal failure, hypokalemia, hypomagnesaemia, polyneuropathy, anemia, and hearing loss [10]. By the end of 2017, 127 WHO Member States have reported XDR TB cases [14]. Treatment of MDR and XDR TB is complicated, and adverse drug reactions are common. Newer drugs, shorter regimens, and all-oral MDR regimens are improving treatment outcomes, but these drugs are costly and associated with adverse effects.

In addition to the symptoms, side effects of treatment, and costs associated with TB, many patients with TB face isolation and stigma. Understanding the infectious nature of TB and associating oneself as a disease vector lead to feelings of guilt and physical and emotional isolation [23]. Many patients have reported TB-related stigma similar to that faced by HIV-positive patients [23]. In audiovisual interviews, patients with MDR and XDR TB expressed their experiences facing stigma, stating that the disease "limits you daily" and "makes your world smaller" and feeling isolated: "I am living in a box, watching the world pass me by" [10]. When looking at how TB burden has influenced disability-adjusted life-years (DALYs), the Global Burden of Disease Study in 2016 found that drug-susceptible tuberculosis resulted in 39.9 million DALYs (38.1 million to

41.9 million), multidrug-resistant tuberculosis without extensive drug resistance 3.32 million (2.79 million to 3.91 million), and extensively drug-resistant tuberculosis 369,000 (301,000–445,000) [24]. In comparison, in the same year, for the 10 NTDs defined in the London Declaration (i.e., human African trypanosomiasis, Chagas disease, Guinea worm disease, leprosy, lymphatic filariasis, onchocerciasis, schistosomiasis, soil-transmitted helminths, blinding trachoma, and visceral leishmaniasis) total DALYs were estimated at 9.0 million (5.3 million to 14.5 million) [24].

33.3 Influence of Poverty and Development

Like NTDs, TB has long been defined as a disease of poverty. Globally, distinct patterns of TB disease emerge in relation to poverty and development. In 2019, of the estimated 10 million new cases of TB globally, approximately 5.4% occurred in Europe and the Americas combined, whereas Africa and South-East Asia accounted for 69% [9]. Africa has the highest TB morbidity and mortality rates, and the proportion of drug-resistant TB cases is increasing in Eastern Europe [25]. These regional disparities can be correlated with patterns of poverty and development. An ecological analysis of the incidence of TB and per capita gross domestic product (GDP) found that doubling GDP was associated with a 38.5% decrease in TB incidence [26]. However, even in high-income countries, independent of ethnicity, TB is strongly associated with poverty [27].

Poor nutrition is associated with poverty and TB disease and accelerates TB disease progression. Malnutrition can lead to secondary immunodeficiency, increasing susceptibility to TB infection [28]. The reactivation of latent TB infection may also be related to malnutrition, as chronic disease can lead to wasting in TB patients. Among tuberculin skin test positive U.S. navy recruits, TB risk was nearly four times higher among underweight men [29]. Another study in Norway found that the relative risk of TB among persons in the lowest body mass index (BMI) category was more than five times higher than the highest BMI category, independent of sex, age, and radiographic findings [30]. The link between malnutrition and TB is bi-directional. In patients with TB, reduced appetite, poor nutrient and micronutrient absorption, and altered metabolism can lead to wasting [28, 31].

Poverty is linked with substandard or inadequate housing, along with crowding and poor indoor air quality, which are all drivers for TB disease. Overcrowded housing conditions can increase TB exposure and the probability of transmission. A study across seven First Nations communities in Canada found that an increase in 0.1 persons per room was associated with a 40% increase in the risk of >2 TB cases occurring [32]. Another study looking at household contacts aged <15 years in Thailand found the risk of positive tuberculin skin testing in household contacts increased with household crowding; children living in crowded households were five times more likely to have TB infection [33]. Many studies

have also implicated tobacco smoke and indoor air pollution as risk factors for TB infection, disease, and death [34]. Chronic exposure to tobacco and environmental air pollutants impairs the normal clearance of secretions on the tracheobronchial mucosal surface, which may allow *Mycobacterium tuberculosis* to bypass defense mechanisms [35].

Cultural and social barriers can trap populations disproportionately affected in a cycle of poverty and disease. TB disease is not just a product of poverty but also can lead to poverty as evidenced by the large proportion of TB patients experiencing catastrophic TB treatment costs. People in all age groups are affected by TB, but most TB cases are among working-age adults [36]. Additionally, lack of basic health services or access, poor nutrition, and inadequate living conditions contribute to TB transmission and morbidity and mortality rates. Poverty contributes to the spread of TB, and TB contributes to the persistence of poverty.

33.4 Neglect

A huge obstacle in TB disease reduction and elimination is neglect—both in terms of funding and political will. Although funding for TB prevention, diagnosis, and treatment services has more than doubled since 2006, the Stop TB Partnership's Global Plan to End TB 2016–2020 still estimated a gap of US$3.5 billion in TB funding in 2018 [36]. TB control funding comes from three sources: government (including loans, such as from the World Bank); the Global Fund to fight AIDS, TB, and Malaria (Global Fund); and donor agencies. Global health funding by disease highlights the neglect of TB. For TB, most funding comes from domestic sources, unlike HIV and malaria funding, therefore TB control can be a major burden for low-income countries with relatively small health budgets for competing priorities and diseases [37]. The Global Fund receives and distributes donor money from developed nations and private foundations and is the largest source of TB financing. The Global Fund disburses 18% of funding to TB and 5% to TB/HIV, compared to 29% for malaria and 48% for HIV [37]. In addition to contributions to the Global Fund, the United States government spends about 2% of its global health funding on TB, compared to 9% for malaria and 48% for HIV [38]. Additionally, the United States government funding for TB could decrease; the President's fiscal year 2021 request proposes $283 million for TB, a decrease of $38 million from previous fiscal years, in which TB control represented only 3% of the U.S. global health budget [38].

Neglect is also evidenced by slow TB vaccine and drug development. The Bacillus Calmette-Guérin (BCG) vaccine, first introduced in 1921, is still the only approved TB vaccine and only is effective at preventing severe disease in children [39]. The main anti-TB drugs (rifampicin, isoniazid, pyrazinamide, ethambutol, and streptomycin) were introduced between 1948 and 1963 [40]. Until the FDA approved bedaquiline in 2012 for MDR TB, no new anti-TB drugs with a novel mechanism of action were approved for four decades [41]. More recently in 2019,

the FDA approved pretomanid for MDR TB [42]. Although other anti-TB drugs are in development, there are far fewer drug candidates for TB than for other infectious diseases, especially HIV [43]. Unfortunately, TB does not represent a substantial disease burden in high-income countries and thus is not viewed as a lucrative investment in the US, European, and Japanese pharmaceutical markets; in developing markets, new therapies are welcomed, but only if they are affordable.

Although TB targets have been set and WHO declared TB a global health emergency in 1993, lack of political will has stalled progress. In 2015, the number of TB deaths surpassed those of HIV deaths, resulting in TB as the leading global cause of infectious disease-related death. In 2015, the End TB Strategy set targets of a 90% decrease in incidence rate and 95% decrease in TB deaths (compared to 2015) by 2035 [44]. To reach these targets, the TB incidence rate needs to decrease 4–5% annually and the mortality rates need to be 10% by 2020 [36]. However, the TB incidence rate is decreasing by only 2% annually, and though decreasing, the mortality rate is 16%, which is not enough to reach the End TB Strategy targets [36].

In 2018, heads of state gathered to discuss a concrete strategy for action and resource commitment at the United Nations High-Level Meeting (UNHLM) on TB [45]. This, in conjunction with the Multisectoral Accountability Framework, is aimed at ensuring accountability for commitments by governments and the health sector [46]. The UNHLM on TB set the target of diagnosing 40 million people with TB by 2022, including 3.5 million children and 1.5 million people with drug-resistant TB. An integral step in reaching the UNHLM TB targets is improving the accuracy and turnaround time of TB diagnostic tests. Until a decade ago, diagnosis of TB disease in most high-burden countries depended on clinical examination and a century-old testing technique, AFB smear microscopy. Although rapid TB diagnostic tests are available, most people in high-burden countries continue to be tested for TB with AFB smear microscopy, which has low sensitivity and specificity and cannot detect drug-resistant TB [10]. The advent of liquid culture media for *M*. *tuberculosis* has improved turnaround time, but, while highly accurate, can take 6 weeks for a result and requires advanced laboratories. Existing rapid diagnostics are faster, sensitive, and allow for the simultaneous detection of rifampin-resistant TB (i.e., Xpert MTB/RIF) or MDR TB (i.e., GenoType MTBDR *plus*). To reach the diagnosis commitments set by UNHLM, countries need 400% more GeneXpert modules and 600% more Xpert MTB/RIF cartridges per year—this is an incremental cost of approximately $586 million, which is 5 times more than current investments [39]. Other targets set at the UNHLM, include treating 40 million people who receive a TB diagnosis from 2018 to 2022, including 1.5 million people with drug-resistant TB, and providing TB preventive therapy (TPT) to at least 30 million people, including 4 million children and 20 million other household contacts and 6 million HIV-positive people. Although the UNHLM targets represent focused political attention on TB and hope for ending TB, these targets will require financial resources and continued political will to achieve.

33.4.1 Applying NTD Lessons in Order to End TB

The situation for NTDs has greatly improved in the past decades, total DALYs from the London Declaration have declined 21.1% from 2010 to 2016; the success largely based on a deliberate decision to utilize population-wide preventive approaches, such as mass drug administration of preventive chemotherapy combined with broader interventions on social environmental, and economic determinants of health [24, 47]. There are overarching principles for the design and implementation of NTD control programs that could be applied to ending TB [47]. First, as both NTDs and TB affect the poor, interventions must be affordable and, if possible, made available free-of-charge. Second, interventions must be simple, safe, and undemanding since people at higher risk may live beyond the reach of effective health systems. Third, since there are few market incentives for research and development for diseases concentrated among the poor, programs should continue to move forward with what already exists in parallel with using field experience to inform ideal new interventions. Finally, community engagement is key to generate grassroots demand for treatment and reduce stigma.

In consideration of these overarching principles from NTD successes, the lens of affordability, simplicity, and grassroots demand generation should be applied to TB therapies, such as engagement of affected communities and civil society. The scale-up of therapies to treat MDR or latent TB infection provide hope for addressing TB burden. In 2019, WHO issued new guidelines for a shorter, all-oral, bedaquiline-containing regimen for the treatment of MDR TB, after programmatic data from South Africa demonstrated significantly better adherence and treatment outcomes [48]. The U.S. President's Emergency Plan for AIDS Relief (PEPFAR) has committed to providing TB preventative therapy (TPT) to all eligible persons receiving antiretroviral therapy by 2021. In 2 years, across 16 PEPFAR countries, TPT initiations have increased 32%, and TPT completions increased 56% [49]. Additionally, through other services provided by PEPFAR and partners, several high HIV-burden countries are now on pace to control their HIV epidemic, also improving the outlook for the TB epidemic [50]. However, in contrast to increased TPT initiations and completions among people living with HIV, there is still a discrepancy in the implementation of TPT for contacts, particularly for exposed or infected children [36].

Similarly, continued demand and push for new TB diagnostics is important. New point-of-care diagnostics, scale-up of proven therapies, and epidemic control of HIV in some countries, are prospects for addressing challenges to end TB. The Xpert MTB/RIF Ultra assay (Ultra; Cepheid, Sunnyvale, CA), uses the same GeneXpert module as Xpert MTB/RIF but has improved sensitivity and a lower limit of detection of *M. tuberculosis*. The increased sensitivity is important for diagnosis of TB and detection of rifampin resistance, especially among patients with smear-negative results, which often is the case for HIV-positive individuals and children [51]. The recently released lateral flow urine lipoarabinomannan assays (LF-LAM) is recommended by WHO for the diagnosis and screening of

TB in HIV-positive individuals with low CD4 counts or who are seriously ill [52]. Urine is easier to collect and store and lacks the infection risks associated with sputum collection. However, the sensitivity of the LF-LAM assay has yet to be optimized for use among the general population. Use of these diagnostics, especially in vulnerable populations, improves detection and early initiation of treatment.

While certain lessons learned from tackling NTDs can be applied to ending TB, it is too simplistic to suggest that TB can be ended through solely overcoming neglect, increasing funding, and boosting political will. While these factors are certainly paramount, it is important to recognize that TB vaccine development efforts have been hampered by the complexity of TB immunology and limited knowledge of clearly established correlates of protection. Additionally, civil conflicts, discrimination, and war interfere with access to care, and as long as there exist unstable political situations in which the population cannot access health care or networks of health care structures are destroyed, control of TB may not be possible. Finally, education and learning from history is important. For example, the consequence of inadequate education and mismanagement of TB patient care is a reason for the increase in MDR-TB prevalence in certain regions of the world.

33.5 Conclusions

There are key similarities TB shares with NTDs, including the basis of its underlying burden of disease, influence and effect on poverty and development, and neglect through political will and funding. In presenting TB within the framework of NTDs, the intention is to gain insights from the successes in battling NTDs and how lessons learned can help global health programs change the trajectory of the TB epidemic, decrease TB morbidity and mortality rates and reach the End TB goals.

Abbreviations

AFB: acid-fast bacillus
BMI: body mass index
DALYs: disability-adjusted life-years
GDP: gross domestic product
LF-LAM: lipoarabinomannan assays
MDR: multidrug-resistant
NTDs: neglected tropical diseases
PEPFAR: President's Emergency Plan for AIDS Relief
TB: tuberculosis
TPT: tuberculosis preventive therapy
UNHLM: United Nations High-Level Meeting
WHO: World Health Organization
XDR: extensively drug-resistant

Disclosures and Conflict of Interest

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Chapter 34

Current Issues in Antibiotic Antimicrobial Resistance*

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Keywords: amphotericin B, amphotericin B-resistant *Candida auris*, antibiotic resistance (AR), antimicrobial resistance, *Aspergillus fumigatus,* asthma, azithromycin, azithromycin-resistant *Neisseria gonorrhoeae*, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae*, carbapenem-resistant *Enterobacteriaceae* (CRE), caspofungin, caspofungin-resistant *Candida*, ceftazidime-avibactam, ceftazidime-avibactam-resistant KPC-producing *Klebsiella pneumoniae*, ciprofloxacin, ciprofloxacin-resistant *Neisseria gonorrhoeae*, daptomycin, daptomycin-resistant methicillin-resistant *Staphylococcus aureus*, diabetes, drug resistance, extended-spectrum beta-lactamase-producing *Escherichia coli*, extended-spectrum cephalosporins, fluconazole, fluconazole-resistant *Candida*, imipenem, methicillin, methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin, penicillinase-producing *Neisseria gonorrhoeae*, penicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, plasmid-mediated vancomycin-resistant *Enterococcus faecium*, rheumatoid arthritis (RA), *Salmonella* Heidelberg bacteria, vancomycin, vancomycin-resistant *Staphylococcus aureus*

34.1 About Antibiotic Resistance

Antibiotic resistance happens when germs like bacteria and fungi develop the ability to defeat the drugs designed to kill them. That means the germs are not killed and continue to grow. Infections caused by antibiotic-resistant germs are difficult, and sometimes impossible, to treat. In most cases, antibiotic-

*This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the Centers for Disease Control and Prevention.

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resistant infections require extended hospital stays, additional follow-up doctor visits, and costly and toxic alternatives. Antibiotic resistance does not mean the body is becoming resistant to antibiotics; it is that bacteria have become resistant to the antibiotics designed to kill them.

34.2 Antibiotic Resistance Threatens Everyone

Antibiotic resistance is often also referred to as antimicrobial resistance or drug resistance. Antibiotic resistance has the potential to affect people at any stage of life, as well as the healthcare, veterinary, and agriculture industries, making it one of the world's most urgent public health problems. Each year in the U.S., at least 2.8 million people are infected with antibiotic-resistant bacteria or fungi, and more than 35,000 people die as a result. No one can completely avoid the risk of resistant infections, but some people are at greater risk than others (for example, people with chronic illnesses). If antibiotics lose their effectiveness, then we lose the ability to treat infections and control public health threats. Many medical advances are dependent on the ability to fight infections using antibiotics, including joint replacements, organ transplants, cancer therapy, and treatment of chronic diseases like diabetes, asthma, and rheumatoid arthritis.

34.3 Brief History of Resistance and Antibiotics

Penicillin, the first commercialized antibiotic, was discovered in 1928 by Alexander Fleming. Ever since, there has been discovery and acknowledgement of resistance alongside the discovery of new antibiotics. In fact, germs will always look for ways to survive and resist new drugs. More and more, germs are sharing their resistance with one another, making it harder for us to keep up.

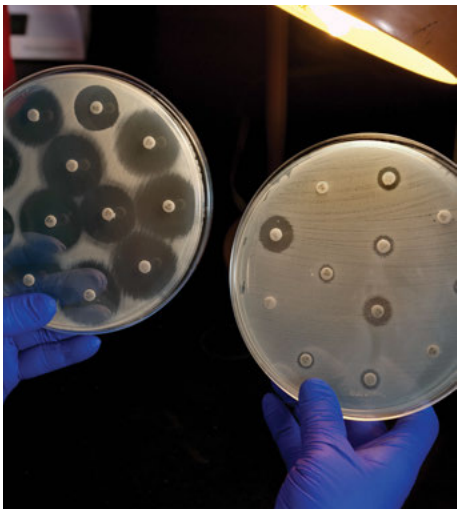
Table 34.1 Select germs showing resistance over time

Antibiotic approved or released	Year released	Resistant germ identified	Year identified
Penicillin	1941	Penicillin-resistant *Staphylococcus aureus*	1942
		Penicillin-resistant *Streptococcus pneumoniae*	1967
		Penicillinase-producing *Neisseria gonorrhoeae*	1976
Vancomycin	1958	Plasmid-mediated vancomycin-resistant *Enterococcus faecium*	1988
		Vancomycin-resistant *Staphylococcus aureus*	2002
Amphotericin B	1959	Amphotericin B-resistant *Candida auris*	2016

(*Continued*)

Antibiotic approved or released	Year released	Resistant germ identified	Year identified
Methicillin	1960	Methicillin-resistant *Staphylococcus aureus*	1960
Extended-spectrum cephalosporins	1980 (Cefotaxime)	Extended-spectrum beta-lactamase-producing *Escherichia coli*	1983
Azithromycin	1980	Azithromycin-resistant *Neisseria gonorrhoeae*	2011
Imipenem	1985	*Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae*	1996
Ciprofloxacin	1987	Ciprofloxacin-resistant *Neisseria gonorrhoeae*	2007
Fluconazole	1990 (FDA approved)	Fluconazole-resistant *Candida*	1988
Caspofungin	2001	Caspofungin-resistant *Candida*	2004
Daptomycin	2003	Daptomycin-resistant methicillin-resistant *Staphylococcus aureus*	2004
Ceftazidime-avibactam	2015	Ceftazidime-avibactam-resistant KPC-producing *Klebsiella pneumoniae*	2015

34.4 Fighting Antibiotic Resistance



What antibiotic resistance looks like. The bacteria on the right plate almost touch the discs of antibiotics, meaning they are antibiotic resistant. The antibiotics are ineffective against the bacteria. The bacteria on the left plate can't grow next to the discs of antibiotics, meaning the bacteria are susceptible and can be treated with antibiotics.

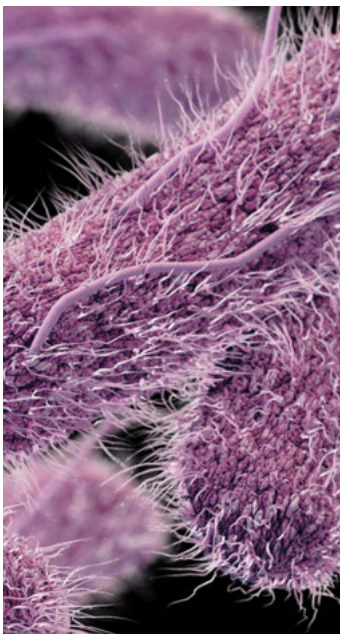
34.4.1 How We Keep Americans Safe

Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics. Antibiotic resistance (AR) is the ability of microbes to resist the effects of drugs—that is, the germs are not killed, and their growth is not stopped. At least 23,000 people die each year as a direct result of these infections. Although some people are at greater risk than others, no one can completely avoid the risk of antibiotic-resistant infections. Infections with resistant organisms are difficult to treat, requiring costly and sometimes toxic alternatives. Some of the current trends in resistance:

- Antibiotics are among the most commonly prescribed drugs used in human medicine, but up to 50% of the time antibiotics are not prescribed properly (for example, often given when not needed or with incorrect drug dosing or duration).
- The germs that contaminate food can become resistant because of the use of antibiotics in food animals as well as people. Studies have shown that the use of antibiotics in food animals can lead to resistant infections, like those caused by *Salmonella* and *Campylobacter* in people.
- The other major factor in the alarming growth of antibiotic resistance is spread of resistant strains of bacteria from person to person, or from contaminated sources in the environment.

34.4.2 ARLAB Network

In 2016, Congress appropriated $160 million for CDC to fight antibiotic resistance. With this investment, NCEZID is working with others to detect, respond, contain, and prevent resistant infections. Some key activities include:



- **Investing in states and communities** across the US for better response, containment, and prevention of AR threats.
- Establishing **the AR Lab Network** made up of CDC labs, 7 regional labs in health departments, and expanded capacity of all state and local health department labs. The new lab network will conduct nationwide testing to fill data gaps and inform how we respond to some of the most serious antibiotic-resistant threats like the nightmare bacteria CRE (carbapenem-resistant Enterobacteriaceae), gonorrhea, and *Salmonella*.
- **Funding innovation** to understand how antibiotics disrupt a healthy microbiome, the community of naturally occurring microbes in and on our bodies.

- One way CDC is working to slow the spread of drug-resistant *Salmonella* is by increasing the ability of states to detect drug resistance through DNA sequencing.

34.5 Antibiotic Resistance: 5 Things to Know

1. Antibiotic resistance occurs when germs defeat the drugs designed to kill them. It does NOT mean the body is resistant to antibiotics.
2. Antibiotic resistance can affect people at any stage of life. Infections caused by resistant germs are difficult—sometimes impossible—to treat. In many cases, these infections require extended hospital stays, additional follow-up doctor visits, and the use of treatments that may be costly and potentially toxic to the patient.
3. Healthy habits can protect you from infections and help stop germs from spreading. Get recommended vaccines, keep hands and wounds clean, and take good care of chronic conditions, like diabetes.
4. Antibiotics save human and animal lives. Any time antibiotics are used, they can lead to side effects and resistance. Antibiotics do not work on viruses, such as colds and the flu. Talk to your healthcare provider or veterinarian about whether antibiotics are needed.
5. Antibiotic resistance has been found in all regions of the world. Modern trade and travel mean AR can move easily across borders. It can spread in places like hospitals, farms, the community, and the environment. Tell your healthcare provider if you recently traveled to or received care in another country.

34.6 Antibiotic-Resistant Infections Threaten Modern Medicine

Millions of people in the United States receive care that can be complicated by bacterial and fungal infections. Without antibiotics, we are not able to safely offer some life-saving medical advances.

34.7 Examples of How Antibiotic Resistance Affects Humans, Animals and the Environment

People

Some types of antibiotic-resistant germs can spread person to person. “Nightmare bacteria” carbapenem-resistant Enterobacteriaceae (CRE) can also survive and

grow in sink drains at healthcare facilities and spread to patients and to the environment through the wastewater.

Animals

Resistant germs can spread between animals and people through food or contact with animals. For example, *Salmonella* Heidelberg bacteria can make both cattle and people sick.

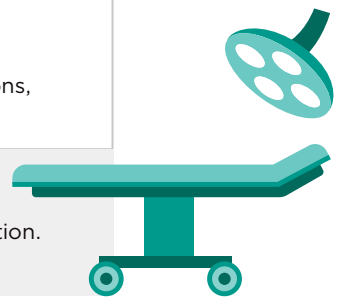
Sepsis Treatment

Anyone can get an infection and almost any infection can lead to sepsis — the body's extreme response to an infection. Without timely treatment with antibiotics, sepsis can rapidly lead to tissue damage, organ failure, and death.

AT LEAST 1.7M adults develop sepsis each year.

Surgery

Patients who have surgery are at risk for surgical site infections. Without effective antibiotics to prevent and treat surgical infections, many surgeries would not be possible today.

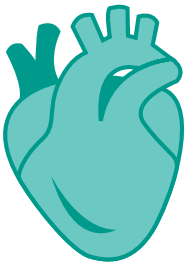


1.2M women had a cesarean section (C-section) in 2017. Antibiotics are recommended to help prevent infection.

Chronic Conditions

Chronic conditions (e.g., diabetes) put people at higher risk for infection. These conditions and some medicines used to treat them can weaken the immune system (how the body fights infection).

MORE THAN 30M people have diabetes. Antibiotics are used to treat common infections in these patients.



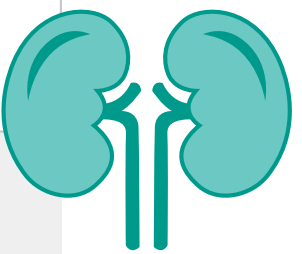
Organ Transplants

Organ transplant recipients are more vulnerable to infections because they undergo complex surgery. Recipients also receive medicine to suppress (weaken) the immune system, increasing risk of infection.

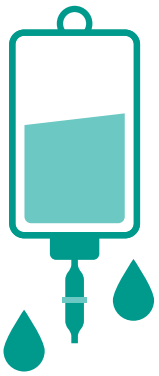
MORE THAN 33,000 organ transplants were performed in 2016. Antibiotics help organ transplants remain possible.

Dialysis for Advanced Kidney Disease

Patients who receive dialysis treatment have a higher risk of infection, the second leading cause of death in dialysis patients.



MORE THAN 500,000 patients received dialysis treatment in 2016. Antibiotics are critical to treat infections in patients receiving life-saving dialysis treatment.



Cancer Care

People receiving chemotherapy for cancer are often at risk for developing an infection during treatment. Infection can quickly become serious for these patients.

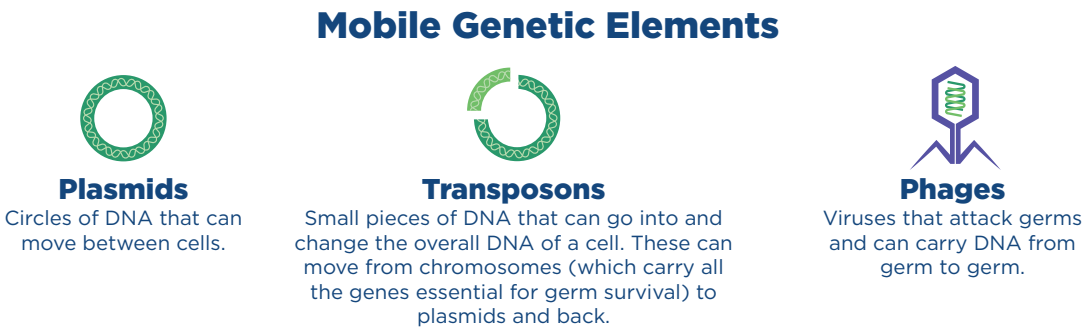
AROUND 650,000 people receive outpatient chemotherapy each year. Antibiotics are necessary to protect these patients.

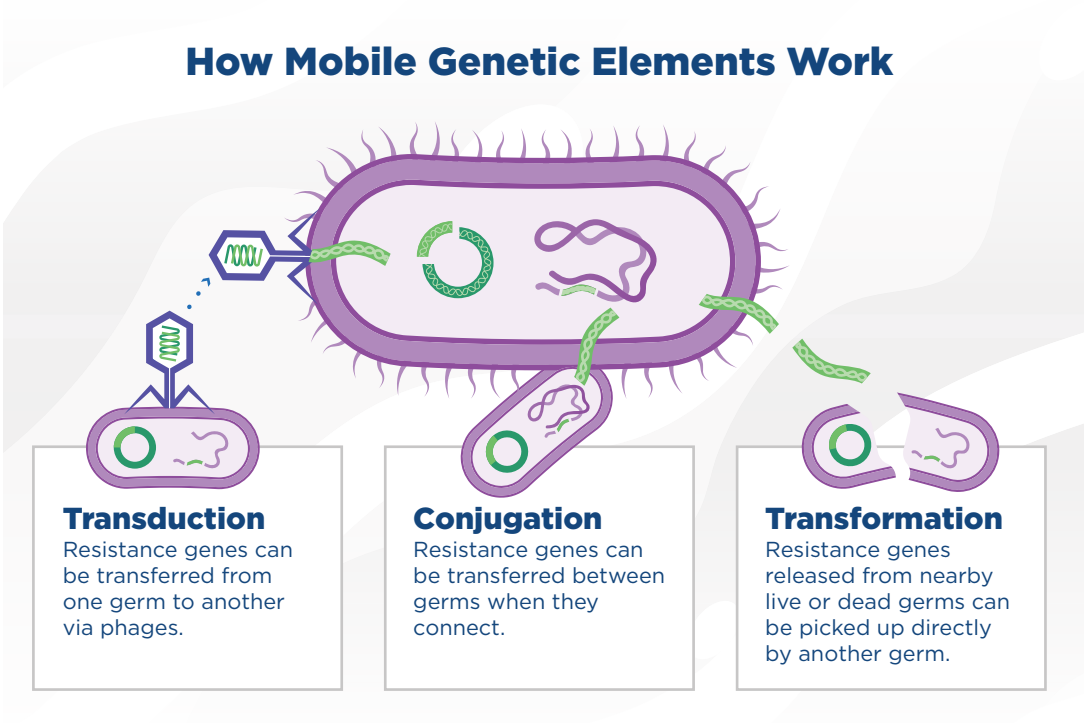
Environment

Antibiotic-resistant germs can spread in the environment. *Aspergillus fumigatus*, a common mold, can make people with weak immune systems sick. In 2018, resistant *A. fumigatus* was reported in three patients. It was also found in U.S. crop fields treated with fungicides that are similar to antifungals used in human medicine.

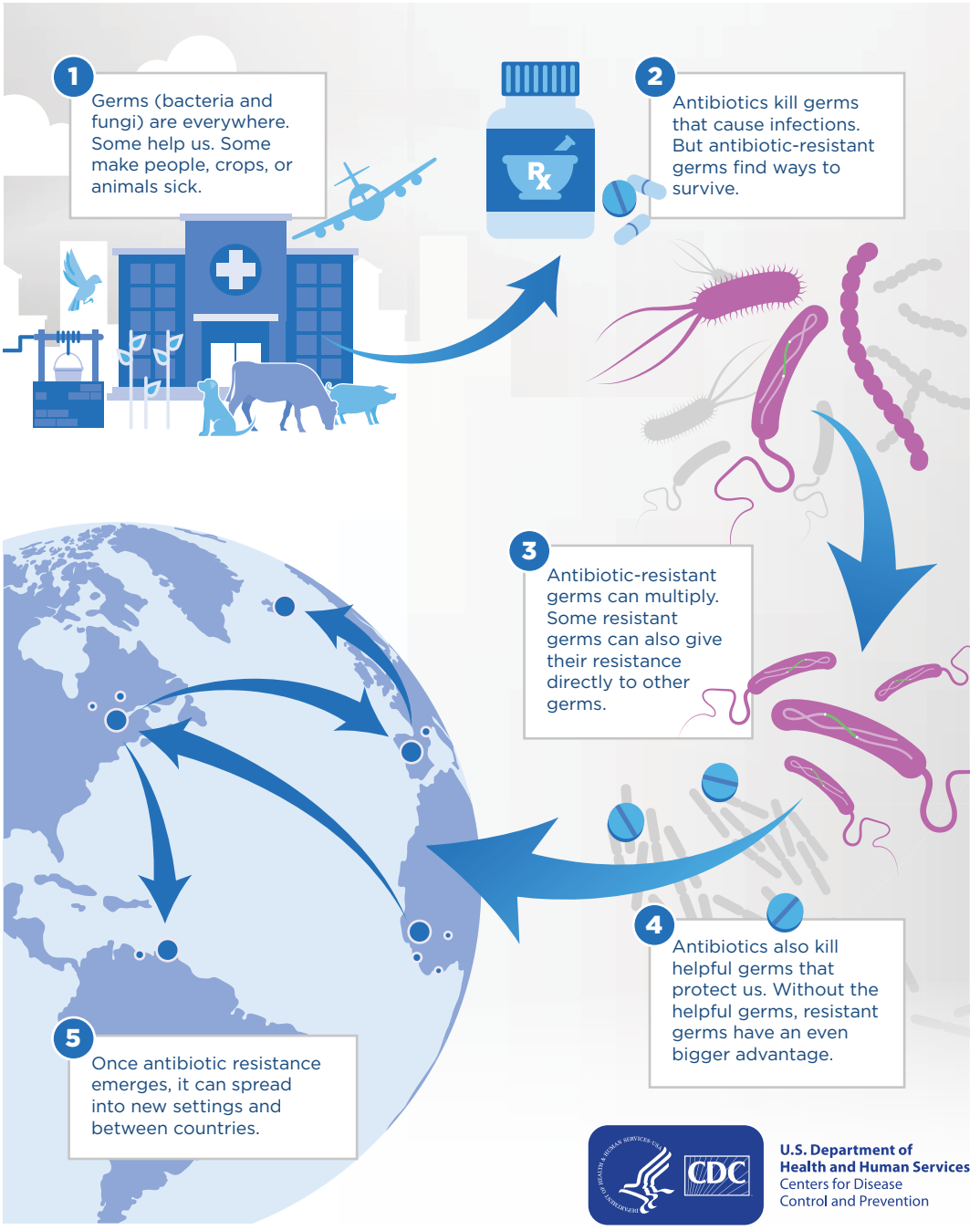
34.8 How Antibiotic Resistance Moves Directly Germ to Germ

Any antibiotic use can lead to antibiotic resistance. Antibiotics kill germs like bacteria and fungi, but the resistant survivors remain. Resistance traits can be inherited generation to generation. They can also pass directly from germ to germ by way of mobile genetic elements.



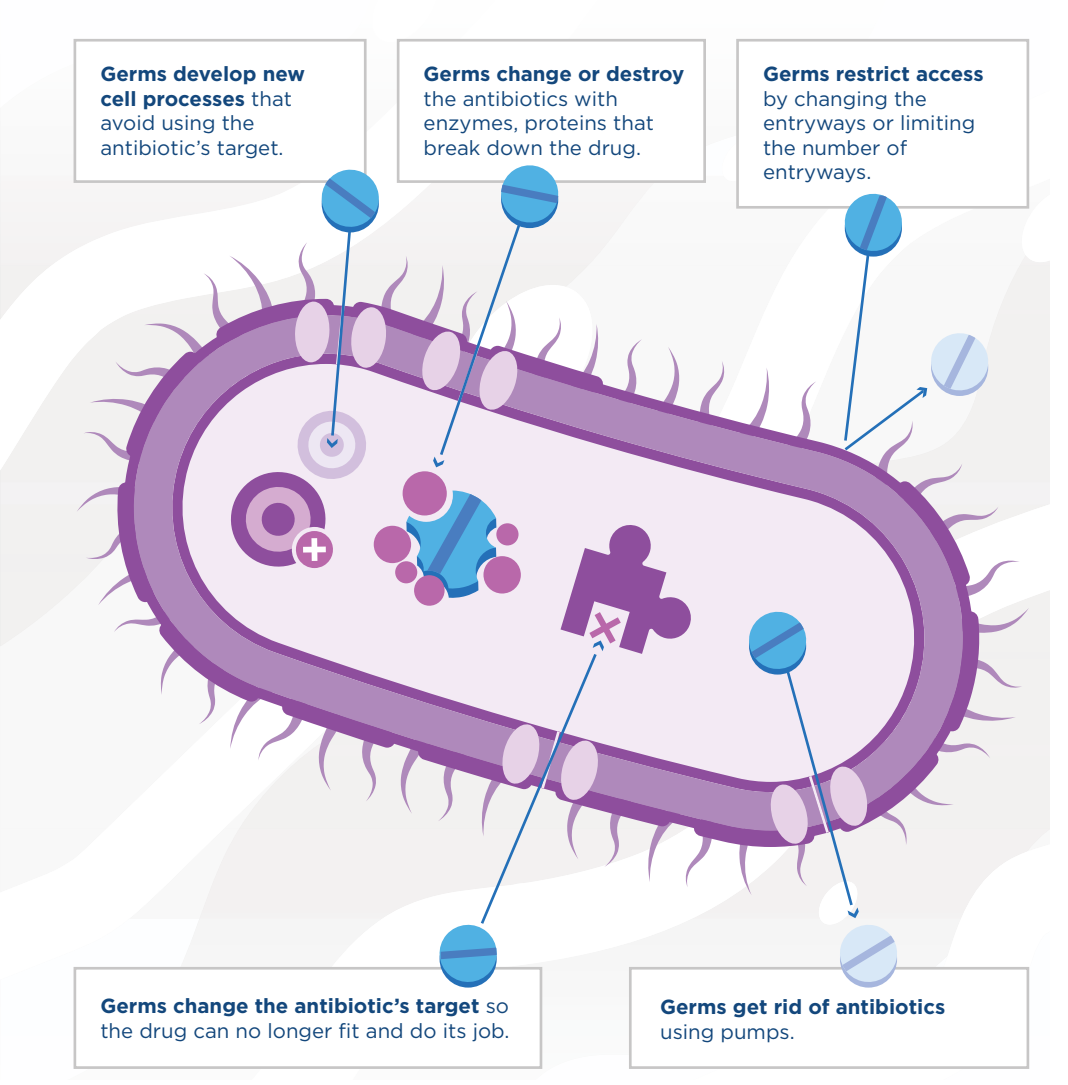


How Antibiotic Resistance Spreads



How Bacteria and Fungi Fight Back Against Antibiotics

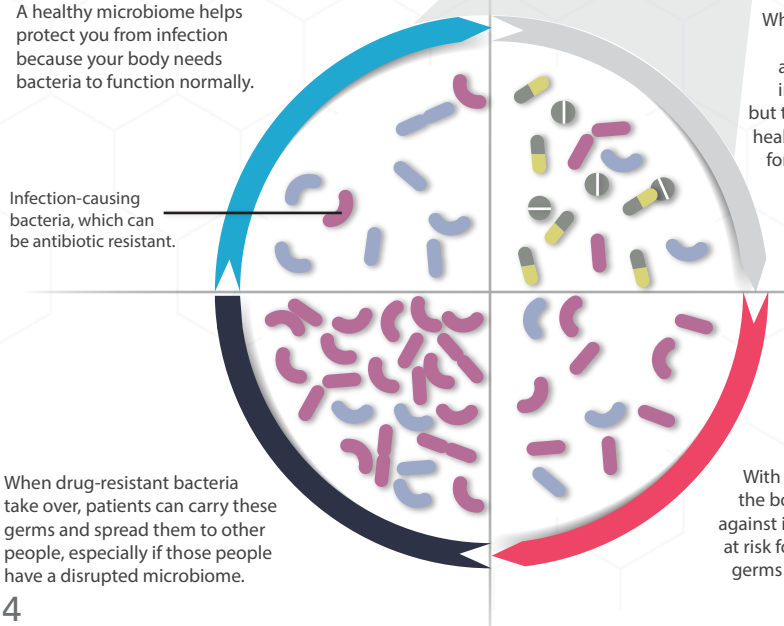
Antibiotics fight germs (bacteria and fungi). But germs fight back and find new ways to survive. Their defense strategies are called **resistance mechanisms**. Only germs, not people, become resistant to antibiotics.



CDC's Antibiotic Resistance (AR) Solutions Initiative: Microbiome

CDC's applied research on the human microbiome aims to identify effective public health approaches to protect people, their microbiomes, and the effectiveness of antibiotics.

Bacteria, fungi, viruses, and other microbes (germs) live naturally on our skin and in our gut and other places within our body. These microbes make up a community called the microbiome. Antibiotics can destroy your microbiome the way a wildfire can destroy a forest.



1

A healthy microbiome helps protect you from infection because your body needs bacteria to function normally.

Infection-causing bacteria, which can be antibiotic resistant.

2

When you take antibiotics to treat an infection, the antibiotics not only kill the infection-causing bacteria, but the bacteria that keep you healthy can also be destroyed for several months. This can disrupt, or unbalance, a healthy microbiome.

When drug-resistant bacteria take over, patients can carry these germs and spread them to other people, especially if those people have a disrupted microbiome.

4

With a disrupted microbiome, the body is less able to defend against infection, putting people at risk for infections from deadly germs like *C. difficile* and MRSA.

3

When antibiotics are needed, the benefits outweigh the risks of side effects or antibiotic resistance. When antibiotics aren't needed, those risks come with no benefits. By only using antibiotics when needed, we can avoid unnecessary microbiome disruption and risk for getting or spreading infections.

221

New nationwide testing in 2017 uncovered 221 instances of unusual resistance genes in "nightmare bacteria."

1 in 10

11% of screening tests, in people with no symptoms, found a hard-to-treat germ that spreads easily.

1st

The Containment Strategy keeps new threats from spreading. Launch at the first sign of unusual resistance.

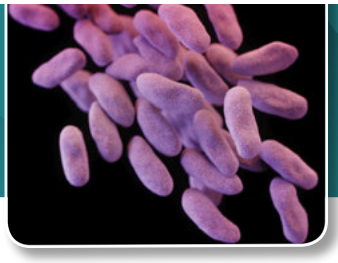
Containing Unusual Resistance

Early, aggressive action can prevent spread

More than 23,000 Americans die each year from infections caused by germs resistant to antibiotics. While antibiotic resistance (AR) threats vary nationwide, AR has been found in every state. And unusual resistance germs, which are resistant to all or most antibiotics tested and are uncommon or carry special resistance genes, are constantly developing and spreading. Lab tests uncovered unusual resistance more than 200 times in 2017 in "nightmare bacteria" alone. With new resources nationwide, early and aggressive action—when even a single case is found—can keep germs with unusual resistance from spreading in health care facilities and causing hard-to-treat or even untreatable infections. For example, CDC estimates show that this aggressive approach could prevent 1,600 cases of CRE* in one state over three years. Health departments can lead the Containment Strategy and act swiftly with health care facilities and CDC at the first sign of unusual resistance.

State and local health departments can:

- Make sure all health care facilities know what state and local lab support is available and what isolates (pure samples of a germ) to send for testing. Develop a plan to respond rapidly to unusual genes and germs when they first occur.
- Assess the quality and consistency of infection control in health care facilities across the state. Help improve practices.
- Coordinate with affected health care facilities, the new AR Lab Network regional labs, and CDC for every case of unusual resistance. Investigations should include onsite infection control assessments and colonization screenings for people who might have been exposed. They could spread it to others. Continue until spread is controlled.
- Provide timely lab results and recommendations to affected health care facilities and providers. If the patient came from or was transferred to another facility, alert that facility.



PROBLEM:

Antibiotic-resistant germs can spread like wildfire.

Germs constantly develop resistance against new and older antibiotics. Antibiotic-resistant germs can cause difficult-to-treat or untreatable infections. Some types of antibiotic resistance are already widespread.

Once antibiotic resistance spreads, it is harder to control–like a wildfire.

Finding and responding to unusual resistance early, before it becomes common, can help stop its spread and protect people.

New or rare types of antibiotic resistance can be easier to contain when found rapidly–like a spark or campfire.

UNUSUAL ANTIBIOTIC-RESISTANT GERMS

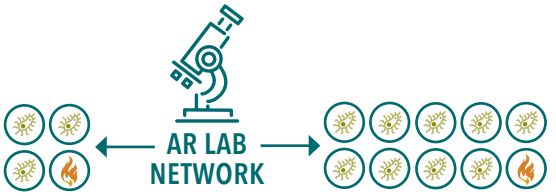
Resistant to all or most antibiotics tested, making them hard to treat, and

Uncommon in a geographic area or the US, or

Have special genes that allow them to spread their resistance to other germs

Examples of unusual resistance: Vancomycin-resistant *Staphylococcus aureus* (VRSA), *Candida auris*, and certain types of "nightmare bacteria" such as carbapenem-resistant Enterobacteriaceae (CRE).

CDC'S AR LAB NETWORK UNCOVERS ANTIBIOTIC RESISTANCE & SILENT SPREAD



1 IN 4 GERMS TESTED WAS POSITIVE.
25% of the germs had special genes that allow them to spread their resistance to other germs. In response, many investigations were conducted and screening tests were performed.

1 IN 10 SCREENING TESTS WAS POSITIVE.
If left undetected, patients without symptoms could continue spreading rare, hard-to-treat germs in the health care facility.

ANTIBIOTIC RESISTANCE CAN SPREAD

- From people with and without symptoms of infection
- Between facilities
- Between germs

PREVENTING AN UNUSUAL ANTIBIOTIC RESISTANCE WILDFIRE

Rapid Response in **Tennessee**

- Health department identified an unusual resistance germ in a patient who recently received health care outside the US.
- Health department and the facility in Tennessee did infection control assessments and colonization screenings within 48 hours. No spread found.
- Moving forward, CDC's AR Lab Network regional labs expanded services to test patients in the US with recent health care outside the country.

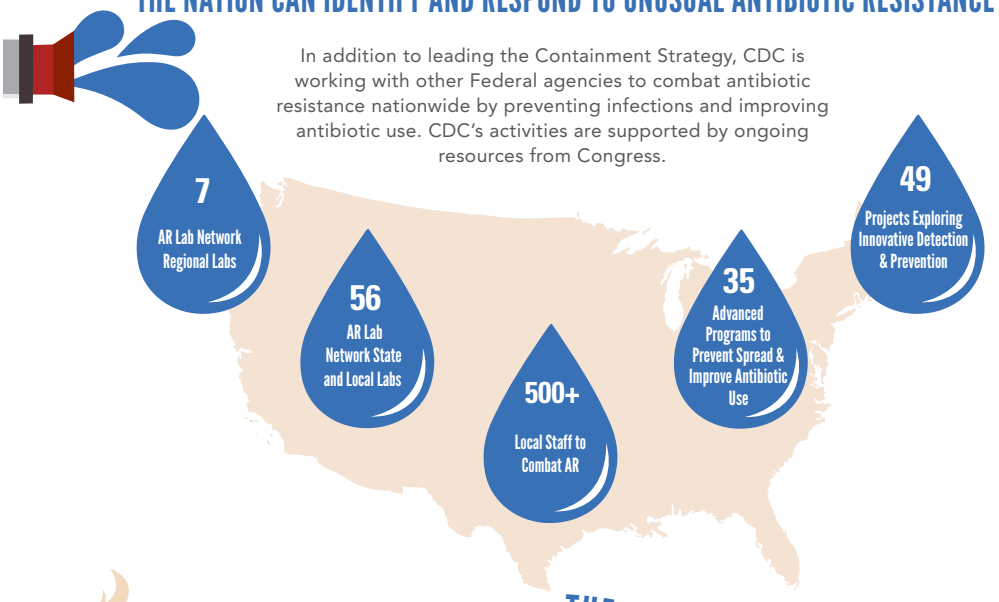
Ongoing Vigilance in **Iowa**

- Health department identified an unusual resistance germ in a nursing home patient.
- Health department and the facility did infection control assessments and screened 30 patients for colonization. Investigation revealed the germ may have spread to 5 additional people.
- Facility used infection control and contact precautions, such as gloves and gowns, to help stop spread.
- No further spread found during follow-up assessments.

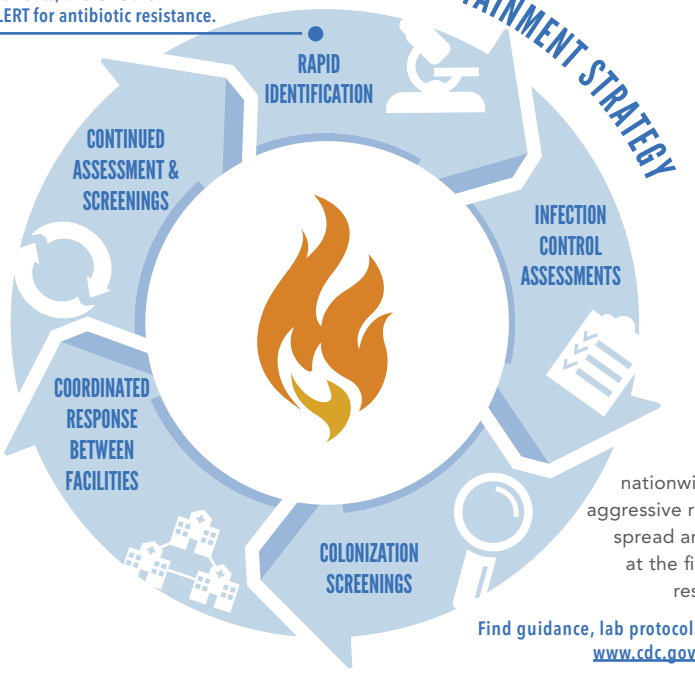
Containment Strategy: Be on guard to contain the first spark.

THE NATION CAN IDENTIFY AND RESPOND TO UNUSUAL ANTIBIOTIC RESISTANCE

In addition to leading the Containment Strategy, CDC is working with other Federal agencies to combat antibiotic resistance nationwide by preventing infections and improving antibiotic use. CDC's activities are supported by ongoing resources from Congress.



Health care facilities, health departments, and CDC are ON ALERT for antibiotic resistance.



Public health teams nationwide can launch early, aggressive responses to contain spread and protect people—at the first sign of antibiotic resistance, every time.

Find guidance, lab protocols, and more resources:
www.cdc.gov/HAI/Outbreaks/MDRO

WHAT CAN BE DONE

THE FEDERAL GOVERNMENT IS:

- Monitoring resistance and sounding the alarm when threats emerge. CDC develops and provides new lab tests so health departments can quickly identify new threats.
- Improving identification through CDC's new AR Lab Network in all 50 states, 5 large cities, and Puerto Rico, including 7 regional labs and a national tuberculosis lab for specialty testing.
- Supporting prevention experts and programs in every state, and providing data and recommendations for local prevention and response.
- Testing innovative infection control and prevention strategies with health care and academic partners.

STATE AND LOCAL HEALTH DEPARTMENTS AND LABS CAN:

- Make sure all health care facilities know what state and local lab support is available and what isolates (pure samples of a germ) to send for testing. Develop a plan to respond rapidly to unusual genes and germs when they first appear.
- Assess the quality and consistency of infection control in health care facilities across the state, especially in facilities with high-risk patients and long stays. Help improve practices.
- Coordinate with affected health care facilities, the new AR Lab Network regional lab, and CDC for every case of unusual resistance. Investigations should include onsite infection control assessments to find spread. Consider colonization screenings. Continue until spread is controlled.
- Provide timely lab results and recommendations to affected health care facilities and providers. If the patient came from or was transferred to another facility, alert that facility.
- Find resources: www.cdc.gov/hai/outbreaks/mdro

HEALTH CARE FACILITIES CAN:

- Plan for unusual resistance arriving in your facility. Find resources: www.cdc.gov/hai/outbreaks/mdro
- **Leadership:** Work with the health department to stop spread of unusual resistance. Review and support infection control in the facility.
- **Clinical labs:** Know what isolates to send for testing. Establish protocols that immediately notify the health department, healthcare provider, and infection control staff of unusual resistance. Validate new tests to identify the latest threats. If needed, use isolates from www.cdc.gov/ARIsolateBank.
- **Healthcare providers, epidemiologists, and infection control staff:** Place patients with unusual resistance on contact precautions, assess and enhance infection control, and work with the health department to screen others. Communicate about status when patients are transferred. Continue infection control assessments and colonization screenings until spread is controlled. Ask about any recent travel or health care to identify at-risk patients.

EVERYONE CAN:

- Inform your healthcare provider if you recently received health care in another country or facility.
- Talk to your healthcare provider about preventing infections, taking good care of chronic conditions and getting recommended vaccines.
- Practice good hygiene, such as keeping hands clean with handwashing or alcohol-based hand rubs, and keep cuts clean until healed.

For more information, please contact
Telephone: 1-800-CDC-INFO (232-4636)
TTY: 1-888-232-6348 | Web: www.cdc.gov

COMBAT ANTIBIOTIC RESISTANCE

Protect Yourself & Your Family

Infections caused by antibiotic-resistant germs are difficult, and sometimes impossible, to treat—but we can help stop the spread of these germs. Antibiotic resistance happens when germs like bacteria and fungi develop the ability to defeat the drugs designed to kill them.

No one can completely avoid getting an infection, but there are steps you can take to reduce your risk.

Know Your Risks, Ask Questions, & Take Care

Ask your healthcare provider about risks for certain infections and sepsis. Speak up with questions or concerns. Keep cuts clean and covered until healed, and take good care of chronic conditions, like diabetes or heart disease.

Clean Your Hands

Keeping your hands clean is one of the best ways to prevent infections, avoid getting sick, and prevent spreading germs.

Get Vaccinated

Vaccines are an important step to prevent infections, including resistant infections.

Be Aware of Changes in Your Health

Talk to your healthcare provider about how to recognize signs and symptoms of infections, or if you think you have an infection. If an infection isn't stopped, it can lead to additional complications like sepsis, a life-threatening medical emergency.

Use Antibiotics Appropriately

Talk with your healthcare provider or veterinarian about the best treatment when you, your family, or your animal is sick. Antibiotics save lives, but any time they are used they can cause sides effects and lead to antibiotic resistance.

Practice Healthy Habits Around Animals

Always clean your hands after touching, feeding, or caring for animals, and keep your animals healthy.

Prepare Food Safely

Follow four simple steps to avoid foodborne infections. Clean your hands, cooking utensils, and surfaces. Separate raw meat from other foods. Cook foods to safe temperatures. Chill leftovers and other foods promptly.

Stay Healthy When Traveling Abroad

Be vigilant when traveling abroad. Know what vaccinations are needed, check health alerts, stick to safe food and drinks, plan in advance in case you get sick, and learn about the risks of medical tourism.

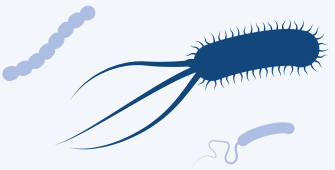
Prevent STDs

Gonorrhea, a common STD, can be resistant to the drugs designed to treat it. The only way to avoid STDs is to not have sex. If you have sex, lower your risk by choosing safer sexual activities and using condoms the right way from start to finish. You and your partner should be treated right away if you test positive to keep from getting infected again.

PROTECT YOUR PATIENTS, COMBAT ANTIBIOTIC RESISTANCE

Actions For Healthcare Providers

You can protect your patients from antibiotic-resistant germs such as bacteria and fungi, which can cause difficult and sometimes impossible to treat infections.



Prevent Infections & the Spread of Germs

Follow infection prevention and control recommendations, including screening at-risk patients when indicated.

Ask patients if they recently received care in another facility or traveled to another country (germs can be spread easily across borders).

Ensure your patients receive recommended vaccines.

Alert receiving facilities when transferring patients who are colonized or infected with antibiotic-resistant germs.

Educate patients on ways to prevent spread.

Stay informed of current outbreaks.

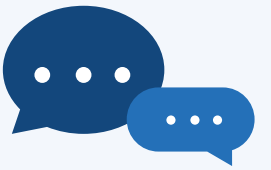
Improve Antibiotic Prescribing

Follow clinical and treatment guidelines. Support CDC's Core Elements of Antibiotic Stewardship to ensure appropriate antibiotic use.

Consider fungal infections for patients with respiratory infections that do not respond to antibiotics.

Watch for signs and symptoms of sepsis. If you suspect sepsis, start antibiotics as soon as possible and reassess antibiotic therapy.

Perform appropriate diagnostic tests to guide antibiotic therapy, including correct drug, dose, and duration.



Be Alert & Take Action

Be aware of infections and resistance patterns in your facility and community.

Ensure you are notified by the lab immediately when antibiotic-resistant germs are identified in your patients.

Inform patients and families if they have an antibiotic-resistant infection, as well as sexual partners when appropriate (e.g., gonorrhea).

Know when to report cases and submit resistant isolates to the health department to help identify unusual resistance or treatment failures.

34.9 Protect People and Animals, Combat Antibiotic Resistance: Actions for Livestock and Poultry Producers

Livestock (e.g., cattle, swine, sheep, goat) and poultry producers are key in helping to reduce the development and spread of antibiotic resistance. Adopting these practices to continue protecting the health of animals and people who work on farms, the community, and our food supply. (https://www.cdc.gov/drugresistance/food.html)

Work Closely with Your Veterinarian

Communicate often with your veterinarian, who will decide when antibiotics are needed to treat, control, and/or prevent disease in animals. Ask for advice related to preventing antibiotic-resistant infections specific to your farm size, animal species, and environment.

Keep Animals Healthy

Follow good husbandry practices and implement biosecurity practices (http://www.cfsph.iastate.edu/pdf/fad-prep-nahems-guidelines-biosecurity)—techniques to prevent the introduction and spread of diseases, including cleaning procedures—on the farm, during transport, and in production facilities. Adhere to animal welfare standards (https://www.nal.usda.gov/awic/standards-and-guidelines) including a safe drinking water supply, good nutrition, clean airflow, and avoid overcrowding. Give veterinarian-recommended vaccinations to prevent diseases and separate sick animals right away to prevent the spread of disease. Adopt best practices in waste management (https://www.epa.gov/agriculture/agricultural-animal-production#inspection) and follow manufacturer recommendations to clean equipment.

Use Antibiotics Exactly as Prescribed

Follow your veterinarian's instructions on the dose (amount), duration (period of time), and route of administration (how to give antibiotics to selected animals). Keep a record of all antibiotic use, including dates and times, and follow up with your veterinarian about how the treatment is working. **Safely dispose** of (https://www.avma.org/PracticeManagement/Facilities/Pages/disposal-unwanted-medications.aspx) unused or expired antibiotics. Find local drug disposal programs. Never pour or flush unused drugs down drains or toilets.

PROTECT YOUR PATIENTS, COMBAT ANTIBIOTIC RESISTANCE

Actions For Veterinarians

Veterinarians are leaders and stewards in preserving the effectiveness of antibiotics for animals and people. Working with animal owners and producers, veterinarians can slow antibiotic resistance by implementing disease prevention strategies and improving the use of antibiotics while also guaranteeing high-quality medical care for animal patients.

Prevent Disease

Implement best practices for animal husbandry, vaccination, nutrition, and biosecurity (e.g., infection control). Educate people who engage with animals on how to prevent disease.

Clean Your Hands & Equipment

Wash your hands regularly to remove germs, avoid getting sick, and prevent spread of germs between animals and people. Disinfect equipment to help prevent spread among animals and between farms.

Maintain Accurate Records of Treatment & Outcomes

Document and review diagnostic test results and patient response to therapy. Re-evaluate reason for prescribing, dose, and duration as needed.

Select & Use Antibiotics Appropriately

Follow regulatory requirements (antibiotic use should involve veterinary oversight per U.S. guidance). Use current established guidelines and diagnostic tests to assess the need, selection, dose, frequency, and duration of antibiotics.

Stay Current

Stay up-to-date on disease prevention tools; consensus and prescribing guidelines; local, state, and federal requirements; and professional standards for antibiotic use.

Prevent Environmental Contamination

Dispose of unused or expired antibiotics appropriately.

Commit to Antibiotic Stewardship

Implement practice-level stewardship activities, including documenting antibiotic use data, examining use practices, and serving as an educational resource for clients. Engage veterinary diagnostic labs to provide antibiograms to help determine which antibiotics will effectively treat infections. Become familiar with and use the American Veterinary Medical Association established antibiotic use principles to build an antibiotic stewardship plan for your practice settings.

Do antibiotics have side effects?

BE ANTIBIOTICS AWARE
SMART USE, BEST CARE

Any time antibiotics are used, they can cause side effects. However, antibiotics can save lives. When you need antibiotics, the benefits outweigh the risks of side effects. If you don't need antibiotics, you shouldn't take them because they can cause harm.

Common side effects of antibiotics include:


Rash


Dizziness


Nausea


Yeast Infection


Diarrhea

Get immediate medical help if you experience severe diarrhea. It could be a symptom of a ***C. difficile* infection** (also called ***C. diff***), which can lead to severe colon damage and death. People can also have severe and life-threatening allergic reactions.

If you experience side effects, follow up with your healthcare professional.

1 out of 5
medication-related visits to the emergency room are from reactions to antibiotics.

Protect Yourself and Your Workers

Make sure farms have one toilet and handwashing station with soap (https://www.osha.gov/laws-regs/regulations/standardnumber/1928/1928.110) and running water for every 20 workers, within a 5-minute walk of their work area. Confirm workers have appropriate vaccinations (https://www.cdc.gov/vaccines/schedules/hcp/imz/adult.html) and receive safety and hygiene training specific to the work they perform. When needed, wear and clean personal protective equipment (http://www.nasdonline.org/browse/14/personal-protective-equipment-ppe.html) appropriately (e.g., gloves, boots, and face and breathing masks).

Viruses or Bacteria What's got you sick?

Antibiotics are often prescribed when they are not needed for respiratory infections. Antibiotics are only needed for treating certain respiratory infections caused by bacteria. Viral illnesses cannot be treated with antibiotics. When an antibiotic is not prescribed, ask your healthcare professional for tips on how to relieve symptoms and feel better.

Common Respiratory Infections	Common Cause			Are Antibiotics Needed?
	Virus	Virus or Bacteria	Bacteria	
Common cold/runny nose	✓			No
Sore throat (except strep)	✓			No
COVID-19	✓			No
Flu	✓			No
Bronchitis/chest cold (in otherwise healthy children and adults)*		✓		No*
Middle ear infection		✓		Maybe
Sinus infection		✓		Maybe
Strep throat			✓	Yes
Whooping cough			✓	Yes

* Studies show that in otherwise healthy children and adults, antibiotics for bronchitis won't help you feel better.

Chapter 35

Ending AIDS as a Public Health Threat by 2030: Time to Reset Targets for 2025

Paul R. De Lay, MD,[a] Adèle Benzaken, MD,[b] Quarraisha Abdool Karim, PhD,[c,d] Sani Aliyu, MD,[e] Carolyn Amole,[f] George Ayala, MD,[g] Kalipso Chalkidou, MD, PhD,[h] Judy Chang, MSc,[i] Michaela Clayton, LLB,[j] Aleny Couto, MD,[k] Carl Dieffenbach, PhD,[l] Mark Dybul, MD,[m] Wafaa El Sadr, MD, MPH,[n] Marelize Gorgens, PhD,[o] Daniel Low-Beer, PhD,[p] Smail Mesbah, MD,[q] Jorge Saveedra, MD, MPH,[r] Petchsri Sirinirund, MD,[s] John Stover, MA,[t] Omar Syarif,[u] Aditia Taslim,[v,‡] Safiatou Thiam, PhD,[w] Lucy Wanjiku Njenga,[x] Peter D. Ghys, MD, PhD,[y] Jose Antonio Izazola-Licea, MD, PhD,[y] Luisa Frescura, MSc,[y] Erik Lamontagne, PhD,[y] Peter Godfrey-Faussett, MD,[y] Christopher Fontaine, MSc,[y] Iris Semini, MSc,[y,‡] and Shannon Hader, MD, MPH[y]

[a]*Independent Consultant, Washington, DC, USA*
[b]*AIDS Healthcare Foundation, Manaus, Brazil,*
[c]*Centre for AIDS Programme of Research in South Africa (CAPRISA), Nelson Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*
[d]*Mailman School of Public Health, Columbia University, New York, USA*
[e]*Department of Microbiology, Cambridge University Hospitals, UK*
[f]*Clinton Health Access Initiative, Boston, Massachusetts, USA*
[g]*Alameda County Public Health Department, California, USA*
[h]*Department of Infectious Disease Epidemiology, School of Public Health, Imperial College, London, UK*
[i]*International Network of People Who Use Drugs (INPUD), London, UK*
[j]*Independent Consultant, Windhoek, Namibia*
[k]*STI/HIV Program at Ministry of Health, Mozambique*
[l]*Division of AIDS, National Institute for AIDS and Infectious Disease (NIAID), Maryland, USA*
[m]*Department of Medicine, Georgetown University, DC, USA*
[n]*ICAP, Columbia University, New York, USA*
[o]*Health, Nutrition and Population Global Practice, World Bank Group, Washington, DC, USA*
[p]*World Health Organization, Geneva, Switzerland*
[q]*Algiers Faculty of Medicine, People's Democratic Republic of Algeria*
[r]*AHF Global Public Health Institute, University of Miami, Florida, USA*
[s]*Consultant, Bangkok, Thailand*
[t]*Avenir Health, Glastonbury, Connecticut, USA*
[u]*The Global Network of People Living with HIV (GNP+), Zaandam, the Netherlands*
[v]*Independent Consultant (previously with Rumah Cemera), West Java, Indonesia*
[w]*National AIDS Council, Dakar, Senegal*
[x]*Positive Young Women Voices, New York, USA*
[y]*Joint UN Programme on HIV/AIDS (UNAIDS), Geneva, Switzerland*
[‡]*Unavailable*

pdelay1@gmail.com

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35.1 Introduction

In 2015, the United Nations' (UN) Sustainable Development Goal (SDG) 3 established that by 2030, the world would "end the epidemics of AIDS, tuberculosis, malaria …" [1]. As part of the SDG strategy, The Joint United Nations Programme on HIV/AIDS (UNAIDS) and partners developed the "Fast Track Response Strategy" in 2016 and, using standardized epidemiologic guidelines, defined "ending AIDS as a public health threat" as a 90% reduction in HIV incidence and mortality by the year 2030, compared to a baseline year of 2010 [2].

The "Fast Track Response Strategy" envisioned an accelerated ramping up of resources and programs with a set of intermediate targets for 2020, which, if achieved, would enable the attainment of the overall goals and top-line targets of 2030. There were 10 major targets and commitments established for 2020, which would result in a major reduction in HIV incidence to make the response sustainable. The Fast Track Strategy has fostered some major successes, especially around the 90–90–90 testing and treatment targets, i.e., for 90% of persons living with HIV (PLHIVs) to be aware of their status, 90% of those aware of their HIV–positive status to be on antiretroviral therapy and 90% of the latter to have achieved viral suppression. A number of countries achieved or nearly achieved these coverage levels [3]. However, overall, the global community has yet to fulfill the set of recommendations and goals of the Fast Track Strategy [4].

Over the past 5 years, funding for the global AIDS response has declined. In 2019, the global resources available for the AIDS response in low- and middle-income countries amounted to $19.8 billion, compared to the global target of $26 billion [5]. Recent data indicate that many countries were only able to reach some of their 2020 programmatic goals. The 90–90–90 testing and treatment targets have been effective globally in nearly reaching the 2020 mortality reduction target. However, mixed treatment coverage, lower emphasis on primary prevention, and insufficient tailoring of these programs to those most in need have resulted in only a 19% reduction (2019) in new HIV infections, compared to a 2020 Fast Track target of a 75% reduction [6]. Inadequate attention toward equity of access, inclusion, dealing with stigma and discrimination, social and sexual determinants of infection, achieving social justice, and implementing societal enablers, including support for community-led programs, have limited access to effective prevention, treatment, and support programs. And, finally, the world is now in the midst of the Coronavirus Disease 2019 (COVID-19) pandemic, and its implications for the public health systems and individual access to health services are evident.

Thus, in light of all of these challenges, is it still realistic to end AIDS as a public health threat by 2030 [7]? Recognizing the challenges and to ensure that we do not reverse the gains made to date, what do we need to do to get back on track to meet the UN 2030 goal to end AIDS as a public health threat?

In 2018, UNAIDS and partners initiated a new strategic planning process, which would examine progress thus far and determine where the global community has succeeded and where we are falling behind. For this process, the most current and comprehensive evidence base was elicited to identify the most effective interventions that can contribute to achieving impact over the next 5 years. This analysis was conducted in a multistakeholder, participatory process and gathered the latest effectiveness and costing data for a range of programmatic interventions. It also sought to improve the ways that mathematical modeling could be performed to translate programmatic targets into epidemiologic impact and to estimate the resources needed to achieve the new targets in low- and middle-income countries. The primary goal for this multiyear endeavor was to reexamine the range of critical targets that needed to be achieved and to assess the impact of a more comprehensive and differentiated approach to addressing the HIV epidemic that would efficiently achieve the 2030 goal. One primary output of this process was to describe a new set of interim targets for 2025, which would recalibrate the global response and assure that the 2030 goals could be achieved. We are therefore announcing a PLOS Collection in which articles will include a more detailed description of the process and present some of the conclusions.

35.2 The Process and Structures

UNAIDS has produced projections of resource needs with accompanying targets 4 times since its inception in 1995. The initial projections performed in 2001 [8] and 2006 [9] focused primarily on levels of service provision and their associated cost. More recently (2011 [10] and 2016 [11]), the projections have included estimated impact of these services on incidence and mortality. This current process builds upon this previous work.

The Steering Committee, established to guide this process, initially met in July 2018, followed by 3 annual meetings. A multiyear time frame allowed for broader and more inclusive participation of critical constituencies and technical groups and provided technical groups adequate time for more extensive data gathering, building of the evidence base, and incorporation of these findings into the modeling process. A diagram of the process is provided in Fig. 35.1.

The tasks for the Steering Committee included the framing of the exercise, the creation of technical consultative groups (TCGs), reviewing the output from these consultations, and determining how to incorporate these data into the modeling process to assess the overall programmatic strategy, the target populations, the rate of scale-up, the expected impact, and the estimated resources needed. The membership of the Steering Committee included country

representatives, civil society and key population–led organizations, HIV program managers, technical experts, and relevant UN organizations.

Six TCGs were convened, each of which focused on key components of the programmatic response. These were responsible for identifying new evidence on the most effective interventions, improved methods for delivering and linking of services, relevant costs, and gathering available data on the potential impact of these programs, especially on incidence of new HIV infections and AIDS-related mortality. Participants in the TCGs included members of affected populations, program managers, researchers, service providers, strategic planners, and specialists of public health, human rights, and financial management.

A team of experts formed the Program Impact Modeling Advisory Group (PIMAG), which provided guidance and advice on modeling approaches.

The UNAIDS Secretariat provided extensive support throughout the entire process and also provided ongoing updates to a Stakeholder group consisting of primary donors and briefings of the Geneva Missions for highly impacted countries.

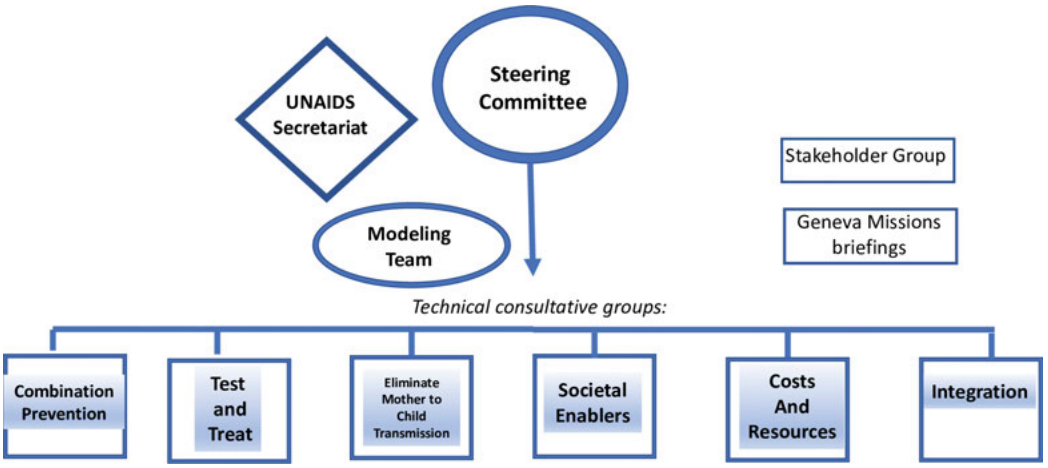

Figure 35.1 Setting AIDS 2025 targets—Functional components.

35.3 Addressing the Key Challenges, Concerns, and Priorities

The articles in this Collection address the rationales for this target setting, selected inputs for key areas, the epidemiological impact modeling, and the associated resource needs estimates. These articles also describe an inventory of the most effective programmatic and social interventions, specific target populations, the evidence base for estimating program effectiveness, the lessons learned from recent trends of program scale-up, the resources needed, and the challenges presented by the concomitant COVID-19 epidemic. The Steering Committee, with guidance from the TCGs, made a number of key decisions, which would inform the inputs used in the modeling exercise.

The UN 2030 goal of ending AIDS as a public health threat should be maintained and the primary focus of the 2025 target setting be used for recalibrating the response to reach the 2030 goal and top-line targets. Given the diversity of epidemics and progress within and between countries, it was also decided to provide global-level and country-level epidemiologic impact targets, but that resource needs estimates would be limited to low- and middle-income countries.

It is important to focus on "people-centered" prevention and treatment services that reach all populations in need, using appropriate venues and approaches for delivering services, with a special focus on specific populations and subpopulations who have not been reached with traditional approaches utilized to date.

The target-setting process must build on closing the testing and treatment gaps to further enhance reductions in morbidity and mortality for all populations in need and realize the secondary prevention potential of treatment. New approaches must be used to increase treatment uptake, introduction of new drugs and drug delivery mechanisms, addressing structural and social barriers, tackling comorbidities from aging, and improvements in service delivery.

The 2 decades of experience with projection and costing models was expanded to examine the role for improving efficiencies in program implementation and how best to achieve them.

The modeling includes anticipated new technologies for prevention and treatment, including new regimens and delivery methods for treatment and preexposure prophylaxis (PrEP) that could potentially be available by 2025.

Notably, the 2025 target setting and modeling drew on the increasing body of evidence that demonstrates the critical role for actions that decrease stigma, discrimination, and criminalization of key populations and how these impact on HIV program uptake and performance and strengthen the investment case for "societal enabler" programs.

Specific attention and consideration must be placed on universal health coverage and building on how HIV response to date has supported health systems strengthening and particularly on how to optimize effectiveness and efficiencies of service integration.

A consultation of costing experts agreed on the methods for determination of the unit costs for programmatic interventions and agreed on assumptions regarding future trajectories of the costs and the benefits from achieving the targets in terms of extending lives.

Finally, it was important to understand the impact that the COVID-19 pandemic has had on the delivery of health services. UNAIDS, with multiple partners, conducted a real-time, multicountry analysis on the effect of the pandemic on various components of the HIV response, particularly examining changes in the levels of testing, uptake of treatment, and continuity of treatment.

35.4 Conclusions

The new set of 2025 targets, including those addressing financial needs and societal enablers, has been incorporated in the UNAIDS Strategy for 2021 to 2026, which was approved by the UNAIDS Programme Coordinating Board in March 2021, and which is expected to guide and influence countries, major donors, and implementing organizations. The target-setting process has pinpointed what needs to be achieved by 2025, including the focus on people-centered and integrated services, on combination prevention, on societal enablers for the very first time, and on increased granularity to reach the SDG of ending AIDS by 2030. Achieving the targets will put the world again on track to end the AIDS epidemic as a public health threat and ensure the gains made to date are not reversed or, even worse, that the HIV/AIDS response joins the ranks of other unfinished pandemics and epidemics that the global community faces.

Disclosures and Conflict of Interest

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Chapter 36

9 Questions to Help Make Sense of Health Research[1]

National Center for Complementary and Integrative Health,
National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: basic research, clinical trials, clinically significant, complementary therapies, conflict of interest, control group, health research, meta-analyses, minimize bias, pilot studies, placebo, placebo-controlled trials, randomized trials, replication of a study, research studies, scientific paper, scientific research article, sham procedure, sham treatment, standard care, statistically significant, systematic reviews, translational research

Introduction

Almost every day, new findings on medical research are published, some of which may include complementary health approaches. Research studies about medical treatments and practices published in scientific journals are often the sources of news stories and can be important tools in helping you manage your health.

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at the NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH.

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But finding scientific journal articles, understanding the studies they describe, and interpreting the results can be challenging. One way to make it easier to understand information you find in a scientific journal is to share the information with your health care providers and get their opinions. Once you understand the basics and terminology of scientific research, you have one more tool to help you make better, informed decisions about your health. Here are 9 questions that can help you make sense of a scientific research article.

1. What are the parts of a scientific paper?

Most articles in scientific journals have a similar basic structure or format. There are five important parts of a research paper, which most articles contain.

2. What were the goals of the study?

Basic research aims to understand fundamental biological processes, or the mechanisms by which a treatment might affect the body.

Translational research aims to create the "building blocks" of information necessary for rigorous investigation of the effects and safety of an intervention in human clinical trials. Both basic and translational research may employ studies done in the laboratory, or with volunteers in a clinical setting.

Clinical trials aim at testing whether an intervention is useful and safe in humans. They may vary in size and type.

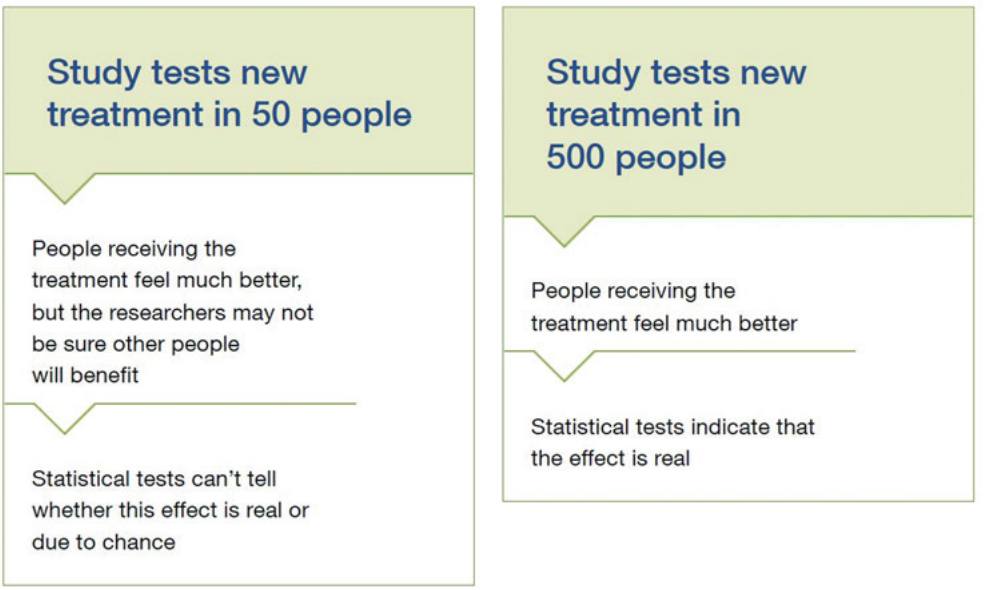
- Preliminary, exploratory, or pilot studies provide essential stepping stones of information about the potential safety and usefulness of an intervention, and help scientists determine whether to perform larger, more definitive clinical trials.
- Well-planned clinical trials give the clearest information about whether a treatment or a lifestyle change is effective and safe. However, because they are complicated, lengthy, and awfully expensive, they're usually done only after smaller preliminary studies have been completed and have shown some promise that the treatment may be helpful to patients.

While all studies, from basic to translational to clinical, are extremely important, clinical trials (because they are done in people) are the types of studies you probably hear about most often in the news and can have the most

immediate impact on improving health and treating disease. Therefore, the remaining questions focus on clinical trials.

3. How large was the study?

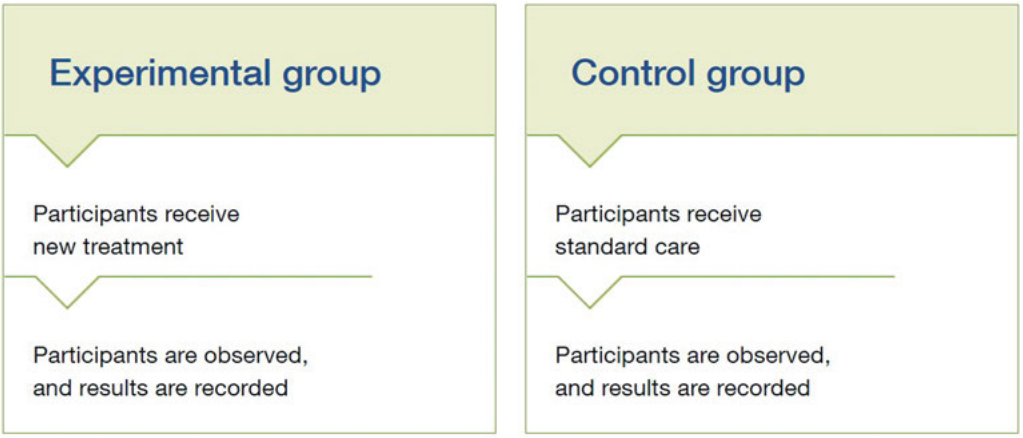
Studies with large numbers of people generally get results that are more reliable than studies with small pools of participants. Larger studies can increase the accuracy of the study findings and reduce the probability that any effect observed in the study was due to chance. Too few participants may make the entire study a failure—it may only produce inconclusive results. Statisticians and scientists have tools to figure out how many volunteers are needed for a clinical study to be meaningful.



In general, a large study with many volunteers may be able to produce conclusive results when a small study may not.

4. Was the study a controlled clinical trial?

In a controlled clinical trial, investigators compare the effects of different treatments in groups of study participants who are as identical as possible in all other respects. For example, the outcomes in one group of participants who receive a new "experimental" treatment may be compared with the results of another group who received standard care, the "control group." In effect the control group provides a "yardstick" for measuring the effects of the new treatment. In this case standard care is the "control" intervention.



Design of a clinical trial in which a new treatment is compared to standard care.

What did the control group receive?

There are many kinds of control groups. Ideally, participants are assigned randomly to one of the study groups. This helps ensure that the two groups are as identical in all respects as possible except for the intervention they receive. Other kinds of control groups are sometimes used, but they have an increased likelihood that factors other than the intervention affected the results.

Design of a clinical trial using a placebo or sham treatment.

In placebo-controlled trials, the control group receives an inactive treatment designed to resemble the treatment being studied. One example of a placebo is a pill that is medically inert (inactive) but looks like the experimental medicine being studied. Another example, called a sham, is used when the treatment being studied is a procedure (e.g., acupuncture), not a product. A sham procedure is designed to simulate the active treatment but does not have any active treatment qualities. When possible, the "placebo" treatment and "experimental" active treatment are delivered in a "double-blind" fashion. That is, neither the investigator delivering the treatment, nor the volunteer know what they are getting. This reduces the possibility the volunteers know what they are receiving.

5. Have steps been taken to minimize bias?

It can be surprisingly difficult to avoid bias in clinical trials. For example, if patients know what treatment they received or if the investigators know which treatment a patient received, it may affect their impression of whether the patient improved–no matter how hard they try to avoid it. Therefore, it is important to ask what steps were taken to minimize bias. For example, was the trial "blinded" or "masked" so that neither the participants nor the investigators knew who was receiving which treatment? Researchers must always work to make sure that the study is objective, and the results reflect the data accurately.

6. Are there potential conflicts of interest?

In viewing the results of any study, it is important to look for potential conflicts of interest or other sources of bias. It is useful to understand who funded the study. How removed were the sponsor and the investigators from any financial or reputational "stake" in the study outcome? Is there similar evidence from other independent sources? Fortunately, most medical journal articles now include information about relevant financial relationships.

7. How do the reported results compare with previous studies?

The strongest evidence about whether an intervention is useful and safe consists of results from several studies by different investigators. Rarely does a single study provide a final, definitive answer. There is a need for a study to be replicated, which involves repeating a study using the same methods but with different volunteers and investigators. Replication of a study gives more confidence that the results are reliable and valid. In addition, independent evaluations that compile the results of multiple studies and rigorously assess the quality of the data from them are especially useful. These evaluations are called systematic reviews and meta-analyses.

8. What does it mean when the results of a study are described as statistically significant but not clinically significant?

"Statistically significant" means the finding in the difference between the study groups is not likely to be due to chance. "Clinically significant" is a measure of the size of the effects observed in the study. For example, a study can find statistically significant differences between two treatment groups, but the differences are so small that they do not have clinical significance in terms of usefulness for patients or safety.

9. How old is the study?

Look at the date of the study. Was it conducted in the last few years? Have there been more recent studies? You can search the National Library of Medicine's PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) for published studies.

Sometimes, new research can dramatically change scientists' view of a topic. For example, older pilot studies may have suggested that a particular complementary approach may be helpful for a certain medical problem, but a new, large clinical trial might show that it doesn't have beneficial effects. The GEM study, on *Ginkgo biloba* for dementia, is one example. This study was the largest clinical trial ever to evaluate ginkgo's effect on the occurrence of dementia, and although the results failed to show benefit of ginkgo in preventing dementia in older adults, the study confirmed the importance of randomized trials in determining the therapeutic benefit for complementary therapies. From a research point of view, the study also provided researchers with vital information about how to design and conduct large dementia prevention trials in older adults.

Chapter 37

Transdisciplinary Research and Clinical Priorities for Better Health

Luigi Fontana, MD, PhD,[a,b,c] Alessio Fasano, MD,[d,e] Yap Seng Chong, MD,[f,g] Paolo Vineis, PhD,[h] and Walter C. Willett, MD, Dr PH[i,j]

[a]*Charles Perkins Centre, Faculty of Medicine and Health, University of Sydney, Sydney, New South Wales, Australia*
[b]*Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia*
[c]*Department of Clinical and Experimental Sciences, Brescia University Medical School, Brescia, Italy*
[d]*Division of Pediatric Gastroenterology and Nutrition and Mucosal Immunology and Biology Research Center, Mass General Hospital for Children, Harvard Medical School, Boston, Massachusetts, USA*
[e]*European Biomedical Research Institute of Salerno (EBRIS), Salerno, Italy*
[f]*Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research (A*STAR), Singapore*
[g]*Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore*
[h]*MRC Centre for Environment and Health, School of Public Health, Imperial College London, London, UK*
[i]*Harvard T. H. Chan School of Public Health, Boston, Massachusetts, USA*
[j]*Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA*

luigi.fontana@sydney.edu.au

Keywords: bioaccumulation, cardiovascular disease (CVD), chronic diseases, Coronavirus Disease 2019 (COVID-19), ecological footprint, environmental degradation, epigenetics, greenhouse gas, health literacy, health promotion, insulin-like growth factor-binding protein 1 (IGFBP1), intergenerational health, lifestyle intervention, Mediterranean-like diet, microbiome, nonalcoholic fatty liver disease (NAFLD), personalized medicine, preventative medicine, preventive science, public health, science policy, sex hormone–binding globulin (SHBG), universal health coverage, wastewater treatment, World Health Organization (WHO)

Environmental degradation, global warming, and rising pollution are impairing planetary health even as lifestyle- and age-related chronic diseases and emerging infectious diseases are devastating human lives. These are among the greatest challenges facing society today, since people are living longer but often not

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healthier lives. More than 65% of people over 65 years have 2 or more chronic diseases [1, 2]. The current epidemic of obesity, beginning in children, is laying the foundation for even greater problems in the near future, including a reduction in healthy life expectancy. Governmental health expenditure as a percentage of gross domestic product is expected to more than double by 2050, making many existing health funding models unsustainable [3]. Additionally, the present medical approach to chronic diseases in the United States and other affluent countries has vast consequences on planetary health and global economic development. In brief, this reactive "sick-care" medical system is not efficient, equitable, or even viable. Similar problems are now affecting low-income countries, where the epidemiological transition to noncommunicable diseases is coupled with a still high incidence of infectious diseases, dramatic environmental dilapidation, lack of medical resources, and limited support for social and health promotion activities, resulting in increasing inequalities and poverty.

37.1 Lifestyle and Prevention of Chronic Diseases

Thanks in part to extraordinary advances in public health and medicine, life expectancy has more than doubled in the last 150 years, even if this trend is starting to reverse, and the incidence of cardiovascular disease (CVD) and obesity-related cancer is increasing in recent birth cohorts [4–6]. However, many of these chronic diseases are not easily curable because they are multifactorial and are usually controlled with lifelong use of often expensive medications and other devices. Moreover, modern medicine focuses on diagnosing and treating clinically evident diseases one at a time, mainly with drugs and surgery. This approach does not consider that many chronic diseases begin early in life and progress over decades of unhealthy lifestyles, which trigger a wide range of physiologic, metabolic, and molecular alterations, deeply influencing their initiation, progression, prognosis, and therapeutic options (Fig. 37.1).

We believe that universal health coverage should be a right of every human being, but we argue that this is achievable only if we invest adequate resources in preventive science and medicine, and not only in finding new pharmacological targets, an approach that further increases health inequities between rich and poor countries. According to WHO, at least 80% of CVD and diabetes and 40% of cancers are preventable [7]. We believe that these numbers are realistic and probably conservative, because experimental studies have shown that the accumulation of molecular damage can be prevented or much delayed by dietary, genetic, and pharmacological manipulations that down-regulate key cellular nutrient-sensing and inflammatory pathways [8]. In rodents and monkeys, dietary restriction with optimal nutrient intake protects against obesity, diabetes, cancer, CVD, brain aging, and frailty [9, 10], and in humans, this induces biological adaptations that protect against those illnesses as well as liver and kidney diseases [11]. Minimizing weight gain during adulthood through regular exercise and a healthy Mediterranean-like diet is key, but specific modulation

of other nutritional factors (e.g., specific amino acids, fatty acids, vitamins, and phytochemicals) directly and/or through gut microbiome metabolism may potentiate their beneficial effects [12]. Cognitive training, avoidance of smoking and excessive alcohol consumption, reducing stress, and improving sleep duration and quality are also crucial in preventing harmful physiological alterations [13]. In one study, US men and women who adopted healthier lifestyle behaviors lived about 10 years longer than those who did not, free of major chronic disease [14].

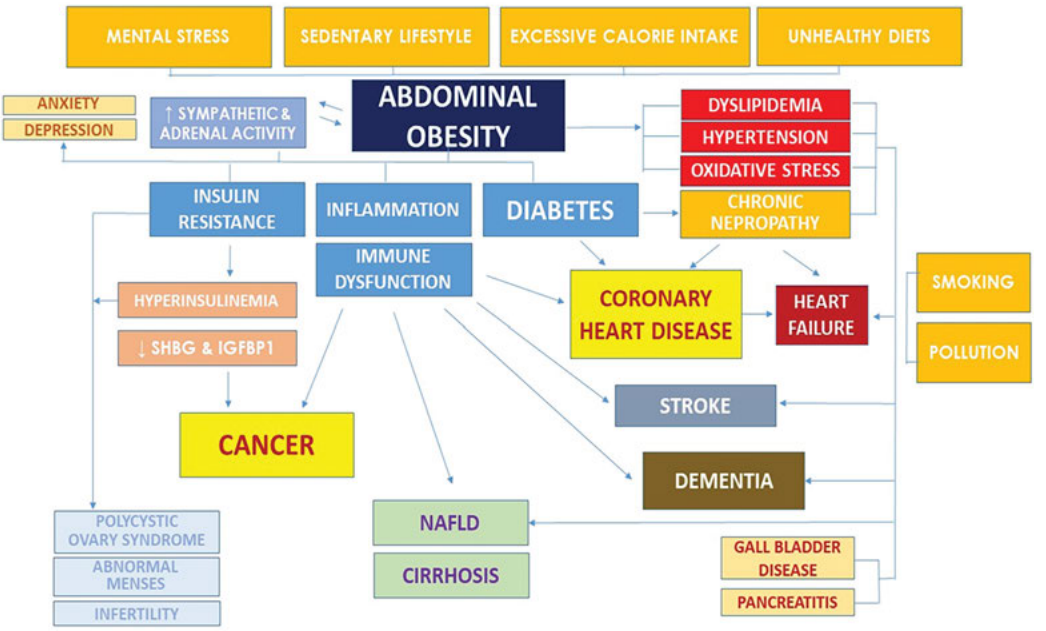

Figure 37.1 Most prevalent chronic diseases share a common metabolic substrate. The unhealthy lifestyle effectors, including excessive calorie intake, poor diets, sedentary lifestyle, mental stress, smoking, and pollution, modulate important metabolic and hormonal factors associated with the development of the most common age-associated chronic diseases. Abdominal obesity is a central node, but many effects of these unhealthy lifestyle are mediated through other metabolic and inflammatory pathways as well. *Abbreviations*: IGFBP1, insulin-like growth factor-binding protein 1; NAFLD, nonalcoholic fatty liver disease; SHBG, sex hormone–binding globulin.

37.2 Intergenerational and Life Course Consequences of Preconception and *in utero* Health

The epigenetic theory of disease is another reason why the current medical approach has limited success. Preconception parental environmental factors (diet, fitness, and metabolic and mental health) affect children's chronic disease risk and influence epigenetic heritage [15]. A high proportion of women experience emotional distress in pregnancy, which seems to be associated with deficits in

children's neurodevelopment [16]. The first 1,000 days of life shaped by maternal diet also influence fetal development, newborn well-being, and health trajectory for the entire life span [17]. This may be in part mediated by shaping the gut, vaginal, and skin microbiome that seems to influence a child's immunity (and autoimmunity) and cognitive development [18]. Noncommunicable diseases result from multistage processes beginning early in life, but clinical medicine usually intervenes in late stages when therapeutic options are limited and chances of cure are highly decreased.

37.3 Ecological Footprint of Modern Medical Systems

The huge progress of medical sciences in treating morbid conditions is undisputed. However, the healthcare sector is responsible for 3% to 10% of CO_2 emissions, about 10% to 18% of which are due to drug prescription [19]. Moreover, the consumption of old and new medications by a growing number of older adults affected by multiple chronic diseases has caused a 10- to 20-fold increase in aquatic levels of pharmaceutical residues and by-products over the past 20 years, which can now be detected in freshwater and marine organisms, and in crops irrigated with reclaimed water and soil amended with wastewater treatment products [20]. The increasing bioaccumulation and exposure to traces of these pharmaceuticals via food webs, even at low concentrations, raises potential serious concerns for human and environmental health.

37.4 Intensive Animal Farming and Pollution

Diets rich in ultraprocessed and animal foods have deleterious effects not only on humans but also on environmental health. Industrial animal farming account for 70% of freshwater use, with 70% of all land under tillage used to feed livestock. Today, the globalization of agriculture based on extensive production of monoculture crops, together with excessive and inappropriate use of agrochemicals, contribute to deforestation; soil degradation and erosion; groundwater depletion; contamination of rivers, lakes, and aquifers; and release of toxic substances from chemical fertilizers [21, 22]. About 15% of greenhouse gas and 20% to 30% of PM10 and PM2.5 in some heavily farmed parts of the world is produced by livestock emissions and by the nitrogen fertilizers [23]. We do not all need to become vegetarians, but substantial movement toward a Mediterranean-like diet, emphasizing whole-plant foods produced with sustainable agriculture practices, would be good for us and the planet [24]. Unfortunately, consumption of meat and dairy is on the rise particularly in low- and middle-income countries [25]. Finally, high-density poultry and swine production creates large reservoirs for the development of antibiotic-resistant bacteria and new viral strains with zoonotic and potential human pandemic effects [26, 27]. Although these trends have enormous implications for health, the medical community involvement in mitigation efforts has been limited.

37.5 Benefits of Investing in Preventive Science, Education, and Medicine

Epidemiological, mechanistic, and translational studies are exponentially elucidating the processes driving the accumulation of organismal damage. Our failure is not due to lack of knowledge, but how we use it. Waiting for millions of people, who eat unhealthy food and engage in harmful lifestyles, to end up in outpatient clinics or hospitals with symptoms of chronic diseases is unethical and financially and environmentally unsustainable. We argue that we should use our accumulating scientific and technological knowledge to increase health equity despite social, economic, and geographical disparities and to minimize the risk of developing diseases, and not only to treat illness after it has clinically occurred.

Transitioning from a primarily disease-centered medical system to a balanced preventive and personalized treatment healthcare system is key. While it is not new to highlight how healthier lifestyles and food systems can address some of these issues, little research and no unifying framework exist to harmonize these concepts of sustainable system management across diverse scientific and medical fields into a coherent theoretical or operational body. Insights beyond reductionist views are needed to encourage integrated changes in the use of our limited financial and human resources, with the aim of achieving their wiser and more productive use.

The real costs of the effects of our dysfunctional medical, food, and agriculture systems, and of our fossil fuel–driven economy, are largely unmeasured and have little or no impact on producers or societal choices about production, distribution, and consumption of goods. Full accounting must become the basis of policy, ethics, and action. This can lead to a range of scientific-based opportunities for cost-effective actions and policies that can be afforded worldwide and add years of healthy life without adding years burdened by disease [14]. Prevention, through the multiple actions we propose in this chapter, would have the additional advantage of reducing social disparities in health, now exacerbated by unequal access to education, healthcare, and health-promoting environments [28].

The Coronavirus Disease 2019 (COVID-19) pandemic has also taught us some key lessons that are relevant not only in infection control but also to health in general. First, dealing with emerging diseases requires an open mind, fast insight, scientific rigor, adaptability, and resilience. Second, it demands strong leadership, political restraint, and effective population engagement. Third, it reminds us that health crises exact the greatest toll on the disenfranchised and disadvantaged, both at the individual and country levels. Fourth, it highlights that borders are meaningless in our highly interconnected world and that global solidarity is vital. Finally, we have to learn from the present and the past to develop and implement high-level systems and organizational policies and practices that enhance health literacy. This passage is imperative to move from a doctor-to-patient 1-way model to a continuous 2-way exchange in which the patient takes ownership of his/her health aided by a multidisciplinary healthcare team that facilitates personalized and preventive care.

37.6 Conclusions and Future Directions

Integrating health literacy as soon as possible into education is key because it shapes health and well-being across people's lives. However, despite the wealth of mechanistic knowledge linking nutrition, exercise, sleep, and cognitive training and health, these topics receive little or no attention in primary, secondary, and tertiary education, including medical schools. Schools and universities should not be a loose collocation of specialized academic silos but transformative engines that provide not only the expertise needed to have successful careers but also knowledge and practical skills on the mechanisms and interventions linking diet and other lifestyles to human and planetary health.

We need to invest more public and private resources to strengthen existing programs of disease prevention and create more complex and transnational scientific and economic analytic models based on multiple objectives and constraints. Additional resources are needed to develop science-based strategies, effective policies, and structural reforms that encourage integrated pro-health changes, with the aim of achieving better healthcare, diets, and farming systems not only in high-income nations but also in low- and middle-income countries. This includes refining our comprehension of facilitating strategies and barriers to implementation, field testing novel eHealth intervention procedures and materials for efficacy and acceptability by target populations, and improving research and assessment procedures in real-world settings.

Critical global goals, which will require political engagement, should be to develop and deploy evidence-based interventions aimed at reducing behaviors associated with poor health prospects or environmental degradation. These could include, for example, lowering taxes and health insurance premiums for people with healthy lifestyles; making healthy foods more affordable relative to less healthy food, thus reducing health inequalities related to income disparities; taxing not only carbon but also animal and ultraprocessed foods and beverages; ending direct and indirect subsidies for crops fed to animals and intensive animal farming; and restricting advertisement of unhealthy foods to children, implementing front-of-package nutrient warning labels, and enhancing food quality in schools to help curb the growing pandemic of child obesity.

These steps are important individually but take on particular significance when integrated by guiding principles for the design of an entirely new disease prevention–centered science, educational, and healthcare system that maximizes both human and planetary health. Scientific and health organizations should play crucial roles in promoting national and international actions needed to confront challenges to our shared human and environmental health.

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Chapter 38

Current Issues about Health News Stories[1]

National Institutes of Health, Bethesda, Maryland, USA
info@nccih.nih.gov

38.1 When Clinical Research is in the News

Have you ever heard that something, like a gene or toxin, can cause disease? Or that a drug can prevent illness? How about a behavior, like too much sitting, that's "linked" to health problems? Such news reports are often based on some type of clinical research, which is the study of health and illness in people. There are many types of clinical studies. Each has its own strengths and weaknesses. Knowing more about the different types can help you think critically about the health and research news you see and hear. The figures below serve as a guide that outlines different types of clinical studies and explains why scientists might use them. The figures note that a study's strength depends on its size, methods, and design. The ideal way to prove that a treatment works is through a well-designed "randomized controlled trial." In such trials, people are randomly assigned to either a "treatment" or a "control" group for comparison. Other clinical studies involve observing people to find associations or links. For instance, a "cohort study" may follow many people over time to learn how a disease arises and find possible risk factors. These observational studies can find "links" but can't prove the cause of disease.

[1]This chapter has been compiled and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Institutes of Health (NIH). A part of the U.S. Department of Health and Human Services, NIH is the largest biomedical research agency in the world. NIH's mission is to seek fundamental knowledge about the nature and behavior of living systems and the application of that knowledge to enhance health, lengthen life, and reduce illness and disability. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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HOW RESEARCH WORKS

UNDERSTANDING THE PROCESS OF SCIENCE

Scientists ask questions about how the world works. Each research advance builds on past discoveries, often in unexpected ways. This process isn't always a straight path. But here's a general overview:

DIFFERENT ANGLES DIFFERENT TECHNIQUES

Scientists with diverse skills and training can look at a question from different angles. They review past research and design new experiments to test their ideas.

EVIDENCE ACCUMULATES

Scientists collect data from their experiments and evaluate what their findings might mean. That may lead to new ideas to test–or new ways to test older ideas.

SHARING DATA

To tell other scientists what they've found, researchers give presentations at meetings and publish papers in scientific journals.

THE BIG PICTURE

Each finding is often a small piece of a larger puzzle. It may take data from many different researchers to start piecing the full puzzle together. Science is constantly evolving, and our understanding changes.

Research results sometimes seem to contradict each other. This can happen when scientists use different methods or timeframes. Reality is often more complex than the findings of a single study. That's why it's important to consider how all research results fit together.

FORMING CONCLUSIONS

Over time, enough evidence accumulates to point toward an explanation of all the different findings on a topic.

MORE QUESTIONS

Some research might not answer the scientists' original questions. But the knowledge gained may help answer other questions. And new findings raise new questions.

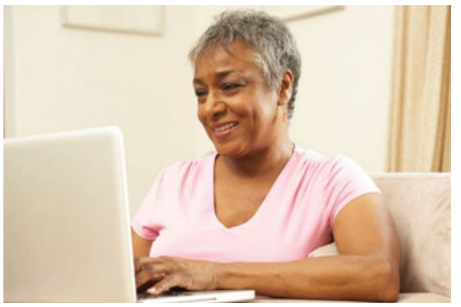
38.2 Health Approaches in the News

News stories about health are often on television, the Internet, and in magazines and newspapers.

In fact, the media is one of our main sources of information when we make decisions about health approaches. While many news reports are reliable, some are missing important information, and some are confusing, conflicting, or misleading.

38.3 Information Missing from Health Stories

Health stories in the media teach us about the importance of health issues and change how we think and what we do about our health. High-quality news reports give us realistic expectations and inform the medical community about medical advances. But news stories about health approaches often lack details that could help us make good decisions about our health.

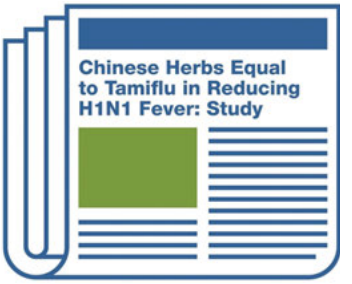


38.3.1 What Information is Sometimes Missing from News Stories?

- How well one approach works compared with another
- The side effects of an approach
- Whether a study's results are "statistically significant"—meaning they didn't happen just by chance
- Whether the study was done in animals or in people

38.4 What's Missing: Important Details!

A 2011 news story reported on a study comparing the conventional flu medicine Tamiflu, a common Chinese herbal flu product called maxingshigan–yinqiaosan, and the combination of the two. Compared with no treatment, the combination helped relieve participants' fevers sooner, the news story explained.



38.4.1 But What Was Missing from the News Story?

The story didn't say that participants' fevers went down only about 11 hours sooner with the combination of products, which readers may want to know when they're deciding whether to use the products. The story also didn't mention that none of the products helped with symptoms such as cough and sore throat.

38.5 What's Missing: Information on Side Effects!

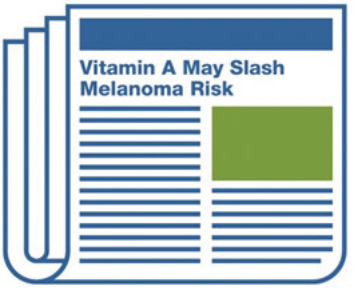
Potential harmful side effects of a health approach and the quality of the evidence supporting a study's findings are sometimes left out or not fully explained in health news stories. One lengthy news story from 2010 talked about using fish oil during pregnancy.

What was missing from the news story?

This story did not mention the possible side effects of fish oil, like upset stomach and interactions with prescription or over-the-counter medications.

38.6 What's Missing: The Full Story!

Another news story reported that men and women in a study who took vitamin A were 60 percent less likely to develop melanoma than those participants who did not take it. But as the story points out "the reduced risk was more pronounced in women than men."



What was missing from the news story?

What the story didn't say was that the reduced risk for men was not "statistically significant," meaning that the reduction was so small it might have been due to chance. That's very different than a 60 percent reduction. The study's authors estimated that among women in the general population, regular vitamin A use would reduce the risk for melanoma by 35 to 90 percent.

38.7 What's Missing: Humans!

Media stories sometimes report the results of studies done only on animals without explaining that such basic science may have little immediate significance to people. For example, a 2010 news story reported that dark chocolate may help guard against brain injury after a stroke.

What was missing from the news story?

The story did not say that the study was done on mice.

38.8 Conflicting Health News

Question: Media reports about new medical research findings sometimes give conflicting information. You may see a news report that a health product or approach is good for you, and later see another news report that it's not. Why do you think there is conflicting information in media reports?

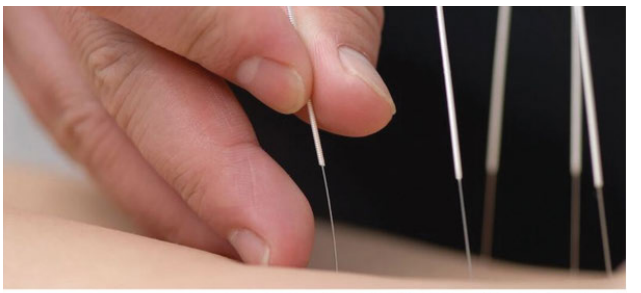
Answer: Sometimes results of new research disagree with earlier studies. Researchers report their findings as they complete their studies, and new studies sometimes disagree with earlier discoveries. For example, studies have shown that red wine has some heart-healthy benefits, and that resveratrol (an ingredient found in red wine) may slow the growth of breast cancer cells. However, previous studies have shown that alcohol consumption of any type can modestly increase breast cancer risk. It's a good idea to look for statements by medical experts, who often put the new study and any conflicting messages into context. Once there has been enough research conducted, experts evaluate all of the research together.

38.9 Accuracy in the Media

Unless you read and understand the original sources for the story, it can be difficult to know whether a news story is misleading. But the likelihood that the story is correct increases if it:

- Comes from a media outlet, like a news station or Web site, that isn't trying to promote a point of view or cause
- Was written by a science or health reporter trained to understand medical findings
- Includes quotes from experts not connected to the study, for a more objective take on the findings or to show another point of view.

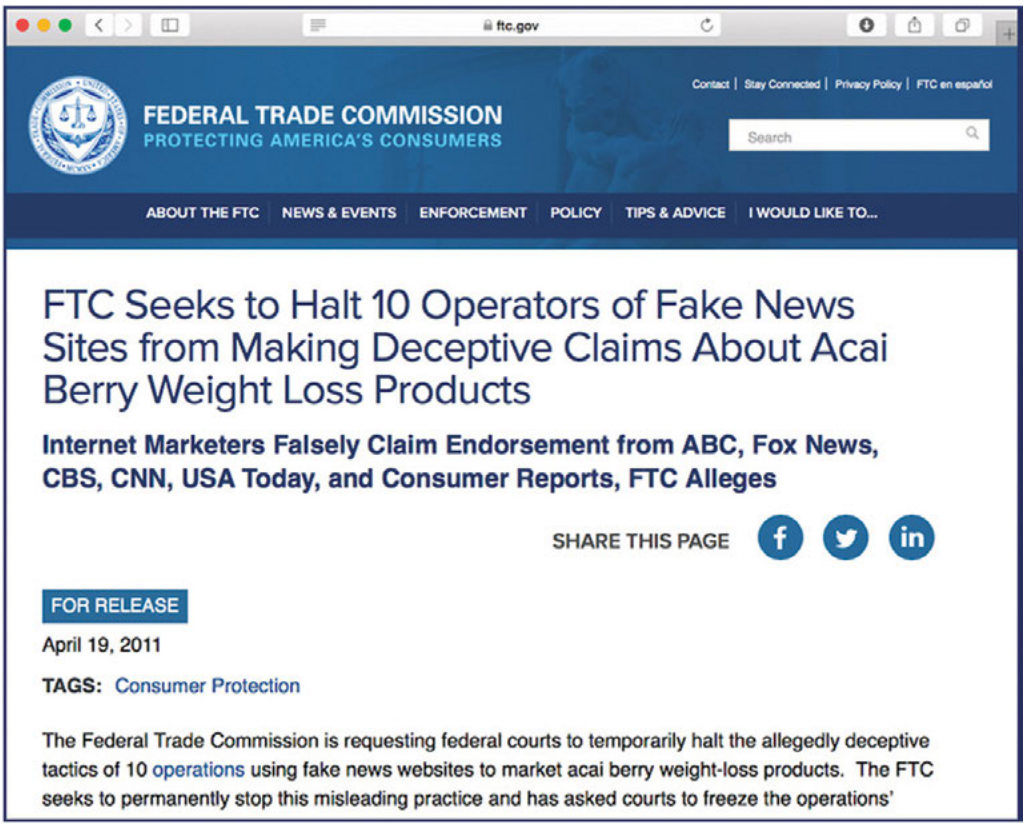
38.10 What the Media Says about Complementary Health Approaches



There's not a lot of research about how the media reports on complementary health approaches. However, existing research has found that:

- Many news stories say complementary health approaches should be used with conventional medicine not instead of it.
- Media reports tend to describe complementary approaches as a treatment for an illness or specific symptoms, even though people also use complementary approaches to try to improve their overall health and to prevent illness.
- The tone of news reports on complementary approaches is generally positive, but that may be because the reports often leave out potential risks.

38.11 Is It Real Online News? Or Just Advertising?



In April 2011, the Federal Trade Commission warned the public about fake online news sites promoting an acai berry "weight-loss" product.

On a typical fake "news" site, a story described an investigation in which a reporter used the product for several weeks, with "dramatic" results. The site looked real, but it was actually an advertisement. Everything was fake: there was no reporter, no news organization, and no investigation. The only real things

were the links to a sales site that appeared in the story and elsewhere on the Web page.

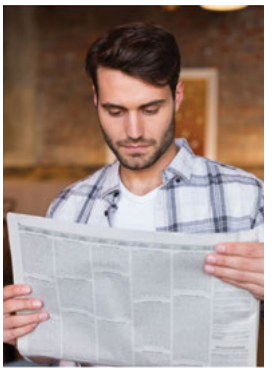
The following may be an indication that a "news" site is fake:

- The site endorses a product.
- The site quotes only people who say good things about the product.
- The site discusses only positive research results.
- The site contains links to a sales site.

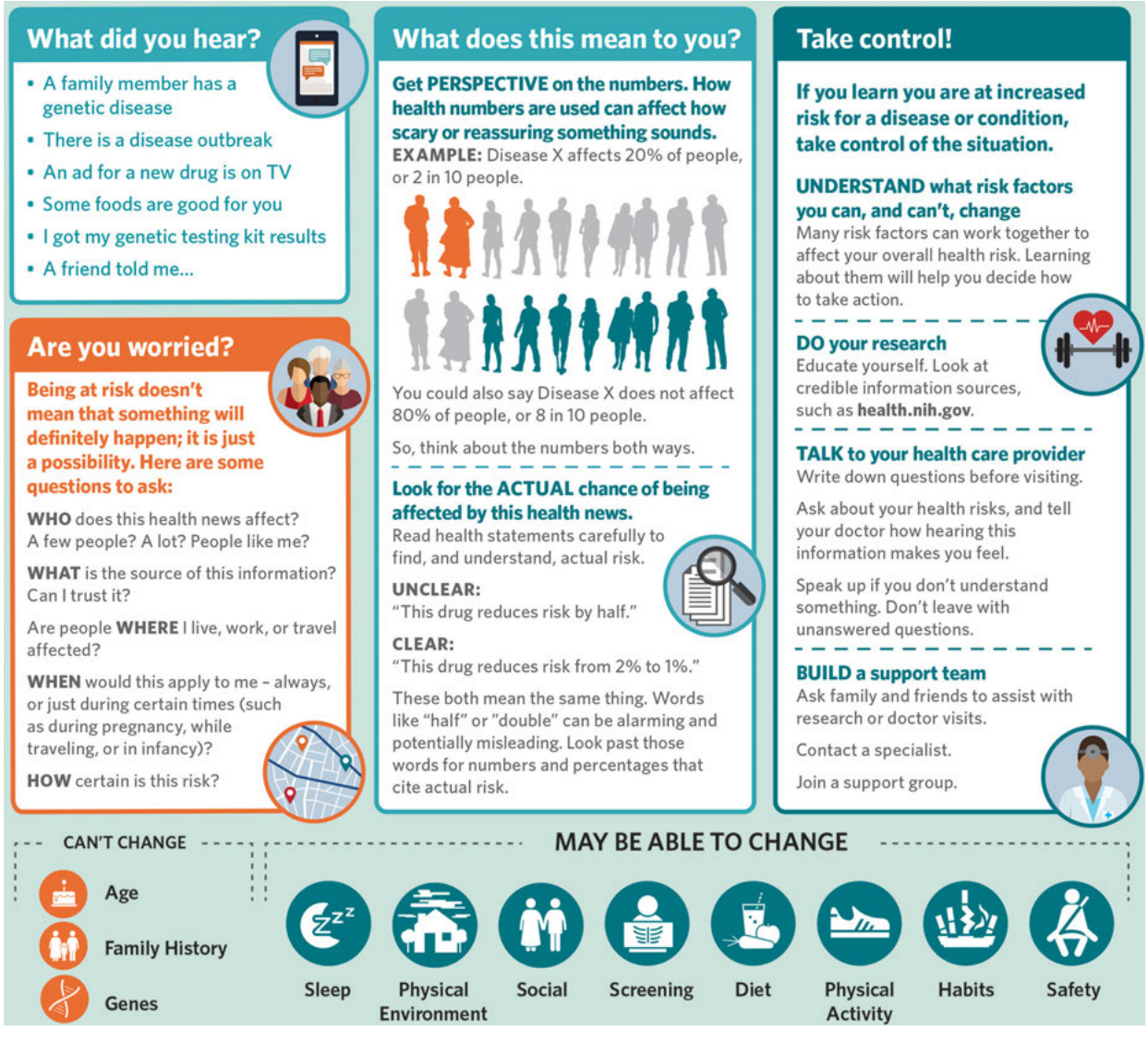
38.12 Checklist for Understanding Health News Stories

To figure out if a news report about a health approach is giving you the full story, you should ask yourself these questions.

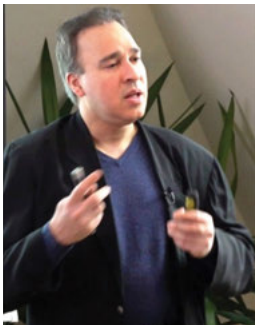
9 Questions to Ask



1. Was the product, procedure, or device tested on people? Findings from animal or laboratory research may not be immediately meaningful to your health.
2. Are there alternatives to the approach being discussed? You want to know what is already available, so you can compare your options.
3. Were enough people studied? When the number of people in a study is small, the results aren't as strong.
4. Were the results big enough to be meaningful to you? A small difference between two approaches might interest scientists but be of little importance to your health or quality of life.
5. Did the researchers consider the many things that can influence results, such as participants' general health or health habits, or discuss the limitations of their results?
6. Were the study participants similar to you in ways that may matter, such as age, race, or gender?
7. Was the study lengthy enough to show long-term benefits or risks? Natural products may take time before they show benefits; some side effects may take months or years to show up.
8. Have other researchers had similar results? One study rarely proves anything.
9. Was the study funded by a group that would profit financially from the study findings? If so, you should be wary of the results.


What did you hear?
• A family member has a genetic disease
• There is a disease outbreak
• An ad for a new drug is on TV
• Some foods are good for you
• I got my genetic testing kit results
• A friend told me...
Are you worried?
Being at risk doesn't mean that something will definitely happen; it is just a possibility. Here are some questions to ask:
WHO does this health news affect? A few people? A lot? People like me?
WHAT is the source of this information? Can I trust it?
Are people WHERE I live, work, or travel affected?
WHEN would this apply to me – always, or just during certain times (such as during pregnancy, while traveling, or in infancy)?
HOW certain is this risk?
What does this mean to you?
Get PERSPECTIVE on the numbers. How health numbers are used can affect how scary or reassuring something sounds.
EXAMPLE: Disease X affects 20% of people, or 2 in 10 people.
You could also say Disease X does not affect 80% of people, or 8 in 10 people.
So, think about the numbers both ways.
Look for the ACTUAL chance of being affected by this health news.
Read health statements carefully to find, and understand, actual risk.
UNCLEAR:
"This drug reduces risk by half."
CLEAR:
"This drug reduces risk from 2% to 1%."
These both mean the same thing. Words like "half" or "double" can be alarming and potentially misleading. Look past those words for numbers and percentages that cite actual risk.
Take control!
If you learn you are at increased risk for a disease or condition, take control of the situation.
UNDERSTAND what risk factors you can, and can't, change
Many risk factors can work together to affect your overall health risk. Learning about them will help you decide how to take action.
DO your research
Educate yourself. Look at credible information sources, such as health.nih.gov.
TALK to your health care provider
Write down questions before visiting.
Ask about your health risks, and tell your doctor how hearing this information makes you feel.
Speak up if you don't understand something. Don't leave with unanswered questions.
BUILD a support team
Ask family and friends to assist with research or doctor visits.
Contact a specialist.
Join a support group.
CAN'T CHANGE
Age
Family History
Genes
MAY BE ABLE TO CHANGE
Sleep
Physical Environment
Social
Screening
Diet
Physical Activity
Habits
Safety

38.13 Remember…



Reading, watching, or listening to health news about health approaches can help you learn and stay informed about new medical findings. However, there's a lot of important information to consider before you try a therapy featured in the news. Remember, no matter how promising an approach may sound, it's important to talk about it with your health care providers before you try it.

38.14 Supplementary Information

What does "homeopathic" mean?

Homeopathic medicine is an alternative medical system that was developed in Germany more than 200 years ago and is based in part on the principle called "law of minimum dose," which is the notion that the lower the dose of the medication, the greater its effectiveness. Many homeopathic remedies are so diluted that no molecules of the original substance remain; however, some products labeled as "homeopathic" can contain substantial amounts of active ingredients and therefore could cause side effects and drug interactions. The U.S. Food and Drug Administration has warned consumers about different products labeled as homeopathic (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm523468.htm).

What does "holistic" mean?

The word holistic (sometimes spelled wholistic) often refers to the treatment of the whole person—body, mind, emotions, and spirit—to achieve wellness and good health. People who practice holistic medicine may use conventional medications to treat a person's ailment, but they will take into consideration other factors such as sleep habits, lifestyle, stress, and diet to improve health and relieve symptoms.

What does "natural" mean?

When it comes to medicine, there is no official definition for use of the term natural. And it's important to know that a medicinal product labeled natural does not always mean safe. For example, the herbs comfrey and kava may be considered natural products, but they can cause serious harm to the liver. An herbal or botanical supplement labeled natural may contain dozens of chemical compounds and all of its individual components may not be known.

What does "naturopathic" mean?

Naturopathic medicine is a medical system that has evolved from a combination of traditional practices and health care approaches popular in Europe during the 19th century. In the United States, naturopathic practitioners use many different treatment approaches, including dietary and lifestyle changes, stress reduction or relaxation techniques, herbs or botanicals, dietary supplements, and other natural products, homeopathy, manipulative therapies, counseling, conventional medicine, and others.

What does "complementary health approaches" mean?

Complementary health approaches include a broad range of practices, interventions, and natural products, which are not typically part of conventional medical care, or which may have origins outside of usual Western practice. Complementary health approaches can be roughly divided into two major groups—mind and body practices, and natural products

What does "alternative medicine" mean?

Like complementary health approaches, alternative medicine are products or practices developed outside of mainstream Western, or conventional, medicine, but they are used *in place of* conventional medicine.

What is clinical research?

Clinical research is research conducted with human subjects, or material of human origin, in which the researcher directly interacts with human subjects. Clinical research helps doctors and researchers to find new and better ways to understand, detect, control, and treat illness. A clinical research study is a way to find answers to difficult scientific or health questions. For example, the study might explore the best ways to treat people with colon cancer. By studying cancer cells from patients, researchers may be able to determine the specific genetic mutations (changes in gene sequence) that caused the normal, healthy cells to become cancerous, and may help doctors decide on the best drugs to prescribe or surgeries to perform. Clinical research today may help other doctors in the future screen their healthy patients before they ever develop cancer.

What is a protocol?

All clinical studies are based on a set of rules or directions called a protocol. A protocol describes what types of people are eligible to participate in the study; determines the schedule of tests, procedures, medications, and dosages; and sets the length of the study.

What is a clinical trial?

A clinical trial is a research study in which one or more human subjects are prospectively assigned to one or more interventions (which may include placebo or other control) to evaluate the effects of those interventions on health-related biomedical or behavioral outcomes.

What are clinical trial "phases?"

Clinical trials of experimental drugs proceed through four phases: In Phase I clinical trials, researchers test a new drug or treatment for the first time in a small group of normal, healthy volunteers (about 20 to 80) to evaluate its safety, determine a safe dosage range, and identify side effects. In Phase II clinical trials, the study drug or treatment is given to a larger group of people (about 100 to 300), including patients with the particular disease, to see if the drug or treatment is effective, and to further evaluate its safety. In Phase III clinical trials, the study drug or treatment is given to large groups of people (from 1,000 to 3,000), including patients, to confirm its effectiveness, monitor side effects, compare it to other commonly used treatments, and collect information that will allow the drug or treatment to be used safely. Phase IV clinical trials are done after the drug or treatment has been approved by the FDA and marketed for public use. These studies continue testing the drug or treatment to collect information about its effect in various populations and gather data on any side effects associated with long-term use.

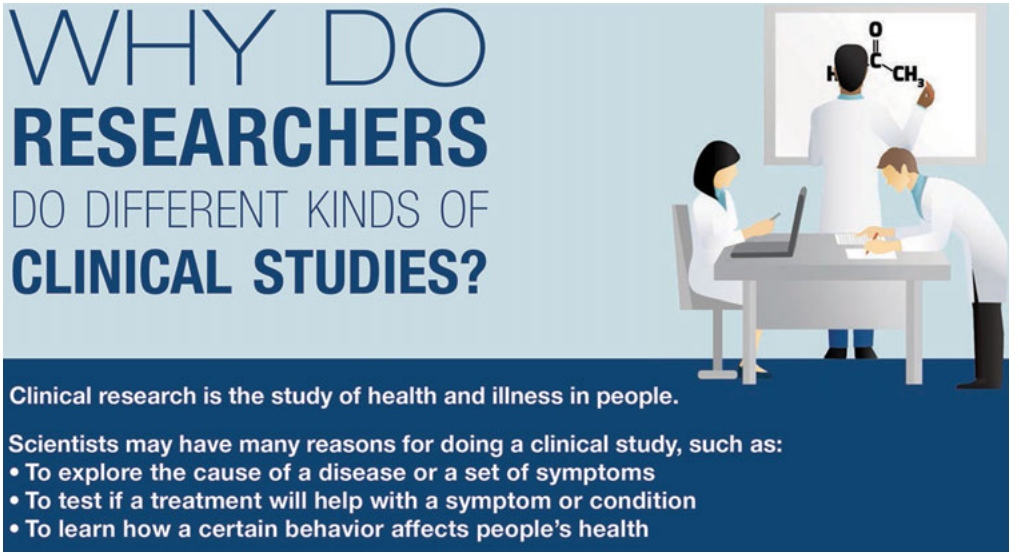
What are "blind" or "masked" studies?

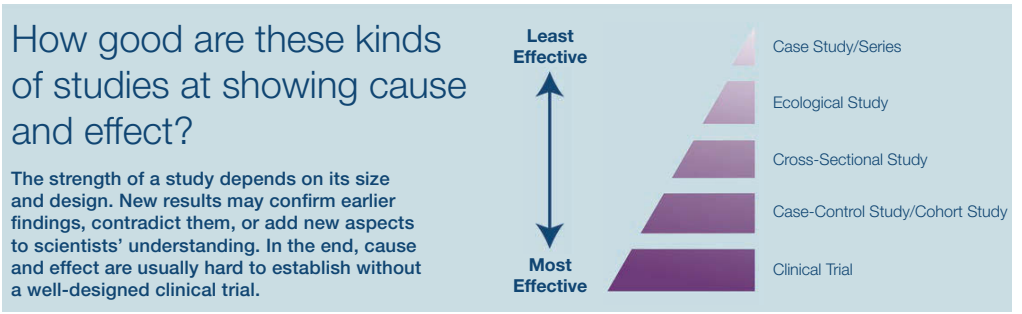
In many clinical trials, one group of patients will be given an experimental drug or treatment, while a control group is given either a standard treatment for the illness, or a placebo (a harmless "fake" drug), or no treatment at all. In a "blinded" or "masked" study, participants do not know whether they are getting the drug being tested, or whether they are in the control group. The goal is to prevent the so-called "placebo effect" from influencing the results of the experiment. The placebo effect is the phenomenon of patients feeling better simply because they think they are receiving a helpful drug or treatment. Sometimes, clinical trials are "double-blind" or "double-masked." That means that neither the participants, nor the study staff members, know who is receiving the experimental drug and who is in the control group. Studies are performed in this way so that neither the patients' nor the doctors' expectations about the experimental drug can influence the observations and results.

Should I volunteer for clinical research?

Clinical research is a vital part of finding new treatments and cures for diseases. Carefully conducted clinical studies are the fastest way to find treatments that

are safe and effective. By volunteering for a clinical study, you would be participating in research that may result in a new treatment for a deadly or debilitating disease. Before you agree to participate in a study, you must be given complete information about the study, known as "informed consent." Informed consent involves two essential components: a document and a process. The informed consent document gives a summary of the research project (including the study's purpose, research procedures, potential benefits and risks, etc.) and explains the individual's rights as a research participant. This document is part of an informed consent process, which consists of conversations between the research team and the participant, and may include other supporting material such as study brochures. The informed consent process provides research participants with ongoing explanations that will help them make informed decisions about whether to begin or continue participating in the research project.





Different types of clinical studies are used in different circumstances. Depending on what is known and what isn't, scientists may even study the same research question using different kinds of studies and in different groups of people. Here are different types of clinical studies and why they might be used.

Observational Studies

In many studies, researchers do not do experiments or test new treatments; they *observe*. Observational studies help researchers understand a situation and come up with hypotheses that can be put to the test in clinical trials. Observational studies can find associations between things but can't prove that one thing causes another. Types include:

Case Study/Case Series

A detailed description of one or more patients. By documenting new and unusual cases, researchers start to generate hypotheses about causes or risk factors.

Ecological Study

Compares the rate of a disease or condition for groups of people, such as towns in different climates or with different average incomes.

Cross-Sectional Study

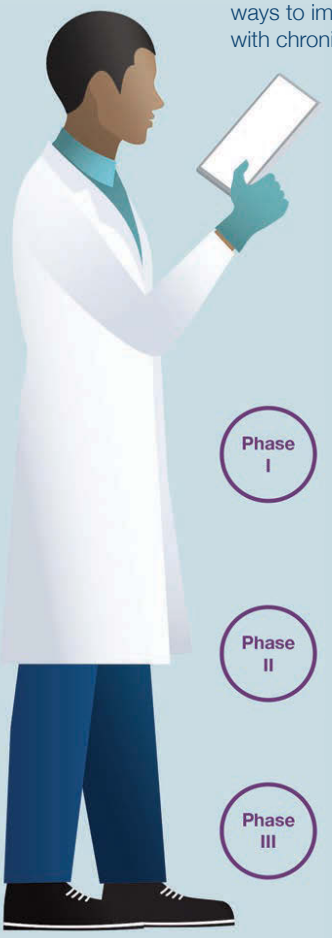
A snapshot of many people at one moment in time. These studies can show how common a condition is and help identify factors associated with it.

Case-Control Study

A group of people who have a condition is compared to a control group of people who don't. Possible causes or risk factors can emerge.

Cohort Study

A large group of people is observed over time. Some eventually develop a disease or condition. Researchers can learn how often the condition occurs and find possible causes or risk factors.



Clinical Trials

In these studies, researchers test new ways to prevent, detect, or treat disease. Treatments might be new drugs or combinations of drugs, new surgical procedures or devices, or new ways to use existing treatments. Clinical trials can also test other aspects of care, such as ways to improve the quality of life for people with chronic illnesses.

A well-designed clinical trial is the gold standard for proving that a treatment or medical approach works, but clinical trials can't always be used. For example, scientists can't randomly assign people to live in different places, or ask people to start smoking or eating an unhealthy diet. Clinical trials are conducted in phases:

Phase I

Phase I

- Purpose: Find out whether a medical approach (e.g., drug, diagnostic test, device) is safe, identify side effects, and figure out appropriate doses.
- Number of people: Typically fewer than 100

Phase II

Phase II

- Purpose: Start testing whether a medical approach works. Continue monitoring for side effects; get information that goes into designing a large, phase III trial.
- Number of people: Typically 100-300

Phase III

Phase III

- Purpose: Prove whether a medical approach works; continue monitoring side effects.
- Number of people: As many as needed or able to enroll—can be 1,000 or more

Phase IV

Phase IV

- Purpose: When a medical approach is being marketed, continue gathering information on its effects.
- Number of people: Thousands

Chapter 39

Complementary, Alternative, or Integrative Health: What's in a Name?[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: acupuncture, acupuncturists, Alexander technique, alternative health, Ayurvedic medicine, chiropractic and osteopathic manipulation, chiropractors, complementary health, dance therapies, dietary supplements, Feldenkrais method, functional medicine, herbs, homeopathy, hypnosis, hypnotherapy, integrative health, massage therapists, microbial-based therapies, mindful eating, music therapies, naturopathy, phytochemicals, Pilates, prebiotics, probiotics, qi gong, relaxation therapies, Rolfing Structural Integration, spinal manipulation, tai chi, traditional Chinese medicine, traditional healers, Trager psychophysical integration, whole person health, yoga

We've all seen the words "complementary," "alternative," and "integrative," but what do they really mean? This chapter looks into these terms to help you understand them better and gives you a brief picture of the mission and role of the National Center for Complementary and Integrative Health (NCCIH) in this area of

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at the NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH.

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research. The terms "complementary," "alternative," and "integrative" are continually evolving, along with the field, but the descriptions of these terms here are how we at the National Institutes of Health currently define them.

39.1 Complementary versus Alternative

According to a 2012 national survey, many Americans—more than 30 percent of adults and about 12 percent of children—use health care approaches that are not typically part of conventional medical care or that may have origins outside of usual Western practice. When describing these approaches, people often use "alternative" and "complementary" interchangeably, but the two terms refer to different concepts:

- If a non-mainstream approach is used **together with** conventional medicine, it's considered "complementary."
- If a non-mainstream approach is used **in place of** conventional medicine, it's considered "alternative."

Most people who use non-mainstream approaches also use conventional health care.

In addition to the terms complementary and alternative, you may also hear the term "functional medicine." This term sometimes refers to a concept similar to integrative health (described below), but it may also refer to an approach that more closely resembles naturopathy (a medical system that has evolved from a combination of traditional practices and health care approaches popular in Europe during the 19th century) (https://www.nccih.nih.gov/health/naturopathy).

39.2 Integrative Health

Integrative health brings conventional and complementary approaches together in a coordinated way. Integrative health also emphasizes multimodal interventions, which are two or more interventions such as conventional medicine, lifestyle changes, physical rehabilitation, psychotherapy, and complementary health approaches in various combinations, with an emphasis on treating the whole person rather than, for example, one organ system. Integrative health aims for well-coordinated care among different providers and institutions by bringing conventional and complementary approaches together to care for the whole person.

The use of integrative approaches to health and wellness has grown within care settings across the United States. Researchers are currently exploring the potential benefits of integrative health in a variety of situations, including pain management for military personnel and veterans, relief of symptoms in cancer patients and survivors, and programs to promote healthy behaviors.

What is whole person health?

Whole person health refers to helping individuals improve and restore their health in multiple interconnected domains—biological, behavioral, social, environmental—rather than just treating disease. Research on whole person health includes expanding the understanding of the connections between these various aspects of health, including connections between organs and body systems.

39.2.1 Integrative Approaches for Pain Management for Military Personnel and Veterans

- Chronic pain is a common problem among active-duty military personnel and veterans. NCCIH, the U.S. Department of Veterans Affairs, and other agencies are sponsoring research to see whether integrative approaches can help. For example:
  - An NCCIH-funded study is developing an innovative, collaborative treatment model involving chiropractors, primary care providers, and mental health providers for veterans with spine pain and related mental health conditions.
  - Other NCCIH-funded studies are testing the effects of adding mindfulness meditation, self-hypnosis, or other complementary approaches to pain management programs for veterans. The goal is to help patients feel and function better and reduce their need for pain medicines that can have serious side effects.
- For more information on pain management for military personnel and veterans, see NCCIH's Complementary Health Practices for U.S. Military, Veterans, and Families webpage (https://www.nccih.nih.gov/health/military-veteran).

39.2.2 Integrative Approaches for Symptom Management in Cancer Patients and Survivors

- Cancer treatment centers with integrative health care programs may offer services such as acupuncture and meditation to help manage symptoms and side effects for patients who are receiving conventional cancer treatment. Although research on the potential value of these integrative programs is in its early stages, some studies have had promising results. For example, NCCIH-funded research has suggested that:
  - Massage therapy may lead to short-term improvements in pain and mood in patients with advanced cancer.

    - Yoga may relieve the persistent fatigue that some women experience after breast cancer treatment, according to the results of a preliminary study.
    - Tai chi or qi gong have shown promise for managing symptoms such as fatigue, sleep difficulty, and depression in cancer survivors.
- For more information, see NCCIH's fact sheet on cancer (https://www.nccih.nih.gov/health/cancer-in-depth).

39.2.3 Integrative Approaches and Health-Related Behaviors

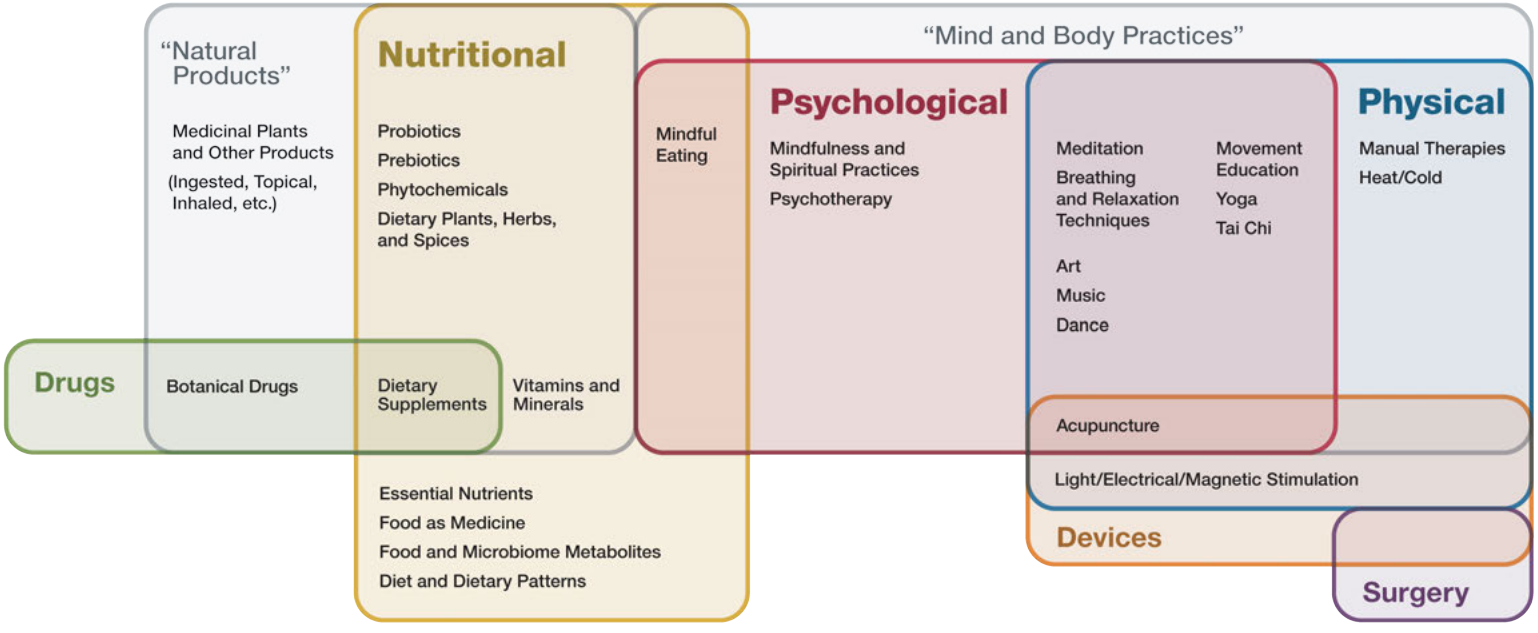
- Healthy behaviors, such as eating right, getting enough physical activity, and not smoking, can reduce people's risks of developing serious diseases. Research is looking at whether complementary and integrative approaches may have a role in promoting healthy behaviors. For example:
    - Preliminary research suggests that yoga and meditation-based therapies may help smokers quit.
    - In a study funded by the National Cancer Institute, complementary health practitioners (chiropractors, acupuncturists, and massage therapists) were successfully trained to provide evidence-based smoking cessation interventions to their patients.
    - An NCCIH-funded study is testing whether a mindfulness-based program that involves the whole family can improve weight loss and eating behavior in adolescents who are overweight.
- For more information, see the NCCIH Quitting Smoking (https://www.nccih.nih.gov/health/quitting-smoking) and Weight Control webpages (https://www.nccih.nih.gov/health/weight-control).

39.3 Complementary Health Approaches

Complementary approaches can be classified by their primary therapeutic input (how the therapy is taken in or delivered), which may be:

- Nutritional (e.g., special diets, dietary supplements, herbs, probiotics, and microbial-based therapies).
- Psychological (e.g., meditation, hypnosis, music therapies, relaxation therapies).
- Physical (e.g., acupuncture, massage, spinal manipulation).
- Combinations such as psychological and physical (e.g., yoga, tai chi, dance therapies, some forms of art therapy) or psychological and nutritional (e.g., mindful eating).

Nutritional approaches include what NCCIH previously categorized as natural products, whereas psychological and/or physical approaches include what was referred to as mind and body practices.

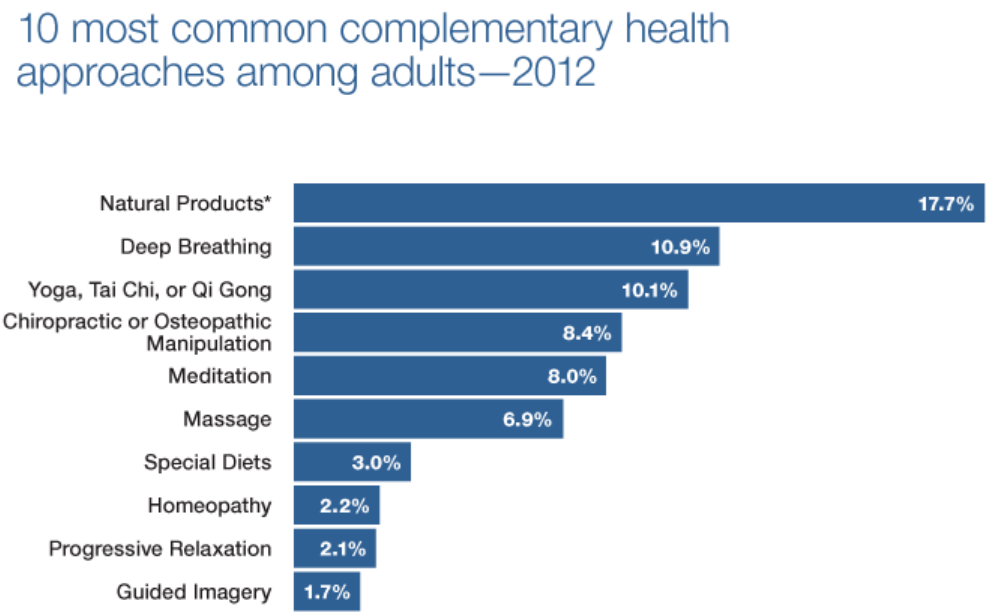


Examples of complementary health approaches that fall within the categories: psychological, physical, and nutritional.

39.3.1 Nutritional Approaches

These approaches include a variety of products, such as **herbs** (also known as botanicals; https://www.nccih.nih.gov/health/herbsataglance), **vitamins and minerals** (https://www.nccih.nih.gov/health/vitamins-and-minerals)**,** and **probiotics** (https://www.nccih.nih.gov/health/probiotics-what-you-need-to-know). They are widely marketed, readily available to consumers, and often sold as **dietary supplements** (https://www.nccih.nih.gov/health/dietary-and-herbal-supplements).

According to the 2012 National Health Interview Survey (NHIS), which included a comprehensive survey on the use of complementary health approaches by Americans, 17.7 percent of American adults had used a dietary supplement other than vitamins and minerals in the past year. These products were the most popular complementary health approach in the survey. (See chart below) The most commonly used nonvitamin, nonmineral dietary supplement was fish oil.



*Dietary supplements other than vitamins and minerals.

Source: Clarke TC, Black LI, Stussman BJ, Barnes PM, Nahin RL. Trends in the use of complementary health approaches among adults: United States, 2002–2012. National health statistics reports; no 79. Hyattsville, MD: National Center for Health Statistics. 2015.

Researchers have done large, rigorous studies on a few dietary supplements, but the results often showed that the products didn't work for the conditions studied. Research on others is in progress. While there are indications that some may be helpful, more needs to be learned about the effects of these products in the

human body, and about their safety (https://www.nccih.nih.gov/health/safety) and potential interactions with medicines (https://www.nccih.nih.gov/health/know-science/how-medications-supplements-interact) and other natural products.

39.3.2 Psychological and Physical Approaches

Complementary physical and/or psychological approaches include **tai chi** (https://www.nccih.nih.gov/health/tai-chi-and-qi-gong-in-depth), **yoga** (https://www.nccih.nih.gov/health/yoga-what-you-need-to-know), **acupuncture** (https://www.nccih.nih.gov/health/acupuncture-in-depth), **massage therapy** (https://www.nccih.nih.gov/health/massage-therapy-what-you-need-to-know), **spinal manipulation** (https://www.nccih.nih.gov/health/spinal-manipulation-what-you-need-to-know), **art therapy, music therapy, dance, mindfulness-based stress reduction,** and many others. These approaches are often administered or taught by a trained practitioner or teacher. The 2012 NHIS showed that yoga, **chiropractic and osteopathic manipulation** (https://www.nccih.nih.gov/health/spinal-manipulation-what-you-need-to-know), and **meditation** (https://www.nccih.nih.gov/health/meditation-in-depth) are among the most popular complementary health approaches used by adults. According to the 2017 NHIS (https://www.nccih.nih.gov/research/statistics/NHIS/2017), the popularity of yoga has grown dramatically in recent years, from 9.5 percent of U.S. adults practicing yoga in 2012 to 14.3 percent in 2017. The 2017 NHIS also showed that the use of meditation increased more than threefold from 4.1 percent in 2012 to 14.2 percent in 2017.

Other psychological and physical approaches include **relaxation techniques** (such as breathing exercises and guided imagery) (https://www.nccih.nih.gov/health/relaxation-techniques-for-health), **qi gong** (https://www.nccih.nih.gov/health/tai-chi-and-qi-gong-in-depth)**, hypnotherapy** (https://www.nccih.nih.gov/health/hypnosis)**, Feldenkrais method, Alexander technique, Pilates, Rolfing Structural Integration,** and **Trager psychophysical integration**.

Research findings suggest that several psychological and physical approaches, alone or in combination, are helpful for a variety of conditions. A few examples include the following:

- **Acupuncture** may help ease types of pain that are often chronic, such as low-back pain, neck pain, and osteoarthritis/knee pain. Acupuncture may also help reduce the frequency of tension headaches and prevent migraine headaches.
- **Meditation** may help reduce blood pressure, symptoms of anxiety and depression, and symptoms of irritable bowel syndrome and flare-ups in people with ulcerative colitis. Meditation may also benefit people with insomnia.
- **Tai chi** appears to help improve balance and stability, reduce back pain and pain from knee osteoarthritis, and improve quality of life in people with heart disease, cancer, and other chronic illnesses.

- **Yoga** may benefit people's general wellness by relieving stress, supporting good health habits, and improving mental/emotional health, sleep, and balance. Yoga may also help with low-back pain and neck pain, anxiety or depressive symptoms associated with difficult life situations, quitting smoking, and quality of life for people with chronic diseases.

The amount of research on psychological and physical approaches varies widely depending on the practice. For example, researchers have done many studies on acupuncture, yoga, spinal manipulation, and meditation, but there have been fewer studies on some other approaches.

Other Complementary Health Approaches

Some complementary approaches may not neatly fit into either of these groups—for example, the practices of **traditional healers, Ayurvedic medicine** (https://www.nccih.nih.gov/health/ayurvedic-medicine-in-depth), **traditional Chinese medicine** (https://www.nccih.nih.gov/health/traditional-chinese-medicine-what-you-need-to-know), **homeopathy** (https://www.nccih.nih.gov/health/homeopathy), **naturopathy** (https://www.nccih.nih.gov/health/naturopathy)**,** and functional medicine.

Chapter 40

Current Issues in Complementary and Integrative Health[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: 5-hydroxytryptophan (5-HTP), acetaminophen, acupuncture, Age-Related Eye Disease Studies (AREDS and AREDS2), age-related macular degeneration (AMD), aloe vera, Asian ginseng, atopic eczema, attention-deficit hyperactivity disorder (ADHD), avocado oil, avocado/soybean unsaponifiables (ASU), Ayurveda, Ayurvedic medicine, benign prostatic hyperplasia (BPH), biofeedback, Bromelain, butterbur, cataract, chamomile, chiropractic manipulation, chondroitin, cognitive behavioral therapy for SAD (CBT-SAD), complementary health, corticosteroids, cyclosporine, deep breathing exercises, devil's claw, diabetic retinopathy, digoxin, dimethyl sulfoxide (DMSO), echinacea, eosinophilia-myalgia syndrome (EMS), ephedra, evening primrose oil, fenugreek, fibromyalgia, fish liver oils, folk medicine, *Ginkgo biloba,* glaucoma, glucosamine, goldenseal, green tea, guided imagery, hypnosis, hypnotherapy, integrative health, irritable bowel syndrome (IBS), jet lag, kava, light therapy, L-tryptophan, *ma huang,* malaria, melatonin, methylsulfonylmethane (MSM), mindfulness-based stress reduction, mindfulness meditation, naturopathy, nonsteroidal anti-inflammatory drugs (NSAIDS), oil of lemon eucalyptus (OLE), omega-3 fatty acids, osteoarthritis (OA), peppermint oil, phantom limb pain, placebo, polyunsaturated fatty acids, post-traumatic stress disorder (PTSD), probiotics, progressive relaxation, Pycnogenol, *Pygeum africanum,* qigong, *Radix glycyrrhizae,* rheumatoid arthritis (RA), S-adenosyl-L-methionine (SAMe), saw palmetto, seasonal affective disorder (SAD), self-hypnosis, *Serenoa repens,* shingles, spinal manipulation, St. John's wort, tai chi, thunder god vine, turmeric, type 2 diabetes, *Urtica dioica,* valerian, vitamin D, warfarin, willow bark extract, yoga, Zika virus

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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Many Americans use medical treatments that are not part of mainstream medicine. When you are using these types of care, it may be called complementary, integrative, or alternative medicine.

Decisions about your health care are important—including decisions about whether or not to use complementary health approaches. Complementary medicine is used together with mainstream medical care. An example is using acupuncture (https://medlineplus.gov/acupuncture.html) to help with side effects of cancer treatment. When health care providers and facilities offer both types of care, it is called integrative medicine. Alternative medicine is used instead of mainstream medical care.

The claims that non-mainstream practitioners make can sound promising. However, researchers do not know how safe many of these treatments are or how well they work. Studies are underway to determine the safety and usefulness of many of these practices.

To minimize the health risks of a non-mainstream treatment:

- Discuss it with your doctor. It might have side effects or interact with other medicines.
- Find out what the research says about it.
- Choose practitioners carefully.
- Tell all of your doctors and practitioners about all of the different types of treatments you use.

Complementary approaches can be classified by their primary therapeutic input (how the therapy is taken in or delivered), which may be:

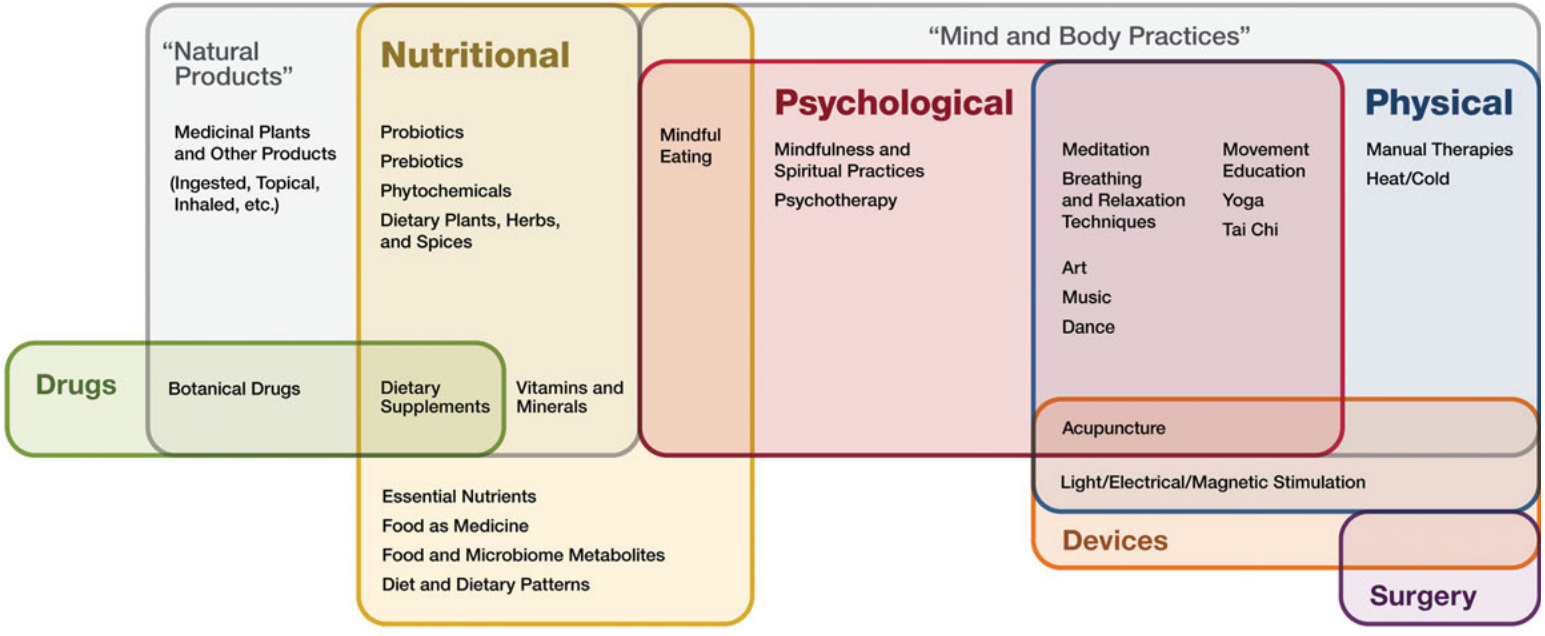
- Nutritional (e.g., special diets, dietary supplements, herbs, probiotics, and microbial-based therapies).
- Psychological (e.g., meditation, hypnosis, music therapies, relaxation therapies).
- Physical (e.g., acupuncture, massage, spinal manipulation).
- Combinations such as psychological and physical (e.g., yoga, tai chi, dance therapies, some forms of art therapy) or psychological and nutritional (e.g., mindful eating).

Nutritional approaches include what the National Center for Complementary and Integrative Health (NCCIH) previously categorized as natural products, whereas psychological and/or physical approaches include what was referred to as mind and body practices.

Some complementary approaches may not neatly fit into any of the groups above—for example, the practices of traditional healers, Ayurvedic medicine, traditional Chinese medicine, homeopathy, naturopathy, and functional medicine.

When considering complementary health approaches, it is important to take charge of your health by being an informed consumer. Find out and consider what scientific studies have been done on the safety and effectiveness of the product or practice that interests you. (For more information on specific complementary health approaches and what the science says about them, the "Health Information" page (https://www.nccih.nih.gov/health) has an A–Z list (https://www.nccih.nih.gov/health/atoz) of various complementary health products and practices.) Discuss the information with your health care provider before making a decision.

When looking for a complementary health practitioner, consider asking your health care provider, a local hospital, or a professional organization. The MedlinePlus® Directories (https://medlineplus.gov/directories.html) page from the National Library of Medicine lists organizations for some professions and provides links to directories of libraries and various types of health professionals, services, and facilities, which might be helpful. (NCCIH does not make referrals to practitioners and does not provide complementary health care or treatments.)



Examples of complementary health approaches that fall within the categories: Psychological, Physical, and Nutritional.

40.1 4 Things to Know about Dietary Supplements for Eye Conditions

As many as 21 million Americans have vision problems and 80 million have potentially blinding eye diseases. Age-related macular degeneration (AMD), cataract, diabetic retinopathy, and glaucoma are the main causes of vision problems and blindness in older Americans. Conventional treatments, such as surgery, are available for some eye conditions (https://www.nccih.nih.gov/health/eye-conditions-at-a-glance), but some people turn to dietary supplements (https://www.nccih.nih.gov/health/dietary-and-herbal-supplements) to prevent them or to delay their progression. Here are 4 things you should know if you are considering taking dietary supplements for eye conditions:

1. Findings from the Age-Related Eye Disease Studies (AREDS and AREDS2) suggest that taking dietary supplements with antioxidant (https://www.nccih.nih.gov/health/antioxidants-in-depth) vitamins and zinc may slow the progression of age-related macular degeneration in people who have intermediate AMD, and those who have late AMD in one eye. Data from other studies do not support using other dietary supplements, such as Ginkgo biloba and omega-3 fatty acids for AMD.
2. Current research does not support the use of dietary supplementation with vitamins A, C, and E as glaucoma treatments. There is also little evidence to support using megavitamins, special diets, acupuncture, relaxation techniques, or therapeutic touch for glaucoma. Early detection and conventional treatment of glaucoma are important.
3. Although there are some data from observational research that dietary vitamin B12 supplements may slow or prevent cataract development, no dietary supplements have been recommended for the treatment of cataracts.
4. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.2 5 Things to Know about Omega-3s for Heart Disease

Omega-3 fatty acids (https://www.nccih.nih.gov/health/omega3-supplements-in-depth) are a group of polyunsaturated fatty acids that are important for a number of functions in the body. They are found in foods such as fatty fish and certain vegetable oils and are also available as dietary supplements. While experts agree that fish rich in omega-3 fatty acids should be included in a heart-healthy diet, there isn't conclusive evidence that shows omega-3s have a protective effect against heart disease.

1. *Experts agree that fish rich in omega-3 fatty acids should be included in a heart-healthy diet.* Much research has been done on fish and heart

disease, and the results provide strong, though not conclusive evidence that people who eat fish at least once a week are less likely to die of heart disease than those who rarely or never eat fish.

2. *Omega-3s in supplement form have not been shown to protect against heart disease.* While there has been a substantial amount of research on omega-3 supplements and heart disease, the findings of individual studies have been inconsistent. In 2012, two combined analyses of the results of these studies did not find convincing evidence that omega-3s protect against heart disease.
3. *Omega-3 supplements may interact with drugs that affect blood clotting.* Omega-3 supplements may extend the time it takes for a cut to stop bleeding. People who take drugs such as anticoagulants ("blood thinners") or nonsteroidal anti-inflammatory drugs should discuss the use of omega-3 fatty acid supplements with a health care provider.
4. *Fish liver oils (which are different from fish oils) contain vitamins A and D as well as omega-3 fatty acids; these vitamins can be toxic in high doses.* The amounts of vitamins in fish liver oil supplements vary from one product to another.
5. *Talk to your health care provider before using omega-3 supplements.* If you are pregnant or nursing a child, if you take medicine that affects blood clotting, if you are allergic to fish or shellfish, or if you are considering giving a child an omega-3 supplement, it is especially important to consult your (or your child's) health care provider.

40.3 6 Tips: How Herbs Can Interact with Medicines

Many people take both dietary supplements and prescription or over-the-counter medicines. But did you know that these medicines and supplements may interact in harmful ways? Some supplements can *decrease* the effects of medicines, while others can *increase* the effects, including unwanted side effects, of medicines. Unfortunately, for many medicines and supplements there's currently little information on possible interactions, and more research is needed. But here are 6 things you should know about herbs that have a high risk of potential interactions with certain medications.

1. St. John's wort (https://www.nccih.nih.gov/health/stjohnswort) interacts with many types of drugs. In most instances, it speeds up the processes that change the drug into inactive substances, leading to a decrease in drug levels in your body. However, St. John's wort can interact with some drugs, including certain types of antidepressants, and can cause harmful side effects.
2. A variety of herbs, including concentrated garlic (https://www.nccih.nih.gov/health/garlic) extracts, can thin the blood in a manner similar to aspirin, which may be a problem during or after surgery.

3. Concentrated green tea (https://www.nccih.nih.gov/health/greentea) supplements can interact with pseudoephedrine (a decongestant).
4. A recent scientific review concluded that the herb goldenseal (https://www.nccih.nih.gov/health/goldenseal) has a high herb-drug interaction risk with some medicines.
5. People who take medicines with a narrow therapeutic index (e.g., digoxin, cyclosporine, warfarin, and others) should take special care to tell their health care providers about their use of herbal supplements. A narrow therapeutic index means that if the amount of the drug is even a little too low or too high, it can cause big problems. People who take herbal supplements such as Asian ginseng (https://www.nccih.nih.gov/health/asianginseng), St. John's wort, and others while taking certain medicines with a narrow therapeutic index should be closely monitored.
6. When you visit your health care providers, it's important to tell them about all the medicines and supplements you take. Bring a written list of everything you take, how often you take them, and the doses you take.

40.4 7 Tips: What You Need to Know about Natural Products for Musculoskeletal Inflammation

There are many natural products sold as dietary supplements that are marketed for inflammatory conditions, such as osteoarthritis (OA), rheumatoid arthritis (RA), and tendinitis. Some of these natural products have a long history of use for treating musculoskeletal inflammation and the pain associated with inflammation. There is some limited evidence that a few natural products may provide modest benefits for these conditions; however, in general, there is insufficient evidence to support the use of many of these natural products for inflammatory disorders. Here are 7 things to know if you are considering using a natural product for inflammation:

1. There is some evidence that omega-3 fatty acids (https://www.nccih.nih.gov/health/omega3-supplements-in-depth) may provide a modest benefit for symptoms of rheumatoid arthritis.
2. Turmeric (https://www.nccih.nih.gov/health/turmeric/ataglance.htm) and willow bark have been used for many years as remedies for the treatment of inflammatory conditions. However, there have only been a few trials of turmeric conducted in people, and there isn't enough evidence to support the use of turmeric supplementation for inflammatory disorders. Research suggests that willow bark is effective as an analgesic and anti-inflammatory and there is moderate evidence of effectiveness for use of willow bark extract for treating low-back pain.
3. Bromelain (https://www.nccih.nih.gov/health/bromelain), which is a mixture of enzymes found in the pineapple plant, is often used as a dietary supplement for nasal swelling and inflammation, osteoarthritis, and muscle

soreness. There is some evidence that bromelain may be helpful along with conventional medicine to help improve acute nasal and sinus inflammation, but there isn't enough evidence as to whether bromelain has any beneficial effects on other inflammatory conditions.

4. There is some moderate evidence that devil's claw (https://medlineplus.gov/druginfo/natural/984.html), an herb native to Africa, is beneficial for osteoarthritis of the spine, hip, and knee.
5. Based on current evidence, it is unclear whether taking ginger (https://www.nccih.nih.gov/health/ginger) supplements is beneficial in treating the symptoms of osteoarthritis, rheumatoid arthritis, or joint and muscle pain.
6. There is some evidence that thunder god vine (https://www.nccih.nih.gov/health/tgvine)—a vine native to China, Japan, and Korea—may reduce some symptoms of rheumatoid arthritis; however, thunder god vine may be associated with some serious adverse side effects.
7. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.5 6 Things You Should Know: The Science of Chronic Pain and Complementary Health Practices

1. Reviews of research on acupuncture, massage, and spinal manipulation for chronic low-back pain (https://www.nccih.nih.gov/health/pain/lowback.htm) have found evidence that these therapies may be beneficial. There is also some evidence that mindfulness-based stress reduction and cognitive behavioral therapy improves pain and functional limitation compared to usual care.

    - Spinal manipulation (https://www.nccih.nih.gov/health/spinalmanipulation): The most recent guidelines from the American Pain Society and the American College of Physicians conclude that spinal manipulation is associated with moderate benefit for chronic low-back pain.

    - Acupuncture (https://www.nccih.nih.gov/health/acupuncture): In many studies, acupuncture has shown some benefit for low-back pain compared to conventional therapy, but simulated (placebo) acupuncture has also shown a similar benefit, suggesting that a component of any benefit from acupuncture may be due to patient expectation or practitioner attention. A 2018 review by the Agency for Healthcare Research and Quality (AHRQ) found that acupuncture was associated with slightly greater effects on chronic low-back pain and function at 1 to 6 months when compared with controls (i.e., simulated acupuncture or usual care).

- Massage (https://www.nccih.nih.gov/health/massage): Studies suggest that massage is associated with short-term beneficial effects in reducing pain and improving function compared to usual care in people with chronic low-back pain.
- Yoga (https://www.nccih.nih.gov/health/yoga): Clinical guidelines issued in 2017 by the American College of Physicians strongly recommended yoga, based on low-quality evidence, as initial treatment for people with chronic low-back pain.
- Mindfulness-based stress reduction (https://www.nccih.nih.gov/health/meditation): A 2018 AHRQ review of nonpharmacologic treatment of chronic pain found that mindfulness-based stress reduction and other therapies improved function and/or pain for at least 1 month.

Recent reviews and clinical trials provide encouraging evidence that practices such as tai chi, yoga, qi gong, acupuncture, mindfulness, and biofeedback may help relieve some fibromyalgia (https://www.nccih.nih.gov/health/fibromyalgia) symptoms.

Clinical practice guidelines issued by the American College of Rheumatology conditionally recommend tai chi, along with other non-drug approaches such as self-management programs and walking aids, for managing knee osteoarthritis (https://www.nccih.nih.gov/health/arthritis). Acupuncture is also conditionally recommended for those who have chronic moderate-to-severe knee pain and are candidates for total knee replacement but can't or won't undergo the procedure.

Available evidence indicates that acupuncture for neck pain may provide better pain relief compared to no treatment. There is also some evidence that spinal manipulation (https://www.nccih.nih.gov/health/spinalmanipulation) may help patients suffering from chronic tension-type or neck-related headaches.

According to a 2016 review of studies performed in the United States, massage therapy may provide short-term relief from neck pain, especially if massage sessions are relatively lengthy and frequent.

As with any treatment, it is important to consider safety before using complementary health products and practices. If you are considering a complementary health practice to help manage your chronic pain, talk with your health care providers first. You can get more information on NCCIH's Web site about the safe use of complementary health products and practices (https://www.nccih.nih.gov/health/safety).

40.6 4 Tips: Mind and Body Practices for Common Aging-Related Conditions

Many older adults are turning to complementary and integrative health approaches to promote health and well-being. Mind and body practices, in particular,

including relaxation techniques and meditative exercise forms such as yoga, tai chi, and qi gong are being used by older Americans, both for fitness and relaxation, and because of perceived health benefits. A number of reviews of the scientific literature point to the potential benefit of mind and body approaches for symptom management, particularly for pain. Check out what the science says about mind and body practices for these 4 common aging-related conditions:

1. *Osteoarthritis.* Practicing tai chi—a traditional Chinese form of exercise—may be helpful for managing osteoarthritis of the knee. Guidelines issued by the American College of Rheumatology conditionally recommend tai chi, along with other non-drug approaches, for this condition.
2. *Menopausal symptoms.* Overall, there is scientific evidence suggesting that some mind and body approaches, such as yoga, tai chi, and meditation may provide some relief from common menopausal symptoms.
3. *Sleep problems.* Using relaxation techniques, (e.g., progressive relaxation, guided imagery, biofeedback, self-hypnosis, and deep breathing exercises) before bedtime can be helpful components of a successful sleep regimen.
4. *Shingles.* Tai chi may help older adults avoid getting shingles by increasing immunity to varicella-zoster virus and boosting the immune response to varicella vaccine in older people. While there have only been a few studies on the effects of tai chi on immunity to varicella, the results so far have been promising.

These mind and body practices are generally considered safe for healthy people when they're performed appropriately. If you have any health problems, talk with both your health care provider and the complementary health practitioner/instructor before starting to use a mind and body practice. For information about natural products for common aging-related conditions, check out these tips (https://www.nccih.nih.gov/health/tips/tips-natural-products-used-for-common-agingrelated-conditions).

40.7 6 Things You Need to Know about Cancer and Complementary Health Approaches

Complementary health approaches may play a role in cancer care, but using them inappropriately can be harmful. Here are 6 things you should know about cancer and complementary health approaches.

1. *If you have cancer, talk to your health care provider before using any complementary health approach.* Some products or practices may interfere with conventional cancer treatment or have other risks. For example, some herbal supplements may interact in harmful ways with drugs used in cancer treatment.

2. *No complementary approach has been shown to cure cancer or cause it to go into remission.* Unproven products or practices should not be used to replace or delay medical treatment for cancer. Delaying treatment can decrease the chances of remission or cure.
3. *Some psychological or physical complementary health approaches (also called mind and body practices) may help people manage cancer symptoms or side effects of treatment, such as nausea, pain, fatigue, and depression*. Practices that have shown promise include acupuncture, hypnosis, mindfulness-based interventions, tai chi/qigong, and yoga.
4. *It's uncertain whether dietary supplements containing ginger are helpful for nausea associated with cancer chemotherapy.* Studies have had mixed results.
5. *Using black salves for self-treatment of skin cancer is a bad idea.* These products may not remove the whole cancer, which may allow it to spread and become more serious. They also can cause scarring and tissue damage, which is sometimes severe and disfiguring.
6. *No dietary supplements have been shown to prevent cancer.* However, there are several other ways to reduce your cancer risk. They include making healthy choices, such as avoiding tobacco and maintaining a healthy weight, getting recommended vaccines against hepatitis B and human papillomavirus (HPV), and getting appropriate cancer screening tests.

40.8 5 Things to Know about Complementary Health Practices for Cognitive Function, Dementia, and Alzheimer's Disease

Many people, particularly older individuals, worry about forgetfulness and whether it is the first sign of dementia or Alzheimer's disease (https://www.nccih.nih.gov/health/alzheimer). In fact, forgetfulness has many causes. It can also be a normal part of aging, or related to various treatable health issues or to emotional problems, such as stress, anxiety, or depression. The National Institute on Aging (https://www.nia.nih.gov/health/alzheimers) has a lot of information on the aging brain as well as cognitive function, dementia, and Alzheimer's disease. Although no treatment is proven to stop dementia or Alzheimer's disease, some conventional drugs may limit worsening of symptoms for a period of time in the early stages of the disease.

Many dietary supplements are marketed with claims that they enhance memory or improve brain function and health. To date, research has yielded no convincing evidence that any dietary supplement can reverse or slow the progression of dementia or Alzheimer's disease. Additional research on dietary supplements, as well as several mind and body practices such as music therapy and mental imagery, which have shown promise in basic research or preliminary clinical studies, is underway.

Here are 5 things to know about current research on complementary health approaches for cognitive function, dementia, and Alzheimer's disease.

1. *To date there is no convincing evidence from a large body of research that any dietary supplement can prevent worsening of cognitive impairment associated with dementia or Alzheimer's disease.* This includes studies of ginkgo (https://www.nccih.nih.gov/health/asianginseng), omega-3 fatty acids/fish oil (https://www.nccih.nih.gov/health/omega3-supplements-in-depth), vitamins B and E, Asian ginseng (https://www.nccih.nih.gov/health/asianginseng), grape seed extract (https://www.nccih.nih.gov/health/grapeseed/ataglance.htm), and curcumin. Additional research on some of these supplements is underway.
2. *Preliminary studies of some mind and body practices such as music therapy suggest they may be helpful for some of the symptoms related to dementia, such as agitation and depression.* Several studies on music therapy in people with Alzheimer's disease have shown improvement in agitation, depression, and quality of life.
3. *Mindfulness-based stress reduction programs may be helpful in reducing stress among caregivers of patients with dementia.* To reduce caregiver stress, studies suggest that a mindfulness-based stress reduction program is more helpful for improving mental health than attending an education and support program or just taking time off from providing care.
4. *Don't use complementary health approaches as a reason to postpone seeing a health care provider about memory loss.* Treatable conditions, such as depression, bad reactions to medications, or thyroid, liver, or kidney problems, can impair memory.
5. *Some complementary health approaches interact with medications and can have serious side effects.* If you are considering replacing conventional medications with other approaches, talk to your health care provider.

40.9 4 Tips: Asthma and Complementary Health Practices

Asthma is a chronic lung disease that affects people of all ages. It causes episodes of wheezing, coughing, shortness of breath, and chest tightness. Although there is no cure, most people are able to control their asthma with conventional therapies and by avoiding the substances that can set off asthma attacks. Even so, some people turn to complementary health practices such as acupuncture, breathing exercises, and herbal supplements in their efforts to relieve symptoms.

If you're thinking about complementary health practices for asthma, here's what you need to know: There is not enough evidence to support the use of any complementary health practices for the relief of asthma symptoms.

1. *At this point, there is little evidence that acupuncture is an effective treatment for asthma.* Although a few studies showed some reduction in medication use and improvements in symptoms and quality of life, most

of the research showed no difference between real acupuncture and sham (fake) acupuncture on asthma symptoms.

2. *A review of research on specific breathing techniques—the Papworth Method and Buteyko Breathing Technique—found a trend toward improvement in asthma symptoms but not enough evidence to draw reliable conclusions.* In spite of increasing patient interest in certain breathing exercises to help with symptoms like hyperventilation and to regulate breathing, there isn't solid evidence to support its use.
3. *There is little or no evidence to support the use of herbs or dietary supplements for asthma.* Some conventional treatments for asthma have their roots in herbal preparations: for example, the bronchodilator theophylline is found in tea leaves, and ephedrine (also a bronchodilator) is a compound in the traditional Chinese herb *ma huang* (ephedra: https://www.nccih.nih.gov/health/ephedra). *Note*: In 2004, the FDA banned the US sale of dietary supplements containing ephedra. The FDA found that these supplements had an unreasonable risk of injury or illness—particularly cardiovascular complications—and a risk of death.
4. Researchers have found little or no evidence of benefit for the relief of asthma symptoms when they studied other herbs and dietary supplements such as boswellia, *tylophora indica*, magnesium supplements, omega-3 fatty acids (https://www.nccih.nih.gov/health/omega3-supplements-in-depth), *Radix glycyrrhizae*, vitamin C, and butterbur (https://www.nccih.nih.gov/health/butterbur).

40.10 10 Things to Know about the Science of Health

It's sometimes difficult to tell the difference between health facts and myths, particularly around complementary and integrative health. Becoming familiar with scientific topics related to health research can help you better understand what you hear and read and make well-informed health decisions.

These 10 facts can give you a start in learning about the science of health. Want to know more? Check out our Know the Science online toolkit (https://www.nccih.nih.gov/health/know-science).

1. *Some online sources of information on complementary health approaches are useful, but others are inaccurate or misleading.* If you're visiting an online health site for the first time or downloading a new app, ask these questions: Who runs or created the site or app? Do its claims seem too good to be true? Is it up-to-date? Where does the information come from? Why does the site or app exist? Is it selling something?
2. *Unless you read and understand the original sources for a health news story, it can be difficult to know whether the story is misleading or has left out important information.* But the likelihood that the story is correct increases if it comes from a media outlet that isn't promoting a point

of view or cause, was written by a trained science or health reporter, and includes quotes from experts not connected to the study.

3. *Sometimes taking a prescription drug and dietary supplement together may increase the drug's effects.* The drug's effects may become too strong, and unwanted side effects may increase.
4. *Sometimes taking a prescription drug and a supplement together may decrease the drug's effects.* This means that you aren't getting the full benefit from the drug that your health care provider wants you to have.
5. *When it comes to medicine, there is no official definition for use of the term natural.* And it's important to know that "natural" does not always mean "safe."
6. *Although many herbal or dietary supplements (and some prescription drugs) come from natural sources, "natural" does not always mean that it's a better option for your health.* Scientists are studying many of these products to identify what ingredients may be active and to better understand their effects in the body.
7. *Clinical trials to test whether a treatment is useful and safe in humans may vary in size and type.* Well-planned clinical trials give the clearest information about whether a treatment is effective and safe. However, because they're complicated, lengthy, and very, very expensive, they're usually done only after smaller preliminary studies have shown some promise that the treatment may be helpful.
8. *Studies with large numbers of people generally get results that are more reliable than those of studies with small pools of participants.* Larger studies can increase the accuracy of the study findings and reduce the probability that any effect observed in the study was due to chance.
9. *The strongest evidence about whether a treatment is useful and safe consists of results from several studies by different investigators.* Rarely does a single study provide a final, definitive answer.
10. *When looking for information from a study published in a medical journal, try to find out if the study has been peer reviewed.* The peer review process subjects a scientist's research to the scrutiny of others who are experts in the same field and is considered necessary to ensure academic scientific quality.

40.11 5 Things to Know about Relaxation Techniques for Stress

When you're under stress, your body reacts by releasing hormones that produce the "fight-or-flight" response. Your heart rate and breathing rate go up and blood vessels narrow (restricting the flow of blood). Occasional stress is a normal coping mechanism. But over the long-term, stress may contribute to or worsen

a range of health problems including digestive disorders, headaches, sleep disorders, and other symptoms.

In contrast to the stress response, the relaxation response slows the heart rate, lowers blood pressure, and decreases oxygen consumption and levels of stress hormones. In theory, voluntarily creating the relaxation response through regular use of relaxation techniques could counteract the negative effects of stress.

1. *Relaxation techniques are generally safe, but there is limited evidence of usefulness for specific health conditions.* Research is under way to find out more about relaxation and health outcomes.
2. *Relaxation techniques include a number of practices such as progressive relaxation, guided imagery, biofeedback, self-hypnosis, and deep breathing exercises.* The goal is similar in all: to consciously produce the body's natural relaxation response, characterized by slower breathing, lower blood pressure, and a feeling of calm and well-being.
3. *Relaxation techniques often combine breathing and focused attention to calm the mind and the body.* These techniques may be most effective when practiced regularly and combined with good nutrition, regular exercise, and a strong social support system.
4. *Most relaxation techniques can be self-taught and self-administered.* Most methods require only brief instruction from a book or experienced practitioner before they can be done without assistance.
5. *Do not use relaxation techniques as a replacement for conventional care or to postpone seeing a doctor about a medical problem.* Talk to your health care providers if you are considering using a relaxation technique for a particular health condition. This will help ensure coordinated and safe care.

40.12 7 Things to Know about Complementary Approaches for Fibromyalgia

Fibromyalgia syndrome is a chronic disorder involving widespread pain and tenderness, fatigue, and other symptoms that can interfere with a person's ability to carry out daily activities. It is estimated that fibromyalgia affects 4 million American adults. Fibromyalgia can affect people of all ages, including children. However, most people are diagnosed during middle age. Women are twice as likely to have fibromyalgia as men.

Treatment of fibromyalgia often involves an individualized approach that may include both medicines and nondrug therapies.

Here are 7 things you should know about what the science says about complementary health approaches for fibromyalgia:

1. *In general, research on complementary health approaches for fibromyalgia is preliminary.* However, the evidence for some approaches is encouraging.

2. *With one possible exception, there is insufficient evidence that any dietary supplements can relieve fibromyalgia pain.* The possible exception is vitamin D supplements, which may reduce pain in people with fibromyalgia who have vitamin D deficiencies.
3. *Meditative movement therapies, such as tai chi, may provide modest relief of some fibromyalgia symptoms.* Some randomized controlled trials have had promising results.
4. *There is limited evidence that massage can be helpful.* Massage therapy or a type of manual therapy called myofascial release, which is directed at connective tissue (fascia), may lead to a small improvement in fibromyalgia symptoms.
5. *Mindfulness meditation may provide short-term improvements in pain and quality of life in people with fibromyalgia.* However, only a small number of studies have been done, and their quality is low.
6. *Biofeedback may have effects on physical functioning, pain, and mood in people with fibromyalgia.* However, the quality of the evidence is low.
7. *Psychological and physical approaches such as tai chi, mindfulness, massage, and biofeedback generally have good safety records when done properly by a trained professional or taught by a well-qualified instructor.* Your medical conditions may affect the safety of a practice. Talk with your health care providers and the practitioner or teacher about your individual needs.

40.13 5 Tips: Natural Products for the Flu and Colds: What Does the Science Say?

It's that time of year again—cold and flu season (https://www.nccih.nih.gov/health/flu/). Each year, approximately 5 to 20 percent of Americans come down with the flu. Although most recover without incident, flu-related complications typically lead to at least 200,000 hospitalizations and between 12,000 and 60,000 deaths each year. Colds generally do not cause serious complications, but they are among the leading reasons for visiting a doctor and for missing school or work.

Some people try natural products such as herbs or vitamins and minerals to prevent or treat these illnesses. But do they really work? What does the science say?

1. *Vaccination is the best protection against getting the flu.* Starting in 2010, the Federal Government's Centers for Disease Control and Prevention has recommended annual flu vaccination (https://www.cdc.gov/flu/season/index.html) for all people aged 6 months and older. There is currently no strong scientific evidence that any natural product is useful against the flu.

2. *Zinc taken orally (by mouth) may help to treat colds, but it can cause side effects and interact with medicines.* Zinc is available in two forms—oral zinc (e.g., lozenges, tablets, syrup) and intranasal zinc (e.g., swabs and gels). A 2015 analysis of clinical trials found that oral zinc helps to reduce the length of colds when taken within 24 hours after symptoms start. Intranasal zinc has been linked to a severe side effect (irreversible loss of the sense of smell) and should not be used.

   A note about safety: Oral zinc can cause nausea and other gastrointestinal symptoms. Long-term use of zinc, especially in high doses, can cause problems such as copper deficiency. Zinc may interact with drugs, including antibiotics and penicillamine (a drug used to treat rheumatoid arthritis).

3. *Vitamin C does not prevent colds and only slightly reduces their length and severity.* A 2013 review of scientific literature found that taking vitamin C regularly did not reduce the likelihood of getting a cold but was linked to small improvements in cold symptoms. In studies in which people took vitamin C only after they got a cold, vitamin C did not improve their symptoms.

   A note about safety: Vitamin C is generally considered safe; however, high doses can cause digestive disturbances such as diarrhea and nausea.

4. *Echinacea has not been proven to help prevent or treat colds.* Echinacea (https://www.nccih.nih.gov/health/echinacea/) an herbal supplement that some people use to treat or prevent colds. Echinacea products vary widely, containing different species, parts, and preparations of the echinacea plant. Reviews of research have found limited evidence that some echinacea preparations may be useful for treating colds in adults, while other preparations did not seem to be helpful. In addition, echinacea has not been shown to reduce the number of colds that adults catch. Only a small amount of research on echinacea has been done in children, and the results of that research are inconsistent.

   A note about safety: Few side effects have been reported in clinical trials of echinacea; however, some people may have allergic reactions. In one large clinical trial in children, those who took echinacea had an increased risk of developing rashes.

5. *The evidence that probiotic supplements may help to prevent colds is weak, and little is known about their long-term safety.* Probiotics (https://www.nccih.nih.gov/health/probiotics-what-you-need-to-know) are a type of "good bacteria," similar to the microorganisms found in the body, and may be beneficial to health. Probiotics are available as dietary supplements and yogurts, as well as other products such as suppositories and creams. Although a 2015 analysis of research indicated that probiotics might help to prevent upper respiratory tract infections, such as the common cold, the evidence is weak and the results have limitations.

   A note about safety: Little is known about the effects of taking probiotics for long periods of time. Most people may be able to use probiotics without

experiencing any side effects—or with only mild gastrointestinal side effects such as gas —but there have been some case reports of serious side effects. Probiotics should not be used by people with serious underlying health problems except with close monitoring by a health care provider.

40.14 5 Things You Should Know about Yoga

Yoga (https://www.nccih.nih.gov/health/yoga-what-you-need-to-know) typically combines physical postures, breathing exercises, and meditation. Researchers are studying how yoga may be used to help improve health. Because many studies of yoga have included only small numbers of people and haven't been of high quality, in most instances we can only say that yoga has shown promise for particular health uses, not that it's been proven to help. If you're thinking about practicing yoga, here are 5 things you should know:

1. *Studies suggest that yoga may be beneficial for a number of health conditions, including low-back pain, neck pain, and menopause symptoms.* It may also help people lose weight or stop smoking.
2. *Yoga may have benefits for healthy people.* Studies show that it may help people manage stress, enhance mental well-being, and improve sleep and balance. Practicing yoga regularly may also be linked with better eating and physical activity habits.
3. *Yoga is generally considered a safe form of physical activity for healthy people when practiced appropriately.* However, as with other forms of physical activity, you can get hurt doing yoga. To reduce your risk of injury, practice yoga under the guidance of a qualified instructor. Practicing yoga by self-study without supervision has been associated with increased risks.
4. *Yoga may have benefits for children.* It is a promising stress management tool for children, and it may also be helpful for weight loss in children who are overweight or obese and for reducing anxiety.
5. *With appropriate precautions, yoga can be safe during pregnancy.* It's usually good to be active when you're pregnant, but you should be evaluated by your health care provider to make sure exercise is safe for you. Some activities, including yoga, may need to be modified during pregnancy (for example, to avoid spending long periods lying on your back). Yoga may have health benefits for pregnant women, including decreasing stress, anxiety, and depression.

40.15 5 Things to Know about Chronic Low-Back Pain and Complementary Health Practices

Low-back pain is a very common condition, but often the cause is unknown. Most people have significant acute back pain at least once in their lives. Usually it resolves on its own without specific treatment.

But for some people, the pain can become chronic or even debilitating, and difficult to treat. Spinal manipulation, acupuncture, massage, and yoga are complementary health approaches often used by people with low-back pain. They are all included in a longer list of treatment options recommended by the American Pain Society and the American College of Physicians for patients whose low-back pain does not improve with more conservative care. Other options include exercise, physical/occupational rehabilitation, cognitive behavioral therapy, and progressive relaxation.

Here's what you need to know about what the science says for chronic low-back pain and some of these practices.

1. *Overall, studies have provided good evidence that spinal manipulation is moderately effective for chronic low-back pain.* Spinal manipulation includes various interventions administered by osteopathic physicians, chiropractors, and physical therapists.
2. *There is fair evidence that acupuncture is helpful in relieving chronic back pain.* Current evidence suggests that factors such as expectations and beliefs of the patient and the provider, rather than acupuncture-specific effects of needling, are primarily responsible for beneficial effects of acupuncture on pain.
3. *There is also fair evidence that massage is helpful in relieving chronic low-back pain*. In general, however, these effects appear to be short term.
4. *Current research, while limited in scope, suggests that a carefully adapted set of yoga poses may reduce low-back pain and improve function.* NCCIH is also supporting research specifically associated with safety of this widely used self-care practice. People with back pain should work with an experienced teacher who can help modify or avoid some yoga poses to prevent adverse effects.
5. *Be sure to tell your health care provider about any complementary health practice you are considering.* This will help ensure coordinated, safe care.

40.16 4 Things to Know about Spinal Manipulation for Low-Back Pain

Back pain is one of the most common health complaints, affecting 8 out of 10 people at some point during their lives. The lower back is the area most often affected.

Spinal manipulation (https://www.nccih.nih.gov/health/chiropractic) may be used by chiropractors, osteopathic physicians, naturopathic physicians, physical therapists, and some medical doctors with a goal of relieving low-back pain (https://www.nccih.nih.gov/health/pain/lowback.htm) and improving physical functioning. These health professionals perform spinal manipulation by using their hands or a device to apply a controlled force to a joint of the spine. Most often they also recommend self-care practices.

But, what does the science tell us?

1. Most acute low-back pain gets better quickly with self-care practices, such as applying heat, using a firm mattress, doing back exercises, or taking pain medications.
2. Overall, studies have shown that spinal manipulation is one of several options—including exercise, massage, and physical therapy—that can provide some individuals with mild-to-moderate relief from low-back pain. Spinal manipulation appears to work about as well as these other treatment approaches.
3. In 2007 guidelines (https://www.acpjournals.org/doi/10.7326/0003-4819-147-7-200710020-00006), the American College of Physicians and the American Pain Society included spinal manipulation as one of several complementary treatment options (others include massage and acupuncture) for practitioners to consider when low-back pain does not improve with self-care. A 2010 Agency for Healthcare Research and Quality (AHRQ) report (http://www.ahrq.gov/clinic/tp/backcam2tp.htm) noted that complementary health therapies, including spinal manipulation, offer additional options to conventional treatments, which often have limited benefit in managing back and neck pain.
4. Reviews of the science have concluded that spinal manipulation for low-back pain is relatively safe when performed by a trained and licensed practitioner (https://www.nccih.nih.gov/health/decisions/practitioner.htm). The most common side effects are generally minor and include feeling tired or temporary soreness. There have been some reports of more serious side effects, but it is unclear if they are actually associated with spinal manipulation. It's important to note that there may be additional risks associated with spinal manipulation affecting the upper (cervical) spine, but that area of the spine is generally not manipulated when treating low-back pain.

40.17 7 Things to Know about Complementary Health Approaches for Autism Spectrum Disorder

The most recent US Government statistics estimate that 1 in 68 children have autism spectrum disorder (ASD). ASD refers to a complex group of generally lifelong developmental disorders, usually diagnosed in childhood. Some of the characteristics of ASD may include problems communicating; difficulty relating to people, things, or events; repetitive movements or behaviors; and problems adjusting to unfamiliar surroundings or routines. Although no cure has been found for ASD, a variety of therapies, including behavioral management therapy and physical therapy, may help address the symptoms associated with ASD.

Many parents try complementary health approaches, usually along with conventional medical care, for their children with ASD to help manage symptoms.

Here are 7 things to know about complementary health approaches for children with ASD:

1. There's very little high quality research on complementary health approaches for ASD.
2. There's no scientific evidence that secretin (a gastrointestinal hormone), hyperbaric oxygen, chelation therapies, or antifungal agents help people with ASD, and they may be dangerous.
3. Melatonin may help with sleep problems in children with ASD. A 2011 review of the scientific literature found that melatonin increased total sleep duration by an average of 73 minutes and decreased sleep latency by an average of 66 minutes. Similar beneficial results were observed when melatonin was compared with placebo.
4. There is some evidence that music therapy may help to improve some social and behavioral skills in children with ASD. A 2014 review of scientific studies concluded that music therapy may help children with ASD to improve their skills in areas such as social interaction and communication, and may also contribute to increasing social adaptation skills.
5. Studies have examined omega-3 fatty acids; acupuncture; a modified version of mindfulness-based therapy; massage therapy, including qi gong massage; and the hormone oxytocin. It's not clear whether they improve ASD symptoms, and they should not be used in place of conventional treatments.
6. Special diets may help some people with ASD but their nutritional well-being needs to be carefully monitored before and while on the diet. There's very limited evidence that the high-fat, very low carbohydrate "ketogenic" diet may help with seizures sometimes associated with autism. People with ASD need to be monitored when they are on a special diet so they avoid any harmful side effects.
7. Talk to your child's health care provider to get help assessing what, if any, complementary approach would help your child, since children respond differently to interventions.

40.18 4 Tips: Start Talking with Your Health Care Providers about Complementary Health Approaches

When patients tell their providers about their use of complementary health practices, they can better stay in control and more effectively manage their health. When providers ask their patients, they can ensure that they are fully informed and can help patients make wise health care decisions.

Here are 4 tips to help you and your health care providers start talking:

1. *List the complementary health practices you use on your patient history form.* When completing the patient history form, be sure to include

everything you use—from acupuncture to zinc. It's important to give health care providers a full picture of what you do to manage your health.

2. *At each visit, be sure to tell your providers about what complementary health approaches you are using.* Don't forget to include over-the-counter and prescription medicines, as well as dietary and herbal supplements. Make a list in advance and take it with you. Some complementary health approaches can have an effect on conventional medicine, so your provider needs to know.
3. *If you are considering a new complementary health practice, ask questions.* Ask your health care providers about its safety, effectiveness, and possible interactions with medications (both prescription and nonprescription).
4. *Don't wait for your providers to ask about any complementary health practice you are using.* Be proactive. Start the conversation.

40.19 7 Things to Know about Omega-3 Fatty Acids

Omega-3 fatty acids are a group of polyunsaturated fatty acids that are important for a number of functions in the body. The omega-3 fatty acids EPA and DHA are found in seafood, such as fatty fish (e.g., salmon, tuna, and trout) and shellfish (e.g., crab, mussels, and oysters). A different kind of omega-3, called ALA, is found in other foods, including some vegetable oils (e.g., canola and soy). Omega-3s are also available as dietary supplements; for example, fish oil supplements contain EPA and DHA, and flaxseed oil supplements contain ALA. Moderate evidence has emerged about the health benefits of consuming seafood. The health benefits of omega-3 dietary supplements are unclear.

Here are 7 things you should know about omega-3s:

1. *Results of studies on diets rich in seafood (fish and shellfish) and heart disease provide moderate evidence that people who eat seafood at least once a week are less likely to die of heart disease than those who rarely or never eat seafood.* The Dietary Guidelines for Americans, 2010 (http://health.gov/dietaryguidelines/dga2010/DietaryGuidelines2010.pdf) includes a new recommendation that adults eat 8 or more ounces of a variety of seafood per week because it provides a range of nutrients, including omega-3 fatty acids. (Smaller amounts are recommended for young children, and there are special recommendations for pregnant or breastfeeding women.
2. *Evidence suggests that seafood rich in EPA and DHA should be included in a heart-healthy diet; however, supplements of EPA and DHA have not been shown to protect against heart disease.* In 2012, two groups of scientists analyzed the research on the effects of EPA/DHA supplements on heart disease risk. One group analyzed only studies in people with a history of heart disease, and the other group analyzed studies in people both with and

without a history of heart disease. Neither review found strong evidence of a protective effect of the supplements.

3. *A 2012 review of the scientific literature concluded that EPA and DHA, the types of omega-3s found in seafood and fish oil, may be modestly helpful in relieving symptoms of rheumatoid arthritis.* In the studies included in the review, many of the participants reported that when they were taking fish oil they had briefer morning stiffness, less joint swelling and pain, and less need for anti-inflammatory drugs to control their symptoms.
4. *The nutritional value of seafood is of particular importance during fetal growth and development, as well as in early infancy and childhood.* Women who are pregnant or breastfeed should consume 8 to 12 ounces of seafood per week from a variety of seafood types that are low in methyl mercury as part of a healthy eating pattern and while staying within their calorie needs. Pregnant or breastfeeding women should limit the amount of white tuna (labeled as "albacore") to no more than 6 ounces per week. They should not eat tilefish, shark, swordfish, and king mackerel because they are high in methyl mercury.
5. *There is ongoing research on omega-3 fatty acids and diseases of the brain and eye, but there is not enough evidence to draw conclusions about the effectiveness of omega-3s for these conditions.* DHA plays important roles in the functioning of the brain and the eye. Researchers are actively investigating the possible benefits of DHA and other omega-3 fatty acids in preventing or treating a variety of brain- and eye-related conditions.
6. *There is conflicting evidence about whether a link might exist between the omega-3 fatty acids found in seafood and fish oil (EPA/DHA) and an increased risk of prostate cancer.* Additional research on the association of omega-3 consumption and prostate cancer risk is under way.
7. *The bottom line: Including seafood in your diet is healthful. Whether omega-3 supplements are beneficial is uncertain.* If you are considering omega-3 supplements, talk to your health care provider. It's especially important to consult your (or your child's) health care provider if you are pregnant or breastfeeding, if you take medicine that affects blood clotting, if you are allergic to seafood, or if you are considering giving a child an omega-3 supplement.

40.20 5 Tips: Natural Products Used for Common Aging-Related Conditions

Many older adults are turning to complementary and integrative health approaches to promote health and well-being. Natural products, often sold as dietary supplements, are frequently used by many older people despite safety

concerns or a lack of evidence to support their use. Although many people believe that natural products are safe, these products can contain pharmacologically active compounds and may interact with prescription medicines or have side effects and risks. Check out what the science says about natural products for these common aging-related conditions, and talk to your health care provider if you are considering taking a natural product. And for information about mind and body approaches for common aging-related conditions, be sure to check out these tips (https://nccih.nih.gov/health/tips/age-mindbody).

1. *Osteoarthritis.* Findings from studies of glucosamine and chondroitin sulfate—taken separately or together—suggest that they do not provide much, if any, meaningful improvement of pain or function for osteoarthritis (OA). Independent clinical practice guidelines published in 2012 by the American College of Rheumatology, and in 2010 by the American Academy of Orthopaedic Surgeons recommend not using glucosamine or chondroitin for OA.
2. *Cognitive decline and Alzheimer's disease.* Although natural products containing fish oils or *Ginkgo biloba* have been widely marketed to improve memory and sharpen the mind, there is a lack of evidence to support the use of these products for the prevention of cognitive decline or dementia.
3. *Sleep problems.* Current research suggests that melatonin (a hormone known to shift circadian rhythms) may be useful in treating several sleep disorders, such as jet lag, delayed sleep phase disorder, and sleep problems related to shift work. Guidelines from the American Academy of Sleep Medicine recommend the use of melatonin supplements to promote daytime sleep among night shift workers. The guidelines also recommend melatonin to reduce symptoms of jet lag and improve sleep following travel across multiple time zones.
4. *Menopausal symptoms.* Many natural products, such as black cohosh, have been studied for their effects on menopausal symptoms, but there is little evidence that they are useful. While some herbs and botanicals are often found in over-the-counter formulas and combinations, many of these combination products have not been studied. It's also important to know that because natural products used for menopausal symptoms can have side effects and can interact with other botanicals or supplements or with medications, research in this area is looking at safety as well as effectiveness.
5. *Benign prostatic hyperplasia (BPH).* Although several small studies have suggested modest benefit of saw palmetto for treating symptoms of BPH, a large study evaluating high doses of saw palmetto found that saw palmetto was not more effective than placebo for treatment of urinary symptoms related to BPH.

40.21 6 Things You Should Know about Dietary Supplements for Osteoarthritis

Osteoarthritis (OA) is the most common type of arthritis—affecting 27 million Americans—and is an increasing problem among older adults. Treatments for osteoarthritis address the symptoms, such as pain, swelling, and reduced function in the joints. Nonmedicinal approaches involve lifestyle changes such as exercise, weight control, and rest. Conventional medicinal treatments for OA include nonsteroidal anti-inflammatory drugs (NSAIDS), acetaminophen (a class of pain reliever), and injections of corticosteroids (anti-inflammatory hormones).

Many people with OA report trying various dietary supplements, including glucosamine and chondroitin, alone or in combination, in an effort to relieve pain and improve function. However, there is no convincing evidence that any dietary supplement helps with OA symptoms or the course of the underlying disease. Here are 6 things you should know about dietary supplements for osteoarthritis:

1. *The majority of research has found little effect of glucosamine or chondroitin on symptoms or joint damage associated with osteoarthritis of the knee or hip.* Studies have found that glucosamine and chondroitin supplements may interact with the anticoagulant (blood-thinning) drug warfarin (Coumadin). But overall, studies have not shown any other serious side effects.
2. *Dimethyl Sulfoxide (DMSO) and Methylsulfonylmethane (MSM) are two chemically related dietary supplements that have been used for arthritic conditions; however, evidence does not suggest that DMSO and MSM are helpful for osteoarthritis symptoms.* Although there are limited safety data available, some side effects from topical DMSO have been reported, including upset stomach, skin irritation, and garlic taste, breath, and body odor. Only minor side effects are associated with MSM in humans including allergy, upset stomach, and skin rashes.
3. *S-Adenosyl-L-methionine (SAMe) is a molecule that is naturally produced in the body and is often taken as a dietary supplement; however, there is not enough evidence to support the use of SAMe for osteoarthritis of the knee or hip.* SAMe is generally considered safe, but common side effects include gastrointestinal problems, dry mouth, headache, sweating, dizziness, and nervousness.
4. *There is preliminary evidence that avocado/soybean unsaponifiables (ASU), supplements made from avocado oil and soybean oil extracts, may have modest beneficial effects on symptoms of osteoarthritis.* Safety information has not been sufficiently available.
5. *Although some results suggest that a few herbs may be beneficial for OA symptoms, the overall evidence is weak, and conclusions among reviews of the literature provide conflicting interpretations.* In general, herbs have not been

studied or prepared in a consistent way. There is also a general lack of safety data available.

6. *If you take, or are considering taking, dietary supplements for osteoarthritis, tell your health care providers.* They can do a better job caring for you if they know what dietary supplements you use.

40.22 7 Tips: Know the Facts about Supplements Marketed for Weight Loss

Although it's easy to be tempted by the "quick fix" claims of dietary supplements marketed for weight loss or appetite suppression, most of these products haven't been proven safe or effective. Many people have the misperception that herbal or traditional products—and those that are "natural"—are safe to use, but research has shown that many of these products carry the same dangers as pharmaceutical agents. So, if you're thinking about a dietary product to help you lose weight, here are some things you should know.

1. *Ask yourself if a product sounds too good to be true***.** Be cautious if the claims for the product seem exaggerated or unrealistic and use phrases like "quick and effective" or "totally safe." Be skeptical about information from personal "testimonials" about the product's benefits. Keep in mind that testimonials, anecdotes, unsupported claims, and opinions are not the same as objective, evidence-based information.
2. *Be aware of the possibility of product contamination***.** The US Food and Drug Administration (FDA) has found weight loss products sold as dietary supplements that contain hidden prescription drugs or other compounds. These tainted products can cause serious harm to unsuspecting consumers.
3. *There is no definitive scientific evidence to support the use of acai berry, bitter orange, and green tea supplements for weight loss.* There is little reliable information about the safety of acai as a supplement, and there have been reports of serious side effects from taking bitter orange supplements and concentrated green tea extracts.
4. *Ephedra is dangerous, and the increased risk of heart problems and stroke far outweighs any potential benefits.* In 2004, the FDA banned the US sale of dietary supplements containing ephedra. The FDA found that these supplements had an unreasonable risk of injury or illness—particularly cardiovascular complications—and risk of death.
5. *Consider a mind and body approach such as mindfulness meditation or yoga.* There is some emerging evidence suggesting that some mind and body approaches are generally safe and may be useful as complements to other weight-loss interventions. Research in this area is in its early stages, but results of studies on yoga and mindful eating are promising.

6. *Make lifestyle changes that work for you, including a healthy eating plan and regular physical activity.* The key to achieving a healthy weight (https://www.nhlbi.nih.gov/health/educational/lose_wt/index.htm) is making changes in your eating and physical activity habits that work for you and that you can maintain for the rest of your life.
7. *Talk with your health care provider.* If your doctor does not ask you about healthy eating, physical activity, and weight management during your regular check-up, you can start the conversation. Your health care provider can assess your weight and health risks, determine whether you need to lose weight, and provide information that will help you make informed decisions about a weight-loss program. You may feel uncomfortable talking about your weight with your health care provider, but remember that he or she is there to help improve your health.

40.23 5 Things to Know about Sleep Disorders and Complementary Health Approaches

Chronic, long-term sleep disorders affect millions of Americans each year. These disorders and the sleep deprivation they cause can interfere with work, driving, social activities, and overall quality of life, and can have serious health implications. Sleep disorders account for an estimated $16 billion in medical costs each year, plus indirect costs due to missed days of work, decreased productivity, and other factors.

People who have trouble sleeping often try various dietary supplements, relaxation therapies, or other complementary health approaches in an effort to fall asleep faster, stay asleep longer, and improve the overall quality of their sleep. Here are 5 things to know about what the science says about sleep disorders and complementary health approaches.

1. *Relaxation techniques may be helpful for insomnia.* Evidence indicates that using relaxation techniques before bedtime can be helpful components of a successful strategy to improve sleep habits. Other components include maintaining a consistent sleep schedule; avoiding caffeine, alcohol, heavy meals, and strenuous exercise too close to bedtime; and sleeping in a quiet, cool, dark room.
2. *Melatonin supplements may be helpful for some people with insomnia or sleep problems caused by shift work or jet lag.* Research on the use of melatonin for children is more limited; available research suggests some benefit in children, but those studies were small and only addressed short-term use of melatonin. The long-term safety of melatonin has not been investigated.
3. *Current evidence regarding other mind and body approaches such as mindfulness-based stress reduction (a type of meditation), yoga, massage therapy, and acupuncture is either too preliminary or inconsistent to draw*

conclusions about whether they are helpful for sleep disorders. These mind and body practices are generally considered safe for healthy people and when performed by an experienced practitioner.

4. *Various herbs and dietary supplements sometimes used as sleep aids, including valerian, kava, chamomile, and L-tryptophan and 5-hydroxytryptophan (5-HTP) have not been shown to be effective for insomnia, and important safety concerns have been raised about a few*. For example, the use of L-tryptophan supplements has been linked to eosinophilia-myalgia syndrome (EMS), a complex, potentially fatal disorder with multiple symptoms including severe muscle pain. Kava supplements have been linked to a risk of severe liver damage.
5. *If you are considering a complementary health approach for sleep problems, talk to your health care providers.* Trouble sleeping can be an indication of a more serious condition, and some prescription and over-the-counter drugs can contribute to sleep problems. So, it's important to discuss your sleep-related symptoms with your health care providers before trying any complementary health product or practice.

40.24 6 Things to Know about Complementary Health Approaches for Seasonal Affective Disorder

Seasonal Affective Disorder (SAD) is a type of depression that comes and goes with the seasons, typically starting in the late fall and early winter and going away during the spring and summer. To be diagnosed with SAD, people must meet full criteria for major depression coinciding with specific seasons (appearing in the winter or summer months) for at least 2 years. Symptoms of the winter pattern of SAD include having low energy, hypersomnia, overeating, weight gain, craving for carbohydrates, social withdrawal (feel like "hibernating"). Some people with SAD may be treated with medication (typically antidepressants) and/or psychotherapy. Others may turn to complementary health approaches such as light therapy or dietary supplements. Here are 6 things to know about complementary health approaches for SAD.

1. There is some evidence that *light therapy* may be useful as a preventive treatment for people with a history of season affect disorder. The idea behind light therapy is to replace the diminished sunshine of the fall and winter months using daily exposure to a light box. Most typically, light boxes filter out the ultraviolet rays and require 20–60 minutes of exposure to 10,000 lux of cool-white fluorescent light, an amount that is about 20 times greater than ordinary indoor lighting.
2. There is some evidence that *cognitive behavioral therapy for SAD (CBT-SAD)* can be effective in reducing the recurrence and remissions of SAD and has been shown last at least between a first and second winter season. Traditional cognitive behavioral therapy has been adapted for use with

SAD (CBT-SAD). CBT-SAD relies on basic techniques of CBT such as identifying negative thoughts and replacing them with more positive thoughts along with a technique called behavioral activation. Behavioral activation seeks to help the person identify activities that are engaging and pleasurable, whether indoors or outdoors, to improve coping with winter.

3. A few small studies suggest that *St. John's wort* may improve some symptoms of SAD; however, there are risks associated with taking St. John's wort. For example, St. John's wort can weaken the effects of many medicines, including antidepressants, contraceptives, cyclosporine, digoxin, indinavir, irinotecan, and anticoagulants. Taking St. John's wort with certain antidepressants or other drugs that affect serotonin may lead to increased serotonin-related side effects, which may be potentially serious.
4. There is some limited evidence (small trials involving few patients) that suggests *melatonin* improves sleep in some patients with SAD. Melatonin appears to be safe when used short-term, but the lack of long-term studies means we don't know if it's safe for extended use.
5. Taking *vitamin D supplements* is not considered an effective SAD treatment. Low blood levels of vitamin D are often found in people with SAD; however, the evidence for its use has been mixed. Although some studies suggest vitamin D supplementation may be as effective as light therapy, others found vitamin D had no effect.
6. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.25 5 Tips: What Consumers Need to Know about Dietary Supplements

Many people take dietary supplements (https://www.nccih.nih.gov/health/supplements) in an effort to be well and stay healthy. Herbal medicines or botanicals, also called "natural products," are one type of dietary supplement. Dietary supplements can come in the form of pills, powders, or liquids and are widely available. While there is a lot of evidence that dietary supplements help in preventing and treating nutrient deficiency, there is much less evidence about their usefulness in preventing or treating other diseases. So, there is a lot we don't know.

If you are thinking about or are using a dietary supplement, here are five tips to consider.

1. *Take charge of your health by being an informed consumer* (https://www.nccih.nih.gov/health/decisions/)**.** The standards for marketing supplements are very different from the standards for drugs. For example, marketers of a supplement do not have to prove to the Food and Drug Administration that it is safe or that it works before it arrives on grocery

store shelves. Find out what the scientific evidence says about the safety (https://www.nccih.nih.gov/health/safety/) of a dietary supplement and whether it works. The resources below can help you.

2. *"Natural" does not necessarily mean "safe."* For example, the herbs comfrey and kava (https://www.nccih.nih.gov/health/kava/) can cause serious harm to the liver. Also, when you see the term "standardized" (or "verified" or "certified") on the bottle, it does not necessarily guarantee product quality or consistency.
3. *Interactions are possible.* Some dietary supplements may interact with medications (prescription or over-the-counter) or other dietary supplements, and some may have side effects on their own. Research has shown that St. John's wort (https://www.nccih.nih.gov/health/stjohnswort/ataglance.htm) interacts with many medications in ways that can interfere with their intended effects, including antidepressants, birth control pills, antiretrovirals used to treat HIV infection, and others.
4. *Be aware of the potential for contamination.* Some supplements have been found to contain hidden prescription drugs or other compounds, particularly in dietary supplements marketed for weight loss, sexual health including erectile dysfunction, and athletic performance or body-building.
5. *Talk to your health care providers.* Tell your health care providers about any complementary health products or practices you use, including dietary supplements. This will help give them a full picture of what you are doing to manage your health and will help ensure coordinated and safe care.

40.26 5 Things to Know about St. John's Wort and Depression

St. John's wort (https://www.nccih.nih.gov/health/stjohnswort), a plant that grows in the wild, has been used for centuries for mental health conditions and is widely prescribed for depression (https://www.nccih.nih.gov/health/depression.htm) in Europe. However, current evidence for using St. John's wort for depression is not conclusive, and the herb can have serious side effects. It is also important to note that in the United States, while there may be public interest in St. John's wort to treat depression, the US Food and Drug Administration has not approved its use as an over-the-counter or prescription medicine for depression.

1. *St. John's wort is not a proven therapy for depression.* Study results on the effectiveness of St. John's wort for depression are not conclusive. While there may be public interest in St. John's wort to treat depression, the US Food and Drug Administration has not approved its use as an over-the-counter or prescription medicine for depression.
2. *St. John's wort is known to affect metabolism of a number of drugs and can cause serious side effects.* Combining St. John's wort with certain antidepressants

can lead to a potentially life-threatening increase of serotonin, a brain chemical targeted by antidepressants. St. John's wort can also limit the effectiveness of many prescription medicines, including birth control pills, digoxin, some HIV drugs and cancer medications, and others.

3. *Do not use St. John's wort to replace conventional care or to postpone seeing your health care provider.* If depression is not adequately treated, it can become severe and, in some cases, may be associated with suicide. Consult a health care provider if you or someone you know may be depressed.
4. *Many dietary supplements have not been tested in pregnant women, nursing mothers, or children.* Little safety information on St. John's wort for pregnant women or children is available, so it is especially important to talk with health experts if you are pregnant or nursing or are considering giving a dietary supplement to a child.
5. *Tell all your health care providers about any complementary health approaches you use.* Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

40.27 6 Things to Know about Massage Therapy for Health Purposes

The term "massage therapy" includes many techniques, and the type of massage given usually depends on your needs and physical condition. In general, massage therapists work on muscle and other soft tissue to help you feel better.

A lot of the scientific research on massage therapy is preliminary or conflicting, but much of the evidence points toward beneficial effect on pain and other symptoms associated with a number of different conditions. Here are six things you should know about massage therapy for health purposes.

1. *Evidence suggests that massage therapy may be useful for some pain conditions such as low-back pain and chronic neck pain.* However, research on massage for headaches is preliminary and only somewhat promising.
2. *Research has suggested that at least for the short-term, massage therapy for cancer patients may reduce pain.* Evidence has suggested that massage therapy may also promote relaxation and boost mood in people with cancer.
3. *A recent review of scientific literature concluded that massage therapy may help to reduce depression.* However, a 2013 review found there is not enough evidence to determine if massage helps pregnant mothers with depression.
4. *A 2010 review concluded that massage therapy may help temporarily reduce pain, fatigue, and other symptoms associated with fibromyalgia, but the evidence is not definitive.* The authors of the review noted that it is important that the massage therapist not cause pain.

5. *Several studies on massage therapy in preterm infants have been conducted to determine if therapeutic massage provides any benefits.* A 2010 review of the scientific literature suggested that massaging preterm infants using moderate pressure may improve weight gain. However, a 2013 review determined that there is not enough evidence to know if massage benefits healthy infants who are developing normally.
6. *Massage therapy appears to have few risks when performed by a trained practitioner.* However, massage therapists should take some precautions with certain health conditions. You should talk with your health care providers to determine if massage therapy is safe for you.

40.28 7 Things to Know about Complementary Health Approaches for ADHD

People with attention-deficit hyperactivity disorder (ADHD) may have trouble paying attention or controlling impulsive behavior, and they may be overly active. Conventional treatment, which may include medication (most often stimulants), behavior therapy, or a combination of both, is helpful for the majority of children and adults with ADHD. Many complementary health approaches have been studied for ADHD, but none has been conclusively shown to be helpful.

Here are 7 things you should know if you are considering a complementary health approach for ADHD:

1. Researchers are studying whether omega-3 fatty acids could be helpful for ADHD, but current evidence is inconclusive.
2. Melatonin has not been shown to relieve ADHD symptoms, but it may help children with ADHD who have sleep problems to fall asleep sooner.
3. Research on other dietary supplements, including L-carnitine, St. John's wort, French maritime pine bark extract (also known as Pycnogenol), and Ginkgo biloba, has not demonstrated that these supplements are helpful for ADHD. Dietary supplements may have side effects and may interact with drugs. In particular, St. John's wort can speed up the process by which the body breaks down many drugs, thus making the drugs less effective.
4. Several mind and body practices, such as acupuncture, massage therapy, and meditation have been studied for ADHD, but the amount of evidence on each of these practices is small, and no conclusions can be reached about whether they are beneficial.
5. Short-term aerobic exercise, including yoga, has shown beneficial effects on symptoms of ADHD such as attention, hyperactivity, and impulsivity.
6. Some research has suggested that neurofeedback, a technique in which people are trained to alter their brain wave patterns, may improve ADHD symptoms, but several small studies that compared neurofeedback to a control procedure did not find differences between the two treatments.

7. If you're considering using any of these complementary health approaches, or others, for ADHD, talk with your (or your child's) health care provider.

40.29 6 Things to Know about Type 2 Diabetes and Dietary Supplements

Diabetes is a group of chronic diseases that affect metabolism—the way the body uses food for energy and growth. Millions of people have diabetes, which can lead to serious health problems if it is not managed well. Conventional medical treatments and following a healthy lifestyle, including watching your weight, can help you prevent, manage, and control many complications of diabetes. Researchers are studying several complementary health approaches, including dietary supplements, to see if they can help people manage type 2 diabetes or lower their risk of developing the disease; however, there is currently not enough scientific evidence to suggest that any dietary supplements can help prevent or manage type 2 diabetes.

Here are 6 things you should know about taking dietary supplements for type 2 diabetes.

1. *A healthy diet, physical activity, and blood glucose testing are the basic tools for managing type 2 diabetes.* Your health care providers will help you learn to manage your diabetes and track how well you are controlling it. It is very important not to replace proven conventional medical treatment for diabetes with an unproven health product or practice.
2. *Some dietary supplements may have side effects, including interacting with your diabetes treatment or increasing your risk of kidney problems.* This is of particular concern because diabetes is the leading cause of chronic kidney disease and kidney failure in the United States. Supplement use should be monitored closely in patients who have or are at risk for kidney disease.
3. *Chromium (an essential trace mineral found in many foods) has been studied for preventing diabetes and controlling glucose levels, but research has found it has few or no benefits.* There have been a few reports of kidney damage, muscular problems, and skin reactions following large doses of chromium.
4. *There is mixed evidence that magnesium helps to manage diabetes—benefits of magnesium supplements on diabetes have been found in some, but not all clinical studies. However, research suggests that people with lower magnesium intake may have a greater risk of developing diabetes.* A large 2007 study found an association between a higher intake of cereal fiber and magnesium and a reduced risk of developing type 2 diabetes. Large doses of magnesium in supplements can cause diarrhea and abdominal cramping, and very large doses—more than 5,000 mg/day per day—can be deadly.

5. *There is no strong evidence that herbs and other dietary supplements, including cinnamon, alpha-lipoic acid, and omega-3s, can help to control diabetes or its complications.* Researchers have found some risks but no clear benefits of cinnamon for people with diabetes. For example, a 2012 review of the scientific literature did not support using cinnamon for type 1 or type 2 diabetes.
6. *Talk with your health care provider before considering any dietary supplement for yourself, particularly if you are pregnant or nursing, or for a child.* Do not replace scientifically proven treatments for diabetes with unproven health products or practices. The consequences of not following your prescribed medical regimen for diabetes can be very serious.

40.30 5 Tips: What You Should Know about Complementary Health Approaches for BPH

Benign prostatic hyperplasia—also called BPH—is a condition in men in which the prostate gland is enlarged but not cancerous. BPH is the most common prostate problem for men over 50. As the prostate enlarges, it presses against and pinches the urethra (the tube that takes urine away from the bladder). Eventually, the bladder may weaken and lose the ability to empty completely, leaving some urine in the bladder. The narrowing of the urethra and urinary retention (the inability to empty the bladder completely) cause many of the problems associated with BPH.

Treatment options for BPH generally include lifestyle changes, medications, minimally invasive procedures, or surgery. The use of some complementary health approaches such as phytotherapy (natural products) for the treatment of lower urinary tract symptoms associated with BPH is common. Although there is limited evidence that some natural products may help improve symptoms related to BPH over the short term, most of the trials conducted have been small in size, of short duration, and used varied doses and preparations. If you are considering a complementary health approach for treating BPH symptoms, here are 5 things you should know:

1. Although several small studies have suggested modest benefit of saw palmetto (*Serenoa repens*) for urinary symptoms associated with BPH, a larger study and a review of the scientific literature found that saw palmetto was not more effective than placebo for these symptoms.
2. There is some limited evidence that *Pygeum africanum* (African plum tree) and *Urtica dioica* (stinging nettle) may improve some lower urinary tract symptoms of BPH over the short term. There is also some limited evidence that *Urtica dioica* and saw palmetto as a combined treatment may improve these symptoms.
3. There isn't sufficient evidence to support the use of lycopene for the prevention or treatment of BPH.

4. There is not enough evidence to determine whether acupuncture is beneficial for symptoms of BPH.
5. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.31 8 Things to Know about Mind and Body Approaches for Health Problems Facing Military Personnel and Veterans

Many military personnel and veterans experience chronic pain, a condition that can be debilitating and often difficult to treat. Service members may have other conditions that are also challenging to treat including, post-traumatic stress disorder (PTSD), anxiety, depression, insomnia, and substance use disorder. Many military personnel, veterans, and their families turn to complementary and integrative health approaches such as mindfulness meditation and other practices to increase their options for the management of pain and associated problems.

Research on complementary health approaches for chronic pain in military populations is currently in progress, but there is very little published information about the effectiveness of these approaches for chronic pain in military populations. However, there is published information on complementary health approaches for PTSD, stress/anxiety, and insomnia in military personnel and veterans, as well as information on chronic pain in non-military populations.

Here are 8 things to know about mind and body approaches for health problems facing military personnel and veterans.

1. Reviews of research on acupuncture, massage, spinal manipulation, and yoga for *chronic low-back pain* in non-military populations have found evidence that these therapies may be beneficial. There is also some evidence that mindfulness-based stress reduction and cognitive behavioral therapy improves pain and function compared to usual care.
    - *Spinal manipulation.* The most recent guidelines from the American College of Physicians conclude that there is some evidence that spinal manipulation may be associated with a small improvement in function, but there may be no difference between spinal manipulation and other active treatments.
    - *Acupuncture.* These same guidelines recommend that patients with chronic low-back pain initially select nonpharmacologic treatment, such as acupuncture, to help manage their pain. In many studies, acupuncture has shown some benefit for low-back pain compared to conventional therapy, but simulated (placebo) acupuncture has also shown a similar benefit, suggesting that a component of any benefit from acupuncture may be due to patient expectation or practitioner attention.

- *Massage*. Studies suggest that massage is associated with short-term beneficial effects in reducing pain and improving function compared to usual care in people with chronic low-back pain.
- *Yoga.* A 2017 review of studies found that there is some evidence that yoga compared to non-exercise (e.g., no treatment, delayed yoga treatment, or patient education) results in small to moderate improvements in back-related function at 3 and 6 months, and may also be slightly more effective for pain, but the effect size was very small.
- *Mindfulness-based stress reduction.* A new study in adults with chronic low-back pain found that mindfulness-based stress reduction and cognitive behavioral therapy resulted in greater improvement in pain and functional limitation compared to usual care.

Available evidence indicates that acupuncture for neck pain may provide better pain relief compared to no treatment. There is some evidence that spinal manipulation may help relieve neck pain, but much of the research on has been of low quality.

According to reviewers who have assessed the research on complementary health practices and fibromyalgia, much of the research is still preliminary, and evidence of effectiveness for the various therapies used is limited. However, research has shown that tai chi may provide a benefit to patients with fibromyalgia.

There is some limited evidence that mind and body practices such as progressive relaxation, hypnosis, imagery, biofeedback, and visual mirror feedback may be useful in reducing phantom limb pain and sensation in amputees, although most studies have been small and of low quality.

Clinical guidelines on the management of post-traumatic stress disorder (PTSD) issued in 2010 by the Department of Veterans Affairs and the Department of Defense indicate that relaxation techniques be considered as a part of treatment approaches for acute stress disorder or PTSD in relieving symptoms associated with physiological hyper-reactivity.

There is some limited evidence that some mind and body approaches, such as yoga, meditation, and relaxation techniques, may have the potential for modest beneficial effects on stress and anxiety in military populations. However, many of the studies have small sample sizes.

There is some evidence to suggest that relaxation techniques, along with behavioral therapies, can be helpful components of a successful strategy to improve sleep, but there have only been a few small studies conducted in military populations. There is also some limited evidence that imagery rehearsal therapy may improve insomnia in nonveteran populations, but only a few small studies have examined imagery rehearsal therapy in combat veterans or active duty military personnel.

Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.32 5 Tips: What You Should Know about Tai Chi for Health

Tai chi is a centuries-old, mind and body practice. It involves certain postures and gentle movements with mental focus, breathing, and relaxation. The movements can be adapted or practiced while walking, standing, or sitting. Several clinical trials have evaluated the effects of tai chi in people with various health conditions. Here are 5 things to know about tai chi for health.

1. Research findings suggest that practicing tai chi may improve balance and stability in older people and reduce the risk of falls. There is also some evidence that tai chi may improve balance impairments in people with mid-to-moderate Parkinson's disease.
2. There is some evidence to suggest that practicing tai chi may help people manage chronic pain (https://www.nccih.nih.gov/health/pain) associated with knee osteoarthritis (https://www.nccih.nih.gov/health/arthritis) and help people with fibromyalgia sleep better and cope with pain, fatigue, and depression.
3. Although tai chi has not been shown to have an effect on the disease activity of rheumatoid arthritis (e.g., tender and swollen joints, activities of daily living) (https://www.nccih.nih.gov/health/arthritis), there is some evidence that tai chi may improve lower extremity (ankle) range of motion in people with rheumatoid arthritis. It is not known if tai chi improves pain associated with rheumatoid arthritis or quality of life.
4. Tai chi may promote quality of life and mood in people with heart failure and cancer. Tai chi also may offer psychological benefits, such as reducing anxiety (https://www.nccih.nih.gov/health/anxiety). However, differences in how the research on anxiety was conducted make it difficult to draw firm conclusions about this.
5. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.33 6 Things to Know about Complementary Health Approaches for Seasonal Allergy Relief

Seasonal allergies, also called hay fever or allergic rhinitis, are triggered each spring, summer, and fall when trees, weeds, and grasses release pollen into the air. When the pollen ends up in your nose and throat, it can bring on sneezing, runny nose, coughing, and itchy eyes and throat. People manage seasonal allergies by taking medication, avoiding exposure to the substances that trigger their allergic reactions, or having a series of "allergy shots" (a form of immunotherapy).

People also try various complementary approaches to manage their allergies. If you are considering any complementary health approach for the relief of seasonal allergy symptoms, here are some things you need to know.

1. *Nasal saline irrigation.* There is some good evidence that saline nasal irrigation (putting salt water into one nostril and draining it out the other) can be useful for modest improvement of allergy symptoms. Nasal irrigation is generally safe; however, neti pots and other rinsing devices must be used and cleaned properly. According to the US Food and Drug Administration (http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm316375.htm), tap water that is not filtered, treated, or processed in specific ways is not safe for use as a nasal rinse.
2. *Butterbur extract.* There are hints that the herb butterbur may decrease the symptoms associated with nasal allergies. However, there are concerns about its safety.
3. *Honey.* Only a few studies have looked at the effects of honey on seasonal allergy symptoms, and there is no convincing scientific evidence that honey provides symptom relief. Eating honey is generally safe; however, children under 1 year of age should not eat honey. People who are allergic to pollen or bee stings may also be allergic to honey.
4. *Acupuncture.* A 2015 evaluation of 13 studies of acupuncture for allergic rhinitis, involving a total of 2,365 participants, found evidence that this approach may be helpful.
5. *Probiotics.* There is some evidence that suggests that probiotics may improve some symptoms, as well as quality of life, in people with allergic rhinitis, but because probiotic formulations vary from study to study, it's difficult to make firm conclusions about its effectiveness.
6. *Talk to your health care provider.* If you suffer from seasonal allergies and are considering a complementary health approach, talk to your health care provider about the best ways to manage your symptoms. You may find that when the pollen count is high, staying indoors, wearing a mask, or rinsing off when you come inside can help.

40.34 7 Tips: What You Should Know about Complementary Health Approaches for Multiple Sclerosis

Multiple sclerosis (MS) is a disease of the central nervous system. In MS, the body's immune system attacks myelin, which coats nerve cells. Symptoms of MS include muscle weakness (often in the hands and legs), tingling and burning sensations, numbness, chronic pain, coordination and balance problems, fatigue, vision problems, and difficulty with bladder control. For more information on MS, visit the National Institute of Neurological Disorders and Stroke's Web site (https://www.ninds.nih.gov/Disorders/All-Disorders/Multiple-Sclerosis-Information-Page).

Although MS has no cure, some conventional treatments can improve symptoms, reduce the number and severity of relapses, and delay the disease's progression. Many people with MS try some form of complementary health approach, often special diets and dietary supplements. Here are 7 things to know about complementary health approaches for MS.

1. Practicing yoga (https://www.nccih.nih.gov/health/yoga) may help with fatigue and mood, but there's no evidence that yoga can help with mobility or thinking ability.
2. Reflexology (applying pressure to the soles of the feet) may reduce a burning or prickling sensation sometimes associated with MS, but larger studies are needed to provide a reliable conclusion.
3. Studies of magnetic therapy, which involves devices that use an electrical current to generate a magnetic field, have shown mixed results for MS-related fatigue.
4. Chemicals in marijuana known as THC/cannabinoids may relieve spasticity and/or pain in people with MS. While no marijuana-derived medications are approved by the US Food and Drug Administration to treat MS in the United States, Canada and some European countries have approved Sativex®, a mouth spray with THC/cannabinoids for muscle control related to MS.
5. Dietary supplements such as fish oil and ginkgo biloba (https://www.nccih.nih.gov/health/ginkgo) have not been shown to be helpful for MS.
6. One large, 5-year study suggests that low blood levels of vitamin D may be a risk factor for long-term disease activity and progression. However, more studies need to be done to determine if vitamin D supplementation is beneficial.
7. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.35 7 Things to Know about Complementary Health Approaches for Anxiety

Occasional anxiety (https://www.nccih.nih.gov/health/anxiety) is a normal part of life. You might feel anxious when faced with a problem at work, before taking a test, or making an important decision. But for some people, anxiety becomes a severe, persistent problem that's hard to control and can interfere with daily activities; this is called an anxiety disorder. Effective conventional treatments for anxiety disorders, including psychotherapy and medication, are available.

Some people turn to complementary health approaches to help them reduce anxiety and cope with stressful situations, such as medical procedures. Certain complementary health approaches may help relieve this type of anxiety; however,

complementary approaches have not been proven effective in treating anxiety disorders. Here are 7 things to know about complementary health approaches for anxiety:

1. Although some studies suggest that acupuncture (https://www.nccih.nih.gov/health/acupuncture) might reduce anxiety, the research is too limited to allow definite conclusions to be reached.
2. In some studies in people with cancer or other medical conditions, massage therapy (https://www.nccih.nih.gov/health/massage/massage-introduction.htm) helped to reduce anxiety; however, other studies did not find a significant beneficial effect. Massage has not been shown to be effective in treating generalized anxiety disorder.
3. Mindfulness meditation (https://www.nccih.nih.gov/health/meditation) may have a small to modest beneficial effect on anxiety-related symptoms. However, meditation has not been shown to be effective in treating anxiety disorders.
4. Relaxation techniques (https://www.nccih.nih.gov/health/stress/relaxation.htm) may reduce anxiety in people with chronic medical problems and those who are having medical procedures. However, conventional psychotherapy (cognitive-behavioral therapy) may be more effective than relaxation techniques in treating anxiety disorders.
5. Kava extract (https://www.nccih.nih.gov/health/kava) may produce moderately beneficial effects on anxiety symptoms; however, the use of kava supplements has been linked to a risk of severe liver damage.
6. There is some evidence that suggests melatonin (https://www.nccih.nih.gov/health/melatonin) may help reduce anxiety in patients who are about to have surgery.
7. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.36 6 Tips: What You Need to Know about Complementary Health Approaches for Skin Conditions

Many people with skin conditions often turn to complementary health approaches, particularly vitamin, mineral, and herbal supplements. In spite of interest in complementary approaches, there have been only a few studies on complementary health approaches for skin conditions, and those that have been conducted have often had methodological problems. Here are 6 things you should know about complementary health approaches for skin conditions, including atopic dermatitis, psoriasis, acne, impetigo, and rosacea:

1. There is some limited evidence that relaxation techniques may help improve symptoms of atopic dermatitis, particularly in the pediatric population, although most studies have had problems with their methodology. The American Academy of Dermatology's clinical practice guidelines for the treatment of atopic dermatitis has stated that there is inconsistent to no evidence to recommend the use of vitamins, minerals and herbal supplements. The guidelines also state that the use of probiotics/prebiotics for the treatment of patients with established atopic dermatitis is not recommended because of inconsistent evidence.
2. There is some evidence that fish oil, Dead Sea climatotherapy, and the topical herbs Mahonia aquifolium and indigo naturalis may be beneficial for the treatment of psoriasis.
3. According to the American Academy of Dermatology's clinical practice guidelines for the treatment of acne, there are currently very limited data regarding the safety and efficacy of herbal and other complementary therapies such as topical tea tree oral or bee venom to recommend their use.
4. There is insufficient evidence to either recommend or dismiss herbal treatments for impetigo, including tea tree oil, garlic, coconut oils, tea effusions, and Manuka honey.
5. Although some natural products have shown promise for improving symptoms of rosacea, there is insufficient evidence to support the use of many of these products for rosacea.
6. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.37 5 Things to Know about Complementary Health Approaches for Parkinson's Disease

Parkinson's disease is a movement disorder that occurs when nerve cells in the brain that make the chemical dopamine stop working normally. The primary symptoms of Parkinson's disease are tremor, or trembling in hands, arms, legs, jaw, and face; rigidity, or stiffness of the limbs and trunk; bradykinesia, or slowness of movement; and postural instability, or impaired balance and coordination. As these symptoms become more pronounced, people may have difficulty walking, talking, or completing other simple tasks. There are currently no blood or laboratory tests that have been proven to help in diagnosing Parkinson's, so it's sometimes hard to diagnose. For more information on Parkinson's disease, visit the NIH's National Institute of Neurological Disorders and Stroke website (https://www.ninds.nih.gov/Disorders/All-Disorders/Parkinsons-Disease-Information-Page).

There is currently no cure for Parkinson's disease, but medications and a surgical procedure called deep brain stimulation may help control symptoms for

a limited period. Some people with Parkinson's disease also try complementary health approaches to help with symptoms of the disease. Here are 5 things to know about complementary health approaches for Parkinson's disease:

1. There is some limited evidence that tai chi may improve some symptoms of Parkinson's disease, such as balance and functional mobility, but study results are mixed.
2. Dance therapy appears to provide short-term benefits for some symptoms of Parkinson's disease, including balance problems.
3. Neither massage nor acupuncture appears to reduce symptoms of Parkinson's disease, but the research on both approaches is limited.
4. No dietary supplements have been shown to help control symptoms of Parkinson's disease.
5. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.38 5 Myths about Popular Natural Products Marketed for Disease Prevention and Wellness

Many natural products sold as dietary supplements are marketed for promoting health and well-being and preventing disease. However, there is often little to no evidence to support these claims. Here are five myths about popular natural products often used for disease prevention and wellness reasons and what the science really says about them for improving health.

Remember, take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

1. Myth: *Herbs such as valerian, chamomile, and kava are effective for insomnia.*

   Fact: Various herbs such as valerian (https://www.nccih.nih.gov/health/valerian), chamomile (https://www.nccih.nih.gov/health/chamomile), and kava (https://www.nccih.nih.gov/health/kava), and homeopathic medicines sometimes used as sleep aids have not been shown to be effective for insomnia, and important safety concerns have been raised about a few. For example, kava supplements have been linked to a risk of severe liver disease. However, there is evidence to suggest that using relaxation techniques (https://www.nccih.nih.gov/health/stress/relaxation.htm), such as progressive relaxation, guided imagery, biofeedback, or deep breathing exercises, before bedtime can be helpful components of a successful strategy to improve sleep habits. Current evidence also suggests that melatonin (https://www.nccih.nih.gov/health/melatonin) may be useful in treating several sleep disorders, including jet lag, delayed sleep phase disorder, and sleep problems related to shift work.

2. Myth: *The herb passionflower can reduce stress and improve overall health.*

   Fact: There are very few studies of passionflower (https://www.nccih.nih.gov/health/passionflower) conducted in people and therefore insufficient evidence to determine whether passionflower is efficacious for any condition. However, there is some scientific evidence to date that suggests mindfulness meditation (https://www.nccih.nih.gov/health/meditation)—a mind and body practice which cultivates abilities to maintain focused and clear attention, and develop increased awareness of the present—may help reduce symptoms of stress, including anxiety and depression. Results from a small body of research suggest that yoga (https://www.nccih.nih.gov/health/yoga) may also affect stress and anxiety symptoms.

3. Myth: *A daily dose of a vitamin C supplement will prevent the onset of the common cold.*

   Fact: Several reviews have concluded that prophylactic vitamin C does not reduce the incidence of colds (https://www.nccih.nih.gov/health/flu) in the general population, but may be useful in reducing incidence of colds for people exposed to brief periods of severe physical exercise (e.g., marathon runners, skiers, and soldiers training in subarctic conditions).

5. Myth: *Taking garlic supplements will prevent heart disease.*

   Fact: There is no evidence that garlic supplements prevent heart disease. However, evidence from small studies is mixed about whether garlic (https://www.nccih.nih.gov/health/garlic) supplements reliably lower cholesterol levels or change other known risk factors for cardiovascular disease.

6. Myth: *Turmeric (curcumin) and Ginkgo biloba supplements can prevent the onset of dementia and Alzheimer's disease in people.*

   Fact: Although there is some evidence in laboratory studies that curcumin (https://www.nccih.nih.gov/health/turmeric/ataglance.htm) may affect brain function and the development of dementia, these results have not been demonstrated in clinical trials. In several large clinical trials, *Ginkgo biloba* (https://www.nccih.nih.gov/health/ginkgo) has not been shown to effective in reducing either the overall incidence rate of dementia or Alzheimer's disease incidence.

40.39 7 Things to Know about Mind and Body Practices for Children and Teens

According to a 2012 national survey, nearly 12 percent of children and teens (about one in nine) in the United States are using some form of complementary health product or practice, such as chiropractic or spinal manipulation, yoga, meditation, or massage therapy. Mind and body practices include a variety of procedures and techniques done or taught by a trained practitioner or teacher

to help improve health and well-being. Older children and teens can do some mind and body activities on their own (or with help from a parent or guardian), such as relaxation techniques and deep breathing. Mind and body practices are generally safe if used appropriately, but the number of studies looking at their safety specifically for children is limited.

Here are 7 things to know about common mind and body practices for children and teens.

1. Biofeedback, guided imagery, mindfulness (https://www.nccih.nih.gov/health/meditation), and yoga (https://www.nccih.nih.gov/health/yoga) are some of the mind and body practices that have the best evidence of being effective for children and are low-risk.
2. Acupuncture (https://www.nccih.nih.gov/health/acupuncture/introduction) appears to be safe for most children, but side effects can occur if it's done by poorly trained practitioners.
3. Massage therapy (https://www.nccih.nih.gov/health/massage) appears to have few risks when done by a trained practitioner. However, massage therapists need to take extra precautions with people who have certain health conditions, such as bleeding disorders.
4. Relaxation techniques (https://www.nccih.nih.gov/health/stress/relaxation.htm) are generally safe for healthy people, including children. However, there have been rare reports that some relaxation techniques might cause or worsen symptoms in people with epilepsy or certain psychiatric conditions, or with a history of abuse or trauma.
5. Spinal manipulation (https://www.nccih.nih.gov/health/spinalmanipulation) is usually safe for healthy people but is also associated with rare but serious complications.
6. Follow the Centers for Disease Control and Prevention's vaccination recommendations (http://www.cdc.gov/vaccines/schedules/easy-to-read/index.html) to safeguard your child against vaccine-preventable diseases. Vaccinating children helps protect our community's and our children's health.
7. It's important that you talk with your child's health care provider about any complementary health approach that you're using or considering for your child, and encourage your teenagers to do the same.

40.40 6 Things to Know about Travel-Related Ailments and Complementary Health Approaches

People planning to travel internationally are often interested in complementary or integrative health approaches for travel-related illnesses and conditions. Some of these approaches for travel-related health problems are promoted widely

in advertising or marketed on the Internet. However, little of this information is supported by research evidence, and some of it is misleading or false. Here are 6 things to know if you are considering using herbal remedies, dietary supplements, or other complementary health approaches for travel-related ailments and hazards.

1. *Malaria.* Although some Web sites and news stories have claimed that using the herb artemisia alone may prevent malaria, studies show it does not. The World Health Organization recommends against using artemisia plant material in any form (including tea) for treating or preventing malaria. Additionally, travelers should not attempt to use quinine (from the cinchona tree) to self-treat or prevent malaria.
2. *Zika virus.* There is no evidence that any herbs or other products, such as activated charcoal or diatomaceous earth, will protect against or treat the Zika virus.
3. *Probiotics.* Research on the use of probiotics in treating acute infectious diarrhea is generally positive. Results from studies on preventing travelers' diarrhea are mixed but encouraging. The US Food and Drug Administration has not approved any health claims for probiotics.
4. *Jet lag.* Melatonin supplements may help with sleep problems caused by jet lag. Relaxation techniques, such as progressive relaxation and mindfulness-based stress reduction, may help with insomnia, but it has not been established whether they are effective for jet lag.
5. *Insect protection.* Laboratory studies found that botanicals, including citronella products, worked for shorter periods than products containing DEET. For people who prefer to use botanicals, the Centers for Disease Control and Prevention recommends Environmental Protection Agency (EPA)-registered products containing oil of lemon eucalyptus (OLE), such as the products Repel and Off! Botanicals.
6. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.41 6 Tips: IBS and Complementary Health Practices

As many as one in five Americans have symptoms of irritable bowel syndrome (IBS). IBS is a chronic disorder that interferes with the normal functions of the colon and is characterized by symptoms such as abdominal pain, cramping, bloating, constipation, and diarrhea. Many people with IBS turn to complementary health practices to help relieve their symptoms, and there is emerging evidence that some of these practices may have modest benefits.

If you are thinking about a complementary health practice for IBS, here are 6 tips:

1. *Hypnotherapy (hypnosis).* This practice involves the power of suggestion by a trained hypnotist or hypnotherapist during a state of deep relaxation, and is the most widely used mind and body intervention for IBS. According to reviews of the scientific literature, hypnotherapy may be a helpful treatment for managing IBS symptoms. Several studies of hypnotherapy for IBS have shown substantial long-term improvement of gastrointestinal symptoms as well as anxiety, depression, disability, and quality of life.
2. *Probiotics* (https://www.nccih.nih.gov/health/probiotics)**.** Probiotics such as *Bifidobacterium* and *Lactobacillus* are live microorganisms that are similar to microorganisms normally found in the human digestive tract. There is some preliminary evidence that suggests some probiotics may improve symptoms of IBS, such as abdominal pain, bloating, and gas; however, there is not enough evidence to support the use of probiotics of IBS, and not all probiotics have the same results.
3. *Peppermint oil* (https://www.nccih.nih.gov/health/peppermintoil). Peppermint oil is one herbal remedy often used to treat IBS for which there are mixed results. There is some evidence that enteric-coated peppermint oil capsules may be modestly effective in reducing several common symptoms of IBS—especially abdominal pain, bloating, and gas. Non-enteric coated forms of peppermint oil may cause or worsen heartburn symptoms, but otherwise appear to be generally safe. (Enteric-coating allows the peppermint oil to pass through the stomach unaltered so it can dissolve in the intestines. However, if coated peppermint oil capsules are taken at the same time as medicines such as antacids, this coating can break down more quickly and increase the risk of heartburn and nausea.)
4. *Herbal remedies.* Herbal remedies are commonly used for IBS symptoms; however, much of the research on these remedies has been done in China. A review of clinical trials for 71 herbal remedies found limited evidence suggesting that a few of these herbal remedies might help improve IBS symptoms including abdominal pain, constipation, and diarrhea. However, the review emphasizes that the studies were generally of poor quality.
5. *Acupuncture* (https://www.nccih.nih.gov/health/acupuncture-in-depth). While a few small studies have indicated that acupuncture has some positive effect on quality of life in people with IBS, reviews of the scientific literature have concluded that there is no convincing evidence to support the use of acupuncture for the treatment of IBS symptoms.
6. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.42 5 Things You Should Know about Dietary Supplements for Hepatitis C

Hepatitis C is a liver disease caused by a virus. It is usually chronic (long-lasting), but most people do not have any symptoms until the virus causes liver damage, which can take 10 or more years to happen. Without medical treatment, chronic hepatitis C can eventually cause liver cancer or liver failure. Conventional medical treatments are available for chronic hepatitis C. Some people with hepatitis C also try complementary health approaches, especially dietary supplements.

If you are considering any dietary supplement for hepatitis C, here are some things you should know.

1. No dietary supplement has been shown to be effective for hepatitis C or its complications.
2. The results of research supported by the National Institutes of Health have shown that silymarin, the active extract of milk thistle and the most popular complementary health product taken by people with liver disease, was no more effective than placebo in people with hepatitis C.
3. Research on other dietary supplements for hepatitis C, such as zinc, licorice root (or its extract glycyrrhizin), SAMe, and lactoferrin, is in its early stages, and no firm conclusions can be reached about the potential effectiveness of these supplements.
4. Colloidal silver is sometimes promoted for treating hepatitis C, but is not safe. Colloidal silver can cause irreversible side effects, including a permanent bluish discoloration of the skin.
5. Check with your health care provider before using any dietary supplement to make sure that it is safe for you and compatible with any medical treatment that you are receiving for hepatitis C or any other health problem.

40.43 5 Things to Know about Probiotics

Probiotics are live microorganisms (e.g., bacteria) that are either the same as or similar to microorganisms found naturally in the human body and may be beneficial to health. If you picture the human body as a "host" for bacteria and other microorganisms, you might have a better understanding of probiotics. The body, especially the lower gastrointestinal tract (the gut), contains a complex and diverse community of bacteria. Although we tend to think of bacteria as harmful "germs," many bacteria actually help the body function properly.

Probiotics are available to consumers in oral products such as dietary supplements and yogurts, as well as other products such as suppositories and creams. It is important to be aware that the US Food and Drug Administration (FDA) has not approved any health claims for probiotics. Here are some other things you should know:

1. There is some evidence that probiotics may be helpful for acute diarrhea, antibiotic-associated diarrhea, and atopic eczema (a skin condition most commonly seen in infants).
2. Although some probiotic formulations have shown promise in research, strong scientific evidence to support other uses of probiotics for most conditions is lacking.
3. Studies suggest that probiotics usually have few side effects. However, the data on safety, particularly long-term safety, are limited, and the risk of serious side effects may be greater in people who have underlying health conditions.
4. Probiotic products may contain different types of probiotic bacteria and have different effects in the human body. The effects also may vary from person to person.
5. If you are considering a probiotic dietary supplement, talk with your health care provider first. Do not replace scientifically proven treatments with unproven products or practices.

40.44 5 Things to Know about Mind and Body Approaches for Substance Use Disorders

Mind and body approaches, such as mindfulness-based interventions, have shown some success when used along with the treatment of substance abuse and addiction. Mindfulness-based approaches, in part, attempt to decrease the impact of negative mood, which is thought to serve as a trigger for substance use. Mind and body approaches can be part of a comprehensive addiction treatment plan that includes behavioral modifications, and may include pharmaceuticals to decrease cravings, group therapy, or counseling.

If you are considering a mind and body approach to help with a substance abuse disorder or addiction, here are 5 things you should know.

1. *Mindfulness-based therapies.* Studies suggest that mindfulness-based therapies may help improve health-related quality of life in people with substance use disorders, and may also help reduce cravings. For people who want to quit smoking, mindfulness training used along with conventional medicine has been shown to help maintain abstinence.
2. *Acupuncture.* There are some results from studies that suggest acupuncture, when used along with conventional medicine, may have positive effects on withdrawal/craving and anxiety symptoms in people with substance use disorders and addiction.
3. *Hypnotherapy.* Findings from some studies suggest that hypnotherapy may help people quit smoking, but there is not enough evidence to show whether hypnotherapy could be as effective as counseling treatment.

4. *Yoga.* Only a few studies have been conducted on the effects of yoga to help people quit smoking. Although preliminary results have been positive, larger, high-quality studies are needed to determine if yoga is an effective treatment.
5. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.45 4 Things to Know about Menopausal Symptoms and Complementary Health Practices

Menopause is the permanent end of a woman's menstrual periods. Menopause (https://www.nccih.nih.gov/health/menopause) can occur naturally or be caused by surgery, chemotherapy, or radiation. During the years around menopause, some women have hot flashes, night sweats, difficulty sleeping, or other bothersome symptoms. Natural products or mind and body practices are sometimes used in an effort to relieve menopausal symptoms such as hot flashes and night sweats.

Here are 4 things to know if you are considering a complementary health approach for managing menopausal symptoms:

1. *Mind and body practices such as hypnosis, mindfulness meditation, and tai chi may help improve some menopausal symptoms.* Researchers looked at mind and body therapies for menopausal symptoms and found that tai chi and meditation-based programs may be helpful in reducing common menopausal symptoms including the frequency and intensity of hot flashes, sleep and mood disturbances, stress, and muscle and joint pain. There is also some evidence that hypnotherapy may help women manage hot flashes.
2. *Many natural products, such as black cohosh, soy isoflavone supplements, and DHEA, have been studied for their effects on menopausal symptoms, but scientists have found little evidence that they are helpful.* There is also no conclusive evidence that the herbs red clover, kava, or dong quai reduce hot flashes.
3. *Natural products used for menopausal symptoms can have side effects and can interact with other botanicals or supplements or with medications.* For example, rare cases of liver damage—some of them very serious—have been reported in people taking commercial black cohosh products. Also, concerns have been raised about the safety of DHEA because it is converted in the body to hormones, which are known to carry risks.
4. *Tell all your health care providers about any complementary health practices you use.* Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

40.46 6 Things to Know When Selecting a Complementary Health Practitioner

If you're looking for a complementary health practitioner to help treat a medical problem, it is important to be as careful and thorough in your search as you are when looking for conventional care.

Here are some tips to help you in your search:

1. *If you need names of practitioners in your area, first check with your doctor or other health care provider.* A nearby hospital or medical school, professional organizations, state regulatory agencies or licensing boards, or even your health insurance provider may be helpful. Unfortunately, the National Center for Complementary and Integrative Health (NCCIH) cannot refer you to practitioners.
2. *Find out as much as you can about any potential practitioner, including education, training, licensing, and certifications.* The credentials required for complementary health practitioners vary tremendously from state to state and from discipline to discipline (https://www.nccih.nih.gov/health/credentialing-licensing-and-education).

   Once you have found a possible practitioner, here are some tips about deciding whether he or she is right for you:
3. *Find out whether the practitioner is willing to work together with your conventional health care providers.* For safe, coordinated care, it's important for all of the professionals involved in your health to communicate and cooperate.
4. *Explain all of your health conditions to the practitioner, and find out about the practitioner's training and experience in working with people who have your conditions.* Choose a practitioner who understands how to work with people with your specific needs, even if general well-being is your goal. And, remember that health conditions can affect the safety of complementary approaches; for example, if you have glaucoma, some yoga poses may not be safe for you.
5. *Don't assume that your health insurance will cover the practitioner's services.* Contact your health insurance provider and ask. Insurance plans differ greatly in what complementary health approaches they cover, and even if they cover a particular approach, restrictions may apply.
6. *Tell all your health care providers about the complementary approaches you use and about all practitioners who are treating you.* Keeping your health care providers fully informed helps you to stay in control and effectively manage your health.

40.47 5 Tips: What You Should Know about Popular Herbs

Herbal or botanical supplements are widely marketed and readily available, often sold as dietary supplements. However, the scientific evidence available about the safety or effectiveness of herbal supplements varies quite a bit. In some cases, there is limited evidence to support a product's use, while in others, evidence has uncovered safety concerns, or is insufficient to draw clear conclusions. Because of this variability, you should carefully review the available information and talk to your health care providers before using a specific product.

Out of the more than 3 million visitors who came to NCCIH's website in 2012, the majority were looking for information on specific herbs and botanicals. The top five searched-for herbs of 2012 that brought people to our site are evening primrose oil (https://www.nccih.nih.gov/health/evening-primrose-oil), St. John's wort (https://www.nccih.nih.gov/health/st-johns-wort), fenugreek (https://www.nccih.nih.gov/health/fenugreek), echinacea (https://www.nccih.nih.gov/health/echinacea), and aloe vera (https://www.nccih.nih.gov/health/aloe-vera). Here are 5 tips about these popular herbal supplements:

1. *Evening Primrose Oil.* Although evening primrose oil has been used as a folk or traditional remedy for eczema, rheumatoid arthritis, and menopausal symptoms, there is not enough evidence to support the use of evening primrose oil for these conditions.
2. *St. John's Wort.* Study results on the effectiveness of St. John's wort for depression are conflicting. While there may be public interest in St. John's wort to treat depression, the US Food and Drug Administration has not approved its use as an over-the-counter or prescription medicine for depression. Importantly, St. John's wort is known to affect metabolism of a number of drugs, such as antiviral medicines, antidepressants, birth control pills, and certain anti-seizure medicines, and can cause serious side effects.
3. *Fenugreek.* Fenugreek is sometimes used as a folk or traditional remedy for diabetes and loss of appetite, and to stimulate milk production in breastfeeding women. However, there is not enough scientific evidence to support the use of fenugreek for these or any health condition. Given its historical use for inducing childbirth, women should use caution when taking fenugreek during pregnancy.
4. *Echinacea.* Overall, the scientific evidence on echinacea for colds is inconclusive. There is limited evidence from some studies that some echinacea preparations might reduce the length or severity of colds in adults, but results from four NCCIH-funded clinical trials of echinacea for colds all indicated that echinacea did not reduce the length or severity of cold

symptoms. Few side effects have been reported in clinical trials of echinacea, but some people may have allergic reactions.

5. *Aloe Vera. Topical use:* A few small studies suggest that topical aloe gel may help heal burns and abrasions. In general, topical use of aloe appears to be safe; one study, however, showed that aloe gel may inhibit healing of deep surgical wounds.

   Oral use: Aloe latex contains strong laxative compounds, and in 2002, the US Food and Drug Administration required that all over-the-counter aloe laxative products be removed from the US market or reformulated because the companies that manufactured them did not provide the necessary safety data.

40.48 8 Things to Know about Meditation for Health

Meditation is a mind and body practice that has a long history of use for increasing calmness and physical relaxation, improving psychological balance, coping with illness, and enhancing overall health and well-being. Many studies have been conducted to look at how meditation may be helpful for a variety of conditions, such as high blood pressure, certain psychological disorders, and pain. A number of studies also have helped researchers learn how meditation might work and how it affects the brain.

Here are 8 things to know about what the science says about meditation for health:

1. *For people who suffer from cancer symptoms and treatment side effects, mind-body therapies, such as meditation, have been shown to help relieve anxiety, stress, fatigue, and general mood and sleep disturbances, thus improving their quality of life.* Evidence-based clinical practice guidelines from the Society for Integrative Oncology recommend meditation, as well as other mind-body modalities, as part of a multidisciplinary approach to reduce anxiety, mood disturbance, chronic pain, and improve quality of life.
2. *There is some evidence that meditation may reduce blood pressure.* A literature review and scientific statement from the American Heart Association suggests that evidence supports the use of Transcendental Meditation as an adjunct or complementary therapy along with standard treatment to lower blood pressure.
3. *A growing body of evidence suggests that meditation-based programs may be helpful in reducing common menopausal symptoms.* A 2010 review of scientific literature found that yoga, tai chi, and meditation-based programs may be helpful in reducing common menopausal symptoms including the frequency and intensity of hot flashes, sleep and mood disturbances, stress, and muscle and joint pain.

4. *There is moderate evidence that meditation improves symptoms of anxiety.* A 2014 review of the literature found that mindfulness meditation programs had moderate evidence of improved anxiety, depression, and pain, and low evidence of improved stress/distress and mental health-related quality of life.
5. *Some studies suggest that mindfulness meditation helps people with irritable bowel syndrome (IBS), but there's not enough evidence to draw firm conclusions.* A 2013 review of the scientific literature concluded that mindfulness training improved IBS patients' pain and quality of life but not their depression or anxiety; however, the amount of improvement was small.
6. *Overall, there is not enough evidence to know whether mind-body practices are as effective as other treatments to help people quit smoking*. To date, there have only been a few studies on mindfulness-based therapies to aid in smoking cessation.
7. *There isn't enough evidence to support the use of meditation for attention deficit hyperactivity disorder (ADHD).* According to a 2010 review of the science, because of the small number of studies conducted on meditation for ADHD, no conclusions could be drawn about its effectiveness for this condition.
8. *Meditation is generally considered to be safe for healthy people*. However, people with physical limitations may not be able to participate in certain meditative practices involving movement.

40.49 6 Things to Know about Complementary Health Approaches for Quitting Smoking

Nearly 70 percent of adult smokers want to quit smoking, according to a national survey. Conventional quit-smoking treatments, including counseling and medication, can increase the chances that a smoker will kick the habit successfully. For more information on quitting smoking, visit smokefree.gov (https://smokefree.gov/), the National Cancer Institute's quit-smoking resource.

Some people also try complementary health approaches to help them quit smoking. Here are 6 things you should know about what the science says about several complementary health approaches for quitting smoking:

1. *Current evidence suggests that some mind and body practices may help people quit smoking.* Studies have found that mindfulness meditation-based therapies (https://www.nccih.nih.gov/health/meditation-in-depth), yoga (https://www.nccih.nih.gov/health/yoga-what-you-need-to-know), and relaxation techniques (https://www.nccih.nih.gov/health/relaxation-techniques-for-health) such as guided imagery and progressive relaxation may help people quit smoking or reduce their cravings for cigarettes.

2. *Firm conclusions cannot be drawn about the effectiveness of hypnotherapy and acupuncture to help people quit smoking.* A 2019 systematic review of the scientific literature on hypnotherapy (https://www.nccih.nih.gov/health/hypnosis) concluded there is not enough evidence to determine whether hypnotherapy is more effective for smoking cessation than other forms of support or unassisted quitting. A 2014 systematic review concluded that acupuncture (https://www.nccih.nih.gov/health/acupuncture-in-depth) might help people stop smoking for short periods of time, but there's no consistent evidence that it helps people quit permanently.
3. *There is no current evidence that the dietary supplements S-adenosyl-L-methionine (SAMe)* (https://www.nccih.nih.gov/health/adenosyllmethionine-same-in-depth), *lobeline (from the herb Lobelia inflata), and St. John's wort* (https://www.nccih.nih.gov/health/st-johns-wort) *help people quit smoking***.** A few studies have been conducted on these dietary supplements, but none has been shown to be effective.
4. *A natural product called cytisine may become an option for smoking cessation treatment in the future.* Several studies have indicated that cytisine, which is used as a smoking cessation aid in some central and eastern European countries, may help people quit smoking. The US Food and Drug Administration has authorized further studies on cytisine, but the product has not yet been approved in the United States.
5. *The mind and body practices discussed here are generally considered safe for healthy people when they're performed appropriately.* If you have any health problems, talk with both your health care provider and the complementary health practitioner/instructor before starting to use a mind and body practice.
6. *If you are considering a dietary supplement, remember that "natural" does not necessarily mean "safe."* Some supplements have side effects, and some may interact with drugs or other supplements to produce adverse effects. In particular, St. John's wort has been shown to interact with many drugs.

40.50 5 Tips: What You Should Know about High Blood Cholesterol

Approximately 13 percent of US adults has high total cholesterol. Lowering cholesterol levels can slow down, reduce, or even stop plaque from building up in the walls of arteries and may decrease the chance of having a heart attack. Mainstays in treating high cholesterol include diet, weight loss, physical activity, and when necessary, drug treatment.

National survey data show that high blood cholesterol is one of the top 10 conditions for which people use complementary health practices such as dietary supplements.

Here are 5 tips about high blood cholesterol:

1. *Work with your health care provider.* Ask your health care provider about proven steps you can take to lower your blood cholesterol levels. And be sure to talk with your provider about any complementary health practice you are considering, including dietary supplements (https://www.nccih.nih.gov/health/supplements). This will help ensure safe and coordinated care.
2. *Change your diet.* (http://www.nhlbi.nih.gov/health/public/heart/chol/chol_tlc.pdf)**.** Saturated fat raises your LDL cholesterol level (often called "bad cholesterol," the main source of cholesterol buildup and blockage in the arteries) more than anything else in your diet. Diets with too much saturated fat and trans fat are the main cause for high blood cholesterol.
3. *Manage your weight.* Losing extra pounds may help lower your LDL and triglycerides (a type of fat found in the blood and in food), while raising your HDL (often called "good cholesterol," helps keep cholesterol from building up in the arteries).
4. *Get moving.* Regular physical activity (such as brisk walking 30 minutes each day) can raise HDL and lower triglycerides, and can help you lose weight and, in that way, help lower your LDL. Aim for a total of at least 150 minutes over the course of a week.
5. *Find out what the science says about dietary supplements marketed for improving cholesterol.* The dietary supplements red yeast rice, flaxseed, and garlic, are among the many supplements that have been studied for lowering cholesterol levels. Unfortunately, there isn't conclusive evidence that any of these supplements are effective in reducing cholesterol levels.

    - *Red yeast rice.* Some red yeast rice products (https://www.nccih.nih.gov/health/redyeastrice) contain substances called monacolins, which are produced by the yeast. Monacolin K is chemically identical to the active ingredient in the cholesterol-lowering drug lovastatin, and can cause the same types of side effects and drug interactions as lovastatin. Other red yeast rice products contain little or no monacolin K, and it is not known whether these products have any effect on cholesterol levels. Unfortunately, there is no way to know how much monacolin K is present in most red yeast rice products. Further, the US Food and Drug Administration has determined that red yeast rice products that contain more than trace amounts of monacolin K cannot be sold legally as dietary supplements.

- *Flaxseed.* Studies of flaxseed (https://www.nccih.nih.gov/health/flaxseed/ataglance.htm) preparations to lower cholesterol levels report mixed results. A 2009 review of the scientific research of flaxseed for lowering cholesterol found modest improvements in cholesterol, seen more often in postmenopausal women and in people with high initial cholesterol concentrations.
- *Garlic.* Some evidence indicates that taking garlic (https://www.nccih.nih.gov/health/garlic/ataglance.htm) supplements can slightly lower blood cholesterol levels; however, an NCCIH-funded study on the safety and effectiveness of three garlic preparations (fresh garlic, dried powdered garlic tablets, and aged garlic extract tablets) for lowering blood cholesterol levels found no effect. Although garlic supplements appear to be safe for most adults, they can thin the blood in a manner similar to aspirin, so use caution if you are planning to have surgery or dental work. Garlic supplements have also been found to interfere with the effectiveness of saquinavir, a drug used to treat HIV infection.

40.51 8 Things to Know about Depression and Complementary Health Approaches

Many people with depression turn to complementary health approaches in addition to or in place of conventional treatment. Research suggests that some approaches may be modestly helpful in reducing depression symptoms. For other approaches, benefits are uncertain or there are safety concerns.

Here are 8 things you should know about complementary health approaches for depression:

1. Depression can be a serious illness. Don't use a complementary health approach to replace conventional care or to postpone seeing a health care provider about symptoms of depression.
2. Some evidence suggests *acupuncture* may modestly reduce depression symptoms.
3. *Music therapy* may provide short-term benefits for people with depression.
4. Studies in adults, adolescents, and children have suggested that yoga (https://www.nccih.nih.gov/health/yoga-what-you-need-to-know) may be helpful in reducing depressive symptoms.
5. It's uncertain whether omega-3 fatty acid supplements (https://www.nccih.nih.gov/health/omega3-supplements-in-depth) are helpful for symptoms of depression.
6. Some research on the herb St. John's wort *(Hypericum perforatum)* (https://www.nccih.nih.gov/health/st-johns-wort) has suggested that it may be helpful for depression symptoms, but not all studies agree. There's an

important concern about the safety of St. John's wort: it can interact in dangerous, sometimes life-threatening ways with a variety of medicines.

7. Current scientific evidence does not support the use of other dietary supplements, including S-adenosyl-L-methionine (SAMe) (https://www.nccih.nih.gov/health/sadenosyllmethionine-same-in-depth) or **inositol,** for depression.
8. Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

40.52 10 Things to Know about Dietary Supplements for Children and Teens

According to a 2012 national survey, nearly 12 percent of children (about one in nine) in the United States use a complementary health approach, such as dietary or herbal supplements (https://www.nccih.nih.gov/health/supplements). Some teens use products advertised as dietary supplements for weight loss or bodybuilding. Increasingly, products sold as dietary supplements, particularly for weight loss and bodybuilding, contain ingredients that could be harmful, including prescription drug ingredients and controlled substances. In addition, many dietary supplements haven't been tested in children. Because children's bodies aren't fully developed, the side effects of these products on children and adults may differ. For more information, see the National Center for Complementary and Integrative Health's fact sheet *Using Dietary Supplements Wisely* (https://nccih.nih.gov/health/supplements/wiseuse.htm).

Here are 10 things to know about dietary supplements for children and teens.

1. Although many dietary supplements come from natural sources, "*natural*" *does not necessarily mean* "*safe*."
2. Federal regulations for dietary supplements are less strict than those for prescription and over-the-counter drugs.
3. Dietary and herbal supplements may be poor quality and contain contaminants, including drugs, chemicals, or metals. Studies of dietary supplements have found significant differences between what's on the label and what's in the bottle of some supplements.
4. Dietary supplements may interact with other products or medications or have unwanted side effects on their own.
5. About 4,600 children go to the emergency room every year because of dietary supplements. Most took a vitamin or mineral when unsupervised. Child-resistant packaging isn't required for dietary supplements.
6. Certain homeopathic products (called "nosodes" or "homeopathic immunizations") are promoted as substitutes for conventional immunizations, but they haven't been shown to protect children against diseases. Follow the

Centers for Disease Control and Prevention's vaccination recommendations (http://www.cdc.gov/vaccines/schedules/easy-to-read/index.html) to safeguard your children against vaccine-preventable diseases. Vaccinating children helps protect our community's and our children's health.

Here's safety information for some common supplements:

- St. John's wort (https://www.nccih.nih.gov/health/stjohnswort) interacts with many medications, including antidepressants, birth control pills, and seizure and cancer treatments.
- Melatonin (https://www.nccih.nih.gov/health/melatonin-what-you-need-to-know), a hormone used as a sleep aid, appears safe for short-term use but we don't know about its long-term effects.
- Giving probiotics (https://www.nccih.nih.gov/health/probiotics) to children doesn't appear to be risky, but we lack conclusive evidence, particularly for long-term use. Critically ill patients shouldn't use probiotics.
- Omega-3 supplements (https://www.nccih.nih.gov/health/omega3-supplements-in-depth) may cause minor stomach problems, such as belching, indigestion, or diarrhea.
- The American Academy of Pediatrics doesn't recommend multivitamins (https://www.nccih.nih.gov/health/vitamins) for healthy children and teens who eat a varied diet (http://www.healthychildren.org/English/ages-stages/preschool/nutrition-fitness/Pages/A-Vitamin-a-Day.aspx). It's best if they can get their vitamins from foods.

Hidden ingredients are increasingly becoming a problem in products promoted for bodybuilding. Some bodybuilding products marketed as dietary supplements contain steroids or steroid-like substances. These could lead to serious liver injury, stroke, kidney failure, or other serious conditions.

Dietary supplements marketed for rapid weight loss, such as acai and hoodia, don't help keep weight off for the long term and can have side effects. Some supplements have a lot of caffeine or herbs such as guarana that contain caffeine, which can cause life threatening changes in your heart rhythm. The FDA has also found weight loss products tainted with potentially dangerous prescription drugs.

Ask your child's health care provider about the effectiveness and possible risks of any complementary health approaches you are considering or already using for your child. Also, remind your teenagers to talk to their health care providers about complementary health approaches they may use or are considering.

40.53 4 Things To Know About Menopausal Symptoms and Complementary Health Practices

Menopause is the permanent end of a woman's menstrual periods. Menopause (https://www.nccih.nih.gov/health/menopause) can occur naturally or be caused by surgery, chemotherapy, or radiation. During the years around menopause,

some women have hot flashes, night sweats, difficulty sleeping, or other bothersome symptoms. Natural products or mind and body practices are sometimes used in an effort to relieve menopausal symptoms such as hot flashes and night sweats.

Here are 4 things to know if you are considering a complementary health approach for managing menopausal symptoms:

1. *Mind and body practices such as hypnosis, mindfulness meditation, and tai chi may help improve some menopausal symptoms.* Researchers looked at mind and body therapies for menopausal symptoms and found that tai chi and meditation-based programs may be helpful in reducing common menopausal symptoms including the frequency and intensity of hot flashes, sleep and mood disturbances, stress, and muscle and joint pain. There is also some evidence that hypnotherapy may help women manage hot flashes.
2. *Many natural products, such as black cohosh, soy isoflavone supplements, and DHEA, have been studied for their effects on menopausal symptoms, but scientists have found little evidence that they are helpful.* There is also no conclusive evidence that the herbs red clover, kava, or dong quai reduce hot flashes.
3. *Natural products used for menopausal symptoms can have side effects and can interact with other botanicals or supplements or with medications.* For example, rare cases of liver damage—some of them very serious—have been reported in people taking commercial black cohosh products. Also, concerns have been raised about the safety of DHEA because it is converted in the body to hormones, which are known to carry risks.
4. *Tell all your health care providers about any complementary health practices you use.* Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

40.54 Ayurvedic Medicine: In Depth

Is Ayurvedic Medicine Effective?

A few studies suggest that Ayurvedic preparations may reduce pain and increase function in people with osteoarthritis and help manage symptoms in people with type 2 diabetes, but most of these trials are small or not well-designed. There is little scientific evidence on Ayurveda's value for other health issues.

How much do we know about Ayurvedic medicine?

Although Ayurvedic medicine and its components have been described in many scholarly articles, only a small number of clinical trials using these approaches have been published in Western medical journals. About 240,000 American adults use Ayurvedic medicine.

What Is Ayurvedic Medicine?

The ancient Indian medical system, also known as Ayurveda, is based on ancient writings that rely on a "natural" and holistic approach to physical and mental health. Ayurvedic medicine is one of the world's oldest medical systems and remains one of India's traditional health care systems. Ayurvedic treatment combines products (mainly derived from plants, but may also include animal, metal, and mineral), diet, exercise, and lifestyle.

What the Science Says About the Effectiveness of Ayurvedic Medicine

Few well-designed clinical trials and systematic research reviews suggest that Ayurvedic approaches are effective.

- Results from a 2013 clinical trial compared two Ayurvedic formulations of plant extracts against the natural product glucosamine sulfate and the drug celecoxib in 440 people with knee osteoarthritis. All four products provided similar reductions in pain and improvements in function.
- A preliminary and small NCCIH-funded 2011 pilot study with 43 people found that conventional and Ayurvedic treatments for rheumatoid arthritis were similarly effective. The conventional drug tested was methotrexate and the Ayurvedic treatment included 40 herbal compounds.
- Outcomes from a small short-term clinical trial with 89 men and women suggested that a formulation of five Ayurvedic herbs may help people with type 2 diabetes. However, other researchers said inadequate study designs haven't allowed researchers to develop firm conclusions about Ayurveda for diabetes.
- Turmeric, an herb often used in Ayurvedic preparations, may help with ulcerative colitis, but the two studies reporting this were small—one, published in 2005, included 10 people while the other, published in 2006, had 89.

What the Science Says About the Safety of Ayurvedic Medicine

- Some Ayurvedic preparations include metals, minerals, or gems. The US Food and Drug Administration warns that the presence of metals in some Ayurvedic products makes them potentially harmful.
- A 2015 published survey of people who use Ayurvedic preparations showed that 40 percent had elevated blood levels of lead and some had elevated blood levels of mercury. About one in four of the supplements tested had high levels of lead and almost half of them had high levels of mercury.
- A 2015 case report published in the Center for Disease Control's *Morbidity and Mortality Weekly Report* linked elevated blood lead levels in a 64-year-old woman with Ayurvedic preparations purchased on the Internet.
- Some Ayurvedic preparations may contain lead, mercury, or arsenic in amounts that can be toxic.

NCCIH-Funded Research

NCCIH is funding research that:

- Builds on earlier investigations in breast cancer survivors that found a positive effect of integrated Ayurvedic medicine on improved quality of life; new research will evaluate ways to make this intervention easier to incorporate into peoples' lives. The proposed Ayurvedic intervention includes diet, lifestyle, yoga, and pressure point treatment.
- Studies the mechanism by which an extract from *Butea monosperma* (BME) flowers may protect against joint destruction from osteoarthritis (BME is widely used in Ayurveda for arthritis and other inflammatory diseases in India).

More To Consider

- Don't use Ayurvedic medicine to postpone seeing a conventional health care provider about a medical problem.
- If you have a health condition, talk with your conventional health care provider before using Ayurvedic products.
- There is no significant regulation of Ayurvedic practice or education in the United States, and no state requires a practitioner to have a license. For more information on credentialing complementary health practitioners, see the NCCIH fact sheet *Credentialing, Licensing, and Education* (https://www.nccih.nih.gov/health/credentialing-licensing-and-education).
- If you're pregnant or nursing, be sure to consult your (or your child's) health care provider as some Ayurvedic products may contain products that could be harmful.
- Tell all your health care providers about any complementary or integrative health approaches you use. Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

40.55 Terms Related to Complementary and Integrative Health

Note: The following terms and definitions are excerpted from Clarke TC, Black LI, Stussman BJ, Barnes PM, Nahin RL. Trends in the use of complementary health approaches among adults: United States, 2002–2012 (http://www.cdc.gov/nchs/data/nhsr/nhsr079.pdf). National health statistics reports; no 79. Hyattsville, MD: National Center for Health Statistics. 2015. The National Center for Health Statistics is part of the Centers for Disease Control and Prevention. This list is intended to provide a brief introduction to common complementary and integrative health terminology and does not in any way reflect an endorsement of these practices by the National Center for Complementary and Integrative Health (NCCIH).

Acupuncture

A family of procedures involving stimulation of anatomical points on the body by a variety of techniques. American practices of acupuncture incorporate medical traditions from China, Japan, Korea, and other countries. The acupuncture technique that has been most scientifically studied involves penetrating the skin with thin, solid, metallic needles that are manipulated by the hands or by electrical stimulation.

Ayurveda

A medical system that originated in India several thousand years ago. Ayurveda is based on theories of health and illness, and on ways to prevent, manage, or treat health problems. Ayurveda aims to integrate and balance the body, mind, and spirit (thus, some view it as "holistic"). This balance is believed to lead to contentment and health and to help prevent illness. A chief aim of Ayurvedic practices is to cleanse the body of substances that can cause disease, and this is believed to help reestablish harmony and balance.

Biofeedback

A technique that uses simple electronic devices to teach clients how to consciously regulate bodily functions, such as breathing, heart rate, and blood pressure, to improve overall health. Biofeedback is used to reduce stress, eliminate headaches, recondition injured muscles, control asthma attacks, and relieve pain.

Chiropractic manipulation

A form of health care that focuses on the relationship between the body's structure, primarily the spine, and its function.

Deep breathing exercises

An active process that involves conscious control over breathing in and out. This may involve controlling the way in which air is drawn in (for example, through the mouth or nostrils), the rate (for example, quickly or over a length of time), the depth (for example, shallow or deep), and the control of other body parts (for example, relaxation of the stomach).

Energy healing therapy

A technique that involves channeling healing energy through the hands of a practitioner into the client's body to restore a normal energy balance and, therefore, health. Energy healing therapy has been used to treat a wide variety of ailments and health problems, and it is often used with other alternative and conventional medical treatments.

Folk medicine

Systems of healing (such as Curanderismo and Native American healing) that have persisted since the beginning of human culture and flourished long before the development of conventional medicine. Folk healers usually participate in a

training regimen of observation and imitation, with healing often considered a gift passed down through several family generations. Folk healers may employ a range of remedies, including prayer, healing touch or laying on of hands, charms, herbal teas or tinctures, and magic rituals, among other techniques. Folk healers are found in all cultures and operate under a variety of names and labels.

Guided imagery

A practice used for healing or health maintenance that involves a series of relaxation techniques followed by the visualization of detailed images, usually calm and peaceful in nature. If used for treatment, persons will visualize their bodies free of the specific problem or condition. Sessions are typically 20 to 30 minutes in length and may be practiced several times a week.

Homeopathic treatment

A system of medical practices based on the theory that any substance that can produce symptoms of disease or illness in a healthy person can cure those symptoms in a sick person.

Hypnosis

An altered state of consciousness characterized by increased responsiveness to suggestion. This hypnotic state is attained by first relaxing the body, then shifting attention toward a narrow range of objects or ideas as suggested by the hypnotist or hypnotherapist. The procedure is used to effect positive changes and to treat numerous health conditions including ulcers, chronic pain, respiratory ailments, stress, and headaches.

Meditation

A group of techniques, most of which started in Eastern religious or spiritual traditions. In meditation, individuals learn to focus their attention and suspend the stream of thoughts that normally occupy the mind. This practice is believed to result in a state of greater physical relaxation, mental calmness, and psychological balance. Practicing meditation can change how a person relates to the flow of emotions and thoughts in the mind.

Mindfulness meditation

A type of meditation based on the concept of being mindful, or having increased awareness, of the present. It uses breathing methods, guided imagery, and other practices to relax the body and mind and help reduce stress. It is also known as mindfulness relaxation and mindfulness-based stress reduction.

Naturopathy

An alternative medical approach based on the belief that there is a healing power in the body that establishes, maintains, and restores health. Practitioners work with the patient with a goal of supporting this power through treatments such

as nutrition and lifestyle counseling, dietary supplements, medicinal plants, exercise, homeopathy, and treatments from traditional Chinese medicine.

Nonvitamin, nonmineral, dietary supplements

Herbs or other nonvitamin supplements such as pills, capsules, tablets, or liquids that have been labeled as dietary supplements. This category did not include vitamin or mineral supplements, homeopathic treatments, or drinking herbal or green teas.

Osteopathic manipulation

A full-body system of hands-on techniques to alleviate pain, restore function, and promote health and wellbeing.

Progressive relaxation

A technique used to relieve tension and stress by systematically tensing and relaxing successive muscle groups.

Qi gong

An ancient Chinese discipline combining the use of gentle physical movements, mental focus, and deep breathing directed toward specific parts of the body. Performed in repetitions, the exercises are normally performed two times or more a week for 30 minutes at a time.

Spiritual meditation

Meditation techniques performed according to the practices of one of the major religions or within a spiritual tradition. The techniques used may be the same as in other types of meditation (for example, Transcendental Meditation), but the focus is on spirituality (such as repeating a spiritual, meditative phrase).

Tai chi

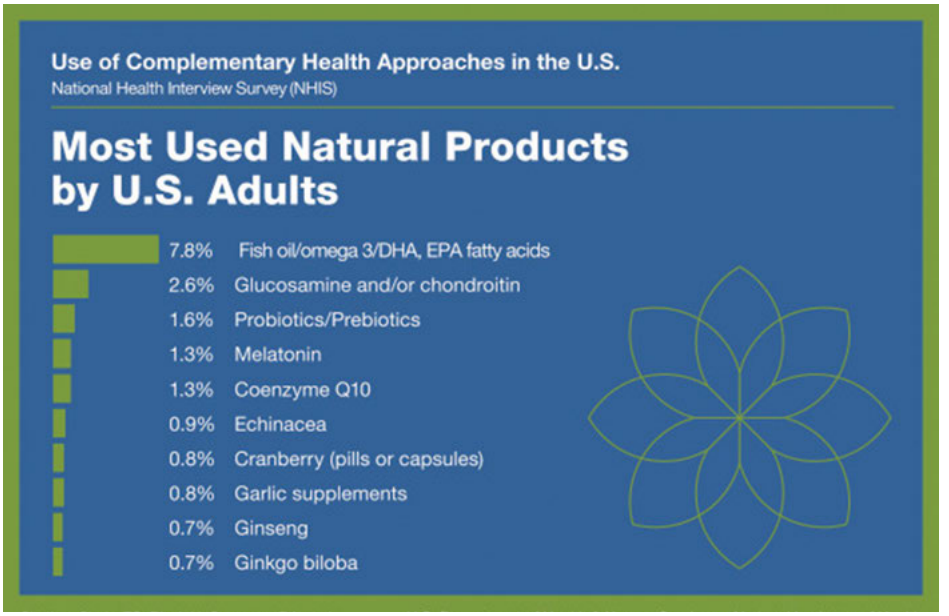
A mind-body practice that originated in China as a martial art. Individuals doing tai chi move their bodies slowly and gently, while breathing deeply and meditating (tai chi is sometimes called moving meditation). Many practitioners believe that tai chi helps the flow throughout the body of a proposed vital energy called "qi." Individuals practicing tai chi move their bodies in a slow, relaxed, and graceful series of movements. One can practice alone or in a group. The movements make up what are called forms (or routines).

Traditional healer

Someone who employs any one of a number of ancient medical practices that are based on indigenous theories, beliefs, and experiences handed down from generation to generation, often orally. The methods employed by each type of traditional healer have evolved to reflect the different philosophical backgrounds and cultural origins of the healer.

Yoga

A combination of breathing exercises, physical postures, and meditation used to calm the nervous system and balance the body, mind, and spirit.



Abbreviations

5-HTP:	5-hydroxytryptophan
ADHD:	attention-deficit hyperactivity disorder
AHRQ:	Agency for Healthcare Research and Quality
AMD:	age-related macular degeneration
AREDS/AREDS2:	age-related eye disease studies
ASD:	autism spectrum disorder
ASU:	avocado/soybean unsaponifiables
BPH:	benign prostatic hyperplasia
CBT-SAD:	cognitive behavioral therapy for seasonal affective disorder
DHA:	docosahexaenoic acid
DMSO:	dimethyl sulfoxide
EMS:	eosinophilia-myalgia syndrome
EPA:	eicosapentaenoic acid/Environmental Protection Agency
FDA:	US Food and Drug Administration
HPV:	human papillomavirus
IBS:	irritable bowel syndrome
MS:	multiple sclerosis
MSM:	methylsulfonylmethane

NCCIH:	National Center for Complementary and Integrative Health
NHLBI:	National Heart, Lung, and Blood Institute
NSAIDS:	nonsteroidal anti-inflammatory drugs
OA:	osteoarthritis
OLE:	oil of lemon eucalyptus
PTSD:	post-traumatic stress disorder
SAD:	seasonal affective disorder
SAMe:	S-Adenosyl-L-methionine

Chapter 41

Skin Conditions and Complementary Health Approaches: What the Science Says[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: acne, atopic dermatitis, azelaic acid, barberry extract, bee venom, borage oil, Chinese herbal medicine, climatotherapy, complementary health, *Curcuma longa,* dexamethasone, dietary supplements, docosahexaenoic acid (DHA), fish oil, gugulipid, hempseed oil, herbal medicine, herbal supplements, impetigo, *Indigo naturalis,* inflammatory skin diseases, *Lactobacillus casei, Lactobacillus rhamnosus,* light therapy, *Mahonia aquifolium,* nodulocystic acne, oral zinc supplementation, pediatric acne vulgaris, primrose oil, probiotics, psoriasis, Psoriasis Area and Severity Index (PASI), rosacea, sea buckhorn oil, sunflower oil, tea tree oil, visible light phototherapy

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at the NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH.

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Research has shown that people with skin conditions often turn to complementary health approaches, particularly vitamin, mineral, and herbal supplements. Despite interest in complementary approaches, there have been only a few studies on complementary health approaches for skin conditions, and those that have been conducted have often had methodological problems. This chapter provides a summary of the current available evidence about complementary health approaches for skin conditions, including atopic dermatitis, psoriasis, acne, impetigo, and rosacea.

41.1 Atopic Dermatitis

According to the American Academy of Dermatology's clinical practice guidelines [1], there is inconsistent to no evidence to recommend the use of fish oils, evening primrose oil, borage oil, multivitamin supplements, zinc, vitamin D, vitamin E, and vitamins B12 and B6 for the treatment of atopic dermatitis. Further, the guidelines state that the use of probiotics/prebiotics for the treatment of patients with established atopic dermatitis is not recommended because of inconsistent evidence.

41.1.1 What Does the Research Show?

- **Dietary Supplements (Oral).** A 2012 review [2] of 11 randomized controlled trials of dietary supplements (e.g., fish oil, vitamin D and vitamin E, vitamin B6, sea buckhorn oil, hempseed oil, sunflower oil, docosahexaenoic acid (DHA), selenium, and zinc sulfate) found no convincing evidence of their benefit for atopic eczema. The American Academy of Dermatology's clinical practice guidelines for the treatment of atopic dermatitis states that "there is inconsistent to no evidence to recommend the use of fish oils, evening primrose oil, borage oil, multivitamin supplements, zinc, vitamin D, vitamin E, and vitamins B12 and B6 for the treatment of atopic dermatitis."
    - **Vitamin D (Oral).** A 2019 systematic review and meta-analysis [3] of 16 studies found that vitamin D supplementation showed clinically relevant improvements in participants with atopic dermatitis at a weighted average dose of 1500–1600 IU for up to 3 months. The reviewers noted that further research is required to establish the efficacy of vitamin D2 versus D3 in reducing AD severity, as well as the effects of vitamin D supplementation on infection rates, including superinfections and topical steroid usage.
- **Probiotics (Oral).** The American Academy of Dermatology's clinical practice guidelines [1] for treatment of atopic dermatitis state that "the use of probiotics/prebiotics for the treatment of patients with established atopic dermatitis is not recommended because of inconsistent evidence (Level of Evidence II; Strength of Recommendation B)."

- There are conflicting data on the efficacy of probiotics for atopic dermatitis in children; overall, evidence suggests that probiotics may be effective for some, but not all, children with atopic dermatitis.
- A 2021 multicenter, randomized, double blind, placebo-controlled study [4] evaluated the effectiveness of *Lactobacillus rhamnosus* and *Lactobacillus casei* strains in children under 2 years of age with atopic dermatitis and a cow's milk protein allergy. After the 3-month intervention, both the probiotic and placebo groups showed a significant decrease in extent and severity of eczema scores, which was maintained 9 months later. The percentage of children who showed improvement was significantly higher in the probiotic than in the placebo group.
- A 2013 systematic review [5] of 21 randomized controlled trials involving 6,859 participants, which included infants or individuals who were either pregnant or breastfeeding, investigated whether nutrient supplementation with probiotics, prebiotics, formula, or fatty acids prevents the development of atopic dermatitis or reduces the severity of the condition in newborns to children under 3 years of age. Data showed that certain types of nutrient supplementation may be an effective method in preventing atopic dermatitis or decreasing its severity. The best evidence, the reviewers found, lies with probiotics supplementation in infants and in pregnant or breastfeeding individuals in preventing the development and reducing the severity of atopic dermatitis.

- **Probiotics (Topical).** A 2021 systematic review [6] of seven studies concluded that preliminary data suggest emollients containing probiotics and bacteria-derived preparations are safe for use in atopic dermatitis; however, well-designed clinical trials are needed to establish the efficacy of topical probiotics on disease severity.
- **Primrose Oil and Borage Oil (Oral)**. A 2013 review [7] of 27 randomized controlled trials involving a total of 1,596 participants found that evening primrose oil and borage oil taken orally had no clinical benefit for the treatment of atopic eczema.
- **Chinese Herbal Medicine (Oral and Topical)**. Another 2013 Cochrane review [7] of 28 randomized controlled trials involving a total of 2,306 participants found no conclusive evidence that oral or topical Chinese herbal medicine could reduce the severity of atopic eczema in children or adults. A 2016 review [8] of 70 studies found some evidence that herbal preparations can have real effects in the treatment of atopic dermatitis; however, the reviewers concluded that the complexity of these preparations and their potential risks make this area inscrutable for most practitioners. They also noted that while there is real evidence of positive effect, there are too many unanswered questions to warrant routine clinical use of such

herbs, and significant further research is needed before widespread clinical adoption can occur.

41.1.2 Safety

- Patients considering the use of Chinese herbal medicine, especially for children, should use caution as they can be potentially hazardous. A 2013 review [9] noted that these medications are easily accessed and not monitored by the U.S. Food and Drug Administration, and that some topical Chinese herbal medicines have been found to include high concentrations of dexamethasone.

41.2 Psoriasis

There is some evidence that fish oil, Dead Sea climatotherapy, and the topical herbs *Mahonia aquifolium* and *indigo naturalis* may be beneficial for the treatment of psoriasis.

41.2.1 What Does the Research Show?

- **Traditional Chinese Medicine (Oral)**. There is some evidence [10] that has shown that the combination of traditional Chinese medicine taken orally with conventional treatments for psoriasis is more efficacious than conventional treatment alone.
- **Dietary Supplements (Oral)**. A 2015 review [11] noted that there has been consistent evidence supporting the efficacy of fish oil supplementation in patients with psoriasis; however, a 2019 meta-analysis [12] of 13 randomized controlled trials involving a total of 625 participants found that fish oil supplementation did not significantly reduce the severity of psoriasis when assessed by Psoriasis Area and Severity Index score. There is conflicting evidence for vitamin D, B12, and selenium supplementation.
- **Herbal Medicine (Topical)**. A 2018 review [13] of eight studies found that *Mahonia aquifolium* leads to a statistically significant improvement of symptoms in psoriasis and atopic dermatitis with minimal side effects. A 2017 randomized, double-blind, placebo-controlled clinical study [14] of 24 participants with moderate plaque psoriasis found that compared with placebo, *indigo naturalis*–treated patients had significant improvement in Psoriasis Area and Severity Index (PASI) scores from baseline. There is a smaller amount of evidence for aloe vera, neem, and extracts of sweet whey.
- **Vitamin D (Topical)**. A 2013 review [7] of 177 studies involving a total of 34,808 people found that topical vitamin D products were superior to placebo, and had similar effects to topical corticosteroids when applied to

the body. However, corticosteroids were superior to vitamin D for scalp psoriasis. Treatment that combined topical vitamin D with a corticosteroid was more effective than topical vitamin D alone and more effective than the topical corticosteroid alone.

- **Climatotherapy.** There is evidence from controlled trials that Dead Sea climatotherapy can improve psoriasis and induce lasting remissions; however, research on other locations of climatotherapy have provided little evidence.
- **Light Therapy.** A 2019 review [15] concluded that based on the efficacy and safety, NB-UVB is the gold standard for treating psoriasis and atopic dermatitis, and the UVB excimer laser and excimer lamp might be the best option for clearing localized therapy-resistant lesions. The reviewers noted that home UV phototherapy systems might improve treatment adherence. However, they also noted that vascular lasers, intense pulse lights, and low-level light treatment cannot currently be recommended for the treatment of inflammatory skin diseases because of the lack of well-controlled studies. Findings from a 2015 randomized controlled trial [16] of 21 patients with plaque psoriasis suggest that moderate to severe plaque psoriasis should show a therapeutic response to orally administered *Curcuma longa* extract if activated with visible light phototherapy. A 2015 randomized controlled trial [16] of 47 patients with mild psoriasis vulgaris evaluated the safety and efficacy of long-term UV-free blue light treatment and found that participants receiving blue light treatment had a significant improvement compared to the control.

41.2.2 Safety

- Some Chinese herbal medicines have been shown to be contaminated with heavy metals or corticosteroids. Other safety concerns include systemic toxicity or contact dermatitis from herbal supplements.
- Ultra-violet light exposure increases the risk of melanoma and non-melanoma skin cancers, so the benefits of climatotherapy should be carefully weighed against the risks for each patient.
- Vitamin D products may cause “local adverse events,” such as skin irritation and burning.

41.3 Acne

According to the American Academy of Dermatology’s clinical practice guidelines for the treatment of acne, there are currently very limited data regarding the safety and efficacy of herbal and other complementary therapies to recommend their use.

41.3.1 What Does the Research Show?

- **Tea Tree Oil, Bee Venom (Topical).** A 2015 Cochrane review [17] of 35 randomized controlled trials involving 3,227 participants concluded that there is some low-quality evidence from single trials that topical tea tree oil and bee venom may reduce total skin lesions in acne, but there is a lack of evidence from the review to support the use of other complementary health approaches, such as herbal medicine, acupuncture, or wet-cupping therapy. A 2019 review [15] of four studies involving pediatric patients with acne concluded that overall, the quality of evidence to support tea tree oil in pediatric acne vulgaris is low.
- **Barberry Extract (Oral).** Other herbal agents, such as oral barberry extract, showed some beneficial effects in a 2012 randomized controlled trial [18] of 49 adolescents with moderate-to-severe acne.
- **Zinc (Oral).** There have been several randomized controlled trials that have examined oral zinc supplementation in the treatment of acne in teenagers and young adults; some have shown efficacy over placebo, while others did not.
- **Gugulipid (Oral).** In one study [19], gugulipid, an extract of gum guggul, given twice daily (25 mg) was compared to oral tetracycline 500 mg twice daily for treatment of nodulocystic acne in patients aged 16 to 25 years. Both interventions resulted in improvement in acne lesions, and the percentage reduction in inflammatory lesions was similar without a statistically significant difference.
- **Probiotics (Oral and Topical).** A 2020 review [20] found that probiotics as adjunct therapy (topical or oral) can play an effective role in managing acne by directly preventing the growth of opportunistic bacteria or by controlling inflammation. The reviewers recommended that interventional studies be conducted using large samples and long follow-ups to demonstrate the effectiveness of these beneficial bacteria and pinpoint their other potential advantages and disadvantages.

41.3.2 Safety

- In a 2012 study [21], oral aqueous extract of barberry was well tolerated, and no notable complications or side effects were reported.
- Tea tree oil contains varying amounts of 1,8–cineole, a skin irritant. Products with high amounts of this compound may cause skin irritation or contact dermatitis in some individuals. Oxidized tea tree oil may trigger allergies more than fresh tea tree oil.
- Tea tree oil should not be swallowed. Poisonings, mainly in children, have caused drowsiness, disorientation, rash, and ataxia. Topical use of diluted tea tree oil is generally considered safe for most adults. Pruritus, burning, stinging, scaling, itch, redness, and dryness have been reported.

- There is a potential for adverse effects from herbal medicines. Patients considering the use of Chinese herbal medicine, especially for children, should use caution as they can be potentially hazardous. A 2013 review [9] noted that these medications are easily accessed and not monitored by the U.S. Food and Drug Administration, and that some topical Chinese herbal medicines have been found to include high concentrations of dexamethasone.

41.4 Impetigo

There is insufficient evidence to either recommend or dismiss herbal treatments for impetigo, including tea tree oil, garlic, coconut oils, tea effusions, and Manuka honey.

41.4.1 What Does the Research Show?

- **Herbal Medicine (Oral and Topical).** A 2003 review [22] of seven randomized and non-randomized studies examining both oral and topical herbal medicines for the treatment of bacterial infections, including impetigo, found some positive results reported for a topical ointment containing tea leaf extract. However, the reviewers concluded that the clinical efficacy of none of the herbal medicines has so far been demonstrated.

41.4.2 Safety

- There is a lack of safety data on herbal medicines for the treatment of impetigo.
- Patients should be encouraged to maintain proper wound care and hand washing and avoid contact with others as the infection can spread.

41.5 Rosacea

Although some natural products have shown promise for improving symptoms of rosacea, there is insufficient evidence to support the use of many of these products for rosacea.

41.5.1 What Does the Research Show?

- **Plant Extracts (Oral and Topical).** A 2015 systematic review [23] of phytochemical and botanical therapies for rosacea found that several botanical therapies may be promising for rosacea symptoms, with several plant extracts and phytochemicals improving facial erythema and papule/pustule counts caused by rosacea. However, many of the studies included in the review were not methodologically rigorous.

- **Azelaic Acid (Topical).** A 2010 review [24] of natural products had similar findings, but noted that based on two randomized trials, topical azelaic acid—a naturally occurring 9-carbon acid found in whole grain cereals and animal products—may provide some benefit for symptoms of rosacea.

41.5.2 Safety

- The 2015 systematic review [23] of various phytochemical and botanical therapies found mild adverse reactions, such as transient burning or pruritus, and noted that several botanicals commonly used for rosacea have not been studied clinically and these may have more significant side effect profiles.
- A 2010 review [24] of two studies found azelaic acid to be generally safe, with mild and transient local adverse reactions, and no difference between azelaic acid and placebo.

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Further Readings

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Chapter 42

Nutrition and Health across the Lifespan: Guidelines and Recommendations[1]

U.S. Department of Agriculture, Washington, DC, USA

Keywords: cardiovascular disease (CVD), chronic disease risk reduction (CDRR), coronary artery disease (CAD), Daily Values (DV), diastolic blood pressure (DBP), Dietary Approaches to Stop Hypertension (DASH), dietary pattern, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), Estimated Energy Requirement (EER), food group recommendations, Generally Recognized as Safe (GRAS), gestational diabetes, Healthy Eating Index (HEI), lacto-ovo vegetarian, LDL cholesterol, methylmercury, monounsaturated fat, Nutrition Facts label, partially hydrogenated oils (PHOs), percent Daily Value (%DV), polyunsaturated fat, portion size, saturated fat, serving size, systolic blood pressure (SBP), tolerable upper intake level (UL), trans fats, type 2 diabetes

42.1 Guideline 1: Follow a Healthy Dietary Pattern at Every Life Stage

A fundamental premise of the *Dietary Guidelines* is that almost everyone, no matter an individual's age, race, or ethnicity, or health status, can benefit from shifting food and beverage choices to better support healthy dietary patterns. Healthy eating starts at birth with the exclusive consumption of human milk, if possible, for about the first 6 months. If human milk is unavailable, infants should be fed an iron-fortified commercial infant formula (i.e., labeled "with iron") regulated by the U.S. Food and Drug Administration (FDA), which are based on standards that ensure nutrient content and safety. Healthy eating continues with the introduction of complementary foods and beverages at about 6 months of age. By 12 months, infants should maintain their healthy eating as they transition to developmentally appropriate foods and beverages. Healthy eating continues in

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Make every bite count
with the *Dietary Guidelines for Americans*. Here's how:
Follow a healthy dietary pattern at every life stage.
1
Customize and enjoy nutrient-dense food and beverage choices to reflect personal preferences, cultural traditions, and budgetary considerations.
2
Focus on meeting food group needs with nutrient-dense foods and beverages, and stay within calorie limits.
3
Limit foods and beverages higher in added sugars, saturated fat, and sodium, and limit alcoholic beverages.
4
DGA
DietaryGuidelines.gov

Key Recommendations

Follow a healthy dietary pattern at every life stage.

At every life stage—infancy, toddlerhood, childhood, adolescence, adulthood, pregnancy, lactation, and older adulthood—it is never too early or too late to eat healthfully.

- **For about the first 6 months of life**, exclusively feed infants human milk. Continue to feed infants human milk through at least the first year of life, and longer if desired. Feed infants iron-fortified infant formula during the first year of life when human milk is unavailable. Provide infants with supplemental vitamin D beginning soon after birth.
- **At about 6 months**, introduce infants to nutrient-dense complementary foods. Introduce infants to potentially allergenic foods along with other complementary foods. Encourage infants and toddlers to consume a variety of foods from all food groups. Include foods rich in iron and zinc, particularly for infants fed human milk.
- **From 12 months through older adulthood**, follow a healthy dietary pattern across the lifespan to meet nutrient needs, help achieve a healthy body weight, and reduce the risk of chronic disease.

Customize and enjoy nutrient-dense food and beverage choices to reflect personal preferences, cultural traditions, and budgetary considerations.

A healthy dietary pattern can benefit all individuals regardless of age, race, or ethnicity, or current health status. The *Dietary Guidelines* provides a framework intended to be customized to individual needs and preferences, as well as the foodways of the diverse cultures in the United States.

Focus on meeting food group needs with nutrient-dense foods and beverages, and stay within calorie limits.

An underlying premise of the *Dietary Guidelines* is that nutritional needs should be met primarily from foods and beverages—specifically, nutrient-dense foods and beverages. Nutrient-dense foods provide vitamins, minerals, and other health-promoting components and have no or little added sugars, saturated fat, and sodium. A healthy dietary pattern consists of nutrient-dense forms of foods and beverages across all food groups, in recommended amounts, and within calorie limits.

The core elements that make up a healthy dietary pattern include:

- Vegetables of all types—dark green; red and orange; beans, peas, and lentils; starchy; and other vegetables
- Fruits, especially whole fruit
- Grains, at least half of which are whole grain
- Dairy, including fat-free or low-fat milk, yogurt, and cheese, and/or lactose-free versions and fortified soy beverages and yogurt as alternatives
- Protein foods, including lean meats, poultry, and eggs; seafood; beans, peas, and lentils; and nuts, seeds, and soy products
- Oils, including vegetable oils and oils in food, such as seafood and nuts

Limit foods and beverages higher in added sugars, saturated fat, and sodium, and limit alcoholic beverages.

At every life stage, meeting food group recommendations—even with nutrient-dense choices—requires most of a person's daily calorie needs and sodium limits. A healthy dietary pattern doesn't have much room for extra added sugars, saturated fat, or sodium—or for alcoholic beverages. A small amount of added sugars, saturated fat, or sodium can be added to nutrient-dense foods and beverages to help meet food group recommendations, but foods and beverages high in these components should be limited. **Limits are:**

- **Added sugars**—Less than 10 percent of calories per day starting at age 2. Avoid foods and beverages with added sugars for those younger than age 2.
- **Saturated fat**—Less than 10 percent of calories per day starting at age 2.
- **Sodium**—Less than 2,300 milligrams per day—and even less for children younger than age 14.
- **Alcoholic beverages**—Adults of legal drinking age can choose not to drink or to drink in moderation by limiting intake to 2 drinks or less in a day for men and 1 drink or less in a day for women, when alcohol is consumed. Drinking less is better for health than drinking more. There are some adults who should not drink alcohol, such as women who are pregnant.

each life stage thereafter. Even though nutrient needs vary across life stages, the foods and beverages that individuals should eat over the lifespan are remarkably consistent. This chapter provides foundational guidance about maintaining a healthy dietary pattern across each life stage—infancy, toddlerhood, childhood, adolescence, adulthood, pregnancy, lactation, and older adulthood.

42.1.1 What Is a Dietary Pattern?

Over the course of any given day, week, or year, individuals consume foods and beverages[2] in combination—a dietary pattern. A dietary pattern represents the totality of what individuals habitually eat and drink, and the parts of the pattern act synergistically to affect health. As a result, the dietary pattern may better predict overall health status and disease risk than individual foods or nutrients. A healthy dietary pattern consists of nutrient-dense forms of foods and beverages across all food groups, in recommended amounts, and within calorie limits. Achieving a healthy dietary pattern at each life stage not only supports health at that point in time, but also supports health in the next life stage and possibly for future generations. If healthy dietary patterns can be established early in life and sustained thereafter, the impact on health could be significant. Establishing and maintaining a healthy dietary pattern can help minimize diet-related chronic disease risk. Conversely, consuming foods and beverages that are not nutrient-dense may lead to disease expression in later years. High intakes of such foods (i.e., an unhealthy dietary pattern) throughout the lifespan can increase the risk of developing chronic diseases. The good news is that at any stage of life, individuals can make efforts to adopt a healthy dietary pattern and improve their health. The Healthy U.S.-Style Dietary Pattern, USDA's primary Dietary Pattern, provides a framework for healthy eating that all Americans can follow. It is based on the types and proportions of foods Americans of all ages, genders, races, and ethnicities typically consume, but in nutrient-dense forms and appropriate amounts. The Healthy U.S.-Style Dietary Pattern is carried forward from the *2015–2020 Dietary Guidelines for Americans*. The 2,000-calorie level of the pattern is shown in Table 42.1. The Healthy Mediterranean-Style Dietary Pattern and the Healthy Vegetarian Dietary Pattern— also carried forward from the *2015–2020 Dietary Guidelines for Americans*—are variations of the Healthy U.S.-Style Dietary Pattern that have the same core elements. The USDA Dietary Patterns are meant to be tailored to meet cultural and personal preferences and used as guides to plan and serve meals for individuals, households, and in a variety of institutions and other settings (https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf). The Dietary Approaches to Stop Hypertension (DASH) dietary pattern is an example of a healthy dietary pattern and has many of the same characteristics as the Healthy U.S.-Style Dietary Pattern. Additional details on DASH are available at http://www.nhlbi.nih.gov/health-topics/dash-eating-plan.

[2]If not specified explicitly, references to "foods" refer to "foods and beverages."

Table 42.1 Healthy U.S.-Style Dietary Pattern at the 2,000-Calorie Level, With Daily or Weekly Amounts From Food Groups, Subgroups, and Components

FOOD GROUP OR SUBGROUP[a]	Daily Amount[b] of Food From Each Group (Vegetable and protein foods subgroup amounts are per week.)
Vegetables (cup eq/day)	**2 ½**
	Vegetable Subgroups in Weekly Amounts
Dark-Green Vegetables (cup eq/wk)	1 ½
Red and Orange Vegetables (cup eq/wk)	5 ½
Beans, Peas, Lentils (cup eq/wk)	1 ½
Starchy Vegetables (cup eq/wk)	5
Other Vegetables (cup eq/wk)	4
Fruits (cup eq/day)	**2**
Grains (ounce eq/day)	**6**
Whole Grains (ounce eq/day)	≥ 3
Refined Grains (ounce eq/day)	< 3
Dairy (cup eq/day)	**3**
Protein Foods (ounce eq/day)	**5 ½**
	Protein Foods Subgroups in Weekly Amounts
Meats, Poultry, Eggs (ounce eq/wk)	26
Seafood (ounce eq/wk)	8
Nuts, Seeds, Soy Products (ounce eq/wk)	5
Oils (grams/day)	**27**
Limit on Calories for Other Uses (kcal/day)[c]	**240**
Limit on Calories for Other Uses (%/day)	12%

[a]Definitions for each food group and subgroup are provided throughout the chapter and are compiled in Appendix 3 at https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf.

[b]Food group amounts shown in cup or ounce equivalents (eq). Oils are shown in grams. Quantity equivalents for each food group are defined in Appendix 3 at https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf. Amounts will vary for those who need <2,000 or >2,000 calories per day.

[c]Foods are assumed to be in nutrient-dense forms, lean or low-fat and prepared with minimal added sugars, refined starches, saturated fat, or sodium. If all food choices to meet food group recommendations are in nutrient-dense forms, a small number of calories remain within the overall limit of the pattern (i.e., limit on calories for other uses). The amount of calories depends on the total calorie level of the pattern and the amounts of food from each food group required to meet nutritional goals. Calories up to the specified limit can be used for added sugars, saturated fat, and/or alcohol, or to eat more than the recommended amount of food in a food group.

Note: The total dietary pattern should not exceed Dietary Guidelines limits for added sugars, saturated fat, and alcohol; be within the Acceptable Macronutrient Distribution Ranges for protein, carbohydrate, and total fats; and stay within calorie limits. Values are rounded.

42.1.2 The Health Benefits of a Healthy Dietary Pattern

Science is the foundation of the *Dietary Guidelines* recommendations on what Americans should eat and drink to promote health, reduce risk of chronic disease, and meet nutrient needs. The science shows that consuming a healthy dietary

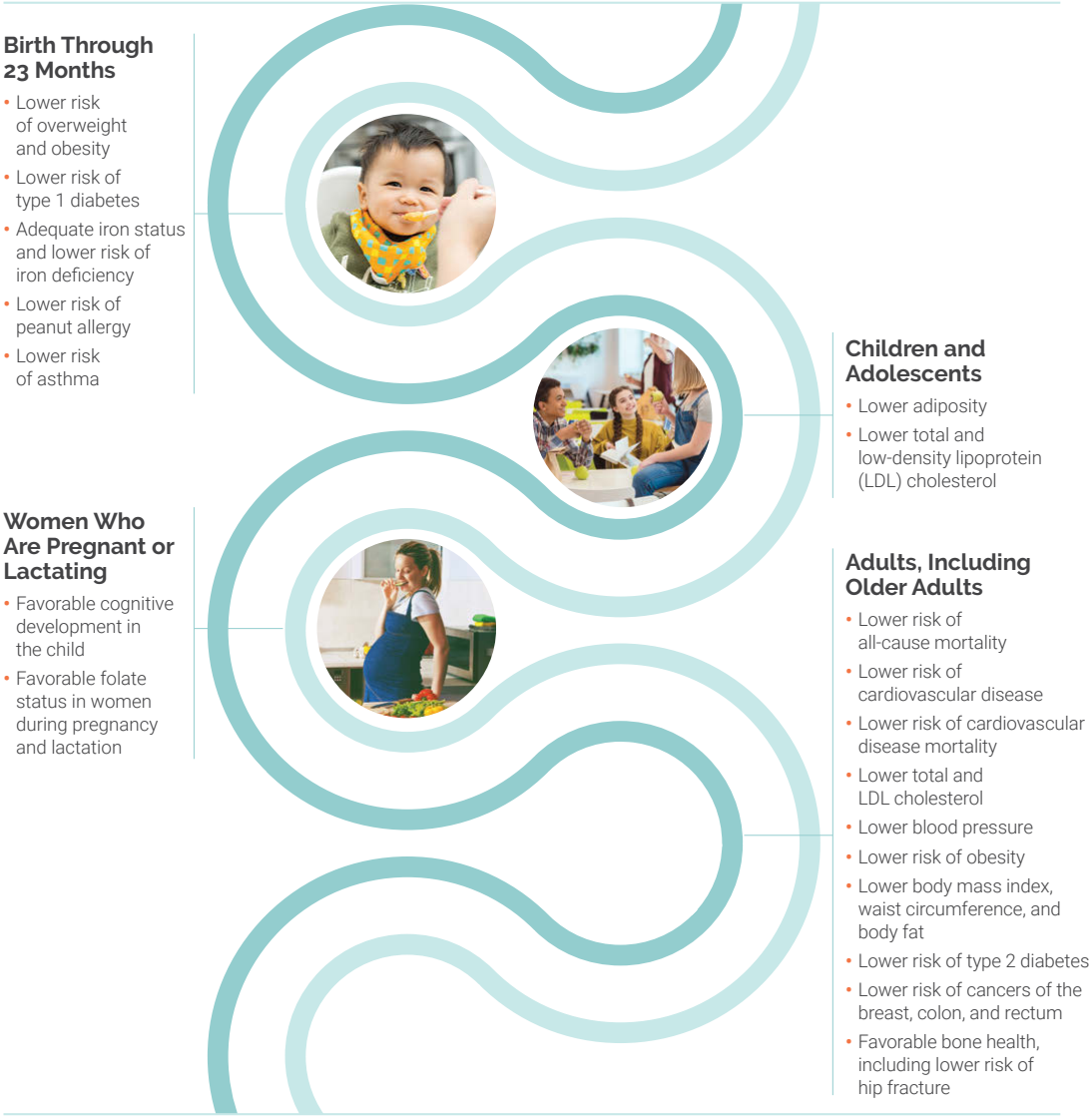


Figure 42.1 The Science Underlying the *Dietary Guidelines* Demonstrates That Healthy Eating Across the Lifespan Can Promote Health and Reduce Risk of Chronic Disease. *Note*: The 2020 Dietary Guidelines Advisory Committee examined the evidence on diet and health across the lifespan. Evidence is not available for all combinations of exposures and outcomes for the population subgroups presented in this figure. The Committee rated the evidence on diet and health as Strong, Moderate, Limited, or Grade Not Assignable. Only outcomes with Strong or Moderate evidence are included in this table.

pattern, meeting food group and nutrient needs with nutrient-dense foods and beverages, and limiting intake of foods and beverages that are not nutrient-dense is related to many health benefits. Science also supports the idea that every life stage provides an opportunity to make food choices that promote health and well-being, achieve and maintain appropriate weight status, and reduce risk of diet-related chronic disease.

The science supporting the *Dietary Guidelines* is extensively documented in the *Scientific Report of the 2020 Dietary Guidelines Advisory Committee*, which describes the state of the science on key topics related to diet and health. Outcomes with Strong or Moderate evidence are provided in Fig. 42.1. The report is available at http://www.dietaryguidelines.gov/.

Evidence on the association between dietary patterns and reduced risk of diet-related chronic diseases has expanded in recent years and supports the use of dietary patterns as a foundation for the recommendations in the *Dietary Guidelines for Americans, 2020–2025*. Consistent evidence demonstrates that a healthy dietary pattern is associated with beneficial outcomes for all-cause mortality, cardiovascular disease, overweight and obesity, type 2 diabetes, bone health, and certain types of cancer (breast and colorectal).

Common characteristics of dietary patterns associated with positive health outcomes include relatively higher intake of vegetables, fruits, legumes, whole grains, low- or non-fat dairy, lean meats and poultry, seafood, nuts, and unsaturated vegetable oils, and relatively lower consumption of red and processed meats, sugar-sweetened foods and beverages, and refined grains. The evidence examined showed broad representation across a number of populations and demographic groups. This suggests a consistent association no matter the region or cultural context in which a healthy dietary pattern is consumed. In addition, dietary patterns characterized by higher intake of red and processed meats, sugar-sweetened foods and beverages, and refined grains are, in and of themselves, associated with detrimental health outcomes.

42.1.3 A Healthy Dietary Pattern Supports Appropriate Calorie Levels

The total number of calories a person needs each day varies depending on a number of factors, namely the person's age, sex, height, weight, level of physical activity, and pregnancy or lactation status. Due to reductions in basal metabolic rate that occur with aging, calorie needs generally decrease for adults as they age. In addition, a need to lose, maintain, or gain weight affects how many calories should be consumed. Estimated amounts of calories needed based on age, sex, and level of physical activity and estimated calorie needs relevant for different ages are distinct at each life stage. These estimates are based on the Estimated Energy Requirement (EER) equations established by the National Academies of Sciences, Engineering, and Medicine (National Academies) using reference heights (average) and reference weights (healthy) for each age-sex group. These amounts are estimates. The best way to evaluate calorie intake, in comparison to calorie needs, is by measuring body weight status.

Rather than focus on weight status at any one point in life, the *Dietary Guidelines* supports healthy weight trajectories at each stage of life—appropriate weight gain during pregnancy and postpartum weight loss, healthy growth and development from infancy through adolescence, weight stability during mid-life, and healthy body composition late in life. Meeting the Dietary Guidelines recommendations within calorie needs can help prevent excess weight gain at every life stage and support overall good health.

Key Dietary Principles

To help people meet the Guidelines and Key Recommendations, the following are important principles when making decisions about nutrient-dense food and beverage choices to achieve a healthy dietary pattern.

Meet nutritional needs primarily from foods and beverages

The *Dietary Guidelines* are designed to meet the Recommended Dietary Allowances and Adequate Intakes for essential nutrients, as well as Acceptable Macronutrient Distribution Ranges, all set by the National Academies. An underlying premise of the *Dietary Guidelines* is that nutritional needs should be met primarily from foods and beverages—specifically, nutrient-dense foods and beverages. In some cases, when meeting nutrient needs is not otherwise possible, fortified foods and nutrient-containing dietary supplements are useful. It is important to note that the nutrient density and healthfulness of what people eat and drink often is determined ultimately by how a food item, dish, or meal is prepared, at home and away from home or produced by a manufacturer. Based on the U.S. food supply and marketplace, the examples of healthy dietary patterns in this edition are achievable through thoughtful, informed choices one decision, one meal, one day at a time—and consistently over time.

Choose a variety of options from each food group

Enjoy different foods and beverages within each food group. This can help meet nutrient needs—and also allows for flexibility so that the *Dietary Guidelines* can be tailored to meet cultural and personal preferences. All forms of foods, including fresh, canned, dried, frozen, and 100% juices, in nutrient-dense forms, can be included in healthy dietary patterns.

Pay attention to portion size

Portion size is a term often used to describe the amount of a food or beverage served or consumed in one eating occasion. It is important to pay attention

to portion size when making food and beverage choices, particularly for foods and beverages that are not nutrient-dense. A concept that can help people choose appropriate portions is *serving size*. This term is included on the Nutrition Facts label and refers to the amount of a food or beverage that is customarily consumed—it is not a recommendation of how much to eat or drink. Consuming less than the stated serving size results in consuming fewer calories and other nutrients or food components. Some products may have multiple servings per package.

42.1.4 Most Americans Do Not Follow a Healthy Dietary Pattern

The typical dietary patterns currently consumed by many in the United States do not align with the *Dietary Guidelines* (Fig. 42.2). The Healthy Eating Index (HEI) is a measure of diet quality that can be used to assess compliance with the *Dietary Guidelines*. For Americans ages 2 and older, HEI-2015 scores indicate that intakes are not consistent with recommendations for a healthy dietary pattern.

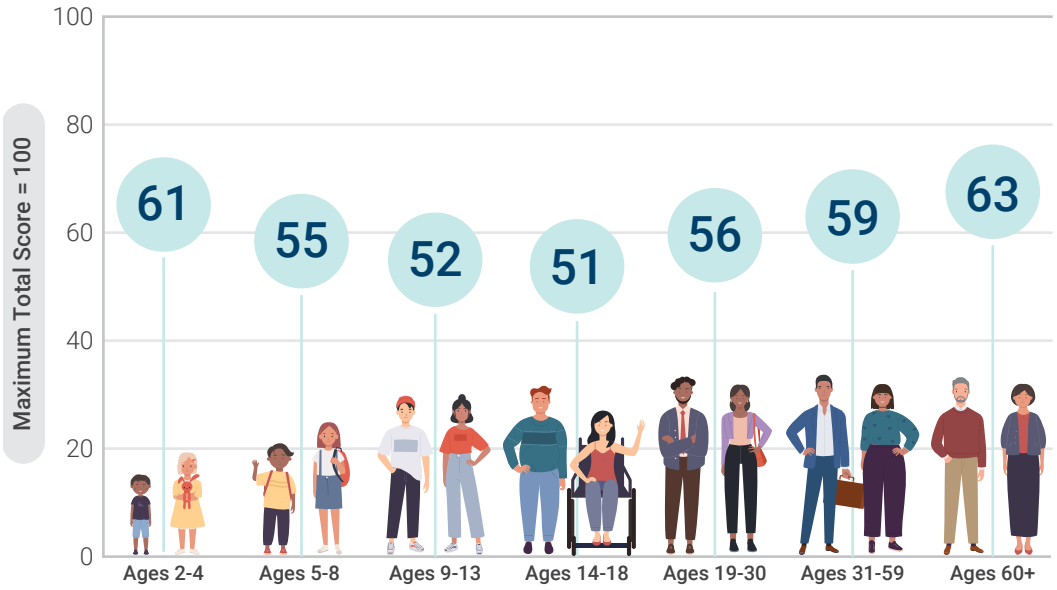


Figure 42.2 Adherence of the U.S. population to the dietary guidelines across life stages, as measured by Average Total Healthy Eating Index-2015 scores. *Note*: HEI-2015 total scores are out of 100 possible points. A score of 100 indicates that recommendations on average were met or exceeded. A higher total score indicates a higher quality diet. *Data Source*: Analysis of What We Eat in America, NHANES 2015-2016, ages 2 and older, day 1 dietary intake data, weighted.

Average diet quality has slightly improved in the past 10 years, but the average score of 59 (on a scale from 0 to 100) indicates that people have much room for improvement. Differences in overall HEI scores are seen across age, sex, race-ethnic, and income subgroups and by pregnancy and lactation status, though poor diet quality is observed across all groups. With each step closer to a diet that aligns with the core elements of a healthy dietary pattern, HEI scores will increase and risk for chronic disease will decrease.

In addition, the high percentage of the population with overweight or obesity suggests that many people in the United States consume foods and beverages that contribute to a calorie imbalance, a situation more likely to occur with low physical activity. As shown in Table 42.2, 74 percent of all adults and 40 percent of all children and youth in the United States have either overweight or obesity.

Table 42.2 Facts about nutrition-related health conditions in the United States

Health conditions	Statistics
Overweight and Obesity	• About 74% of adults are overweight or have obesity. • Adults ages 40 to 59 have the highest rate of obesity (43%) of any age group with adults 60 years and older having a 41% rate of obesity. • About 40% of children and adolescents are overweight or have obesity; the rate of obesity increases throughout childhood and teen years.
Cardiovascular Disease (CVD) and Risk Factors: • Coronary artery disease • Hypertension • High LDL and total blood cholesterol • Stroke	• Heart disease is the leading cause of death. • About 18.2 million adults have coronary artery disease, the most common type of heart disease. • Stroke is the fifth leading cause of death. • Hypertension, high LDL cholesterol, and high total cholesterol are major risk factors in heart disease and stroke. • Rates of hypertension and high total cholesterol are higher in adults with obesity than those who are at a healthy weight. • About 45% of adults have hypertension.[a] • More Black adults (54%) than White adults (46%) have hypertension. • More adults ages 60 and older (75%) than adults ages 40 to 59 (55%) have hypertension. • Nearly 4% of adolescents have hypertension.[b] • More than 11% of adults have high total cholesterol, ≥240 mg/dL. • More women (12%) than men (10%) have high total cholesterol, ≥240 mg/dL. • 7% of children and adolescents have high total cholesterol, ≥200 mg/dL.
Diabetes	• Almost 11% of Americans have type 1 or type 2 diabetes. • Almost 35% of American adults have prediabetes, and people 65 years and older have the highest rate (48%) compared to other age groups. • Almost 90% of adults with diabetes also are overweight or have obesity. • About 210,000 children and adolescents have diabetes, including 187,000 with type 1 diabetes. • About 6–9% of pregnant women develop gestational diabetes.

Health conditions	Statistics
Cancer[c] • Breast Cancer • Colorectal Cancer	• Colorectal cancer in men and breast cancer in women are among the most common types of cancer. • About 250,520 women will be diagnosed with breast cancer this year. • Close to 5% of men and women will be diagnosed with colorectal cancer at some point during their lifetime. • More than 1.3 million people are living with colorectal cancer. • The incidence and mortality rates are highest among those ages 65 and older for every cancer type.
Bone Health and Muscle Strength	• More women (17%) than men (5%) have osteoporosis. • 20% of older adults have reduced muscle strength. • Adults over 80 years, non-Hispanic Asians, and women are at the highest risk for reduced bone mass and muscle strength.

[a]For adults, hypertension is defined as systolic blood pressure (SBP) >130 mm Hg and/or a diastolic blood pressure (DBP) >90 mm Hg.

[b]For children, hypertension was defined using the 2017 American Academy of Pediatrics (AAP) Clinical Practice Guideline.

[c]The types of cancer included here are not a complete list of all diet- and physical activity-related cancers.

Even from the youngest ages, almost all Americans should shift to healthier food and beverage choices and consume smaller portions to achieve a healthy dietary pattern within an appropriate number of calories. It is never too early or too late to improve intake and establish a healthy dietary pattern.

42.2 Guideline 2: Customize and Enjoy Food and Beverage Choices to Reflect Personal Preferences, Cultural Traditions, and Budgetary Considerations

Eating should be enjoyed, and a healthy dietary pattern can be enjoyable, from early life to older adulthood. The science reviewed to inform the *Dietary Guidelines* (https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf) represents the diversity of Americans, including all ages and life stages, different racial and ethnic backgrounds, and a range of socioeconomic statuses. A healthy dietary pattern can benefit all individuals regardless of age, race or ethnicity, or current health status. These *Guidelines* provides a framework intended to be customized to fit individual, household, and Federal program participants' preferences, as well as the foodways of the diverse cultures in the United States. The U.S. population is diverse in myriad ways. The *Dietary Guidelines* framework purposely provides recommendations by food groups and subgroups—not specific foods and beverages—to avoid being prescriptive. This framework approach ensures that people can "make it their own" by selecting healthy foods, beverages, meals, and snacks specific to their needs and preferences. The food groups include a broad variety of nutrient-dense

food and beverage choices. In every setting, across all cultures, and at any age or budget, there are foods and beverages that can fit within the *Guidelines* framework.

Start with Personal Preferences

Exposure to different types of food is important early in life to better develop a child's interest and willingness to eat and enjoy a variety of foods. Through each life stage that follows, a key starting point for establishing and maintaining a healthy dietary pattern is to ensure that individual and/or family preferences—in nutrient-dense forms—are built into day-to-day choices.

Incorporate Cultural Traditions

Cultural background can have significant influence on food and beverage choices. Customizing the *Dietary Guidelines* framework to reflect specific cultures and traditions is an important strategy to help communities across the country eat and enjoy a healthy dietary pattern. Nutrient-dense culturally relevant foods and beverages are part of all of the food groups. Spices and herbs can help flavor foods when reducing added sugars, saturated fat, and sodium, and they also can add to the enjoyment of nutrient-dense foods, dishes, and meals that reflect specific cultures. Relying on the expertise of professionals in nutrition and in specific cultural foodways can help people prepare foods healthfully while retaining heritage.

Consider Your Budget

Despite a common perception that eating healthfully is expensive, a healthy dietary pattern can be affordable and fit within budgetary constraints. There are a range of strategies that can be used to help individuals and families follow a healthy dietary pattern including advanced planning; considering regional and seasonal food availability; and incorporating a variety of fresh, frozen, dried, and canned options. The USDA Food Plans—Thrifty, Low-Cost, Moderate-Cost, and Liberal-Cost food plans (https://www.fns.usda.gov/cnpp/usda-food-plans-cost-food-reports)—each represent a nutritious diet at a different cost level.

Vegetables

- **Dark-Green Vegetables:** All fresh, frozen, and canned dark-green leafy vegetables and broccoli, cooked or raw: for example, amaranth leaves, bok choy, broccoli, chamnamul, chard, collards, kale, mustard greens, poke greens, romaine lettuce, spinach, taro leaves, turnip greens, and watercress.
- **Red and Orange Vegetables:** All fresh, frozen, and canned red and orange vegetables or juice, cooked or raw: for example, calabaza, carrots, red or orange bell peppers, sweet potatoes, tomatoes, 100% tomato juice, and winter squash.
- **Beans, Peas, Lentils:** All cooked from dry or canned beans, peas, chickpeas, and lentils: for example, black beans, black-eyed peas, bayo beans, chickpeas (garbanzo beans), edamame, kidney beans, lentils, lima beans, mung beans, pigeon peas, pinto beans, and split peas. Does not include green beans or green peas.
- **Starchy Vegetables:** All fresh, frozen, and canned starchy vegetables: for example, breadfruit, burdock root, cassava, corn, jicama, lotus root, lima beans, plantains, white potatoes, salsify, taro root (dasheen or yautia), water chestnuts, yam, and yucca.
- **Other Vegetables:** All other fresh, frozen, and canned vegetables, cooked or raw: for example, asparagus, avocado, bamboo shoots, beets, bitter melon, Brussels sprouts, cabbage (green, red, napa, savoy), cactus pads (nopales), cauliflower, celery, chayote (mirliton), cucumber, eggplant, green beans, kohlrabi, luffa, mushrooms, okra, onions, radish, rutabaga, seaweed, snow peas, summer squash, tomatillos, and turnips.

Fruits

- All fresh, frozen, canned, and dried fruits and 100% fruit juices: for example, apples, Asian pears, bananas, berries (e.g., blackberries, blueberries, currants, huckleberries, kiwifruit, mulberries, raspberries, and strawberries); citrus fruit (e.g., calamondin, grapefruit, lemons, limes, oranges, and pomelos); cherries, dates, figs, grapes, guava, jackfruit, lychee, mangoes, melons (e.g., cantaloupe, casaba, honeydew, and watermelon); nectarines, papaya, peaches, pears, persimmons, pineapple, plums, pomegranates, raisins, rhubarb, sapote, and soursop.

Figure 42.3 Customizing the *Dietary Guidelines* Framework. The *Dietary Guidelines* approach of providing a framework–not prescriptive details–ensures that its recommendations can "meet people where they are," from personal preferences to cultural foodways, and including budgetary considerations. The examples below are a sample of the range of options in each food group—to be eaten in nutrient-dense forms.

Grains

- **Whole grains:** All whole-grain products and whole grains used as ingredients: for example, amaranth, barley (not pearled), brown rice, buckwheat, bulgur, millet, oats, popcorn, quinoa, dark rye, whole-grain cornmeal, whole-wheat bread, whole-wheat chapati, whole-grain cereals and crackers, and wild rice.
- **Refined grains:** All refined-grain products and refined grains used as ingredients: for example, white breads, refined-grain cereals and crackers, corn grits, cream of rice, cream of wheat, barley (pearled), masa, pasta, and white rice. Refined-grain choices should be enriched.

Dairy and Fortified Soy Alternatives

- All fluid, dry, or evaporated milk, including lactose-free and lactose-reduced products and fortified soy beverages (soy milk), buttermilk, yogurt, kefir, frozen yogurt, dairy desserts, and cheeses. Most choices should be fat-free or low-fat. Cream, sour cream, and cream cheese are not included due to their low calcium content.

Protein Foods

- **Meats, Poultry, Eggs:** Meats include beef, goat, lamb, pork, and game meat (e.g., bison, moose, elk, deer). Poultry includes chicken, Cornish hens, duck, game birds (e.g., ostrich, pheasant, and quail), goose, and turkey. Organ meats include chitterlings, giblets, gizzard, liver, sweetbreads, tongue, and tripe. Eggs include chicken eggs and other birds' eggs. Meats and poultry should be lean or low-fat.
- **Seafood:** Seafood examples that are lower in methylmercury include: anchovy, black sea bass, catfish, clams, cod, crab, crawfish, flounder, haddock, hake, herring, lobster, mullet, oyster, perch, pollock, salmon, sardine, scallop, shrimp, sole, squid, tilapia, freshwater trout, light tuna, and whiting.
- **Nuts, Seeds, Soy Products:** Nuts and seeds include all nuts (tree nuts and peanuts), nut butters, seeds (e.g., chia, flax, pumpkin, sesame, and sunflower), and seed butters (e.g., sesame or tahini and sunflower). Soy includes tofu, tempeh, and products made from soy flour, soy protein isolate, and soy concentrate. Nuts should be unsalted.

Figure 42.3 (*continued*)

42.3 Guideline 3: Focus on Meeting Food Group Needs With Nutrient-Dense Foods and Beverages, and Stay Within Calorie Limits

The *Dietary Guidelines* include recommendations for food groups—vegetables, fruits, grains, dairy, and protein foods—eaten at an appropriate calorie level and in forms with limited amounts of added sugars, saturated fat, and sodium. Science shows that these same core elements of a healthy dietary pattern are consistent across each life stage.

However, as shown in Fig. 42.4, when compared to the Healthy U.S.-Style Dietary Pattern, most Americans have substantial room for improvement:

- More than 80 percent have dietary patterns that are low in vegetables, fruits, and dairy.
- More than half of the population is meeting or exceeding total grain and total protein foods recommendations, but are not meeting the recommendations for the subgroups within each of these food groups.

About Beans, Peas, and Lentils

"Beans, peas, and lentils" is a new name for the vegetable subgroup formerly called "legumes (beans and peas)." Beans, peas, and lentils, which also are known as pulses, include the dried edible seeds of legumes. The foods in this vegetable subgroup have not changed. However, the new name of the subgroup more accurately reflects the category of foods included. Beans include varieties such as kidney beans, pinto beans, white beans, black beans, lima beans, and fava beans. Also included are dried peas (e.g., chickpeas, black-eyed peas, pigeon peas, and split peas) and lentils. Edamame, which is the soybean in the pod, is counted in the beans, peas, and lentils subgroup even though it is eaten fresh and not dried. Because beans, peas, and lentils have a similar nutrient profile to foods in both the vegetable group and the protein foods group, they may be thought of as either a vegetable or a protein food when aiming to meet recommended intakes. Green peas and green (string) beans are not counted in the beans, peas, and lentils subgroup because the nutrient content of these vegetables is more similar to vegetables in other subgroups. Green peas, which are not dried before consumption, are grouped with starchy vegetables and green beans are in the other vegetables subgroup, which includes onions, iceberg lettuce, celery, and cabbage. Generally, foods made from processed soybeans are a part of the nuts, seeds and soy products protein foods subgroup.

Figure 42.4 Dietary intakes compared to recommendations: percent of the U.S. population ages 1 and older who are below and at or above each dietary goal. **Note*: Recommended daily intake of whole grains is to be at least half of total grain consumption, and the limit for refined grains is to be no more than half of total grain consumption. Data Source**:** Analysis of What We Eat in America, NHANES 2013-2016, ages 1 and older, 2 days dietary intake data, weighted. *Recommended Intake Ranges*: Healthy U.S.-Style Dietary Patterns (see Appendix 3 at https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf.

The following sections use the Healthy U.S.-Style Dietary Pattern to show how people can make shifts in their choices to achieve a healthy dietary pattern.

Eating an appropriate mix of foods from the food groups and subgroups—within an appropriate calorie level—is important to promote health at each life stage. Each of the food groups and their subgroups provides an array of nutrients, and the amounts recommended reflect eating patterns that have been associated with positive health outcomes. Foods from all of the food groups should be eaten in nutrient-dense forms. The following sections describe special considerations related to each food group.

42.3.1 Vegetables

Healthy dietary patterns include a variety of vegetables from all five vegetable subgroups—dark green; red and orange; beans, peas, and lentils; starchy; and other. These include all fresh, frozen, canned, and dried options in cooked or raw forms, including 100% vegetable juices. Vegetables in their nutrient-dense forms have limited additions such as salt, butter, or creamy sauces. Examples of vegetables in each of the subgroups are available in Appendix 3 at https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020–2025.pdf.

Almost 90 percent of the U.S. population does not meet the recommendation for vegetables. In addition, with few exceptions, the U.S. population does not meet intake recommendations for any of the vegetable subgroups. About 45 percent of all vegetables are eaten as a separate food item; about 40 percent as part of a mixed dish; and the remainder are mostly consumed as snack foods and condiments. Vegetables, when consumed on their own, are generally consumed in forms with additional sodium either from salt added in cooking or added sauces such as soy sauce or bottled stir-fry sauces. Many vegetables are consumed as part of mixed dishes like sandwiches, pasta with a tomato-based sauce, or casseroles that may have other ingredients that are sources of saturated fat and/or sodium.

For most individuals, following a healthy eating pattern will require an increase in total vegetable intake and from all vegetable subgroups, shifting to nutrient-dense forms, and an increase in the variety of different vegetables consumed over time. Vegetables can be part of many types of mixed dishes, from burgers, sandwiches, and tacos, to pizza, stews, pasta dishes, grain-based casseroles, and soups. Strategies to increase vegetable intake include increasing the vegetable content of mixed dishes or eating less of a main dish to allow for more vegetables as side dishes—keeping these nutrient dense.

42.3.2 Fruits

The fruit food group includes whole fruits and 100% fruit juice. Whole fruits include fresh, canned, frozen, and dried forms. Whole fruits can be eaten in various forms, such as cut, sliced, diced, or cubed. At least half of the recommended

amount of fruit should come from whole fruit, rather than 100% juice. When juices are consumed, they should be 100% juice and always pasteurized or 100% juice diluted with water (without added sugars). Also, when selecting canned fruit, choose options that are canned with 100% juice or options lowest in added sugars.

About 80 percent of the U.S. population does not meet fruit recommendations. Over 60 percent of all fruit intake comes from whole forms—fresh, canned, frozen, or dried—or 100% juice. Fruit is generally consumed in nutrient-dense forms such as plain bananas, apples, oranges, or grapes. However, some fruit is consumed as part of foods that may not be nutrient-dense, such as fruit pie or similar desserts.

Most people would benefit from increasing their intake of fruit, mostly as whole fruits in nutrient-dense forms. A wide variety of fruits are available in the U.S. marketplace, some year-round and others seasonally. Strategies to help achieve this shift include choosing more whole fruits as snacks and including them in meals.

42.3.3 Grains

Healthy dietary patterns include whole grains and limit the intake of refined grains. At least half of total grains should be whole grains. Individuals who eat refined grains should choose enriched grains. Individuals who consume all of their grains as whole grains should include some that have been fortified with folic acid. Grain-based foods in nutrient-dense forms limit the additions of added sugars, saturated fat, and sodium.

A food is a 100% whole-grain food if the only grains it contains are whole grains. A 1 ounce-equivalent of 100% whole grains has 16 grams of whole grains. The recommendation to consume at least half of total grains as whole grains can be met in a number of ways.

- Choose 100% whole-grain foods for at least half of all grains consumed. The relative amount of whole grain in the food can be inferred by the placement of the grain in the ingredient list. The whole grain should be the first ingredient—or the second ingredient after water. For foods with multiple whole-grain ingredients, they should appear near the beginning of the ingredient list.
- Choose products with at least 50 percent of the total weight as whole-grain ingredients. If a food has at least 8 grams of whole grains per ounce-equivalent then half of the grains are whole-grain ingredients.

Most Americans meet recommendations for total grain intakes, although 98 percent fall below recommendations for whole grains and 74 percent exceed limits for refined grains. Almost half of all intake of refined grains is from mixed dishes, such as sandwiches, burgers, tacos, pizza, macaroni and cheese, and spaghetti

with meatballs. About 20 percent of intake of refined grains comes from snacks and sweets, including crackers, pretzels, cakes, cookies, and other grain desserts. The remaining refined grains are generally eaten as separate food items, such as pancakes, cereals, breads, tortillas, pasta, or rice. About 60 percent of whole-grain intake in the United States is from individual food items, mostly cereals and crackers, rather than mixed dishes. Grains are generally consumed in forms with higher amounts of sodium (e.g., breads, tortillas, crackers) and added sugars (e.g., grain-based desserts, many ready-to-eat breakfast cereals) rather than the nutrient-dense forms. Further, grains are often consumed as part of mixed dishes, such as pasta dishes, casseroles, and sandwiches that may have other ingredients that are not in nutrient-dense forms.

Shifting from refined to whole-grain versions of commonly consumed foods—such as from white to 100% whole-wheat breads, and white to brown rice where culturally appropriate—would increase whole-grain intakes and lower refined grain intakes to help meet recommendations. Additionally, shifting to more nutrient-dense forms of grains, such as ready-to-eat breakfast cereals with less sugar, will help meet healthy dietary patterns. With careful planning, limited amounts of salt, butter, or sources of added sugars can be used to make some grain-based foods more palatable while staying within calorie and nutrient limits, but most grains should be eaten in their most nutrient-dense forms. Reducing intakes of cakes, cookies, and other grain desserts will also support reducing refined grain intakes and staying within calorie needs.

Dairy and Fortified Soy Alternatives

Healthy dietary patterns feature dairy, including fat-free and low-fat (1%) milk, yogurt, and cheese. Individuals who are lactose intolerant can choose low-lactose and lactose-free dairy products. For individuals who choose dairy alternatives, fortified soy beverages (commonly known as “soy milk”) and soy yogurt—which are fortified with calcium, vitamin A, and vitamin D—are included as part of the dairy group because they are similar to milk and yogurt based on nutrient composition and in their use in meals.

Other products sold as “milks” but made from plants (e.g., almond, rice, coconut, oat, and hemp “milks”) may contain calcium and be consumed as a source of calcium, but they are not included as part of the dairy group because their overall nutritional content is not similar to dairy milk and fortified soy beverages.

Therefore, consuming these beverages does not contribute to meeting the dairy group recommendation.

About 90 percent of the U.S. population does not meet dairy recommendations. The percent of Americans who drink milk as a beverage on a given day is 65 percent among young children, 34 percent in adolescents, and about 20 percent for adults. Dairy is generally consumed in forms with higher amounts of sodium (e.g., cheeses as part of mixed dishes such as sandwiches, pizza, and pasta dishes) and saturated fat (e.g., higher fat milks and yogurts) and can be a source of added sugars such as flavored milk, ice cream, and sweetened yogurts.

Most individuals would benefit by increasing intake of dairy in fat-free or low-fat forms, whether from milk (including lactose-free milk), yogurt, and cheese, or from fortified soy beverages or soy yogurt. Strategies to increase dairy intake include drinking fat-free or low-fat milk or a fortified soy beverage with meals or incorporating unsweetened fat-free or low-fat yogurt into breakfast or snacks.

42.3.4 Protein Foods

Healthy dietary patterns include a variety of protein foods in nutrient-dense forms. The protein foods group comprises a broad group of foods from both animal and plant sources, and includes several subgroups: meats, poultry, and eggs; seafood; and nuts, seeds, and soy products. As noted previously, beans, peas, and lentils may be considered a part of the protein foods group as well as the vegetable group. Protein also is found in some foods from other food groups, such as dairy. Meats and poultry vary in fat content and include both fresh and processed forms. Most intake of meats and poultry should be from fresh, frozen, or canned, and in lean forms (e.g., chicken breast or ground turkey) versus processed meats (e.g., hot dogs, sausages, ham, luncheon meats).

A healthy vegetarian dietary pattern can be achieved by incorporating protein foods from plants. Compared with the Healthy U.S.-Style Dietary Pattern, the Healthy Vegetarian Dietary Pattern is higher in soy products (particularly tofu and other processed soy products); beans, peas, and lentils; nuts and seeds; and whole grains. Inclusion of dairy and eggs make this an example of a lacto-ovo vegetarian pattern. Meats, poultry, and seafood are not included.

Seafood, which includes fish and shellfish, is a protein foods subgroup that provides beneficial fatty acids (e.g., eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]). In addition, mercury, in the form of methylmercury, is found in seafood in varying levels. The U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA) provide joint advice regarding seafood consumption to limit methylmercury exposure for women who might become or are pregnant or lactating and young children.[3] Seafood choices higher in EPA and DHA and lower in methylmercury are encouraged. Seafood varieties commonly consumed in the United States that are higher in EPA and DHA and lower in methylmercury include salmon, anchovies, sardines, Pacific oysters, and trout.

[3]Available at FDA.gov/fishadvice and EPA.gov/fishadvice.

Tilapia, shrimp, catfish, crab, and flounder are commonly consumed varieties that also are lower in methylmercury.

Intakes of protein foods are close to the target amounts, but many Americans do not meet recommendations for specific protein subgroups. About three-quarters of Americans meet or exceed the recommendation for meats, poultry, and eggs. However, almost 90 percent do not meet the recommendation for seafood and more than half do not meet the recommendation for nuts, seeds, and soy products. Slightly less than half (43%) of all protein foods are consumed as a separate food item, such as a chicken breast, a steak, an egg, a fish filet, or peanuts. About the same proportion are consumed as part of a mixed dish (48%), with the largest amount from sandwiches including burgers and tacos. Protein foods are generally consumed in forms with higher amounts of saturated fat or sodium and often part of mixed dishes (e.g., sandwiches, casseroles, pasta dishes) that include other ingredients that are not in nutrient-dense forms.

Shifts are needed within the protein foods group to add variety to subgroup intakes. Selecting from the seafood subgroup or the beans, peas, and lentils subgroup more often could help meet recommendations while still ensuring adequate protein consumption. Replacing processed or high-fat meats (e.g., hot dogs, sausages, bacon) with seafood could help lower intake of saturated fat and sodium, nutrients that are often consumed in excess of recommended limits. Replacing processed or high-fat meats with beans, peas, and lentils would have similar benefits, as well as increasing dietary fiber, a dietary component of public health concern.

Follow Food Safety Recommendations

An important part of healthy eating is keeping food safe. Individuals in their own homes can help keep food safe by following safe food handling practices. Four basic food safety principles work together to reduce the risk of foodborne illness—Clean, Separate, Cook, and Chill.

Some eating behaviors, such as consuming raw, undercooked, or unpasteurized food products, increase the risk of contracting a foodborne illness. Populations at increased risk of foodborne illness, or those preparing food for them, should use extra caution. These include women who are pregnant, young children, and older adults. Individuals with weakened immune systems are also at increased risk for foodborne illness. More information about food safety is available at:

- Your Gateway to Food Safety: https://www.foodsafety.gov/
- USDA Food Safety Education campaigns: https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/teach-others/fsis-educational-campaigns
- Fight BAC!®: http://fightbac.org/ and for Babies and Toddlers: http://fightbac.org/kids/
- CDC 4 Steps to Food Safety: http://cdc.gov/foodsafety
- FDA: Buy, Store & Serve Safe Food at https://www.fda.gov/food/consumers/buy-store-serve-safe-food

42.3.5 Oils

Oils are important to consider as part of a healthy dietary pattern as they provide essential fatty acids. Commonly consumed oils include canola, corn, olive, peanut, safflower, soybean, and sunflower oils. Oils also are naturally present in nuts, seeds, seafood, olives, and avocados. The fat in some tropical plants, such as coconut oil, palm kernel oil, and palm oil, are not included in the oils category because they contain a higher percentage of saturated fat than do other oils.

Strategies to shift intake include cooking with vegetable oil in place of fats high in saturated fat, including butter, shortening, lard, or coconut oil. However, some foods, such as desserts and sweet snacks, that are prepared with oils instead of fats high in saturated fat are still high in added sugars, and are thus not a nutrient-dense food choice.

42.3.6 Beverages

When choosing beverages in a healthy dietary pattern, both the calories and nutrients that they provide are important considerations. Beverages that are calorie-free—especially water—or that contribute beneficial nutrients, such as fat-free and low-fat milk and 100% juice, should be the primary beverages consumed. Coffee, tea, and flavored waters also are options, but the most nutrient-dense options for these beverages include little, if any, sweeteners or cream. For discussion on sugar-sweetened beverages or alcohol, see Section 42.4.1, "Added Sugars," and Section 42.4.2, "Alcoholic Beverages," respectively.

42.3.6.1 Caffeine

Caffeine is a dietary component that functions in the body as a stimulant. Most intake of caffeine in the United States comes from coffee, tea, and soda. Caffeine is a substance that is Generally Recognized as Safe (GRAS) in cola-type beverages by the U.S. Food and Drug Administration (FDA). For healthy adults, the FDA has cited 400 milligrams per day of caffeine as an amount not generally associated with dangerous, negative effects.

Beverages and Added Sugars

Examples of beverages that often have added sugars are regular soda (i.e., not sugar-free), fruit drinks, sports drinks, energy drinks, sweetened waters, and coffee and tea beverages with added sugars. Coffee and tea beverages from restaurants can contain many extra calories because of the addition of cream or milk and sugar. See below for examples of 12-ounce beverages showing the added sugars and total calories.

Drink (12-ounce serving)	Total calories	Added sugars (grams)	Added sugars (teaspoons)
Plain water	0	0	0
Unsweetened tea	0	0	0
Sports drinks	97	20	5
Cafe mocha	290	21	5
Chai tea latte	180	23	5 ½
Sweetened tea	115	29	7
Regular soda	156	37	9
Lemonade	171	43	10
Fruit drinks	238	59	14

Data Source: U.S. Department of Agriculture, Agricultural Research Service. 2020. *USDA Food and Nutrient Database for Dietary Studies and USDA Food Patterns Equivalents Database 2017–2018.* Food Surveys Research Group Home Page, http://ars.usda.gov/nea/bhnrc/fsrg.

42.3.7 Dietary Components of Public Health Concern for Underconsumption

Current inadequate intake of nutrient-dense foods and beverages across food groups has resulted in underconsumption of some nutrients and dietary components. Calcium, potassium, dietary fiber, and vitamin D are considered dietary components

of public health concern for the general U.S. population because low intakes are associated with health concerns.

If a healthy dietary pattern is consumed, amounts of calcium, potassium, and dietary fiber can meet recommendations. Individuals should be encouraged to make shifts to increase the intake of vegetables, fruits, beans, whole grains, and dairy to move intakes of these under-consumed dietary components closer to recommendations. In some cases, fortified foods and dietary supplements may be useful in providing one or more nutrients that otherwise may be consumed in less than recommended amounts. Vitamin D recommendations are harder to achieve through natural sources from diet alone and would require consuming foods and beverages fortified with vitamin D. In many cases, taking a vitamin D supplement may be appropriate especially when sunlight exposure is limited due to climate or the use of sunscreen. Lists of dietary sources of calcium, potassium, dietary fiber, and vitamin D are available at http://www.dietaryguidelines.gov/.

42.4 Guideline 4: Limit Foods and Beverages Higher in Added Sugars, Saturated Fat, and Sodium, and Limit Alcoholic Beverages

A healthy dietary pattern is designed to meet food group and nutrient recommendations while staying within calorie needs. Additionally, a healthy dietary pattern is designed to not exceed the Tolerable Upper Intake Level (UL) or Chronic Disease Risk Reduction (CDRR) level for nutrients. To achieve these goals, the pattern is based on consuming foods and beverages in their nutrient-dense forms—forms with the least amounts of added sugars, saturated fat, and sodium.

Most of the calories a person needs to eat each day—around 85 percent—are needed to meet food group recommendations healthfully, in nutrient-dense forms. The remaining calories—around 15 percent—are calories available for other uses, including for added sugars or saturated fat beyond the small amounts found in nutrient-dense forms of foods and beverages within the pattern, to consume more than the recommended amount of a food group, or for alcoholic beverages. This equates to 250 to 350 remaining calories for calorie patterns appropriate for most Americans.

As such, a nutrient-dense diet, where most nutritional needs are met by 85% of the calories consumed, offers a small amount of leeway to add minimal amounts of added sugars or saturated fat to the diet. For example, one way to use remaining calories is to add small amounts of added sugars or saturated fat to *some* nutrient-dense foods to help make some foods more palatable while working towards meeting food group recommendations—for example, oatmeal with a

small amount of brown sugar or vegetables prepared with small amounts of butter. However, to achieve a healthy dietary pattern, all (or mostly all) food group recommendations should be met with foods and beverages that are in nutrient-dense forms.

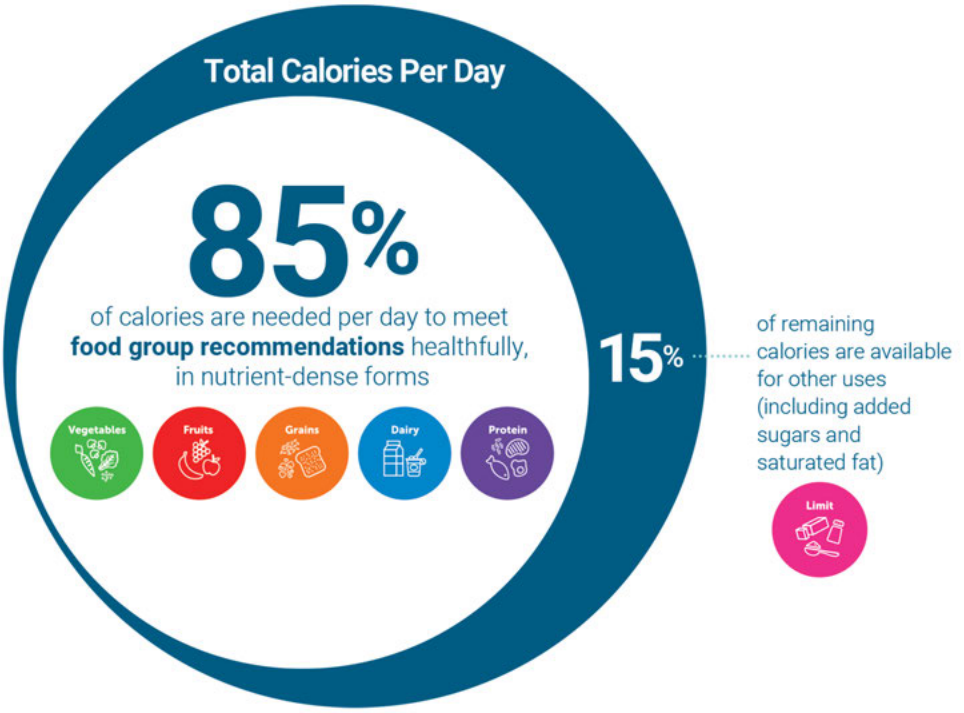


Figure 42.5 The 85–15 guide: percentage of calories needed to meet food group needs with nutrient-dense choices and percentage left for other uses.

A healthy dietary pattern has little room available for foods and beverages high in added sugars, saturated fat, and/or sodium. Intakes of foods and beverages high in these components should be limited. These foods and beverages should be occasional choices— consumed in small portions.

While intakes of added sugars, saturated fat, and sodium should be limited, the guidance below is intended to allow programs and individuals to have some flexibility to choose a healthy dietary pattern within calorie limits that fits personal preferences and cultural traditions—and allows day-to-day flexibility to support a healthy dietary pattern over time. Additionally, if alcoholic beverages are consumed, intakes should be within the limits described in this chapter, and calories should be accounted for to keep total calorie intake at an appropriate level.

42.4.1 Added Sugars

A healthy dietary pattern limits added sugars to less than 10 percent of calories per day. Added sugars can help with preservation; contribute to functional attributes

such as viscosity, texture, body, color, and browning capability, and/or help improve the palatability of some nutrient-dense foods. In fact, the nutrient-dense choices included in the Healthy U.S.-Style Dietary Pattern are based on availability in the U.S. food supply and include 17–50 calories from added sugars, or 1.5–2 percent of total calories.

Foods and beverages high in calories from added sugars should be limited to help achieve healthy dietary patterns within calorie limits. When added sugars in foods and beverages exceed 10 percent of calories, a healthy dietary pattern within calories limits is very difficult to achieve. Most Americans have less than 8 percent of calories available for added sugars, including the added sugars inherent to a healthy dietary pattern. The limit for added sugars is based on the following assumptions:

Most calorie levels have less than 15 percent of calories remaining after meeting food group recommendations through nutrient-dense choices.

- Approximately half of remaining calories are consumed as saturated fat and half consumed as added sugars.
- Total saturated fat intakes meet the recommendation for less than 10 percent of total calorie intake.
- No alcoholic beverages are consumed.
- Overall calorie intake does not exceed intake needs to maintain or achieve a healthy weight.

Based on the assumptions above, an individual who needs 2,000 calories per day (based on age, sex, and physical activity level) has less than 7 percent of calories available for added sugars. Individuals who need 2,800 calories per day or less have less than 8 percent of calories available for added sugars. Individuals who need more than 3,000 calories may have a total of 9 to 10 percent of calories available for added sugars. In this portion of the population that requires high calorie intake, an upper limit of 10 percent of calories from added sugars may be consumed while still meeting food group recommendations in nutrient-dense forms. The 10 percent added sugar limit allows for flexibility in food choices over time but also requires careful planning. For example, if one chooses to eat less than the allotted amount of calories for saturated fat, 10 percent of added sugars may fit in a healthy dietary pattern. Added sugars account on average for almost 270 calories—or more than 13 percent of total calories—per day in the U.S. population. The major sources of added sugars in typical U.S. diets are sugar-sweetened beverages, desserts and sweet snacks, sweetened coffee and tea, and candy. Together, these food categories make up more than half of the intake of all added sugars while contributing very little to food group recommendations.

Individuals have many potential options for reducing the intake of added sugars, including reducing the intake of major sources of added sugars. Strategies include reducing portions, consuming these items less often, and selecting options low in added sugars. For those with a weight loss goal, limiting intake of foods and beverages high in added sugars is a strategy to help reduce calorie intake. It should be noted that replacing added sugars with low- and no-calorie sweeteners may reduce calorie intake in the short-term and aid in weight management, yet questions remain about their effectiveness as a long-term weight management strategy. For additional information about high-intensity sweeteners permitted for use in food in the United States, see http://www.fda.gov/food/food-additives-petitions/high-intensity-sweeteners.

42.4.2 Saturated Fat

For those 2 years and older, intake of saturated fat should be limited to less than 10 percent of calories per day by replacing them with unsaturated fats, particularly polyunsaturated fats. Although some saturated fat is inherent in foods (e.g., high-fat meat), some sources are added (e.g., butter on toast). Similar to added sugars, some of the nutrient-dense choices included in the Healthy U.S.-Style Dietary Pattern include saturated fat. Approximately 5 percent of total calories inherent to the nutrient-dense foods in the Healthy U.S.-Style Dietary Pattern are from saturated fat from sources such as lean meat, poultry, and eggs; nuts and seeds; grains; and saturated fatty acids in oils. As such, there is little room to include additional saturated fat in a healthy dietary pattern while staying within limits for saturated fat and total calories. Current average intakes of saturated fat are 11 percent of calories. Only 23 percent of individuals consume amounts of saturated fat consistent with the limit of less than 10 percent of calories. The main sources of saturated fat in the U.S. diet include sandwiches, including burgers, tacos, and burritos; desserts and sweet snacks; and rice, pasta, and other grain-based mixed dishes. Saturated fat is commonly found in higher amounts in high-fat meat, full-fat dairy products (e.g., whole milk, ice cream, cheese), butter, coconut oil, and palm kernel and palm oil. Strategies to lower saturated fat intake include reducing intakes of dessert and sweet snacks by consuming smaller portion sizes and eating these foods less often. Additional strategies include reading food labels to choose packaged foods lower in saturated fats and choosing lower fat forms of foods and beverages (e.g., fat-free or low-fat milk instead of 2 percent or whole milk; lean rather than fatty cuts of meat). When cooking and purchasing meals, select lean meat and lower fat cheese in place of high-fat meats and regular cheese—or replace them with ingredients with oils, such as nuts, seeds, or avocado. Cook and purchase products made with oils higher in polyunsaturated and monounsaturated fat (e.g., canola, corn, olive, peanut, safflower, soybean, and sunflower) rather than butter, shortening, or

coconut or palm oils. A note on *trans* fats and dietary cholesterol: The National Academies recommends that *trans*-fat and dietary cholesterol consumption to be as low as possible without compromising the nutritional adequacy of the diet. The USDA Dietary Patterns are limited in *trans* fats and low in dietary cholesterol. Cholesterol and a small amount of *trans*-fat occur naturally in some animal source foods. As of June 2018, partially hydrogenated oils (PHOs), the major source of artificial *trans*-fat in the food supply, are no longer Generally Recognized as Safe (GRAS). Therefore, PHOs are no longer added to foods.

42.4.3 Sodium

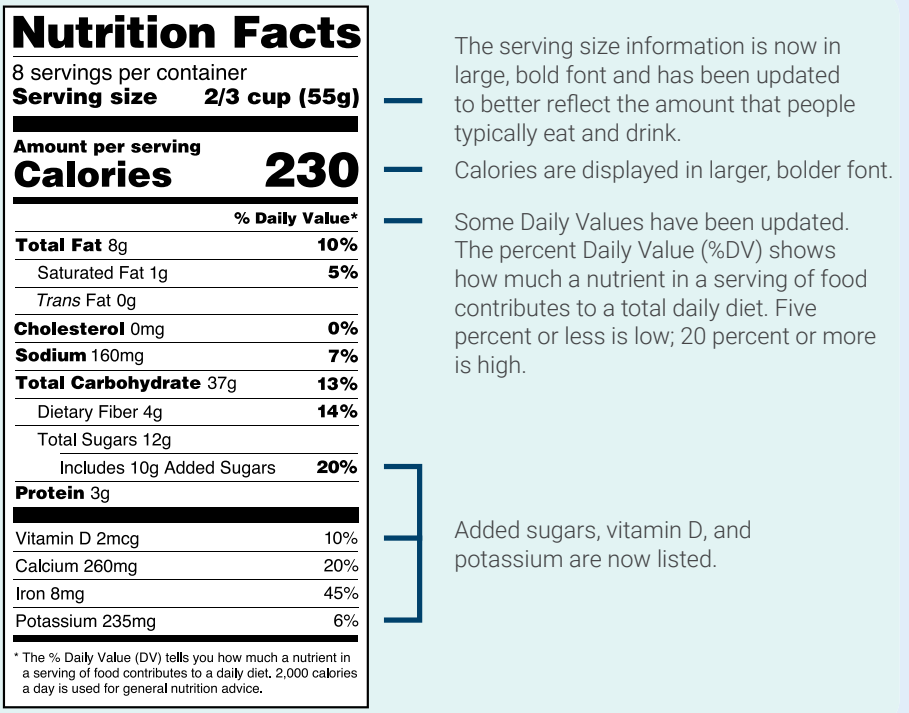
Sodium is an essential nutrient primarily consumed as salt (sodium chloride). Healthy eating patterns limit sodium to the Chronic Disease Risk Reduction (CDRR) levels defined by the National Academies—1,200 mg/day for ages 1 through 3; 1,500 mg/day for ages 4 through 8; 1,800 mg/day for ages 9 through 13; and 2,300 mg/day for all other age groups. The CDRR for sodium was established using evidence of the benefit of reducing sodium intake on cardiovascular risk and hypertension risk.

As a food ingredient, sodium is used in multiple ways, including curing meat, baking, as a thickening agent, as a flavor enhancer, as a preservative, and to retain moisture. The nutrient-dense choices in the Healthy U.S.-Style Dietary Pattern provide approximately 60–100 percent of the age-specific CDRR for sodium across calorie levels with amounts ranging from about 1,000 to 2,200 mg. For most calorie levels and at most ages, there is very little room for food choices that are high in sodium. Average intakes of sodium are high across the U.S. population compared to the CDRRs. Average intakes for those ages 1 and older is 3,393 milligrams per day, with a range of about 2,000 to 5,000 mg per day. Only a small proportion of total sodium intake is from sodium inherent in foods or from salt added in home cooking or at the table. Most sodium consumed in the United States comes from salt added during commercial food processing and preparation, including foods prepared at restaurants. Sodium is found in foods from almost all food categories across the food supply, including mixed dishes such as sandwiches, burgers, and tacos; rice, pasta, and grain dishes; pizza; meat, poultry, and seafood dishes; and soups. Calorie intake is highly associated with sodium intake (i.e., the more foods and beverages

people consume, the more sodium they tend to consume). Because sodium is found in so many foods, multiple strategies should be implemented to reduce sodium intake to the recommended limits. Careful choices are needed in all food groups to reduce intake. Strategies to lower sodium intake include cooking at home more often; using the Nutrition Facts label to choose products with less sodium, reduced sodium, or no-salt-added, etc.; and flavoring foods with herbs and spices instead of salt based on personal and cultural foodways

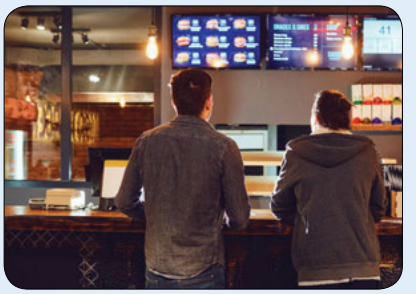
Nutrition Facts Label

The Nutrition Facts label on packaged foods and beverages is a tool for making informed and healthy food choices. For the first time in more than 20 years, the U.S. Food and Drug Administration (FDA) has updated the Nutrition Facts label. There are a number of key changes to the label including:



Along with the updated design, the Nutrition Facts label helps support healthy dietary patterns by providing information on nutrients of public health concern—dietary fiber, vitamin D, calcium, iron, and potassium—and on dietary components to limit, such as added sugars, saturated fat, and sodium. More information on the Nutrition Facts label is available at: https://www.fda.gov/food/nutrition-education-resources-materials/new-nutrition-facts-label.

Menu Nutrition Labeling



Americans eat and drink about one-third of their calories from foods prepared away from home. Usually, these foods provide more calories, saturated fat, and sodium than meals prepared at home. To help individuals make informed and healthy decisions, many food establishments and chain restaurants list calories in foods or beverages on menus or menu boards and additional nutrition information is available upon request. More information is available at https://www.fda.gov/food/nutrition-education-resources-materials/calories-menu.

42.4.4 Alcoholic Beverages

The *Dietary Guidelines* (https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf) does not recommend that individuals who do not drink alcohol start drinking for any reason. There are also some people who should not drink at all, such as if they are pregnant or might be pregnant; under the legal age for drinking; if they have certain medical conditions or are taking certain medications that can interact with alcohol; and if they are recovering from an alcohol use disorder or if they are unable to control the amount they drink. If adults age 21 years and older choose to drink alcoholic beverages, drinking less is better for health than drinking more. Evidence indicates that, among those who drink, higher average alcohol consumption is associated with an increased risk of death from all causes compared with lower average alcohol consumption. Alcohol misuse or consuming alcohol in excess of recommendations increases risk of several other conditions such as liver disease, cardiovascular disease, injuries, and alcohol use disorders. For the purposes of evaluating amounts of alcohol that may be consumed, the *Dietary Guidelines* defines drink equivalents. One alcoholic drink equivalent is defined as containing 14 grams (0.6 floz) of pure alcohol. The following count as one alcoholic drink equivalent: 12 fluid ounces of regular beer (5% alcohol), 5 fluid ounces of wine (12% alcohol), or 1.5 fluid ounces of 80 proof distilled spirits (40% alcohol). To help Americans move toward a healthy dietary pattern and minimize risks associated with drinking, adults of legal drinking age can choose not to drink or to drink in moderation by limiting intakes to 2 drinks or less in a day for men and 1 drink or less in a day for women, on days when alcohol is consumed. This is not intended as an average over several days, but rather the amount consumed on any single day. Binge drinking,[4] defined as 5 or more drinks for the typical adult male or 4 or more drinks for the typical adult female in about 2 hours, should be avoided. Emerging evidence suggests that even drinking

[4]More information is available at http://niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking.

within the recommended limits may increase the overall risk of death from various causes, such as from several types of cancer and some forms of cardiovascular disease. Alcohol has been found to increase risk for cancer, and for some types of cancer, the risk increases even at low levels of alcohol consumption (less than 1 drink in a day). Caution, therefore, is recommended. Alcoholic beverages are not a component of the USDA Dietary Patterns. The amount of alcohol and calories in beverages varies and should be accounted for within the limits of healthy dietary patterns, so that calorie limits are not exceeded (see the box titled "Calories in Alcoholic Beverages"). Approximately 60 percent of adults report alcoholic beverage consumption in the past month. Of those, approximately 30 percent binge drink, sometimes multiple times per month. During days when men and women consume alcohol, their consumption typically exceeds current guidance. Among adults, including those who do not drink, alcoholic beverages contribute approximately 5 percent of calorie intake (3 to 4% of calories for women and 5 to 7% for men); this translates into approximately 9 percent of calories among those who drink. As such, among those who drink, alcoholic beverages, alone, account for most of the calories that remain after meeting food group recommendations in nutrient-dense forms—leaving very few calories for added sugars or saturated fat.

Adults who choose to drink, and are not among the individuals listed above who should not drink, are encouraged to limit daily intakes to align with the *Dietary Guidelines*—and to consider calories from alcoholic beverages so as not to exceed daily calorie limits.

Calories in Alcoholic Beverages

Alcoholic beverages supply calories but few nutrients, and calories from alcoholic beverages should be accounted for to keep total calorie intake at an appropriate level. Alcoholic beverages may contain calories from both alcohol and other ingredients, such as soda, juice, and added sugars. It is important to consider ingredients and portion size. The range of calories in cocktails varies widely depending on serving size and ingredients. Examples of calories contained in alcoholic beverages include:

More information on calories in alcoholic beverages is available at **rethinkingdrinking.niaaa.nih.gov/Tools/Calculators/calorie-calculator.aspx**.

42.5 Support Healthy Dietary Patterns for All Americans

Everyone has a role to play to support access to healthy foods and beverages in multiple settings nationwide where people live, learn, work, play, and gather. Having access to healthy, safe, and affordable food is crucial for an individual to achieve a healthy dietary pattern. Concerted efforts within communities, businesses and industries, organizations, government, and other segments of society are needed to support individuals and families in making lifestyle choices that align with the *Dietary Guidelines* (https://www.dietaryguidelines.gov/sites/default/files/2020-12/Dietary_Guidelines_for_Americans_2020-2025.pdf). Food manufacturers and retail establishments can support Americans in achieving a healthy dietary pattern by providing healthy options in all the places where foods and beverages are purchased. During the past few decades, food products and menus have evolved substantially in response to consumer demand and public health concerns. Food reformulation and menu and retail modification opportunities include offering more vegetables, fruits, whole grains, low-fat and fat-free dairy, and a greater variety of protein foods that are nutrient dense, while also reducing sodium and added sugars, reducing saturated fat and replacing it with unsaturated fats, and reducing added refined starches. Portion sizes also can be reduced to help individuals make choices that better fit within their calorie needs. Food manufacturers are encouraged to consider the entire composition of the food or beverage, and not just individual nutrients or ingredients when developing or reformulating products. Similarly, when developing or modifying menus, establishments can consider the range of offerings both within and across food groups and other dietary components to determine whether the healthy options offered reflect the proportions in healthy dietary patterns. In taking these actions, care should be taken to assess any potential unintended consequences so that as changes are made to better align with the *Dietary Guidelines*, undesirable changes are not introduced. For example, a change made to reduce the amount of added sugars in a product should not come at the expense of increasing the amount of saturated fat or sodium. Food access is influenced by diverse factors, such as proximity to food retail outlets (e.g., the number and types of stores in an area), ability to prepare one's own meals or eat independently, and the availability of personal or public transportation. The underlying socioeconomic characteristics of a neighborhood also may influence an individual's ability to access foods to support healthy eating patterns. In 2019, 10.5 percent of households were food insecure at least some time during the year. Food insecurity occurs when access to nutritionally adequate and safe food is limited or uncertain. Food insecurity can be temporary or persist over time, preventing individuals and families from following a healthy dietary pattern that aligns with the *Dietary Guidelines*. The prevalence of food insecurity typically rises during times of economic downturn as households experience greater hardship. Government and nongovernment nutrition assistance programs help alleviate food insecurity and play an essential role by providing food, meals, and educational resources so that participants can

make healthy food choices within their budget. As discussed in subsequent chapters, everyone has an important role in leading disease prevention efforts within their organizations and communities to make healthy eating an organizational and societal norm. Changes at multiple levels of society are needed, and these changes, in combination and over time, can have a meaningful impact on the health of current and future generations.

Chapter 43

Using Dietary Supplements Wisely[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: botanicals, chondroitin, coenzyme Q10, cranberry (pills or capsules), dietary supplements, docosahexaenoic acid (DHA), echinacea, eicosapentaenoic acid (EPA), fatty acids, fish oil, garlic supplements, *Ginkgo biloba*, ginseng, glucosamine, melatonin, omega 3, Phenytoin, prebiotics, probiotics, St. John's wort, Warfarin

43.1 What's the Bottom Line?

How much do we know about dietary supplements?

The amount of scientific evidence we have on dietary supplements varies widely—we have a lot of information on some and very little on others.

What do we know about the effectiveness of dietary supplements?

- Studies have found that some dietary supplements may have some benefit, such as melatonin for jet lag, and others may have little or no benefit, such as ginkgo for dementia.

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at the NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH.

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- Supplements you buy from stores or online may differ in important ways from products tested in studies.
- Most research shows that taking multivitamins doesn't result in living longer, slowing cognitive decline, or lowering the chance of getting cancer, heart disease, or diabetes.

What do we know about the safety of dietary supplements?

- Taking a multivitamin is unlikely to pose any health risks.
- Dietary supplements may interact with your medications or pose risks if you have certain medical problems or are going to have surgery.
- Many dietary supplements haven't been tested in pregnant women, nursing mothers, or children.
- Some products marketed as dietary supplements—promoted mainly for weight loss, sexual enhancement, and bodybuilding—may contain prescription drugs not allowed in dietary supplements or other ingredients not listed on the label. Some of these ingredients may be unsafe.

43.2 What Are Dietary Supplements?

Federal law defines dietary supplements as products that:

- You take by mouth (such as a tablet, capsule, powder, or liquid)
- Are made to supplement the diet
- Have one or more dietary ingredients, including vitamins, minerals, herbs or other botanicals, amino acids, enzymes, tissues from organs or glands, or extracts of these
- Are labeled as being dietary supplements.

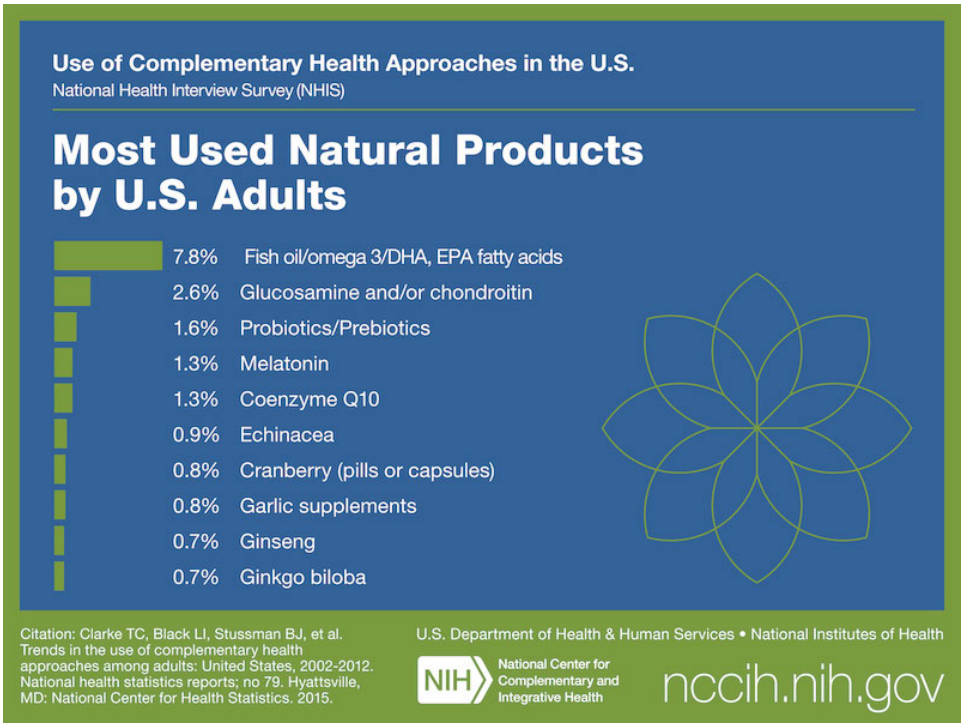
43.3 What Are Herbal Supplements?

Herbal supplements are a type of dietary supplement containing one or more herbs. They are:

- Sometimes called botanicals
- Made from plants, algae, fungi, or a combination of these
- Sold as teas, extracts, tablets, capsules, powders, or in other forms.

43.4 Dietary Supplement Use in the United States

According to the 2012 National Health Interview Survey, which included questions on Americans' use of natural products (dietary supplements other than vitamins and minerals), almost 18 percent of adults and about 5 percent of children used these products in 2012.



More

- The National Health and Nutrition Examination Survey collected data from 2011 to 2012 on the use of all types of dietary supplements. It found that 52 percent of American adults took at least one dietary supplement. Multivitamin or multimineral supplements—a product having 10 or more vitamins or minerals—were one of the most common, and 31 percent of all adults took them. This was a decrease from 1999 to 2000, when 37 percent of American adults reported using multivitamin or multimineral supplements. Women were more likely than men to take dietary supplements.
- For more information on dietary supplement use in the United States, including among children, see the National Center for Complementary and Integrative Health (NCCIH) webpage with statistics about complementary and integrative health approaches (https://www.nccih.nih.gov/research/statistics-on-complementary-and-integrative-health-approaches).

Why Do People Use Dietary Supplements?

American adults often use dietary supplements for wellness. According to the 2012 National Health Interview Survey, 89 percent of American adults who took dietary supplements other than vitamins and minerals gave wellness-related reasons for using them, including:

- General wellness or disease prevention (83 percent)

- Improve immune function (42 percent)
- Improve energy (31 percent)
- Focuses on the whole person—mind, body, and spirit (27 percent)
- Improve memory or concentration (22 percent).

43.5 Federal Regulation of Dietary Supplements

- Federal regulations state that companies are responsible for having evidence that their dietary supplements are safe and for ensuring that product labels are truthful and not misleading. Manufacturers are required to produce dietary supplements in a quality manner, ensure that they don't contain contaminants or impurities, and label them accurately.
- However, rules for manufacturing and distributing dietary supplements are less strict than those for prescription or over-the-counter drugs.
  - The U.S. Food and Drug Administration (FDA), which regulates dietary supplements, requires that companies submit safety data about any new ingredient not sold in the United States in a dietary supplement before 1994. In all other cases, the FDA is not authorized to review dietary supplements for safety and effectiveness before they are marketed.
  - The FDA can take action against adulterated or misbranded dietary supplements only after the product is on the market. In contrast, companies must show the FDA evidence that their prescription and over-the-counter drugs are safe and effective before the drugs are marketed.
- Once a dietary supplement is on the market, the FDA tracks side effects reported by consumers, supplement companies, and others. You can report any safety concerns you may have about a dietary supplement through the U.S. Health and Human Services Safety Reporting Portal (https://www.safetyreporting.hhs.gov/).
- If the FDA finds a product to be unsafe, it can take legal action against the manufacturer or distributor, and may issue a warning or require that the product be removed from the marketplace. However, the FDA says it can't test all products marketed as dietary supplements that may have potentially harmful hidden ingredients.
- For more information on contaminants in dietary supplements, see the FDA's Dietary Supplement Products & Ingredients webpage (http://www.fda.gov/Food/DietarySupplements/ProductsIngredients/default.htm).

Health and Structure/Function Claims

- The labels on dietary supplements cannot claim that the product can diagnose, treat, cure, mitigate, or prevent any disease; claims like these are

only permitted for drugs. However, some types of claims related to health or the way that the product affects the structure or function of the body may appear on dietary supplement labels.

- **Health claims** describe a relationship between a substance in the supplement and reduced risk of a disease or condition. They must be based on scientific evidence. For example, if a supplement label says, "Calcium may reduce the risk of the bone disease osteoporosis," that's a health claim.
- **Structure/function claims** describe the effect of a substance on maintaining the body's normal structure or function. For example, if a supplement label says, "Calcium builds strong bones," that's a structure/function claim. Structure/function claims on dietary supplement labels must be accompanied by this disclaimer: "This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, mitigate, or prevent any disease."
- **Advertising** The U.S. Federal Trade Commission (FTC), which regulates advertising, requires that advertising be truthful and not misleading.

43.6 What the Science Says about the Effectiveness of Dietary Supplements

- Some dietary supplements can be good for your health, while others haven't been proven to work. For information on the effectiveness of different supplements, see the NCCIH webpage about dietary supplements (https://www.nccih.nih.gov/health/supplements).
- Studies of some supplements haven't supported claims made about them. For example, in several studies, echinacea didn't help cure colds and *Ginkgo biloba* wasn't useful for dementia. Many times the research on a dietary supplement is conflicting, such as whether the supplements glucosamine and chondroitin improve symptoms of osteoarthritis.

43.7 What the Science Says about the Safety and Side Effects of Dietary Supplements

- What's on the label may not be what's in the product. For example, the FDA has found prescription drugs, including anticoagulants (e.g., warfarin), anticonvulsants (e.g., phenytoin), and others, in products being sold as dietary supplements. You can see a list of some of those products on the FDA's Tainted Supplements webpage (https://www.accessdata.fda.gov/scripts/sda/sdNavigation.cfm?filter=&sortColumn=1d&sd=tainted_supplements_cder&page=1).

- A 2012 Government study of 127 dietary supplements marketed for weight loss or to support the immune system found that 20 percent made illegal claims.
- Some dietary supplements may harm you if you have a particular medical condition or risk factor or are taking certain prescription or over-the-counter medications. For example, the herbal supplement St. John's wort makes many medications less effective.
- Dietary supplements result in an estimated 23,000 emergency room visits every year in the United States, according to a 2015 study. Many of the patients are young adults having heart problems from weight-loss or energy products and older adults having swallowing problems from taking large vitamin pills.
- Although it's still rare, more cases are being reported of acute (sudden) liver damage in people taking dietary supplements in the United States and elsewhere. The liver injury can be severe, can require an emergency liver transplant, and is sometimes fatal.
- Many dietary supplements (and some prescription drugs) come from natural sources, but For example, the kava plant is a member of the pepper family but taking kava supplements can cause liver disease.
- A manufacturer's use of the term **"standardized" (or "verified" or "certified") does not necessarily guarantee product quality or consistency**.

43.8 Safety Considerations

- If you're going to have surgery, be aware that **certain dietary supplements may increase the risk of bleeding or affect your response to anesthesia**. Talk to your health care providers as far in advance of the operation as possible and tell them about all dietary supplements that you're taking.
- If you're pregnant, nursing a baby, trying to get pregnant, or considering giving a child a dietary supplement, consider that many **dietary supplements have not been tested in pregnant women, nursing mothers, or children**.
- If you're taking a dietary supplement, **follow the instructions on the label**. If you have side effects, stop taking the supplement and contact your health care provider. You may also want to contact the supplement manufacturer.

Take charge of your health—talk with your health care providers about any complementary health approaches you use. Together, you can make shared, well-informed decisions.

For More Information

NCCIH Clearinghouse

The NCCIH Clearinghouse provides information on NCCIH and complementary and integrative health approaches, including publications and searches of Federal

databases of scientific and medical literature. The Clearinghouse does not provide medical advice, treatment recommendations, or referrals to practitioners.

Toll-free in the U.S.: 1-888-644-6226 tty (for deaf and hard-of-hearing callers): 1-866-464-3615

Website: https://nccih.nih.gov/

Email: info@nccih.nih.gov(link sends email)

PubMed®

A service of the National Library of Medicine, PubMed® contains publication information and (in most cases) brief summaries of articles from scientific and medical journals. For guidance from NCCIH on using PubMed, see How To Find Information About Complementary Health Approaches on PubMed.

Website: https://pubmed.ncbi.nlm.nih.gov/

Office of Dietary Supplements (ODS), National Institutes of Health (NIH)

ODS seeks to strengthen knowledge and understanding of dietary supplements by evaluating scientific information, supporting research, sharing research results, and educating the public. Its resources include publications (such as *Dietary Supplements: What You Need To Know*; https://ods.od.nih.gov/factsheets/WYNTK-Consumer/) and fact sheets on a variety of specific supplement ingredients and products (such as vitamin D and multivitamin/mineral supplements).

Website: https://ods.od.nih.gov/

Email: ods@nih.gov(link sends email)

U.S. Food and Drug Administration (FDA)

The FDA oversees the safety of many products, such as foods, medicines, dietary supplements, medical devices, and cosmetics. See its webpage on Dietary Supplements (http://www.fda.gov/Food/DietarySupplements/default.htm).

Toll-free in the U.S.: 1-888-463-6332

Website: https://www.fda.gov/

Center for Food Safety and Applied Nutrition (CFSAN)

Part of the FDA, CFSAN oversees the safety and labeling of supplements, foods, and cosmetics. It provides information on dietary supplements. Online resources for consumers include *Tips for Dietary Supplement Users: Making Informed Decisions and Evaluating Information.*

Toll-free in the U.S.: 1-888-723-3366

Website: https://www.fda.gov/AboutFDA/CentersOffices/OfficeofFoods/CFSAN/

Federal Trade Commission (FTC)

The FTC is the Federal agency charged with protecting the public against unfair and deceptive business practices. A key area of its work is the regulation of advertising (except for prescription drugs and medical devices).

Toll-free in the U.S.: 1-877-382-4357

Website: https://www.ftc.gov/

MedlinePlus

To provide resources that help answer health questions, MedlinePlus (a service of the National Library of Medicine) brings together authoritative information from the National Institutes of Health as well as other Government agencies and health-related organizations.

Website: https://www.medlineplus.gov/

Dietary Supplement Label Database

The Dietary Supplement Label Database—a project of the National Institutes of Health—has all the information found on labels of many brands of dietary supplements marketed in the United States. Users can compare the amount of a nutrient listed on a label with the Government's recommended amounts.

Website: https://dsld.od.nih.gov/

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Chapter 44

Association of Genetic Liability to Smoking Initiation with e-Cigarette Use in Young Adults: A Cohort Study

Jasmine N. Khouja, PhD,[a,b,c] Robyn E. Wootton, PhD,[a,b] Amy E. Taylor, PhD,[b,d] George Davey Smith, MD, DSc,[a,b] and Marcus R. Munafò, PhD[a,c,d]

[a]*MRC Integrative Epidemiology Unit at the University of Bristol, Bristol, UK*
[b]*Bristol Medical School: Population Health Sciences, University of Bristol, Bristol, UK*
[c]*School of Psychological Science, University of Bristol, Bristol, UK*
[d]*NIHR Biomedical Research Centre at the University Hospitals Bristol NHS Foundation Trust and the University of Bristol, Bristol, UK*

jasmine.khouja@bristol.ac.uk

Keywords: attention-deficit/hyperactivity disorder (ADHD), conduct disorder (CD), development and wellbeing assessment (DAWBA), electronic cigarette (e-cigarette), genome-wide association studies (GWAS), horizontal pleiotropy, hyperactivity, Instrument Strength Independent of Direct Effect (InSIDE), Mendelian randomization (MR), minor allele frequency (MAF), negative control, odds ratio (OR), oppositional defiant disorder (ODD), polygenic risk scores (PRS), single nucleotide polymorphisms (SNPs), socioeconomic position (SEP), standard deviation (SD), Strengthening the Reporting of Observational Studies in Epidemiology (STROBE), strengths and difficulties questionnaire (SDQ), vertical pleiotropy

44.1 Introduction

There are an estimated 3.6 million electronic cigarette (e-cigarette) users in Great Britain [1], and evidence is growing that e-cigarettes are effective in helping tobacco smokers quit [2, 3]. The use of e-cigarettes for smoking cessation is common among young adults in the United Kingdom [4]; therefore, it would be logical to assume that smoking causally influences e-cigarette use in this population. However, some studies have shown an association between e-cigarette use and subsequent smoking among nonsmokers, which suggests the possibility

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that e-cigarette use may also act as a gateway to smoking (sometimes referred to as the gateway hypothesis), particularly among adolescents. A recent meta-analysis found that for young people aged 30 years or younger, there is a strong and consistent positive association between e-cigarette use among never smokers and later smoking, but that there is currently insufficient evidence to conclude that this association is causal [5]. Understanding more about the nature of the association between smoking and e-cigarette use, particularly in young adulthood, is vital to inform tobacco control policies that aim to prevent youth smoking initiation by restricting access to e-cigarettes. Specifically, it is important to understand whether the association found among young adults is causal, or due to other factors that influence both smoking and e-cigarette use independently.

For example, there is some evidence for a shared genetic liability to both smoking and e-cigarette use [6]. This could indicate a causal relationship in that genetic variants influence smoking which then increases the probability of e-cigarette use (i.e., vertical pleiotropy), or it could be due to genetic variants that influence a phenotype which consequently influences both behaviours (i.e., horizontal pleiotropy) [7]. One biologically plausible explanation for a genetic link between smoking and e-cigarette use is that they are both influenced by the same genetic variants that influence an individual's response to nicotine or their nicotine metabolism. However, evidence suggests that some of the genetic influence on smoking initiation is mediated by personality traits, such as risk-taking and impulsivity, that influence (among other things) smoking uptake [8]. Allegrini and colleagues [6] suggest that a genetic link between smoking and e-cigarette use may reflect these personality traits (i.e., a genetic liability to take risks may influence an individual's likelihood of initiating smoking and e-cigarette use).

Using genetic variants, we can explore whether smoking is associated with e-cigarette use, and which factors or mechanisms may influence the association. Ideally, we would explore the genetic overlap between smoking and e-cigarette use by comparing the genetic variants identified in genome-wide association studies (GWAS) of each behaviour, but at present, there are no large, well-powered GWAS of e-cigarette use. However, a GWAS of various smoking behaviours has recently been published [9], which identified 378 single nucleotide polymorphisms (SNPs) associated with smoking initiation. Using these SNPs, smoking initiation polygenic risk scores (PRS) can be created and associations between these PRS and a range of outcomes examined.

Causality cannot be inferred from such analyses, but negative control outcomes can be used to inform the overall evaluation of whether an association is causal via a hypothesised route. Negative controls are outcomes which are not plausibly caused by the exposure—for example, smoking is associated with risk of dying by suicide (which is biologically plausible), but equally strongly associated with risk of dying by homicide (which is not), casting doubt on the causal nature of the former association [10]. Triangulating evidence from outcomes where a simple biological pathway from smoking to the outcome is implausible (e.g., gambling), or impossible (e.g., externalising behaviour or socioeconomic position

[SEP] in childhood, before smoking has occurred) can aid consideration of potential pathways by which smoking and e-cigarette use may share a genetic predisposition. These potential pathways (displayed in Fig. 44.1) include a biological pathway from smoking to e-cigarette use (i.e., vertical pleiotropy), a shared genetic predisposition which influences smoking and e-cigarette use independently (i.e., horizontal pleiotropy), or a genetic liability to a broader, risk-taking phenotype (i.e., a shared risk factor) which causes both smoking and e-cigarette use. Alternatively, triangulation could help us consider whether an association is due to a shared genetic predisposition between parents and offspring. Where parents share their offspring's smoking initiation predisposition, they are likely to expose their offspring to cigarette smoke in utero or in childhood. Consequently, an apparent effect of a child's own genetic variants may be a result of their prenatal or postnatal environment due to a dynastic effect of their parents' genetic variants. If associations are only found between smoking initiation PRS and e-cigarette use, but not negative control outcomes, this would strengthen the vertical pleiotropy interpretation; however, if an association is also found with negative control outcomes, this would indicate that horizontal pleiotropy is occurring or that shared parent–offspring genetic predisposition may be confounding the association.

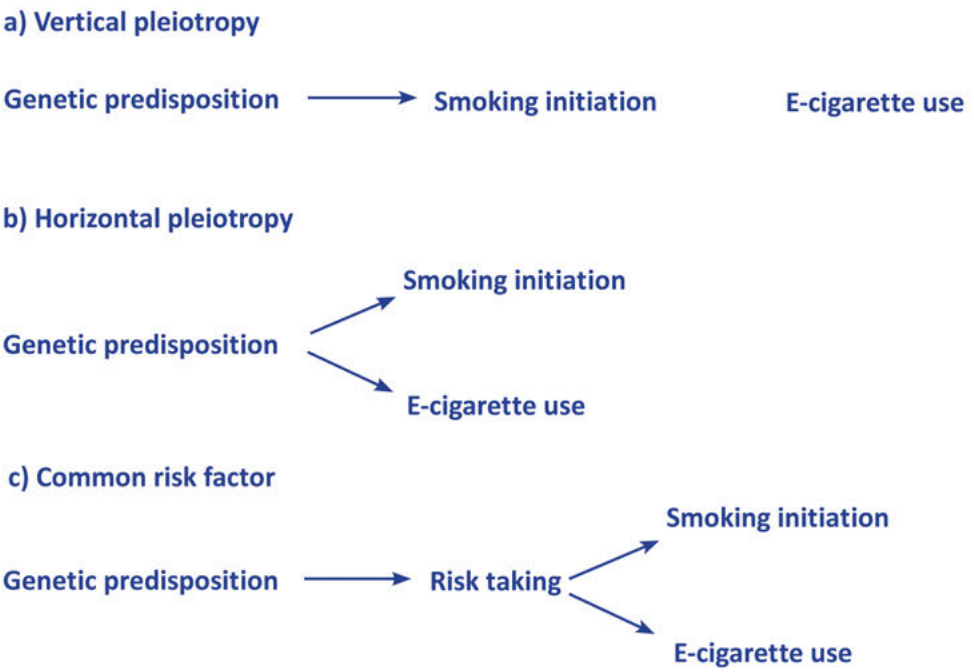

Figure 44.1 Potential models of shared liability for the relationship between genetic predisposition to smoking initiation and e-cigarette use.

Additionally, using varying *p*-value thresholds to create PRS could help to identify the presence of horizontal pleiotropy. Calculating PRS at less strict *p*-value

thresholds than the standard genome-wide significant threshold increases the percentage variance in the phenotype explained by the score, and thus increases power to detect an association. However, using less stringent thresholds will also tend to increase the likelihood of including genetic variants which are related to other factors, making the PRS less specific to the exposure of interest (and may eventually result in PRS which explain less variance in the exposure). The more SNPs included in a PRS, the less likely it is that the effect of each variant on the trait of interest is proportional to the effect of the trait of interest on the exposure, and the more likely it is proportional to the effects on other (horizontally pleiotropic) traits [11], increasing the likelihood that any associations found between the PRS and an outcome could be due to horizontal pleiotropy. Triangulating evidence from a variety of thresholds and a variety of outcomes may provide a clearer picture of the true association. Associations observed when more stringent PRS thresholds are used could be due to a causal effect of smoking, and consistent magnitudes of association at less stringent thresholds could indicate that any associations observed are driven by the effect of the more specific PRS. However, increasing magnitudes of association observed at less stringent thresholds (particularly among negative control outcomes) may indicate horizontal pleiotropy is driving part of the associations observed.

We aimed to investigate whether smoking initiation PRS are associated with ever use of e-cigarettes in young adulthood. Given the possibility of a shared liability mechanism (e.g., an underlying risk-taking phenotype), we also aimed to explore any associations with outcomes that are not plausibly biologically related (e.g., gambling) or that precede smoking (e.g., hyperactivity in childhood), to determine whether the association between smoking and e-cigarette use could reflect a broader risk-taking phenotype captured by the smoking initiation PRS (i.e., a common risk factor). Finally, we aimed to explore whether the smoking initiation PRS may be capturing broader social influences on smoking (e.g., socioeconomic position at birth) which cannot plausibly have been a causal effect of the young adult's own smoking.

44.1.1 Methods

This study is reported as per the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline (S1 STROBE Checklist).

44.1.2 Data Sources

Two data sources were utilised for this study: the GWAS & Sequencing Consortium of Alcohol and Nicotine use (the discovery sample; GSCAN) and the Avon Longitudinal Study of Parents and Children (the target sample; ALSPAC) [9, 12, 13].

GSCAN. GSCAN report summary level statistics from a GWAS of smoking initiation [9]. This GWAS was based on 1,232,091 participants from 29 cohorts.

In order to eliminate data overlap with the target sample, summary statistics were obtained (through correspondence with GSCAN) with ALSPAC participants removed (N = 11,345). 23andMe participants (N = 599,289) were also excluded from this summary data due to data sharing restrictions. The remaining summary data consisted of data from 621,457 participants. Smoking initiation was defined as ever being a regular smoker. The exact definition varied across the cohorts included in the consortium, with 3 different definitions: (1) Have you smoked over 100 cigarettes over the course of your life? (2) Have you ever smoked every day for at least a month? (3) Have you ever smoked regularly? The majority of the SNPs identified were intergenic with no known function, but glutamate and dopaminergic gene pathways were enriched for smoking initiation. Also, the rs6265 variant (a nonsynonymous SNP in the BDNF gene) which has previously been found to be associated with smoking initiation [14] was also associated with smoking initiation in GSCAN. A comprehensive description of the genetic variants, and the genes they are within (e.g., PPP1R1B, GRIN2A, HOMER2), have been described previously [9].

ALSPAC. The target sample consisted of participants from ALSPAC [12, 13], a prospective cohort study with extensive data from birth to young adulthood (including genetic data). This study recruited pregnant women residing in Avon, UK with expected delivery dates between 1 April 1991 and 31 December 1992. The phases of enrolment are described in detail in the cohort profile paper and its update [15]. A total of 15,454 mothers were recruited, resulting in 15,589 foetuses. Of these, 14,901 children were alive at 1 year of age. The study website contains details of all the data that are available via a fully searchable data dictionary and variable search tool (*http://www.bristol.ac.uk/alspac/researchers/our-data/*). After samples that did not pass quality control were removed, genetic data were available for 9,085 young adults. PRS were created for 7,859 unrelated individuals of European ancestry. Of these individuals, 2,905 also had data for our main outcome (e-cigarette use) at 24 years (the most recent time point at which detailed e-cigarette use data were collected prior to analysis). ALSPAC study data from 22 years onwards were collected and managed using REDCap electronic data capture tools hosted at the University of Bristol [16]. Sample sizes varied by outcome due to restrictions (e.g., restricting to never smokers) and differing time points of measurement (i.e., missing data).

44.1.3 Ethics

Ethics approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Written informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time of study initiation (i.e., 1991).

44.1.4 Polygenic Risk Scores

Summary data from GSCAN (excluding ALSPAC and 23andMe, N = 621,457) were used to select SNPs associated with smoking initiation. Betas were converted to log odds ratios (ORs). Each participant was given a score which indicated the average number of risk alleles (0, 1, or 2 effect alleles) they possessed for the selected SNPs. Scores were weighted (i.e., multiplied) by the regression coefficients from the summary statistics (with ALSPAC and 23 and Me removed), then standardised by transforming to z-scores. Five p-value thresholds (5×10^{-8}, 0.0005, 0.005, 0.05, 0.5) were used to determine 5 groups of SNPs to be included in 5 different PRS for each participant. PLINK was used to determine PRS at the $p < 5 \times 10^{-8}$ threshold using the SNPs which met the genome-wide significance threshold in the GSCAN GWAS of smoking initiation [9]. PRSice software was used to calculate the PRS at all other thresholds [17]. The data acquired from GSCAN was pruned for SNPs with a Minor Allele Frequency (MAF) > 0.001 where at least 10% of the maximum sample size had SNP data available in at least 3 of the consortium studies. SNPs were clumped to ensure low linkage disequilibrium ($r^2 < 0.1$).

44.1.5 Outcomes

Detailed information regarding the phenotype data including the questions and answer options provided in the questionnaires are available in Table S1.

E-cigarette use. At 24 years (between 2016 and 2017), outcome data were collected via questionnaire on whether participants had ever used e-cigarettes. Ever use was defined as ever having used/vaped an e-cigarette or other vaping device.

Smoking. Self-reported smoking initiation and ever smoking were included as positive control outcomes (i.e., outcomes for which an association with the exposure is expected). Smoking initiation by 24 years was defined as having smoked 100 or more cigarettes in their lifetime. Ever smoking by 24 years was defined as having ever smoked a whole cigarette (including roll-ups).

Negative controls. Four negative control outcomes at age 23 and 24 were included in the analysis: high number of sexual partners, having been in trouble with the law, ever gambling, and enjoying taking risks. These were selected on the basis of being related to broad risk-taking behaviour, but where a causal pathway from smoking was not considered biologically plausible. Three negative control outcomes at age 7 were included: hyperactivity, conduct disorder (CD), and oppositional defiant disorder (ODD). These externalising disorders are indicators of impulsivity and were selected on the basis that few (if any) children at this age have smoked, ruling out a causal pathway from their own smoking to these outcomes. Parental SEP, which was measured at birth, was also included in the analysis. This outcome was based on highest occupation of both parents at birth (preceding smoking) and was selected on the basis that it could not possibly be caused by a young person's own smoking. Further information regarding the negative controls can be found in S1 Text.

44.1.6 Statistical Analyses

After creating the PRS using PLINK and PRSice software, all analyses were carried out in STATA 15.1 [18]. Using the logistic command, we conducted a series of logistic regressions adjusted for age (in months at the time of the outcome measure), sex, and the first 10 principal components of population stratification (i.e., common subpopulation differences in allele frequencies). We assessed the association between smoking initiation PRS and (i) ever e-cigarette use by age 24 among the full sample and those who had never smoked, (ii) regular e-cigarette use at age 24, (iii) smoking initiation, and (iv) negative control outcomes (risk-taking behaviours, externalising disorders, and SEP). All analyses were repeated for each of the 5 *p*-value thresholds for determining SNP inclusion in the PRS. We also assessed the association between the main outcome of interest (e-cigarette use) and each negative control outcome. These analyses were planned prior to the analysis being conducted and were not data driven; however, the plan was not made publicly available prior to the analysis.

44.2 Results

A total of 378 SNPs were identified as genome-wide significant in the GSCAN GWAS of smoking initiation [9], 356 of which were available in ALSPAC. Nine SNPs were removed at the clumping stage, leaving 347 SNPs included in the most stringent PRS (*p*-value threshold $p < 5 \times 10^{-8}$). The number of SNPs included in each PRS at the less stringent thresholds is shown in Table S2. Of note, PRS calculated at these less stringent thresholds were based on the significance level reported in the restricted sample (excluding ALSPAC and 23andMe) summary data.

Table 44.1 shows the characteristics of the sample; 878 (30%) young adults were self-reported ever e-cigarette users by 24 years, and 1,695 (64%) were self-reported ever smokers. Of those who had ever used an e-cigarette, 95% (n = 830) had ever smoked at least one whole cigarette, and 71% (n = 616) had smoked 100 or more cigarettes. Less than 1% of the sample had used an e-cigarette prior to smoking. Self-reported smoking and e-cigarette use were associated with lower parental SEP and having externalising disorders in childhood (Table S3). Self-reported smoking and e-cigarette use were also associated with increased odds of engaging in risk-taking behaviours (Table S3).

Table 44.1 Characteristics of young adults in ALSPAC

Characteristic	*N* (%)
Ever used an e-cigarette by 24 (used once or more)	878 (30%)
Regularly used an e-cigarette at 24 (used at least once a month)	150 (5%)
Ever smoked by 24 (1 cigarette or more)	1,695 (64%)

(*Continued*)

Table 44.1 (*Continued*)

Characteristic	*N* (%)
Initiated smoking by 24 (100 cigarettes or more)	972 (33%)
Ever used an e-cigarette but not initiated smoking by 24	262 (13%)
High number of sexual partners at 23*	647 (25%)
Been in trouble with the law since 23rd birthday	69 (2%)
Enjoys taking risks at 24	1,618 (55%)
Ever gambled at 24	2,156 (74%)
Hyperactivity at 7	2,219 (42%)
Conduct disorder at 7	1,199 (22%)
Oppositional defiant disorder at 7	1,868 (35%)
Parental SEP (manual)	1,068 (27%)
	Mean (SD)
Age in months at 24-year questionnaire	298 (6)

Sample sizes varied by characteristic due to differing time points of measurement (i.e., missing data).

*Eleven or more sexual partners, determined using the upper quartile for number of lifetime sexual partners in the ALSPAC sample (11 sexual partners).

Abbreviations: ALSPAC, Avon Longitudinal Study of Parents and Children; SEP, socioeconomic position.

44.2.1 Smoking Initiation PRS and Self-Reported Smoking

We observed positive associations between smoking initiation PRS and ever smoking (having smoked at least 1 cigarette in a lifetime) by the age of 24 years ($p < 5 \times 10^{-8}$ threshold OR ($OR_{10\text{-}8}$) = 1.25, 95% CI 1.16 to 1.35, $p < 0.001$) and smoking initiation (having smoked at least 100 cigarettes in a lifetime) by the age of 24 years ($OR_{10\text{-}8}$ = 1.29, 95% CI 1.19 to 1.39, $p < 0.001$). We found strong associations between smoking initiation PRS and self-reported smoking measures at all *p*-value thresholds (Table 44.2).

Table 44.2 Associations between polygenic risk scores for smoking initiation with ever e-cigarette use, ever smoking, and smoking initiation

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
Ever e-cigarette use by age 24	2,894			
5×10^{-8}		1.24	1.14, 1.34	<0.001
0.0005		1.27	1.17, 1.38	<0.001
0.005		1.36	1.26, 1.48	<0.001
0.05		1.39	1.28, 1.51	<0.001
0.5		1.39	1.28, 1.51	<0.001

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
Regular e-cigarette use at age 24 (at least once a month)	2,894			
5×10^{-8}		1.18	1.00, 1.40	0.049
0.0005		1.22	1.03, 1.44	0.019
0.005		1.22	1.04, 1.44	0.017
0.05		1.18	1.00, 1.39	0.051
0.5		1.22	1.04, 1.44	0.018
Ever smoking by age 24 (1 cigarette or more)	2,931			
5×10^{-8}		1.25	1.16, 1.35	<0.001
0.0005		1.27	1.17, 1.38	<0.001
0.005		1.32	1.22, 1.43	<0.001
0.05		1.33	1.23, 1.44	<0.001
0.5		1.34	1.24, 1.44	<0.001
Smoking initiation (100 cigarettes or more) by age 24	2,925			
5×10^{-8}		1.29	1.19, 1.39	<0.001
0.0005		1.38	1.27, 1.49	<0.001
0.005		1.46	1.34, 1.58	<0.001
0.05		1.49	1.37, 1.61	<0.001
0.5		1.49	1.37, 1.39	<0.001
Ever e-cigarette use by age 24 among never smokers (<100 cigarettes)	1,937			
5×10^{-8}		1.10	0.97, 1.26	0.150
0.0005		1.05	0.92, 1.20	0.464
0.005		1.12	0.98, 1.28	0.087
0.05		1.15	1.00, 1.31	0.046
0.5		1.18	1.04, 1.35	0.012

Ever smoking and smoking initiation models were included as positive controls. Analyses were adjusted for age, sex, and principal components 1–10.

Abbreviation: OR, odds ratio.

44.2.2 Smoking Initiation PRS and Self-Reported e-Cigarette use

We observed positive associations between smoking initiation PRS and self-reported ever use of e-cigarettes by the age of 24 years ($OR_{10\text{-}8}$ = 1.24, 95% CI 1.14 to 1.34, $p < 0.001$) and self-reported regular (at least once a month) e-cigarette use at 24 years ($OR_{10\text{-}8}$ = 1.18, 95% CI 1.00 to 1.40, $p = 0.049$). We observed these associations at all *p*-value thresholds (Table 44.2). Among those who had never initiated smoking (i.e., smoked <100 cigarettes in their lifetime), we found no clear

evidence for an association between smoking initiation PRS and ever e-cigarette use at the most stringent *p*-value thresholds. However, we found evidence of a positive association with PRS calculated using less stringent thresholds ($p < 0.5$ threshold OR = 1.18, 95% CI 1.04 to 1.35, $p = 0.012$; Table 44.2). We found similar patterns of association among those who had never smoked any cigarettes (Table S4).

44.2.3 Smoking Initiation PRS and Negative Controls

We observed a positive association between smoking initiation PRS and high number of sexual partners by 23 years ($OR_{10\text{-}8} = 1.15$, 95% CI 1.05 to 1.26, $p = 0.003$) and having ever gambled by 24 years ($OR_{10\text{-}8} = 1.12$, 95% CI 1.03 to 1.22, $p = 0.008$) at all *p*-value thresholds (Table 44.3). We found some evidence of a positive association between smoking initiation PRS and enjoying taking risks at 24 years ($OR_{0.005} = 1.11$, 95% CI 1.03 to 1.19, $p = 0.005$), but this was less clear at the more stringent thresholds (Table 44.3). There was no clear evidence of an association between smoking initiation PRS and having been in trouble with the law since their 23rd birthday (Table 44.3).

Table 44.3 Associations between polygenic risk scores for smoking initiation with negative controls of risky behaviour

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
11 or more sexual partners by age 23*	2,505			
5×10^{-8}		1.15	1.05, 1.26	0.003
0.0005		1.12	1.02, 1.23	0.019
0.005		1.18	1.08, 1.29	<0.001
0.05		1.25	1.14, 1.37	<0.001
0.5		1.30	1.19, 1.43	<0.001
Been in trouble with the law since 23rd birthday	2,928			
5×10^{-8}		1.00	0.79, 1.28	0.988
0.0005		1.12	0.88, 1.43	0.352
0.005		1.11	0.87, 1.41	0.407
0.05		1.04	0.82, 1.33	0.745
0.5		0.90	0.71, 1.15	0.394
Enjoys taking risks at age 24	2,932			
5×10^{-8}		1.06	0.98, 1.14	0.154
0.0005		1.05	0.98, 1.14	0.163

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
0.005		1.11	1.03, 1.19	0.005
0.05		1.09	1.01, 1.17	0.029
0.5		1.08	1.01, 1.16	0.033
Ever gambled by age 24	2,899			
5×10^{-8}		1.12	1.03, 1.22	0.008
0.0005		1.16	1.07, 1.26	0.001
0.005		1.16	1.06, 1.26	0.001
0.05		1.20	1.10, 1.30	<0.001
0.5		1.15	1.06, 1.25	0.001

Number of sexual partners, trouble with the law, enjoying risk-taking, and gambling models were included as negative controls. Analyses were adjusted for age, sex, and principal components 1–10.

*Low (<11) vs. high (11 or more) number of sexual partners, determined using the upper quartile for number of life time sexual partners in the ALSPAC sample (11 sexual partners).

Abbreviations: ALSPAC, Avon Longitudinal Study of Parents and Children; OR, odds ratio.

We found evidence of a positive association between smoking initiation PRS and hyperactivity at 7 years ($OR_{0.0005}$ = 1.10, 95% CI 1.04 to 1.16, p = 0.001) but not at the most stringent threshold (Table 44.4). There was also a positive association with CD at 7 years ($OR_{10\text{-}8}$ = 1.10, 95% CI 1.03 to 1.17, p = 0.004) at all thresholds (Table 44.4). There was some evidence of a positive association between PRS and ODD specifically at the 0.0005 threshold ($OR_{0.0005}$ = 1.08, 95% CI 1.02 to 1.14, p = 0.013). We also found a positive association with lower parental SEP ($OR_{10\text{-}8}$ = 1.08, 95% CI 1.01 to 1.16 p = 0.017) at all thresholds (Table 44.5).

Table 44.4 Associations between polygenic risk scores for smoking initiation with negative controls of externalising disorders in childhood

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
Hyperactivity at age 7	5,227			
5×10^{-8}		1.02	0.96, 1.08	0.511
0.0005		1.10	1.04, 1.16	0.001
0.005		1.14	1.08, 1.20	<0.001
0.05		1.14	1.08, 1.21	<0.001
0.5		1.15	1.08, 1.21	<0.001
Conduct disorder at age 7	5,334			
5×10^{-8}		1.10	1.03, 1.17	0.004
0.0005		1.11	1.04, 1.19	0.001

(*Continued*)

Table 44.4 (*Continued*)

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
0.005		1.11	1.04, 1.18	0.002
0.05		1.08	1.01, 1.15	0.021
0.5		1.08	1.01, 1.15	0.017
Oppositional defiant disorder at age 7	5,325			
5×10^{-8}		1.02	0.96, 1.08	0.496
0.0005		1.08	1.02, 1.14	0.013
0.005		1.04	0.98, 1.10	0.200
0.05		1.04	0.98, 1.10	0.173
0.5		1.02	0.96, 1.08	0.529

Hyperactivity and conduct disorder were assessed using the strengths and difficulties questionnaire (SDQ), and oppositional defiant disorder was assessed using the development and wellbeing assessment (DAWBA). All variables were recoded into binary outcomes (no disorder/symptoms versus borderline/disorder/symptoms).

Abbreviations: DAWBA, development and wellbeing assessment; OR, odds ratio; SDQ, strengths and difficulties questionnaire.

Table 44.5 Associations between polygenic risk scores for smoking initiation with negative controls of socioeconomic indicators

Outcome *p*-value threshold	*n*	OR	95% CI	*p*
Parental SEP (manual)	6,702			
5×10^{-8}		1.08	1.01, 1.16	0.017
0.0005		1.13	1.06, 1.21	<0.001
0.005		1.16	1.09, 1.24	<0.001
0.05		1.11	1.03, 1.18	0.003
0.5		1.13	1.05, 1.20	<0.001

Parental SEP was based on the higher of the mother or partner's occupational social class using the Office of Population Censuses and Surveys (OPCS) occupation codes.

Abbreviations: OPCS, Office of Population Censuses and Surveys; OR, odds ratio; SEP, socioeconomic position.

44.3 Discussion

In this study, we explored the association between smoking initiation PRS and e-cigarette use, using logistic regression. We further explored the findings by observing the association between smoking initiation PRS and positive controls (smoking) and negative controls (e.g., risk-taking), as well as restricting the analysis to never smokers. Smoking initiation PRS were strongly associated with

ever e-cigarette use by 24 years whereby higher genetic liability to smoking initiation was associated with a 24% increase in the likelihood of ever using an e-cigarette (per standard deviation (SD) increase in PRS). As expected, we observed an association of smoking initiation PRS and both ever smoking and smoking initiation. It was notable that the associations of the smoking initiation PRS and both smoking and e-cigarette use were of similar magnitude. Given the small amount of variation in smoking initiation explained by the SNPs (2.3%), and the fact that any causal effect will only explain a proportion of the outcome, these small effect sizes are to be expected. Interestingly, we also observed positive associations between smoking initiation PRS and risk-taking, impulsivity, and parental SEP at birth.

In contrast to the results of Allegrini and colleagues [6], we found an association between ever e-cigarette use and smoking initiation predisposition where they only found an association with smoking heaviness predisposition. This is likely due to the use of different SNPs to create the score; our score was based on the findings of a recent, large GWAS (GSCAN [9]; N = 1,232,091), whereas Allegrini and colleagues [6] based their score on an earlier GWAS with a much smaller sample (the Tobacco and Genetics Consortium [14]; N = 69,207). Thus, there was greater statistical power to detect genome-wide significant associations in the GWAS we based our score on.

The association between smoking initiation PRS and e-cigarette use could be explained by smoking causally influencing e-cigarette use. This hypothesis is supported by observational evidence; use of e-cigarettes for smoking cessation is common among both young adults in the UK [4] and adults in Great Britain [19]. However, the associations observed among the restricted analysis and between the negative control outcomes suggest there may be other factors at play—there may be shared genetic risk factors that influence both behaviours. Among never smokers, we found weak evidence of an association between smoking initiation PRS and e-cigarette use, which suggests that the e-cigarette use is not simply caused by smoking (which has not occurred in these cases) but that there is a shared genetic aetiology influencing both behaviours. Hence, what appears to be a gateway between e-cigarette use and smoking in previous studies [5] could actually be a shared genetic liability, and the order of use is coincidental or due to other factors such as perceived risk or misreporting of smoking status [20].

Alternatively, the smoking initiation PRS may be capturing much more than just smoking or nicotine use. Using less stringent p-value thresholds to create PRS increases the percentage variance in the phenotype explained by the score, and therefore the power to detect an association up to a point; using less stringent thresholds also increases the likelihood of capturing SNPs which are related to other factors, which adds noise and eventually results in less specific PRS that explain less variance in the exposure and more variance in other (horizontally pleiotropic) effects. Increasing magnitudes and strengthened evidence of association with PRS and negative controls at less stringent p-value thresholds suggests that the smoking initiation PRS is capturing, at least in part, a broad

phenotype which is not entirely specific to smoking/nicotine. Although weaker associations were observed between risk-taking factors and PRS for smoking initiation compared to e-cigarette use and smoking, the associations are still relatively strong and consistent. Recent observational evidence also indicated a strong association between e-cigarette use and smoking prior to adjusting for risk-taking behaviours and other shared risk factors but showed no clear evidence of an association after adjusting for risk-taking behaviours and other shared risk factors [21]. We also found an association between the smoking initiation PRS and externalising disorders in childhood (7 years) which precedes the age at which cigarettes are first smoked in the vast majority of cases in this cohort (>99%) and therefore cannot be a causal effect of own smoking. However, this association could potentially be due to causal in utero effects of maternal smoking in pregnancy or maternal smoking in childhood, since maternal and offspring genotype will be correlated. Nevertheless, combined with evidence that liability to attention-deficit/hyperactivity disorder (ADHD) increases the likelihood of smoking initiation and vice versa [22], our results suggest the possibility that the smoking initiation PRS is capturing a broad impulsivity phenotype. The association observed between PRS for smoking initiation and parental SEP also suggests the PRS could be capturing sociodemographic factors as well as smoking. Alternatively, there may be a dynastic effect whereby parental predisposition to smoking (which is correlated with their child's genetic predisposition) influences parental SEP at birth. The apparent association of the child's genotype could actually be an outcome of parental genetic predisposition.

Despite the strengths of this study (which include the use of a well-powered GWAS to create our score, lack of overlap between samples, and use of negative controls to explore potential mechanistic pathways), there are a number of limitations of this study. First, the relatively low sample size—particularly when investigating associations with regular e-cigarette use and restricting to never smokers. Second, restricting analysis to never smokers could introduce collider bias [23]. We found that smoking initiation PRS were strongly associated with smoking initiation; if e-cigarette use causes young adults to smoke, then smoking status is a collider and conditioning on this variable (i.e., restricting analysis to never smokers) may inflate any association between smoking initiation PRS and e-cigarette use. Third, this cohort is not appropriate to directly study the gateway hypothesis as the young adults in ALSPAC were approximately 17 years old when e-cigarettes became widely available and therefore were exposed to cigarettes earlier in their adolescence than e-cigarettes and had more opportunity to smoke than use e-cigarettes than later birth cohorts. Future research should explore this association in a larger sample of individuals with exposure to both cigarettes and e-cigarettes during adolescence. Fourth, the attrition rate in ALSPAC is considerable—only 2,905 of the 7,859 nonrelated participants of European ancestry with genetic data responded to the questions about e-cigarette use in the 24 year questionnaire—and missingness in this cohort has previously been associated with smoking initiation PRS [24]. Replicating the participation scores used by Taylor and colleagues [24], we found that higher

smoking initiation PRS were associated with participating in fewer ALSPAC questionnaires and clinics (change in participation per SD increase in smoking initiation PRS [$p < 5 \times 10^{-8}$ threshold] = −1.15, 95% CI −1.53 to −0.76, $p < 0.001$). Furthermore, we found that those with higher smoking initiation PRS were less likely to have been included in the analysis of smoking initiation PRS and e-cigarette use due to attrition ($OR_{10\text{-}8}$ per SD of smoking initiation PRS = 0.87, 95% CI 0.83 to 0.91, p < 0.001) so our estimates may be biased by selection and the association could be stronger than observed here. However, interpretation of any study including smoking initiation PRS will be difficult as the association between smoking initiation PRS and attrition could induce bias such as collider bias [25]. Fifth, the variability in the nature of the key assessments and the use of self-reports may have resulted in measurement error of the phenotype and outcomes.

The associations observed here may have implications for the use of smoking initiation PRS in Mendelian randomisation (MR) analysis. This method is often implemented to provide unconfounded causal estimates, as long as the assumptions of MR hold [26]. One assumption is that the genetic instrument (e.g., smoking initiation PRS) is not associated with any confounders (e.g., risk-taking, childhood externalising disorders, SEP). The association we observed between smoking initiation PRS and negative control outcomes, even when restricted to only genome-wide significant SNPs, indicates that smoking initiation PRS may not be a valid instrument to use in MR to investigate the causal effects of smoking initiation. This emphasises the importance of using pleiotropy robust methods (e.g., MR Egger). The InSIDE (Instrument Strength Independent of Direct Effect) assumption requires that SN*P*-exposure effects (e.g., the effect of smoking initiation SNPs on smoking initiation) should not be correlated with horizontal pleiotropic effects (e.g., the effect of smoking initiation SNPs on broad risk-taking behaviour). The association observed between the smoking initiation PRS and multiple risk-taking behaviours and externalising disorders in childhood suggests that the smoking initiation SNPs may be capturing a broader phenotype, such as risk-taking, which is not specific to smoking or nicotine, and thus this assumption may be violated. One approach which could be used to address this is Steiger filtering which can be used to exclude SNPs which explain the variance in the outcome over and above the variance in the exposure [11, 27]. The same approach can be applied in MR studies using smoking initiation PRS to remove SNPs which explain more variance in the negative control outcomes used in this study (or other phenotypes/proxies for risk-taking behaviour) than variance in smoking initiation. However, if the InSIDE assumption is perfectly violated (i.e., if the SNP effect on broad risk-taking causes smoking initiation), the smoking initiation PRS will be an invalid instrument using any MR method. At the very least, triangulating evidence across multiple MR methods (e.g., median weighted and mode based) would be advised in MR studies using smoking initiation PRS but, ideally, other causal inference methods should also be used. Further research could also explore the potential mediating effects of the positive and negative controls included in this analysis; if a PRS for e-cigarette initiation is identified

in a GWAS, pleiotropy robust multivariable MR methods [28] could be employed to explore mediating effects using smoking initiation, e-cigarette initiation, and risk-taking PRS (providing the PRS are sufficiently independent from one another).

The results also provide support for a shared genetic liability between e-cigarette use and smoking, which may have implications for policy; strict policies (e.g., bans), which aim to prevent e-cigarette use in order to reduce the risk of smoking initiation among youth and young adults, may not be effective. In fact, they may have the opposite effect; if young people are predisposed to both e-cigarette use and smoking but only cigarettes are available, this could increase their likelihood of smoking because it is the only option available to them. Furthermore, such policies may prevent and discourage adult smokers from accessing an effective smoking cessation tool and hamper smoking cessation attempts and could therefore have a negative impact on smoking rates. Ideally, policy should prevent use by nonsmokers but promote use by smokers for smoking cessation.

In conclusion, we find evidence to suggest there is a shared genetic aetiology between smoking and e-cigarette use but also with risky behaviour, SEP, and externalising disorders in childhood. This suggests the PRS for smoking initiation is not specific to smoking or nicotine use but is capturing something much broader. Future research is needed to explore this in a population which has been exposed to both e-cigarettes and cigarettes in adolescence.

Supporting Information

S1 STROBE Checklist. STROBE Checklist. https://doi.org/10.1371/journal.pmed.1003555.s001.

S1 Text. Supplementary material. https://doi.org/10.1371/journal.pmed.1003555.s002.

S1 Table. Questionnaire items and possible responses. https://doi.org/10.1371/journal.pmed.1003555.s003.

S2 Table. *p*-Value thresholds and number of SNPs included in polygenic risk scores. https://doi.org/10.1371/journal.pmed.1003555.s004.

S3 Table. Association between self-reported e-cigarette use and smoking and risk-taking behaviours, socioeconomic indicators, and externalising disorders in childhood. https://doi.org/10.1371/journal.pmed.1003555.s005.

S4 Table. Association between polygenic risk scores for smoking initiation with ever e-cigarette use among never smokers (smoked <1 cigarette in their lifetime). https://doi.org/10.1371/journal.pmed.1003555.s006.

Abbreviations

ADHD: attention-deficit/hyperactivity disorder
ALSPAC: Avon Longitudinal Study of Parents and Children
CD: conduct disorder

DAWBA: development and wellbeing assessment
GWAS: genome-wide association studies
GSCAN: GWAS & Sequencing Consortium of Alcohol and Nicotine use
InSIDE: Instrument Strength Independent of Direct Effect
MR: mendelian randomization
MAF: Minor Allele Frequency
ORs: odds ratios
ODD: oppositional defiant disorder
PRS: polygenic risk scores
SNPs: single nucleotide polymorphisms
SEP: socioeconomic position
SD: standard deviation
SDQ: strengths and difficulties questionnaire
STROBE: Strengthening the Reporting of Observational Studies in Epidemiology

Disclosures and Conflict of Interest

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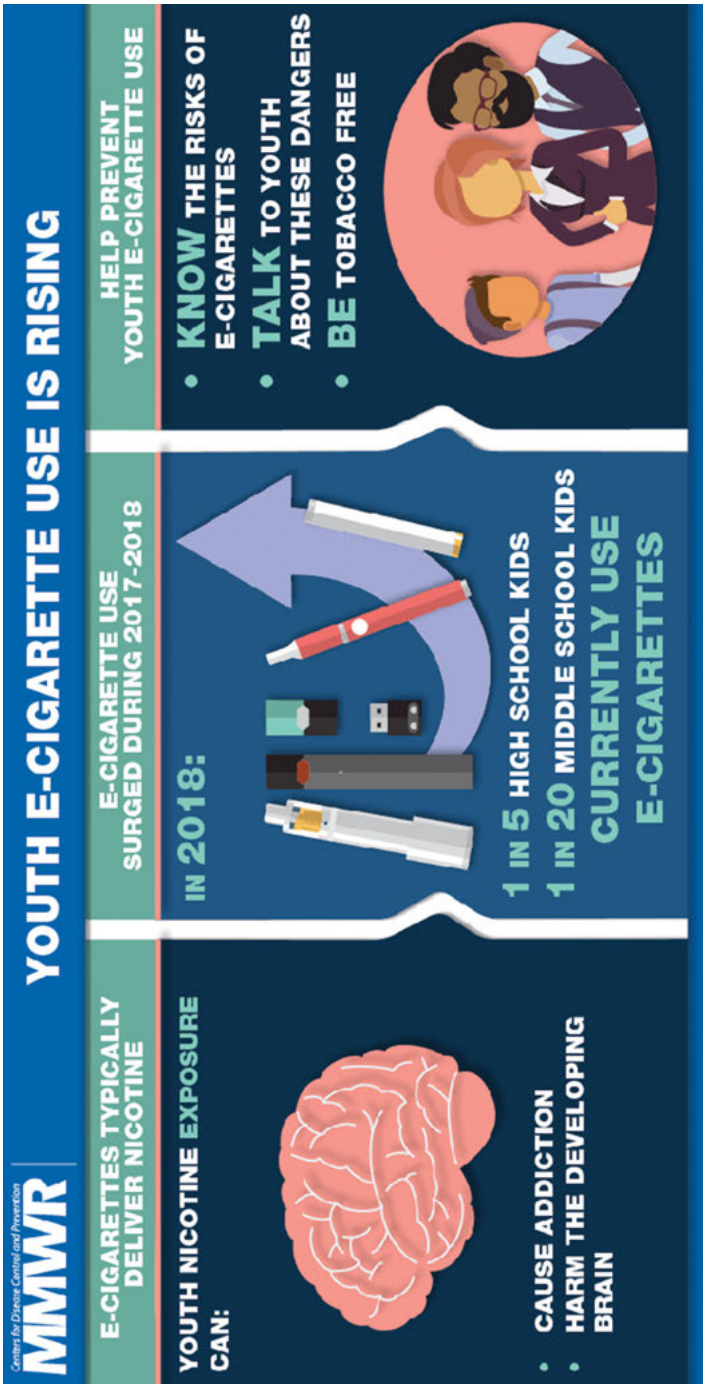
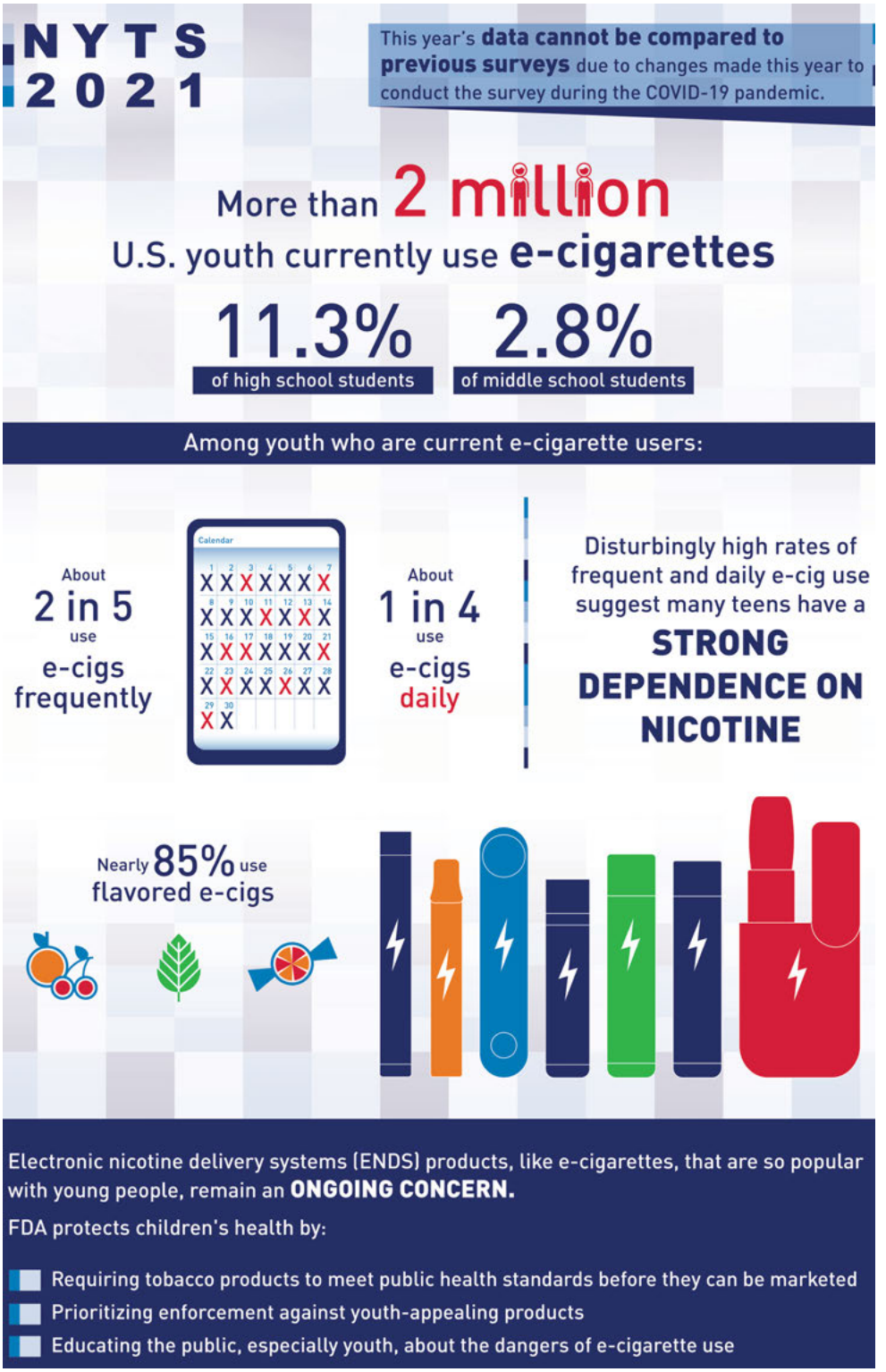
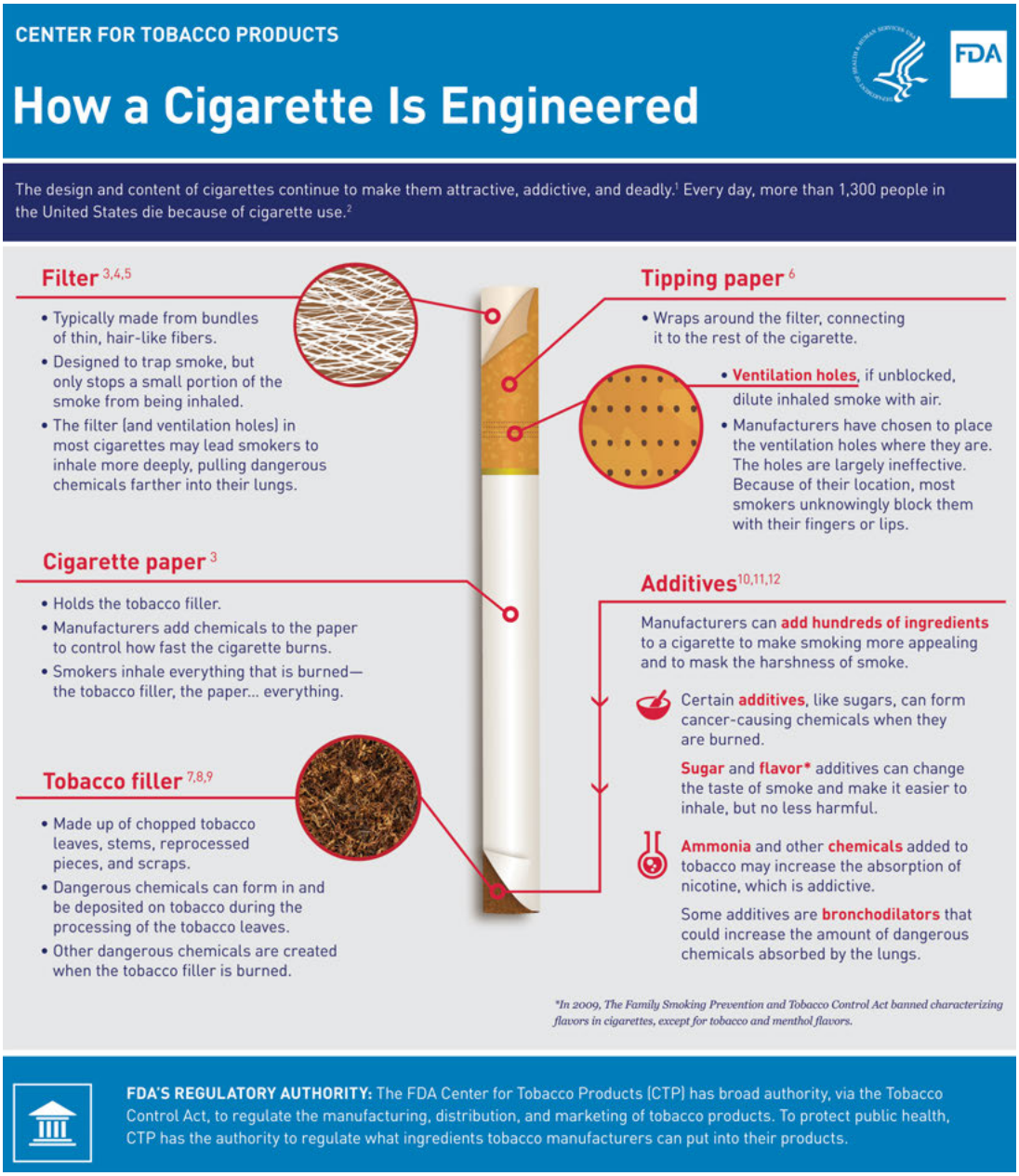
Appendix*



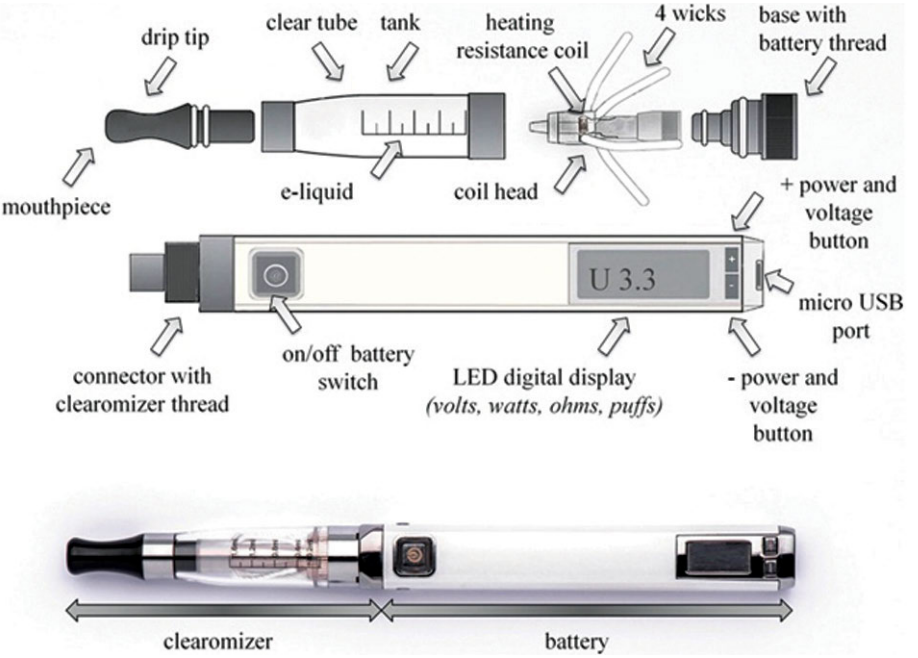
*The Appendix has been compiled by the Series Editor, Raj Bawa, PhD, MD. The materials have been kindly provided to Dr. Bawa by the Centers for Disease Control and Prevention, GA, and Wikimedia Commons.


NYTS 2021
This year's data cannot be compared to previous surveys due to changes made this year to conduct the survey during the COVID-19 pandemic.
More than 2 million
U.S. youth currently use e-cigarettes
11.3%
of high school students
2.8%
of middle school students
Among youth who are current e-cigarette users:
About 2 in 5 use e-cigs frequently
Calendar
About 1 in 4 use e-cigs daily
Disturbingly high rates of frequent and daily e-cig use suggest many teens have a STRONG DEPENDENCE ON NICOTINE
Nearly 85% use flavored e-cigs
Electronic nicotine delivery systems (ENDS) products, like e-cigarettes, that are so popular with young people, remain an ONGOING CONCERN.
FDA protects children's health by:
Requiring tobacco products to meet public health standards before they can be marketed
Prioritizing enforcement against youth-appealing products
Educating the public, especially youth, about the dangers of e-cigarette use

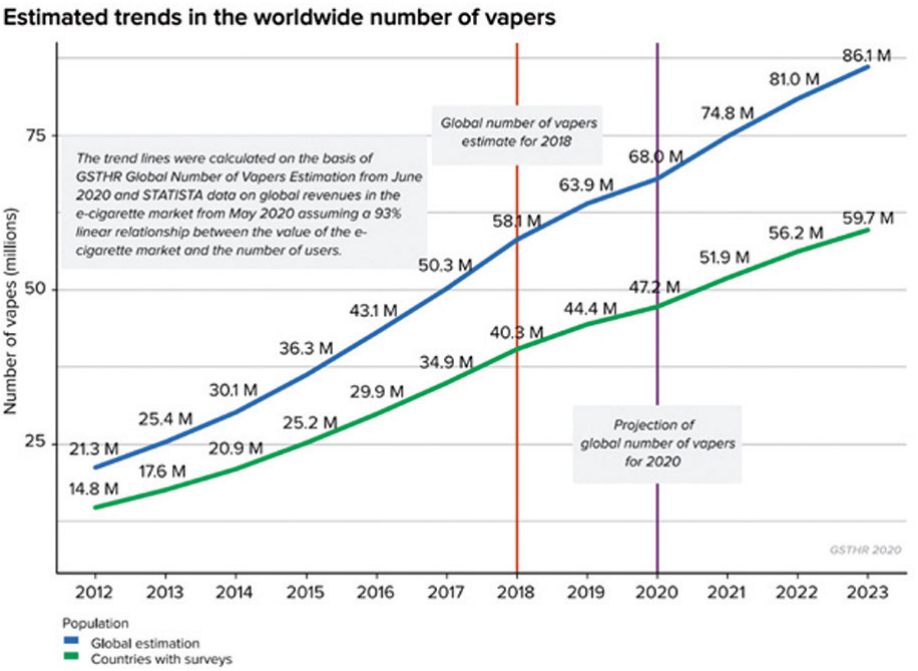

CENTER FOR TOBACCO PRODUCTS
How a Cigarette Is Engineered
FDA
The design and content of cigarettes continue to make them attractive, addictive, and deadly.[1] Every day, more than 1,300 people in the United States die because of cigarette use.[2]
Filter [3,4,5]
• Typically made from bundles of thin, hair-like fibers.
• Designed to trap smoke, but only stops a small portion of the smoke from being inhaled.
• The filter (and ventilation holes) in most cigarettes may lead smokers to inhale more deeply, pulling dangerous chemicals farther into their lungs.
Cigarette paper [3]
• Holds the tobacco filler.
• Manufacturers add chemicals to the paper to control how fast the cigarette burns.
• Smokers inhale everything that is burned—the tobacco filler, the paper... everything.
Tobacco filler [7,8,9]
• Made up of chopped tobacco leaves, stems, reprocessed pieces, and scraps.
• Dangerous chemicals can form in and be deposited on tobacco during the processing of the tobacco leaves.
• Other dangerous chemicals are created when the tobacco filler is burned.
Tipping paper [6]
• Wraps around the filter, connecting it to the rest of the cigarette.
• Ventilation holes, if unblocked, dilute inhaled smoke with air.
• Manufacturers have chosen to place the ventilation holes where they are. The holes are largely ineffective. Because of their location, most smokers unknowingly block them with their fingers or lips.
Additives [10,11,12]
Manufacturers can add hundreds of ingredients to a cigarette to make smoking more appealing and to mask the harshness of smoke.
Certain additives, like sugars, can form cancer-causing chemicals when they are burned.
Sugar and flavor* additives can change the taste of smoke and make it easier to inhale, but no less harmful.
Ammonia and other chemicals added to tobacco may increase the absorption of nicotine, which is addictive.
Some additives are bronchodilators that could increase the amount of dangerous chemicals absorbed by the lungs.
*In 2009, The Family Smoking Prevention and Tobacco Control Act banned characterizing flavors in cigarettes, except for tobacco and menthol flavors.
FDA'S REGULATORY AUTHORITY: The FDA Center for Tobacco Products (CTP) has broad authority, via the Tobacco Control Act, to regulate the manufacturing, distribution, and marketing of tobacco products. To protect public health, CTP has the authority to regulate what ingredients tobacco manufacturers can put into their products.

A first-generation e-cigarette that resembles a tobacco cigarette. The battery portion of the e-cigarette can be disconnected and recharged using the USB power charger. By Ecig Click-Electronic Cigarette and USB Charger, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=52467733.

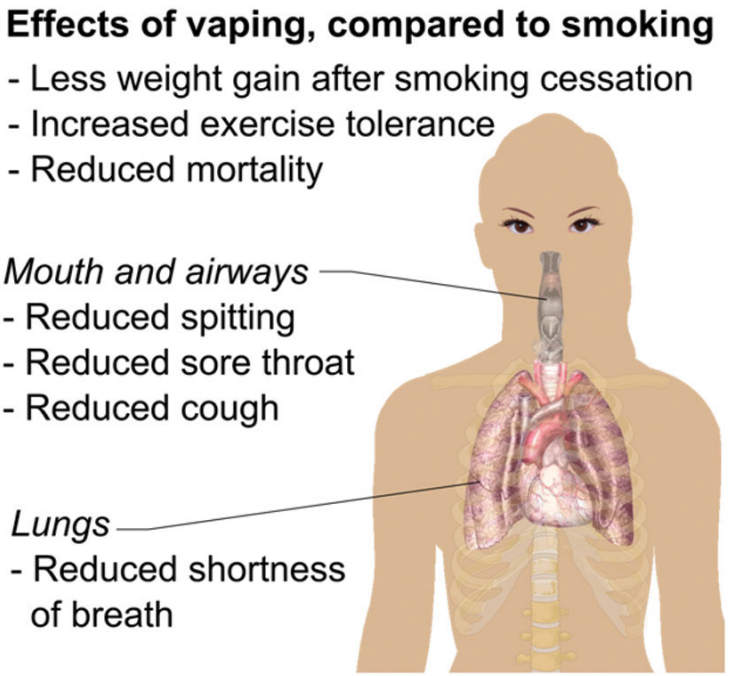
Various types of e-cigarettes, including a disposable e-cigarette, a rechargeable e-cigarette, a medium-size tank device, large-size tank devices, an e-cigar, and an e-pipe. By Centers for Disease Control and Prevention – https://www.cdc.gov/cdcgrandrounds/pdf/archives/2015/october2015.pdf, Public Domain, https://commons.wikimedia.org/w/index.php?curid=54931273.



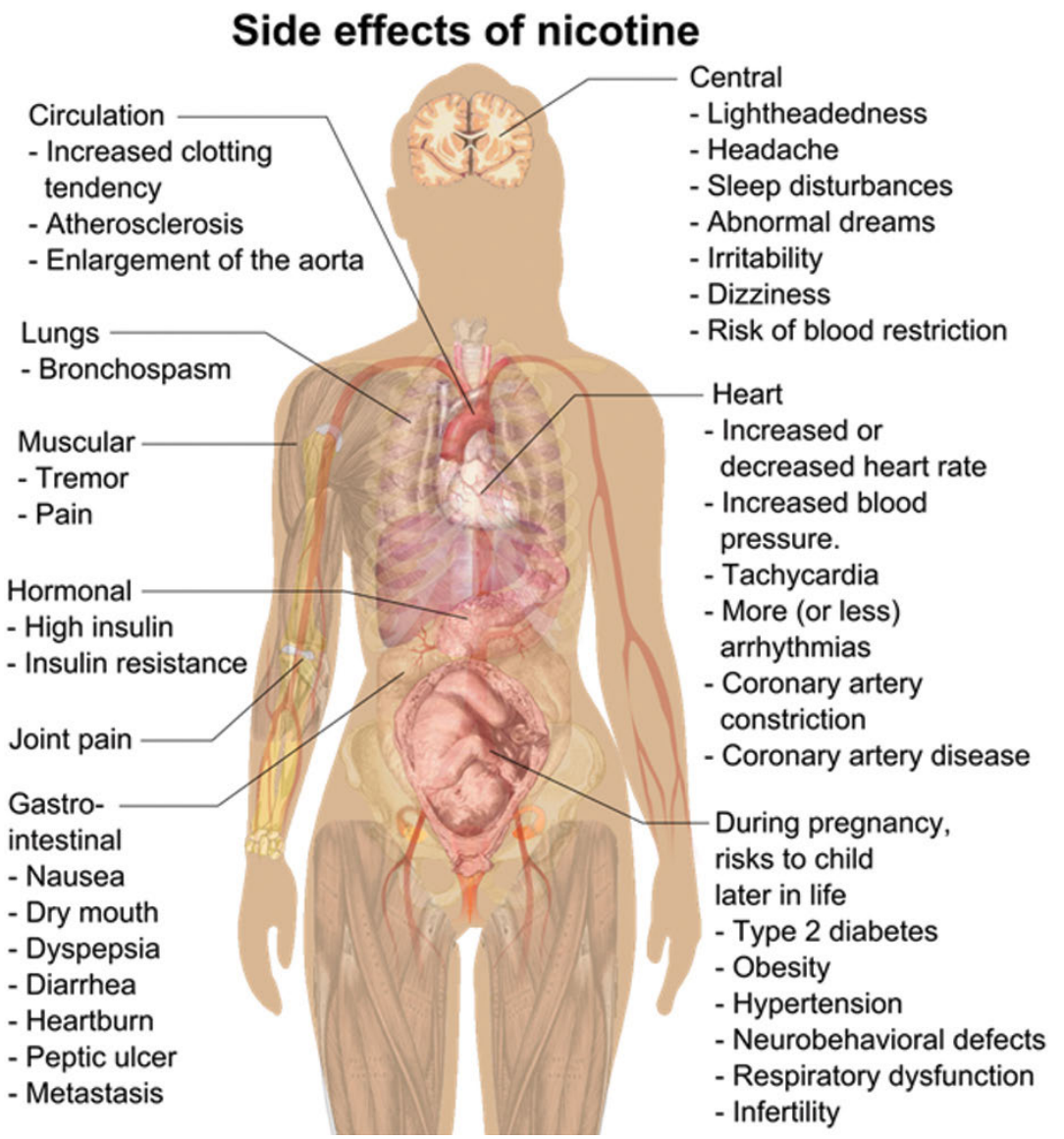
Exploded view of an e-cigarette with transparent clearomizer and changeable dual-coil head. This model allows for a wide range of settings. By Christian Giroud, Mariangela de Cesare, Aurélie Berthet, Vincent Varlet, Nicolas Concha-Lozano, and Bernard Favrat – https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4555324/, CC BY 4.0, https://commons.wikimedia.org/w/index.php?curid=46635108.



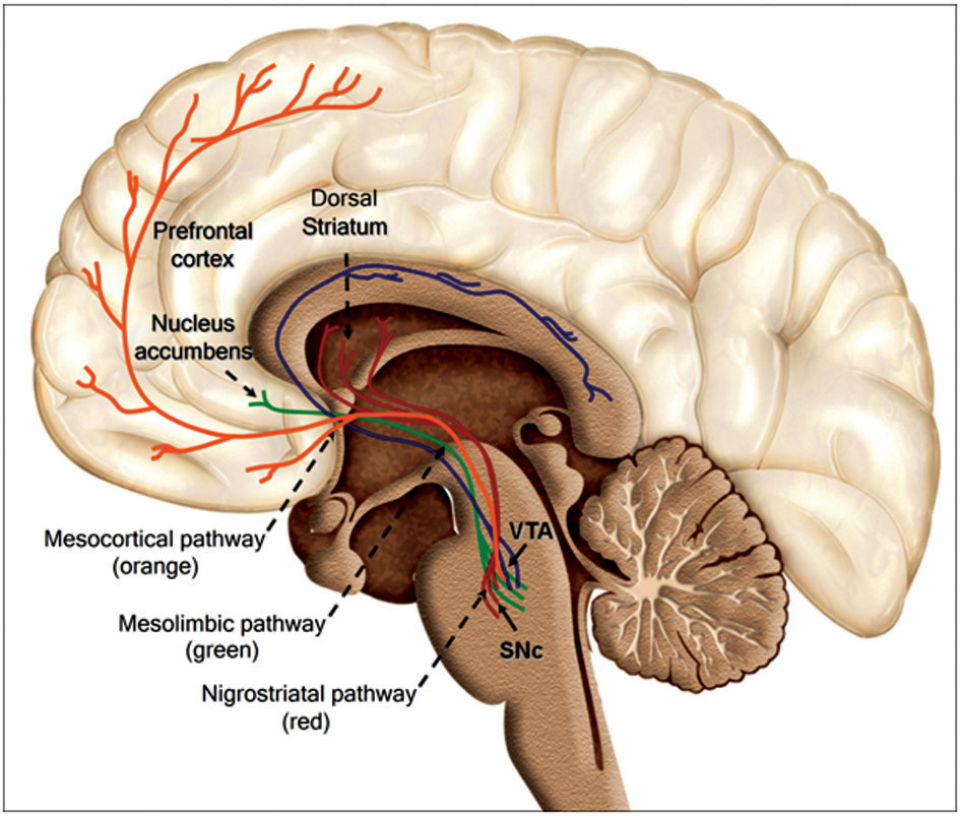
Estimated trends in the global number of vapers. CC BY 4.0, https://commons.wikimedia.org/w/index.php?curid=121107097.



Effects of vaping, compared to tobacco smoking. By Mikael Häggström – All used images are in public domain., Public Domain, https://commons.wikimedia.org/w/index.php?curid=41475856.



Possible side effects of nicotine. By Mikael Häggström, used with permission. All used images are in public domain., Public Domain, https://commons.wikimedia.org/w/index.php?curid=8332393.



The reinforcing effects of addictive drugs, such as nicotine, are associated with its ability to excite the mesolimbic and dopaminergic systems. *How does the nicotine in e-cigarettes affect the brain*? Until about age 25, the brain is still growing. Each time a new memory is created or a new skill is learned, stronger connections – or synapses – are built between brain cells. Young people's brains build synapses faster than adult brains. Because addiction is a form of learning, adolescents can get addicted more easily than adults. The nicotine in e-cigarettes and other tobacco products can also prime the adolescent brain for addiction to other drugs such as cocaine. By Oscar Arias-Carrión, Maria Stamelou, Eric Murillo-Rodríguez, Manuel Menéndez-González and Ernst Pöppel. Substantially modified by Seppi333 - Oscar Arias-Carrión, Maria Stamelou, Eric Murillo-Rodríguez, Manuel Menéndez-González and Ernst Pöppel. Dopaminergic reward system: a short integrative review. *International Archives of Medicine* 2010, 3:24 doi:10.1186/1755-7682-3-24. http://www.biomedcentral.com/1755-7682/3/24/, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=69172180.

Chapter 45

Current Issues in Vaccine Development

Raj Bawa, PhD, MD

Patent Law Department, Bawa Biotech LLC, Ashburn, Virginia, USA
Guanine Inc., Rensselaer, New York, USA
Teva Pharmaceutical Industries Ltd., Israel

bawa@bawabiotech.com

This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the U.S. Government Accountability Office (GAO), Washington DC. GAO supports the U.S. Congress in meeting its constitutional responsibilities, and helps improve the performance and ensures the accountability of the federal government. It provides the U.S. Congress with timely information that is objective, fact-based, nonpartisan, nonideological, and balanced. GAO, often called the "congressional watchdog," is an independent, non-partisan agency that works for Congress. In 2021, the GAO celebrated 100 years.

The CARES Act included a provision for GAO to report on its ongoing monitoring and oversight efforts related to the COVID-19 pandemic. This chapter is excerpted from a November 2021 GAO report that discusses technologies, approaches, and associated challenges for vaccine (1) research and development, (2) testing, and (3) manufacturing, as well as (4) the economic factors that affect vaccine investment. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

Keywords: adjuvants, allergenics, artificial intelligence (AI), bacille Calmette-Guérin (BCG) vaccine, biological products, biologics license application (BLA), bioprocess intensification, bovine spongiform encephalopa, cell cultures, cell density, cell-free synthesis, closed-production system, continuous manufacturing system, Coronavirus Disease 2019 (COVID-19), COVID-19 vaccines, dermal delivery, diphtheria, emergency use authorization (EUA), epidemics, first-to-market entrants, gene therapy, gross domestic product (GDP), H1N1

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influenza pandemic, H1N1 viruses, hepatitis A, hepatitis B, hepatitis B vaccine, human papillomavirus, influenza A viruses, machine learning (ML), measles, microneedles, Middle Eastern Respiratory Syndrome (MERS), modular bioprocessing systems, monoclonal antibodies, mumps, *Mycobacterium leprae, Mycobacterium tuberculosis,* nasal delivery, nasal vaccines, Nipah virus, omics analysis, oral delivery, oral poliovirus vaccine, pandemics, pertussis, phase 1 clinical trials, phase 4 clinical trials, polio, post-marketing surveillance studies, poxvirus, process optimization, push and pull incentives, reverse vaccinology, Rift Valley Fever, rubella, Severe Acute Respiratory Syndrome (SARS), single-use systems, smallpox, smallpox vaccine, somatic cells, suspension cultures, tetanus, T-lymphocytes, vaccination, vaccine, vaccinia virus, variant, viral vector, West Nile Fever

45.1 Vaccine Development: An Introductory Overview

Vaccinations are an important tool for individual and public health and have saved millions of lives. For example, between 1900 and 1980, smallpox killed approximately 300 million people and disfigured millions more. However, by 1980, a successful vaccination campaign eliminated smallpox worldwide. Similarly, in 1988, at the onset of a global campaign to end polio, there were 350,000 polio cases worldwide. According to the World Health Organization (WHO), the number of polio cases has since declined by over 99 percent worldwide.

Infectious diseases carry a high price tag for society. According to the World Bank, the economic losses from six major outbreaks of highly fatal infectious diseases that occurred between 1997 and 2009 amounted to at least $80 billion globally.[1] According to the National Foundation for Infectious Diseases, in the U.S., an average influenza illness can last up to 15 days, typically resulting in 5 or 6 missed work or school days. Adults who contract hepatitis A lose an average of one month of work. Further, when people are not vaccinated, they may be vulnerable to serious infections, such as human papillomavirus and hepatitis B (both can cause cancer), which have significant personal and economic burdens.

Providing the public with safe and effective vaccines[2] is also crucial to mitigating global pandemics. The Coronavirus Disease 2019 (COVID-19) pandemic has highlighted the devastating impact new infectious diseases can have. As of

[1]The infectious diseases included in this study were Nipah Virus (Malaysia), West Nile Fever (U.S.), severe acute respiratory syndrome (Asia, Canada, other), highly pathogenic avian Influenza (Asia, Europe), bovine spongiform encephalopathy (U.S., U.K.), Rift Valley Fever (Tanzania, Kenya, Somalia). See World Bank. *People, Pathogens and Our Planet: The Economics of One Health*. 2012, Washington, DC. Available at: https://openknowledge.worldbank.org/handle/10986/11892 (accessed on November 25, 2022).

[2]In this chapter, the term efficacy refers to the results of adequate and controlled clinical trial studies that evaluate clinical disease endpoints, and effectiveness to refer to the results of studies carried out under field conditions. For regulatory purposes, FDA determines whether a vaccine is safe and effective, and effectiveness is generally based on the results of adequate and well controlled studies evaluating a clinical disease endpoint (efficacy studies) or a well-accepted immune endpoint (effectiveness studies). For more information on the mRNA vaccine candidate that was not as efficacious in clinical trials, see CureVac, *CureVac Provides Update on Phase 2b/3 Trial of First-Generation COVID-19 Vaccine Candidate, CVnCoV* (Tübingen, Germany/Boston, USA, 2021).

November 1, 2021, the Centers for Disease Control and Prevention (CDC) reported that over 745,000 people in the U.S. had died of COVID-19. An October 2020 study estimated the total economic cost of the at more than $16 trillion, or approximately 90 percent of the annual U.S. gross domestic product.[3] In December 2020, the U.S. took an important step to protect the public against COVID-19, as the first COVID-19 vaccines—developed in a shorter time than any previous vaccine—were authorized for emergency use and administered.[4] As of November 2021, three COVID-19 vaccines were available in the United States. One vaccine was licensed by the Food and Drug Administration (FDA) for individuals 16 and older, and was also available for individuals aged 5-15 years under an emergency use authorization (EUA).[5] Since implementation of the COVID-19 vaccine program in December of 2020, the number of new daily cases has declined.[6] For example, the number of COVID-19 cases in the U.S. reached a high of over 290,000 new daily cases on January 8, 2021, but declined to about 75,000 new daily cases as of November 2, 2021.[7]

While the National Institutes of Health (NIH) conducts and supports research that contributes to relevant technological advances, vaccine development overall continues to be a difficult, complex, and costly endeavor, and from an investment standpoint remains highly risky for those who pursue it. Economists we spoke to stated that the benefits that vaccines provide are not necessarily commensurate with the return on investment from developing or manufacturing them and vaccine development from discovery to licensure can cost billions of dollars and

[3]D.M. Cutler and L. Summers The COVID-19 Pandemic and the $16 Trillion Virus*, Journal of the American Medical Association*, Oct. 20, 2020.

[4]The Secretary of Health and Human Services (HHS) may declare that circumstances, prescribed by statute, justify the emergency use of certain medical products, such as vaccines. Once a declaration of an emergency has been made, the Food and Drug Administration (FDA) may temporarily allow use of unlicensed vaccines through an emergency use authorization (EUA). For FDA to issue an EUA for a vaccine, it must be reasonable to believe that the vaccine may be effective and that the known and potential benefits of the vaccine outweigh the known and potential risks, among other statutory criteria. See 21 U.S.C. § 360bbb-3. See also FDA, *Emergency Use Authorization for Vaccines to Prevent COVID-19, Guidance for Industry* (Washington, D.C.: May 2021).

[5]FDA licenses biological products, such as vaccines, through review and approval of a biologics license application (BLA). See 42 U.S.C. § 262. FDA guidance indicates that licensure is the goal for COVID-19 vaccines, including those that first receive an EUA.

[6]COVID-19 vaccine implementation involves the prioritization, allocation, distribution, and administration of vaccine doses.

[7]For more information on the accelerated COVID-19 vaccine development process, see GAO, *COVID-19: Efforts to Increase Vaccine Availability and Perspectives on Initial Implementation*, GAO-21-443, (Washington, D.C.: April 14, 2021), GAO, *Operation Warp Speed: Accelerated COVID-19 Vaccine Development Status and Efforts to Address Manufacturing Challenges*, GAO-21-319, (Washington, D.C.: Feb. 11, 2021), and GAO, *COVID-19: Federal Efforts Accelerate Vaccine and Therapeutic Development, but More Transparency Needed on Emergency Use Authorizations*, GAO-21-207 (Washington, D.C.: Nov. 17, 2020). GAO has also produced an interactive dashboard that integrates multiple data sources to visualize the status of vaccine development, which may be found at https://ows.gaoinnovations.gov/.

can take over 10 years to complete.[8] Vaccine developers face numerous technical challenges related to the biological complexity of some infectious diseases and the maturity of new vaccine technologies. Other challenges include long development time frames, high rates of clinical trial failure, and the lack of incentives to invest in vaccines.

Given the public health consequences of infectious disease, policymakers have a vital interest in developing and maintaining modern, flexible, rapid, and robust vaccine development and manufacturing capabilities.[9] These capabilities will allow for better response to endemic levels of infectious disease, as well as better preparation for potential future epidemics and pandemics.[10] This urgent need comes at a time marked by rapid growth in basic scientific understanding—in areas such as genomics and structural biology that are supporting a new era in vaccine development—as well as ongoing challenges.

In summary, vaccines protect people from disease by preparing the body to respond to an infection. Vaccinations are a key part of individual and community health, but vaccine development remains complex and costly. Innovative technologies and approaches, such as those identified in this report, may enhance the nation's ability to respond to infectious diseases. For example, reverse vaccinology and next-generation platforms—combined with existing research—helped researchers develop some COVID-19 vaccines more quickly and effectively (Fig. 45.1).

However, key challenges may hinder the adoption of these innovative technologies and approaches. Some promising technologies face issues and challenges such as inherent technical limitations and high cost. For example, organ chips (Fig. 45.2) may facilitate testing, but they are not yet able to replicate many of the complex functions of the human immune system. Similarly, single-use systems may increase the flexibility of vaccine manufacturing facilities, but may require extensive testing to ensure that they do not negatively affect the resulting vaccine.

Further, economic challenges may hinder vaccine development. Experts attribute underinvestment in vaccines to market failures (i.e., market interactions that fall short of what would have been socially beneficial). For example, vaccines benefit those who are vaccinated, and, to some degree, those who are not. This additional benefit is not captured in the price, which reduces return on vaccine

[8]D. Gouglas, et al., Estimating the Cost of Vaccine Development Against Epidemic Infectious Diseases: a Cost Minimisation Study. *The Lancet Global Health*, vol. 6, no. 12 (2018): pp. e1386–e1396.

[9]Policymakers is a broad term including, for example, Congress, federal agencies, state and local governments, academic and research institutions, and industry.

[10]Endemic refers to the constant presence or usual prevalence of disease in a population within a geographic area. An epidemic refers to an increase, often sudden, in the number of cases of a disease above what is normally expected in a population and area. A pandemic refers to an epidemic that has spread over several countries or continents, usually affecting a large number of people.

investment. GAO identified nine policy options that may help address challenges hindering the adoption of vaccine development technologies and approaches or economic challenges (Table 45.1).

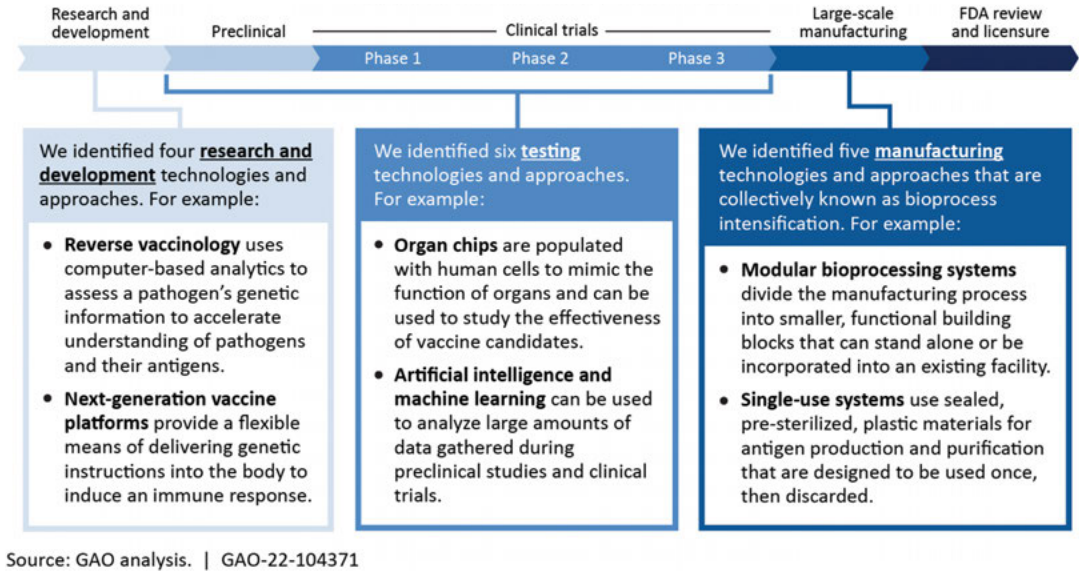


Figure 45.1 Clinical phases of vaccine.

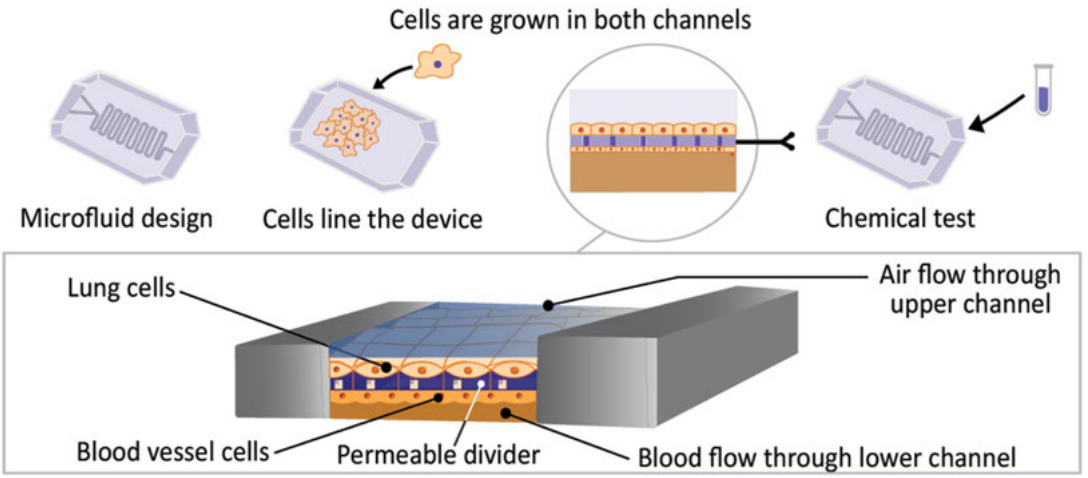


Figure 45.2 Source: GAO analysis, Wyss Institute at Harvard University, and J.E. Sosa-Hernandez et al., "Organs-on-a-Chip-Module: A Review from the Development and Applications Perspective," *Micromachines*, vol. 9, no. 10 (2018): 536. License: CC BY 4.0. https://creativecommons.org/licenses/by/4.0/legalcode.

Table 45.1 Selected policy options to address challenges in vaccine development

	Opportunities	Considerations
Prioritize infectious disease pathogens Policymakers could collaborate across sectors (e.g., government, academia, researchers, industry, and nonprofit organizations) to prioritize infectious disease pathogens with pandemic potential for vaccine R&D. For example, policymakers could develop a working group to prioritize pathogens with pandemic potential and work more closely with international organizations to prioritize vaccine development as well as develop monoclonal antibodies.	• Prioritizing pathogens with pandemic potential could improve strategic vaccine R&D decision-making and help focus resources on developing and adopting key technologies and approaches that most effectively address those pathogens. • Appropriately matching the technologies and approaches to the prioritized potential pandemic pathogens then leveraging technologies may help address certain technical limitations and cost. • With greater leadership and strategic partnerships, policymakers could more quickly address threats to the U.S. population	• As new threats are identified, priorities may change, which may cause uncertainty for vaccine developers. • Policymakers may have different priorities based on their respective missions. • There may be disagreements as to which key technologies should be prioritized and used, resulting in the need for policymakers to weigh the potential advantages and disadvantages associated with various options.
Improve preparedness Policymakers could provide support for public-private partnerships to strategically address potential pandemic pathogens identified as priorities. These partnerships could, for example, develop and test vaccine candidates that may provide protection from pathogens with pandemic potential.	• This early development could provide a coordinated foundation that can be mobilized in an emergency. Such an approach could speed vaccine development as well as potentially reduce risk for vaccine researchers and developers concerning questions of safety, efficacy, and manufacturability.	• The lack of certainty of the commercial market and government funding for vaccines against pathogens with pandemic potential may be too risky for the private sector to undertake.

	Opportunities	Considerations
Further support development of data standards Policymakers could further support coordinated efforts to obtain the views of all stakeholders and to develop standards for health data and their use in clinical trials.	• Integrating researchers' needs into the standards development process could better ensure the necessary data are available. • Access to high-quality data in a standardized format may allow streamlined patient recruitment for clinical trials.	• Expanding access to patient heath data requires attention to ensure privacy. • Developing and implementing standardized data formats and IT infrastructure is time-consuming and costly.
Improve preparedness Policymakers could provide support for public/private partnerships to strategically develop manufacturing capacity to respond to surge requirements. To maintain this capacity, partnerships could manufacture prototype vaccine candidates against high-priority pathogens.	• Manufacturing, testing, and stockpiling vaccines could be mobilized in an emergency and more rapidly mitigate future pandemics. • By leveraging strategic partnerships, policymakers could take steps to increase the availability of vaccines to more quickly address threats to the U.S. population.	• May require new resources or reallocation of resources from other efforts. • There may be a risk that the vaccines manufactured, tested, and stockpiled against prioritized pathogen classes miss certain pandemic pathogens. • The stockpiled vaccines would need to be regularly replenished prior to expiration.
Evaluate factors that inhibit vaccine investment and mechanisms to increase it Policymakers could collaborate across sectors, such as government, academia, and industry, to conduct a systematic evaluation of factors that inhibit developers from investing in new vaccines.	• A clear understanding of the range of factors discouraging vaccine investment would provide the basis for effectively addressing those factors.	• Collaboration between policymakers and other stakeholders to obtain all relevant viewpoints can be time-consuming and it may be hard to reach a consensus.

45.2 How Vaccines Work

Vaccines protect people from disease by triggering the immune system to produce antibodies that will fight a pathogen attacking the body. A pathogen is a bacterium, virus, or other microorganism that can cause disease. Preparing the immune system through vaccination allows the body to respond more quickly if that pathogen infects the individual in the future. During an infection, the immune system responds to specific parts of a pathogen called antigens by producing antibodies—proteins that help bind to and neutralize specific pathogens—to fight the pathogen and in some cases prevent future infections (Fig. 45.3). The immune system also produces specific cells—such as T-lymphocytes—that assist in neutralizing pathogens in a process known as cell-mediated immunity. When the human body is exposed to an antigen for the first time, it takes time for the immune system to respond and produce antibodies specific to that antigen. During that period of time, the individual is susceptible to becoming ill from the disease caused by the pathogen. Once antigen-specific antibodies are produced, they work with the rest of the immune system to destroy the pathogen.

Vaccines mimic this natural process by introducing antigens without necessarily introducing the disease-causing pathogen itself. For example, vaccines can use a weakened or inactive pathogen, a microorganism that is closely related to the pathogen but does not cause disease in humans, or a molecule derived from the pathogen.[11] Vaccines may include a variety of ingredients such as stabilizers, adjuvants, and preservatives to enhance the effectiveness of the vaccine or offer other benefits.[12]

Once an immune response has been generated, if the person is exposed later to the pathogen, their immune system will 'remember' seeing that pathogen and respond more quickly, increasing their chances of fighting off the infection.[13] Additionally, vaccines that protect against one pathogen may also protect against similar pathogens. Vaccines may also reduce the spread of infectious disease, which can convey some level of protection to those who are not vaccinated. For example, the bacille Calmette–Guérin (BCG) vaccine, which contains weakened *Mycobacterium bovis*—a bacterium that causes tuberculosis in cattle—is used to protect humans from *Mycobacterium tuberculosis*—a bacterium that causes

[11]For example, the smallpox vaccine uses a related poxvirus, the vaccinia virus, which is unlikely to cause significant disease in healthy human recipients, but elicits an immune response that is protective for smallpox.

[12]Stabilizers are substances such as amino acids and other substances that help the antigen maintain its effectiveness during storage. Adjuvants are compounds such as aluminum salts that help to enhance the immune response. Preservatives are chemical substances that help to protect against bacterial and fungal growth during storage. Also see, https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/common-ingredients-us-licensed-vaccines.

[13]Vaccine effectiveness varies for each vaccine. For example, according to CDC, the effectiveness of seasonal influenza vaccines ranges from between 40 percent to 60 percent during seasons when influenza vaccine viruses are similar to circulating influenza viruses, while a two–dose course of the measles, mumps, and rubella (MMR) vaccine is 97 percent effective at preventing measles.

tuberculosis in humans. Further, this vaccine may also provide some protection against *Mycobacterium leprae*—a bacterium that causes leprosy in humans.

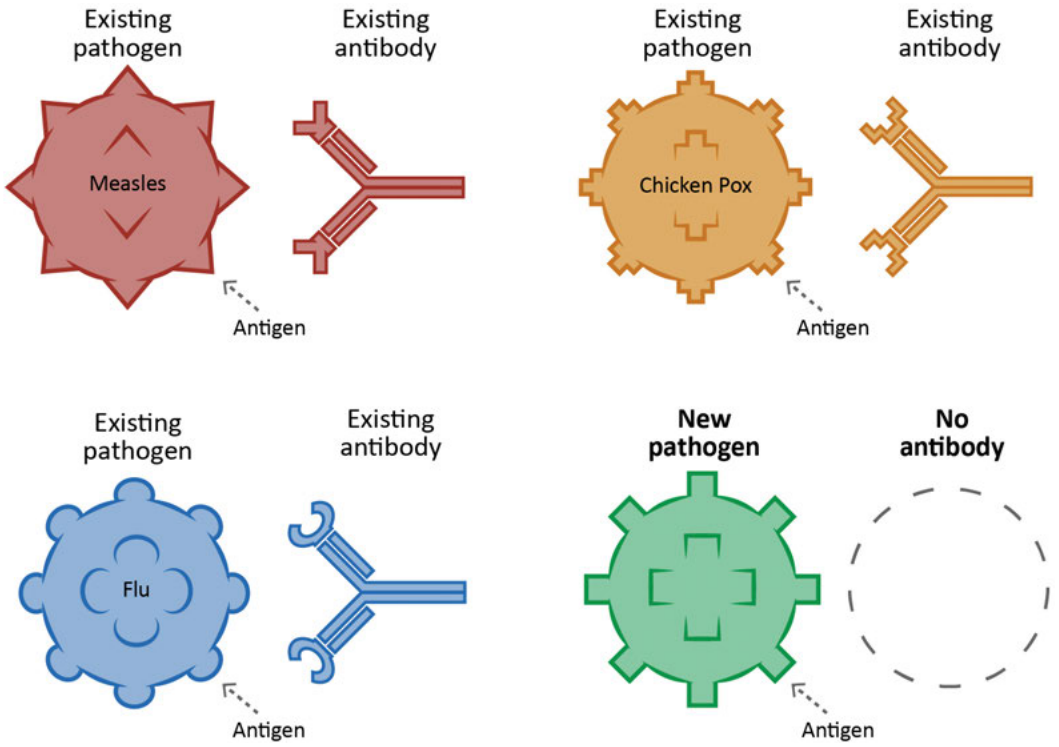
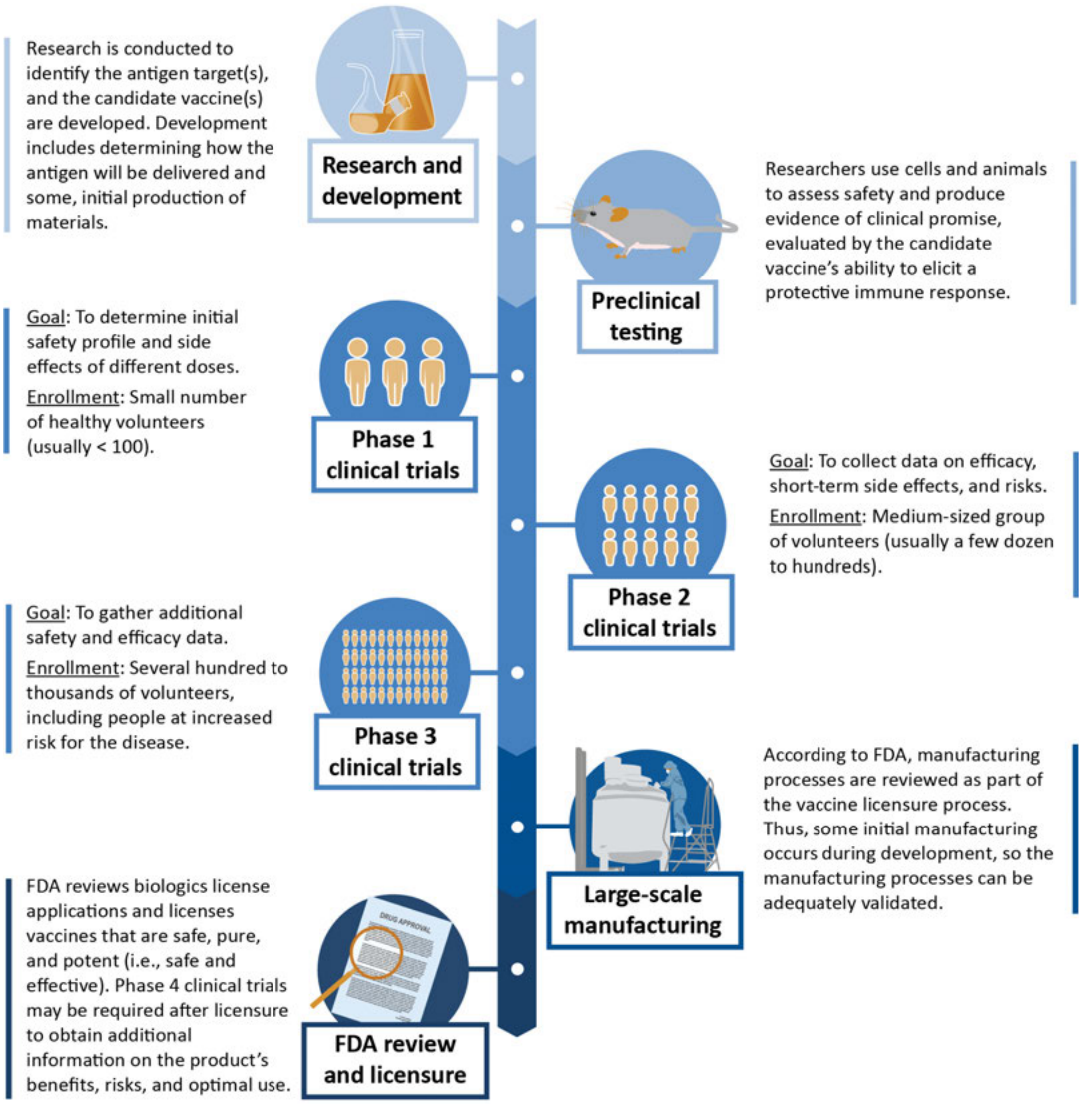


Figure 45.3 How antibodies fight invading pathogens. Source: World Health Organization (WHO), https://www.who.int/news-room/feature-stories/detail/how-do-vaccines-work (accessed on November 20, 2022).

45.3 Vaccine Development

The traditional process for developing a new vaccine is well established and tends to be sequential (Fig. 45.4), although stages sometimes overlap.[14] The purpose of this sequential approach is, in part, to reduce financial risk because each stage is costly—with later stages being especially costly—and each stage improves the understanding of whether the next stage might be successful. However, one expert told us that this is not guaranteed. At any stage, the process can be terminated for a variety of reasons, including detection of adverse events, such as serious side effects or if the evidence suggests that the vaccine is unlikely to be protective.

[14]The GAO has previously reported on the traditional vaccine development timeline compared to a potential timeline for COVID-19 vaccine development. See GAO-21-319.



Source: GAO analysis of information from the Food and Drug Administration (FDA) and literature. | GAO-22-104371

Note: The steps shown in the timeline are not drawn to scale, and the specific development steps for a given vaccine may vary. For example, the federal government accelerated this process for the development of a COVID-19 vaccine under the HHS-DOD COVID-19 Countermeasures Acceleration Group (formerly known as Operation Warp Speed) by overlapping certain phases to speed up the process so the vaccines could be used as quickly as possible to control the pandemic. No trial phases were skipped.

In this chapter, we use the term "efficacy" to refer to the results of adequate and controlled clinical trial studies that evaluate clinical disease endpoints, and "effectiveness" to refer to the results of studies carried out under field conditions. For regulatory purposes, FDA determines whether a vaccine is safe and effective, and effectiveness is generally based on the results of adequate and well controlled studies evaluating a clinical disease endpoint (efficacy studies) or a well-accepted immune endpoint (effectiveness studies).

Figure 45.4 Traditional vaccine development process.

45.4 Improving Vaccine R&D

Technologies and approaches for vaccine R&D may help researchers, developers, or other scientists better identify and characterize pathogens and their antigens—the components of the pathogen that stimulate an immune response—and determine how the human immune system responds to pathogens. This improved understanding may also result in more efficient generation of safe and effective vaccines and other biological products, such as monoclonal antibodies.[15] However, the use of some technologies may be affected by their inherent technical limitations, complexity, and high cost. Further, while policymakers—which include Congress, federal agencies, state and local governments, academic and research institutions, and industry—have supported vaccine R&D for many infectious diseases, it is not clear the extent to which they have prioritized specific potential pandemic pathogens for vaccine R&D.[16] This lack of clarity raises questions about whether vaccine R&D efforts, and the technologies needed to support those efforts, enhance the capabilities to best respond to endemic levels of infectious disease and potential future epidemics and pandemics.

Key factors in conducting vaccine R&D are:

- Identifying and characterizing a pathogen's key antigens and the immune system response
- Applying knowledge about antigens and immune responses to rapidly develop vaccines or other biological products that safely and effectively stimulate or complement an immune response
- Considering the various routes of delivery, including alternative routes such as dermal or oral, that may help maximize vaccination rates

Identifying antigens that stimulate a protective immune response has, traditionally, been a slow process done largely through trial-and-error testing. Any given pathogen may have thousands of potential antigens, and the human immune system includes many different types of cells with different functions,

[15]Biological products, which include a wide range of products—including vaccines, blood and blood components, allergenics, somatic cells, gene therapy, and tissues, among other things—are derived from living sources such as humans, animals, and microorganisms. See 42 U.S.C. § 262(i)(1) and 21 C.F.R.§600.3(h) (2020).

[16]NIH conducts and supports basic, translational, and applied clinical research that contributes to technological advances relevant for vaccine development. For example, NIH officials noted the National Institute of Allergy and Infectious Diseases (NIAID) role in funding research on SARS and Middle East Respiratory Syndrome (MERS) contributed to the successful development of vaccines for COVID-19. However, it is noted that no commercial vaccines for SARS or MERS exist. This is partly attributed to lack of continued investments in a vaccine for SARS, for which cases ceased to be reported, and for MERS, which resulted in relatively few and geographically isolated cases. One expert who had developed a SARS vaccine candidate stated that had that vaccine been able to proceed through phase 1 clinical trials and stockpiled, it could have been beneficial for COVID-19 and accelerated vaccine development.

not all of which are fully understood. To identify the antigens that most effectively stimulate a protective response in the human immune system, researchers typically select and test potential antigens one or a few at a time. Researchers then run laboratory tests or conduct animal studies to see if any of the antigens they selected produces a protective immune response. (See Box 45.1 for one example of how antigens were identified.) It is also difficult to quickly develop vaccines that safely and effectively stimulate an immune response. There are many reasons for this, but two key factors are the time needed to produce antigens and a lack of adaptability. First, traditional vaccine development often requires the growth of pathogens or the use of other cells, such as bacteria, yeast, or insect cells, containing the antigen(s) of interest from the pathogen. Viral pathogens can be grown in eggs or other cells—known as cell cultures. These methods can take months to years to develop. Second, traditional methods of vaccine development may be not highly adaptable for multiple pathogens or diverse antigens. This may limit the ability to quickly develop vaccines when new pathogens emerge or change a vaccine when variants arise.[17]

Finally, administering vaccines by injection using a needle and syringe—the traditional method of delivering vaccines—may affect, for example, some individuals' willingness to get a vaccination due to a fear of needles. Additionally, the need for trained personnel and the costs and potential scarcity of vaccination supplies (e.g., vials, syringes, and needles), particularly during pandemics, can make administration of vaccines using traditional injections difficult.

Box 45.1 Coronavirus research

In the case of COVID-19, identifying and characterizing the spike protein antigen was the result of decades of previous research dating back to human coronavirus research begun in the 1960s, the Severe Acute Respiratory Syndrome (SARS) epidemic in 2003-2005, and the Middle Eastern Respiratory Syndrome (MERS) outbreak of 2012. If earlier research on SARS and MERS had not been funded, development of a COVID-19 vaccine may have taken significantly longer.

Source: GAO analysis of information from literature and an expert meeting.

45.5 Technologies and Approaches for Vaccine R&D

Drawing on information from experts, stakeholders, and related literature, The GAO identified four technologies and approaches that may improve vaccine R&D (Table 45.2). Additional information on these technologies and approaches are provided further down.

[17]A variant is a new form of the same pathogen that arises from distinct changes—also known as mutations—in the genetic sequence of the pathogen.

Table 45.2 Selected technologies and approaches for vaccine research and development (R&D)

Name	Description
Omics	Omics refers to the combined analyses of DNA (genomics and epigenomics), RNA (transcriptomics), proteins (proteomics), other small molecules (metabolomics), and other biological components. In vaccine R&D, omics is meant to improve the understanding of pathogens and host immune responses.
Reverse vaccinology	Reverse vaccinology uses computer-based analytics to assess a pathogen's genetic code and identify potential antigens. Reverse vaccinologyallows researchers to identify potential vaccine antigen candidates without the need to grow the pathogens and develop vaccines that were previously difficult or impossible to make.
Next-generation vaccine platforms	Next-generation vaccine platforms incorporate the genetic information that codes for a pathogen's antigen into a delivery vehicle. A delivery vehicle can be another virus (viral vector), a microparticle, or a lipid nanoparticle. The delivery vehicle protects the genetic information until it is administered into an individual, where the immune response is triggered. The platform may also be able to be used in a plug-and-play fashion to pair a delivery vehicle with different genetic sequences to create new or updated vaccines. Vaccine platforms may have uniform, predictable characteristics, such as safety effects; however, each antigen in a specific platform will have different immune response characteristics.[a]
Routes of vaccination	Traditional vaccinations are delivered by injection either under the skin (subcutaneous) or into muscle (intramuscular). The identification and use of nontraditional vaccine delivery routes, such as dermal (skin) and mucosal (oral, nasal) may offer the potential for better immune responses, increased public acceptance, and lower dosages.

Source: GAO.

[a]According to National Institutes of Health (NIH), vaccine platform technologies are approaches, delivery systems, and other tools that serve as the basis for delivering those vaccine antigen designs and for the development of candidate vaccines. Vaccine prototype design is the research and development that results in a candidate antigen design as the basis of a vaccine.

The technologies and approaches we identified can be applied at different stages of vaccine R&D. For example, in early exploration and research, omics and reverse vaccinology (Fig. 45.5) can help researchers to more rapidly identify antigens and how they stimulate an immune response.

For later stages of vaccine R&D, genetic code for the identified antigens can be quickly incorporated into delivery vehicles, such as lipid nanoparticles or viral vectors, accelerating vaccine testing (Fig. 45.6).

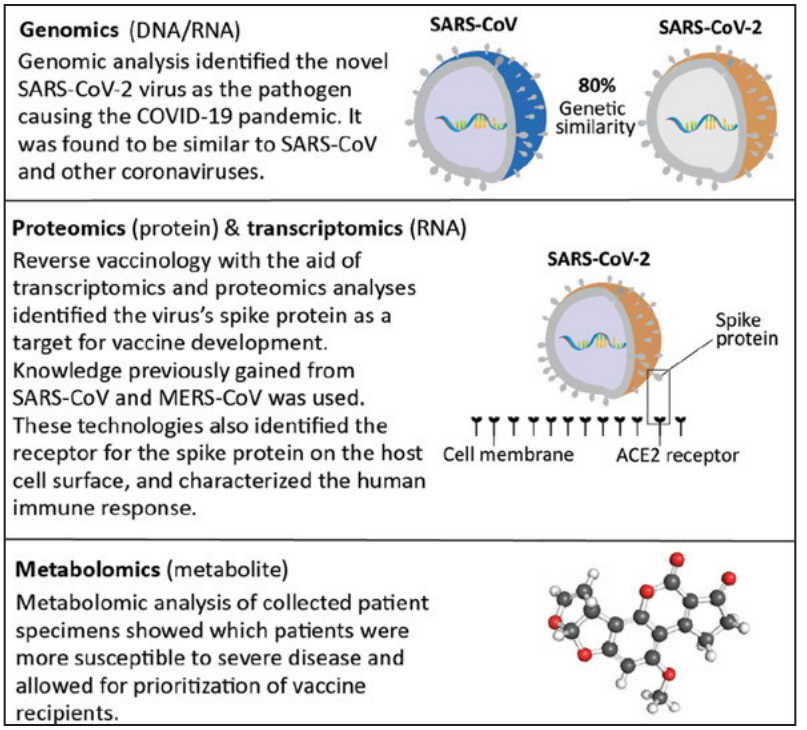


Figure 45.5 Researchers used omics and reverse vaccinology to develop COVID-19 vaccines and prioritize vaccine recipients more quickly and effectively. Source: GAO analysis and gamelover/fizzgig/stock.adobe.com.

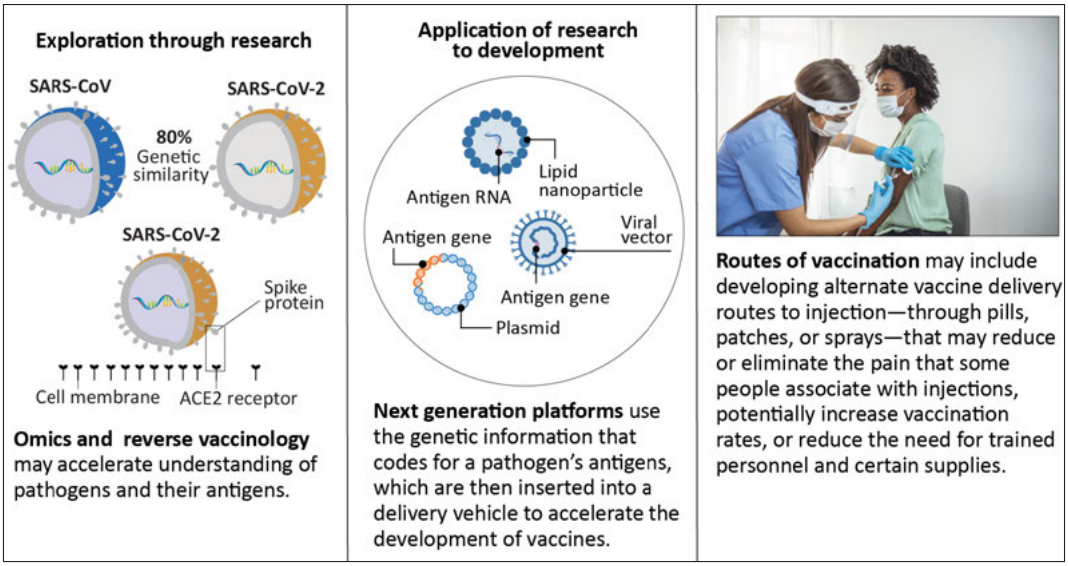


Figure 45.6 Selected application of technologies and approaches used during vaccine research and development. Source: GAO analysis of scientific literature and narstudio/ Dragana Gordic/stock.adobe.com.

Omics and reverse vaccinology aid in the early exploration process by helping researchers to more quickly identify antigens that stimulate a protective immune response by analyzing the pathogen's DNA, RNA, proteins, or other biological molecules. Then, researchers can apply computer simulations to predict more quickly which of the potential antigens may stimulate an immune response and which modifications to the antigens may enhance the immune response. As a result, researchers can more quickly begin testing those antigens that are most likely to work. In this context, see Box 45.2 for an example of how reverse vaccinology has been used.

Box 45.2 Reverse vaccinology and the COVID-19 vaccine

Researchers used reverse vaccinology to accelerate development of some COVID-19 vaccines. When the genetic code of SARS-CoV-2—the virus that causes COVID-19—became available, researchers quickly identified it as a coronavirus similar to the Severe Acute Respiratory Syndrome (SARS) coronavirus. According to the National Institutes of Health (NIH), this enabled researchers to use information gained from prior National Institute of Allergy and Infectious Diseases (NIAID)-funded studies, among others, that used reverse vaccinology and protein structure analysis to characterize the SARS and Middle East Respiratory Syndrome (MERS) spike protein antigens and their human cell receptors. This then helped researchers more quickly identify, assess, and stabilize the spike protein from SARS-CoV-2, which, in turn, allowed for the quick development of potential COVID-19 messenger RNA (mRNA) vaccine candidates, according to NIH. Researchers were able to test mRNA vaccines in a phase 1 clinical trial within 90 days of the SARS-CoV-2 genetic code release. In comparison, the vaccine candidates for MERS and SARS reached clinical trials within about 22 months and 25 months, respectively, after their outbreaks. Dengue, Chikungunya, and Zika vaccine candidates took even longer to reach clinical trials: approximately 52 years, 19 years, and 9 years, respectively, after declaration of major outbreaks. According to Food and Drug Administration (FDA) officials, other differences, including the nature of the pathogens and funding levels, also contributed to the extended development timeframes.

Source: GAO analysis of literature.

Omics can also help researchers characterize the human immune system by allowing researchers to understand which cells provide protection. Omics analysis of the immune system may also enable researchers to predict how individuals may react to vaccines and could allow for tailored vaccines for certain populations.[18]

Next-generation vaccine platforms (Fig. 45.7), such as nucleic acid (e.g., mRNA and DNA) and viral vector platforms, also aid in the development of a vaccine.

[18]For example, researchers used omics analysis to characterize the differences in the immune system to the hepatitis B vaccine in two groups of older adults, one that quickly produces high levels of antibodies and another that does not produce any response. The results of these studies may enable researchers to better predict vaccination outcomes—whether a strong, weak, or no immune response occurs—in individuals and help researchers increase vaccine effectiveness.

Specifically, next-generation platforms can help speed development, particularly when new pathogens emerge, because they rely on the pathogen's genetic information that codes for antigens. This eliminates the need to grow the pathogen or purify antigens. These platforms are also highly adaptable to multiple pathogens, allowing the development of many different vaccines to address a diverse range of pathogens on a single platform. They also have the potential to be used to develop universal vaccines which protect against multiple pathogens from the same or closely related families. However, next-generation platforms may not be practical for certain pathogens or developing countries, according to one expert we spoke to.

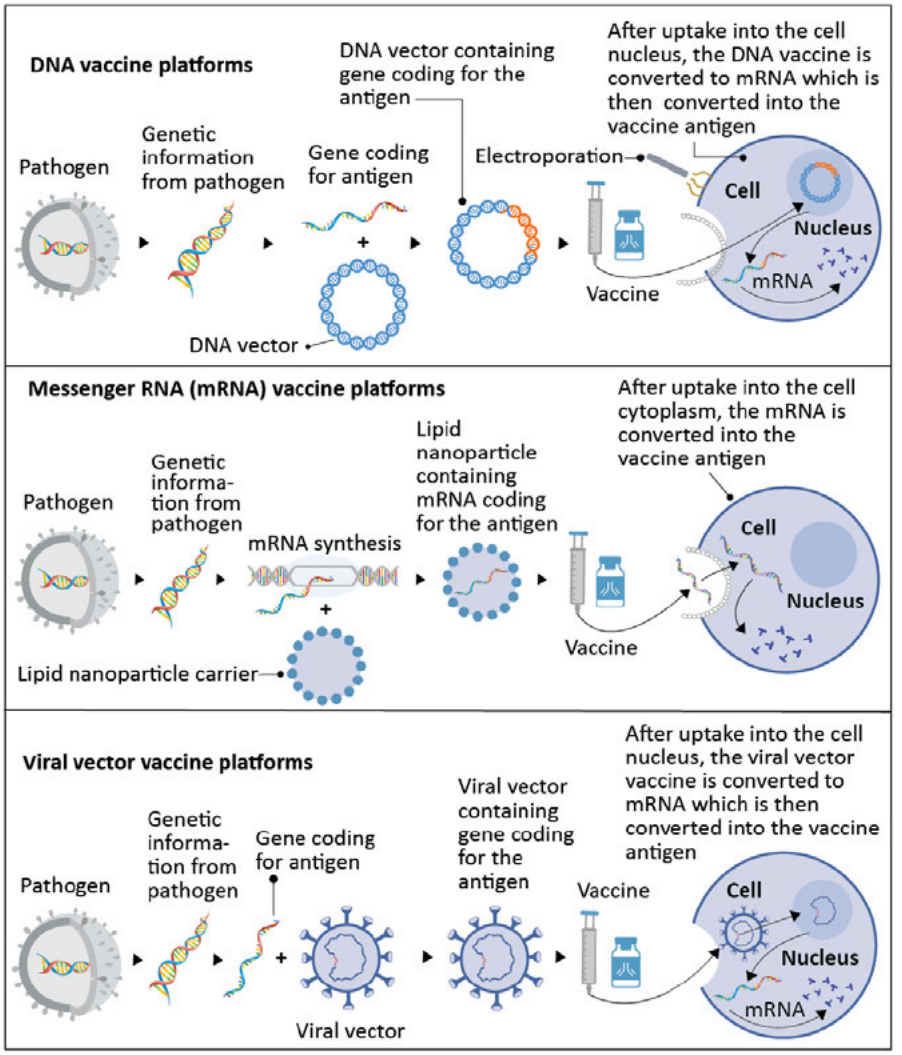


Figure 45.7 Vaccine platforms. Source: GAO (analysis); adaption of images depicting vaccine technologies with permission from Springer Nature: *Nature* ("The Race for Coronavirus Vaccines: A Graphical Guide," Ewin Callaway) © 2020 and MariLee/fizzgig/Sir.Vector/stock.adobe.com.

Three nontraditional routes of vaccination—dermal, nasal, and oral—are an important consideration in vaccine development (Fig. 45.8). Using nontraditional routes of vaccination can reduce or eliminate the pain that some people associate with injections, potentially increasing vaccination rates or reducing the need for trained personnel and certain supplies.

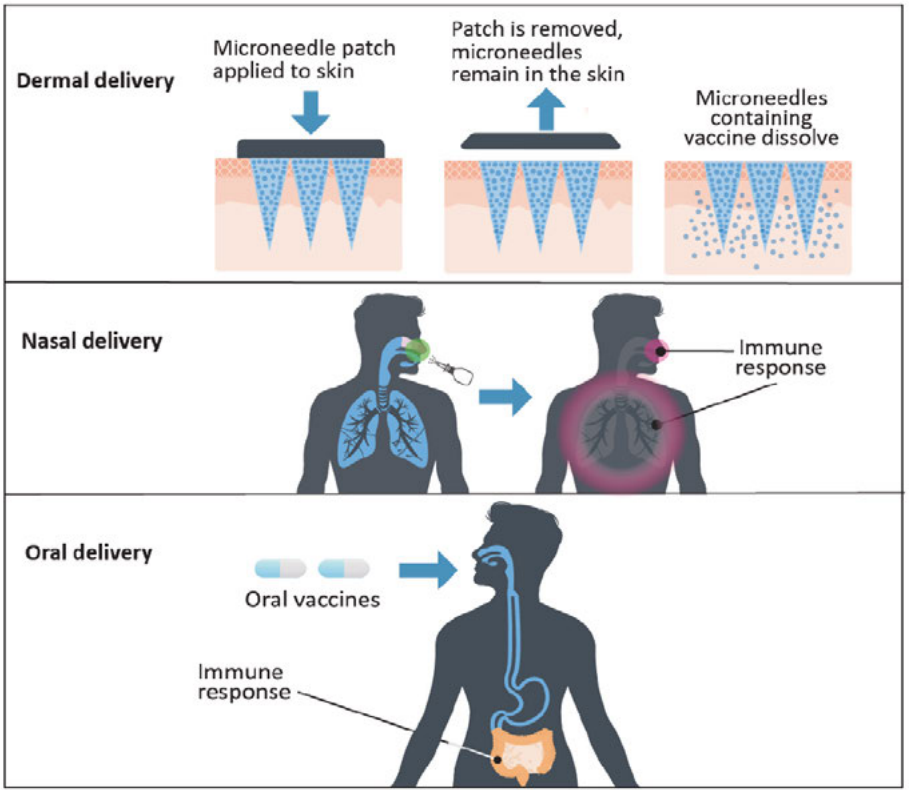


Figure 45.8 Nontraditional vaccination methods. Source: GAO analysis, Harro © magazine by Harro Hoefliger, Bharat Biotech, and Melazerg/Pavlo Plakhotia/stock.adobe.com.

- **Dermal delivery.** Delivering vaccines through the skin can produce strong immune responses at much lower doses than intramuscular and subcutaneous vaccines, making it a good route for vaccination. Microneedles, which can be nearly pain-free, are small structures designed to pierce the skin and deliver vaccines in the epidermis or dermis layers.[19] FDA has licensed one influenza vaccine that uses dermal delivery.
- **Nasal delivery.** Nasal vaccines can induce immunity even at distant sites of the body. The antigens are taken up by cells in the nasal cavity, stimulating a potent antibody in the respiratory tract that prevents the pathogen from entering the body. The antigens also stimulate the body's overall immune response, which may increase the general effectiveness of

[19]The epidermis is the outermost layer of skin, which provides a waterproof barrier and contributes to skin tone. The dermis lies just beneath the epidermis and contains tough connective tissue, hair follicles, and sweat glands.

the vaccine. There are FDA-licensed nasal vaccines for seasonal influenza; however, in the past, one was less effective than an injected vaccine.[20]

- **Oral delivery.** The intestine contains 70 to 80 percent of all antibody-producing cells in the body. Oral vaccination—delivered in a pill or liquid form—can induce a broad protective immune response in the body (including the intestine), which can be difficult to achieve using injections with needles and syringes. However, oral vaccines must be formulated to protect against degradation in the gastrointestinal tract, while still stimulating an effective immune response. Examples of oral vaccines in use today include those for polio, rotavirus, and cholera.[21]

Additionally, experts we spoke with emphasized that monoclonal antibodies are emerging as a potential approach to preventing infections. Monoclonal antibodies are laboratory-produced antibodies that act to mimic the immune system's ability to fight off pathogens. They are not vaccines and have traditionally been used as treatments for individuals that are already infected (Table 45.3; Fig. 45.9). However, many of the same types of technologies—for example, omics and delivery platforms—used for vaccine development could also be used for monoclonal antibodies.

Table 45.3 Monoclonal antibodies and vaccines work differently to protect against disease

	Monoclonal antibodies	Vaccines
What is it?	A protein that binds to a pathogen to mitigate effects of an infection	A modified pathogen or a part of a pathogen that triggers the immune system
How is it used?	To provide treatment or short-term protection to avoid infection	To stimulate the immune system to fight against a pathogen
How quickly does it work?	Immediately	Several weeks after all required doses are given
How long does the protection last?	Weeks to months	Years to lifetime (some vaccines require boosters)

Source: GAO analysis.

[20]After the 2009 H1N1 influenza pandemic, several U.S. studies among 2 through 17-year-olds found that the live attenuated influenza vaccine administered intranasally was as effective against influenza B viruses and influenza A (H3N2) viruses as the traditional injectable vaccine, but it was less effective than the injectable vaccines against the 2009 pandemic H1N1 viruses. These data led CDC to recommend against use of this specific vaccine for the 2016–2017 and 2017–2018 influenza seasons. For more information on the flu mist nasal vaccine, see https://www.fda.gov/vaccines-blood-biologics/vaccines/fda-information-regarding-flumist-quadrivalent-vaccine.

[21]Oral poliovirus vaccine is no longer used in the U.S., but it is used in other countries. The U.S. no longer uses the oral poliovirus vaccine due to the risk of vaccine-derived poliovirus disease in certain individuals. However, other oral vaccines have been shown to be safe when not contraindicated.

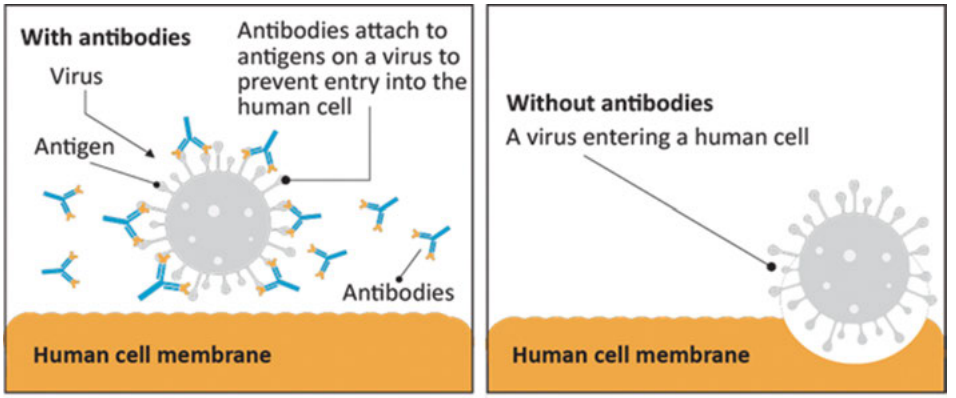


Figure 45.9 Monoclonal antibody targeting SARS-CoV-2, the virus that causes COVID-19. Source: GAO analysis, and Maryland Department of Health Office of Preparedness and Response, and iiierlok_xolms/stock.adobe.com.

Unlike vaccines, which can stimulate an individual's immune system to produce protective antibodies for years, monoclonal antibodies may provide shorter protection against infectious diseases, usually for weeks to months. If able to be developed and used early in a pandemic, monoclonal antibodies may potentially provide some initial benefit. It is for this reason that we include monoclonal antibodies (Box 45.3) as an approach that could be considered alongside traditional vaccine R&D.

Box 45.3 COVID-19 monoclonal antibodies

During the COVID-19 pandemic, the Food and Drug Administration (FDA) issued four emergency use authorizations (EUA) for monoclonal antibodies to treat COVID-19. Two of these four treatments consist of a mixture of two monoclonal antibodies, while the remaining two treatments each consist of a single monoclonal antibody. Additionally, researchers are investigating using monoclonal antibodies to protect against COVID-19 before someone is infected or for individuals with compromised immune systems. For example, one developer recently published clinical trial data showing that a mixture of two monoclonal antibodies reduced the risk of people developing any COVID-19 symptoms by 77 percent.

Source: GAO analysis of literature.

45.6 Addressing Challenges Related to Vaccine R&D: Policy Options

The GAO identified two policy options[22] that may help address challenges related to the adoption of vaccine R&D technologies and approaches (Table 45.4).

[22]The GAO defines policymakers in this chapter as a broad term including, for example, Congress, federal agencies, state and local governments, academic and research institutions, and industry.

Table 45.4 Policy options that may help address challenges with developing vaccines for infectious diseases

Policy option	Opportunities	Considerations
Prioritize infectious disease pathogens *Policymakers could collaborate across sectors (e.g., government, academia, researchers, industry, and nonprofit organizations) to prioritize infectious disease pathogens with pandemic potential for vaccine R&D.* For example, policymakers could develop a working group to prioritize pathogens with pandemic potential and work more closely with international organizations to prioritize vaccine development as well as develop monoclonal antibodies as prophylactics and therapeutics. The working group could also periodically revisit the prioritized list and update as appropriate to ensure newly identified threats are addressed. This could help address the challenges we identified related to appropriately prioritizing potential pandemic pathogen vaccine R&D efforts and the technologies and approaches needed to support those efforts, address technological limitations, and focus the use of costly instruments and personnel.	Prioritizing pathogens with pandemic potential could improve strategic vaccine R&D decision-making and help focus resources on developing and adopting key technologies and approaches that most effectively address those pathogens. Appropriately matching the technologies and approaches to the prioritized potential pandemic pathogen, then leveraging technologies and expertise held by various entities—such as government, private sector, and academic laboratories—may help address certain technological limitations and costs. With greater leadership and strategic partnerships, policymakers could take steps to increase preparedness to more quickly address threats to the U.S. population.	As new threats are identified, priorities may change, which may cause uncertainty for vaccine developers. Policymakers may have different priorities based on their respective missions; for example, private sector priorities may differ from government priorities. There may be disagreements as to which key technologies should be prioritized and used, resulting in the need for policymakers to weigh the potential advantages and disadvantages associated with various options.
Improve preparedness *Policymakers could provide funding and other support for public/private partnerships to strategically address potential pandemic pathogens identified as priorities.* These partnerships could, for example, develop and test vaccine candidates and monoclonal antibodies that may provide protection from high impact pathogens and pathogens with pandemic potential. Leveraging relationships with the private sector may allow for sharing of highly trained personnel and costs of the complex instruments that are critical parts of the pandemic preparedness infrastructure.	This early development of vaccine candidates and monoclonal antibodies could provide a coordinated foundation that can be mobilized in an emergency. Such an approach could speed vaccine development as well as potentially reduce risk for vaccine researchers and developers concerning questions of safety, efficacy, and manufacturability. Assessing the likelihood of outbreaks from known pathogens, and developing vaccines through at least phase 1 clinical trials may make it easier to more rapidly mitigate future pandemics.	The lack of certainty of the commercial market and government procurement for vaccines against pathogens with pandemic potential may be too risky for the private sector to undertake.

Source: GAO.

45.7 Technologies and Approaches That May Enhance Vaccine Testing

Six technologies and approaches that may improve vaccine testing and provide additional methods for assessing data gathered during clinical trials were identified (Table 45.5).

Table 45.5 Selected technologies and approaches for vaccine testing

Name	Description
Organ chips	Populated with cells and used in preclinical studies, organ chips mimic the function of human organs and can be used to study the effect of a vaccine candidate.
Artificial intelligence (AI) and machine learning (ML)	AI and ML systems can analyze large amounts of data gathered during preclinical studies and clinical trials.
Electronic health records (EHR)	An EHR is a digital record of a patient's medical information that can be used to support trials for patient recruitment, clinical data analysis, and post-trial follow-up.
Common control groups	A common control group allows multiple groups of participants in preclinical studies or clinical trials to be compared with a single control group, reducing the number of participants needed or enabling comparison among vaccine candidates.
Standardized assays	Standardized assays are standardized tests or investigative procedures that can potentially be used by different vaccine developers to determine the immune response induced by a vaccine candidate. For example, standardized assays could measure the presence of antibodies in clinical trial participants who have received different vaccines candidates.
Virtual clinical trials and wearable devices	Virtual clinical trials, also referred to as decentralized trials, extend the reach of clinical investigations to where patients live and work. Data for virtual trials can be collected remotely via wearable digital health technologies including watches, bracelets, patches, textiles, and clothing.

Source: GAO.

The technologies and approaches we identified can be applied at different phases of vaccine testing. For example, in the preclinical phase organ chips may be used to determine whether vaccine ingredients have any toxic effects on human cells, which may complement the information gleaned from testing in animals or reduce the need for such testing. Artificial intelligence (AI) and machine learning (ML) may be used for a number of purposes (Fig. 45.10). For example, AI and ML could be used during each phase to predict toxicity in preclinical studies, identify suitable participants for clinical trials, and track long-term side effects during post-marketing surveillance studies (Fig. 45.11), among other things.[23]

[23]Post-marketing surveillance studies, also referred to as phase 4 clinical trials, may be required after licensure to obtain additional information on the product's benefits, risks, and optimal use.

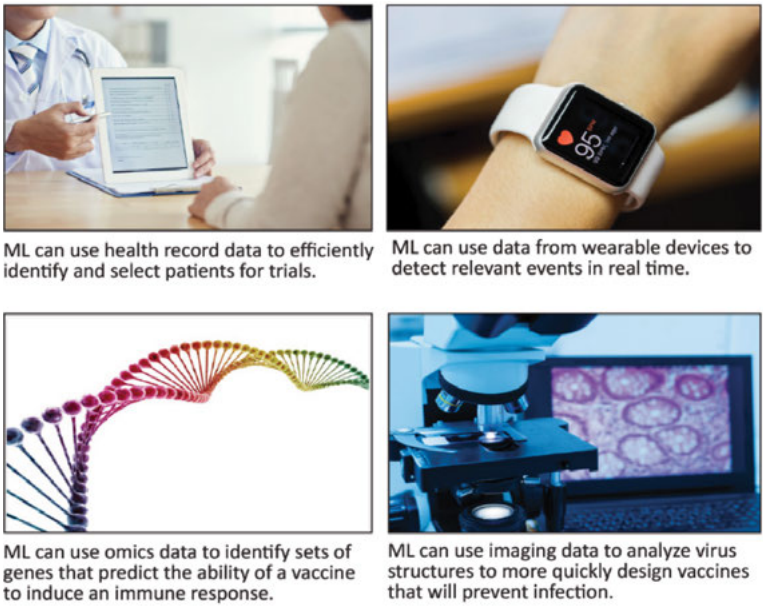


Figure 45.10 Artificial intelligence (AI) and machine learning (ML) can use various types of data to enhance vaccine development. Source: GAO analysis and Dragonimages/ballball14/arcyto/Jezper/stock.adobe.com.

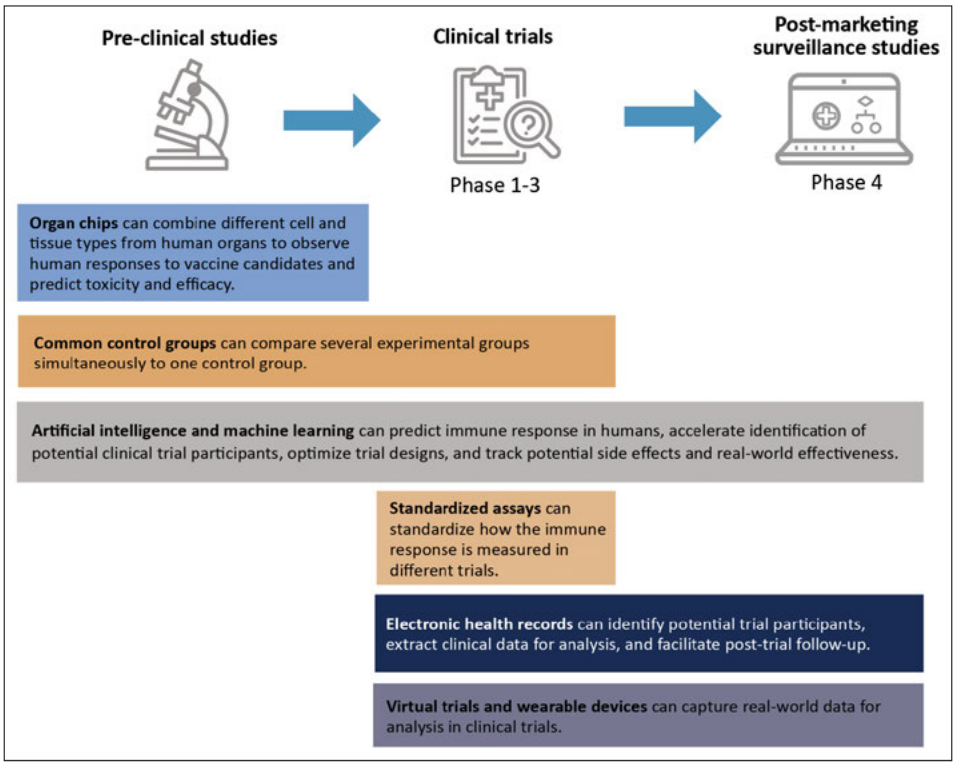


Figure 45.11 Selected applications of vaccine testing technologies and approaches. Source: GAO analysis and Sir.Vector/blankstock/stock.adobe.com.

The technologies and approaches identified may help address factors that affect clinical trials by enabling developers to better collect and analyze safety and efficacy data for various vaccine candidates and more successfully recruit and retain clinical trial participants, among other things (Fig. 45.12).

Technology /approach	Safety monitoring Although safety is monitored in each phase of a clinical trial, issues may only become apparent once larger populations participate in phase 3 trials.	Recruiting and retaining participants A trial that does not enroll enough participants can result in a sample that is too small to yield valid results.	Costs Clinical trials require extensive funding, time, and resources that increase at each successive phase as increasingly larger numbers of volunteers participate.	Demonstrating efficacy The primary reason for clinical trial failure is an inability to demonstrate efficacy.
Organ chips	May complement animal testing to predict a vaccine candidate's toxicity in humans prior to clinical trial testing in humans.	N/A	May reduce the number of animals needed for preclinical studies.	N/A
Artificial intelligence and machine learning	May predict whether a vaccine candidate may cause a low or high number of adverse side effects and may identify rare adverse events from safety reports.	May identify potential participants by assessing suitability from health records and other patient data.	May save money by identifying design efficiencies, such as the ideal number of participants to enroll and the optimal dosage.	May analyze clinical and immunological data from trials and predict antibody response in humans, thereby identifying the immune protection a vaccine candidate may provide.
Common control groups	May allow for evaluating safety data across multiple vaccine candidates.	May increase a patient's chance of receiving a vaccine candidate, which could help with recruiting patients to clinical trials.	May save money since fewer participants are needed when comparing experimental groups to a single control group.	May allow for evaluating efficacy data across multiple vaccine candidates.
Standardized assays	N/A	N/A	N/A	May establish a standard measurement approach that allows for head-to-head vaccine candidate comparisons.
Electronic health records	May be used in safety surveillance to answer specific safety questions about vaccine candidates.	May help identify which individuals receiving care in a health system meet study criteria as eligible participants.	May save money and time by centralizing data collection with an electronic clinical trial management system.	May provide data that can be analyzed for clinical trials.
Virtual trials and wearable devices	May allow for continuous safety monitoring of vaccine candidate side effects.	May expand the pool of potential participants and enable remote data capture, which reduces barriers to participation.	May save money after the initial expense of adopting the technology.	May collect data continuously, potentially providing a more complete picture of participants' health during the trial.

Figure 45.12 Technologies and approaches that may improve vaccine clinical trials. Source: GAO analysis and SergeyCherednichenko/dzm1try/kornkun/blankstock/stock.adobe.com.

45.8 Challenges Related to Vaccine Testing: Policy Options

Two policy options were identified that may help address challenges related to the adoption of testing technologies and approaches (Table 45.6).

Table 45.6 Policy options that may help address challenges related to vaccine testing

Policy option	Opportunities	Considerations
Further support development of data standards *Policymakers could further support coordinated efforts to obtain the views of all stakeholders and to develop standards for health data and their use in clinical trials.* This support could help address the challenge we identified related to ongoing data standards development.	• Integrating researchers' needs into the standards development process could better ensure the necessary data are available. • Data standards could more easily allow researchers to combine different data sets, enabling better transmission of data for analysis in trials. • Interoperable systems may eliminate the manual transcription of data, reducing data entry errors. • Access to high-quality data in a standardized format may allow streamlined patient recruitment for clinical trials. • Improving standards may facilitate identification of differences in clinical trial efficacy across population subgroups (e.g., differences by age, race, and gender) by broadening the pool of patient records available for research.	• Expanding access to patient heath data requires attention to ensure privacy. If patient data have been de-identified, combining data from multiple sources may make it easier to re-identify. • Data from different sources, such as wearable devices and clinical notes, may vary in quality and reliability, making it difficult to use them in combination. • Developing and implementing standardized data formats and IT infrastructure is time-consuming and costly. • Disparities in access to health care, particularly among some population groups, may limit the pool of patient records available for research.
Study feasibility of using common control groups and standardized assays *Policymakers could study the feasibility of collaborating with industry for use of standardized assays and common control groups during pandemic and non-pandemic scenarios.* This option could help address the challenge we identified related to limited stakeholder collaboration and agreement on common approaches to testing.	• Trial logistics could be streamlined, and cost-sharing could produce savings. • Meeting recruitment goals could be faster, which can be important during pandemics. • Trial participants are more likely to receive a vaccine candidate, which may boost recruitment. • Incorporating adaptive trial designs in planned protocols may lessen regulatory review requirements when modifications need to be made. • A head-to-head comparison of vaccine candidates, enabled by following master protocols, improves understanding of efficacy. The master protocol approach may also facilitate the implementation of clinical research across successive outbreaks of a disease since protocols will already be in place.	• Vaccine developers may be unwilling to give up control of designing trials and use of proprietary assays, and may resist having head-to-head comparisons with other vaccine candidates. • If not agreed to in advance, stakeholder coordination, infrastructure requirements, and complex trial design elements could make the start-up time for a master protocol longer than that of a single-purpose trial. • Determining the timing and predicting the required funding level could be difficult if new vaccine candidates will be added to a trial design on an ongoing basis. • Developing assays requires significant effort. Harmonizing results across vaccine candidates, as was done for COVID-19 phase 3 clinical trials, may be more achievable than requiring developers to use a standardized method to measure immune response.

Source: GAO.

45.9 Technologies and Approaches for Vaccine Manufacturing

Certain technologies and approaches for vaccine manufacturing may have the potential to enhance the U.S.'s ability to address infectious diseases and prepare for future epidemics and pandemics. These technologies and approaches may allow for an increase in manufacturing flexibility—the ability to quickly switch from manufacturing one vaccine to another—and an increase in manufacturing productivity. However, challenges such as technical limitations, costs, and the need for highly trained personnel affect vaccine manufacturers' ability to adopt new technologies and approaches. Further, private manufacturers may be reluctant to establish and maintain costly excess manufacturing capacity to address surges in vaccine demand during pandemics. The federal government has attempted to address these issues; however, challenges remain.

Drawing on information from experts, stakeholders, and the scientific literature, five selected technologies and approaches that may improve vaccine manufacturing—collectively known as bioprocess intensification were identified (Fig. 45.13 and Table 45.7). These selected technologies and approaches are not necessarily new: some have been used for years in other industries, including chemical and pharmaceutical manufacturing, and some have already been applied in vaccine manufacturing processes. These technologies and approaches may help vaccine manufacturers increase flexibility and capacity by allowing for rapid switching between different vaccines within the same facility, increasing productivity to help manage surge demand, and potentially distributing manufacturing closer to points of need.

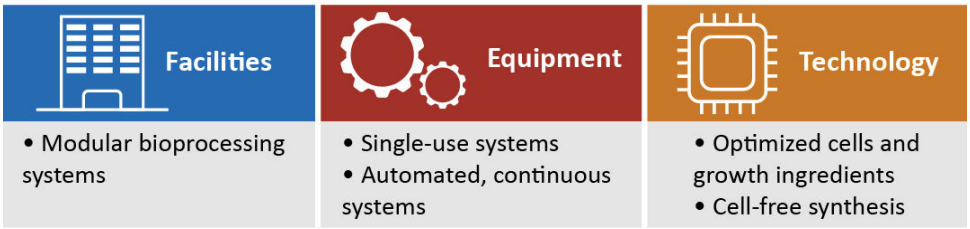


Figure 45.13 The three parts of bioprocess intensification. Source: GAO | GAO-22104371.

The technologies and approaches identified can be applied at different stages of vaccine manufacturing. For example, modular bioprocessing systems may replace fixed, inflexible infrastructure, potentially allowing for rapid switching between vaccines, scale up, and customization. Process optimization can enable vaccine manufacturers to increase antigen yields and use smaller production volumes through, for example, the use of specific cell lines and growth ingredients (Fig. 45.14).

Table 45.7 Selected technologies and approaches for vaccine manufacturing

Name	Description
Single-use systems	Single-use systems refer to bioprocessing equipment that is designed to be used once and then discarded. Such equipment is generally composed of sealed, pre-sterilized, plastic components.
Modular bioprocessing systems	These systems divide the manufacturing process into smaller functional building blocks known as modules, suites, or pods that can stand alone or be incorporated into an existing facility. For example, new modules can be added to quickly expand capacity or switched to rapidly change processes, according to an expert we spoke to.
Cell-free synthesis	Biological enzymes—proteins that cause biochemical reactions—are used to generate antigens, which are then combined with other materials to create vaccines.
Process optimization	This approach improves the cells and growth ingredients—known as medium—and other processing steps. According to an expert we interviewed, this technology may increase productivity and allow manufactures to get more out of the same equipment or facility.
Continuous manufacturing systems	These systems use automated, high-throughput, small-footprint production and purification equipment to manufacture vaccines. In contrast to existing batch processing methods, which use separate tanks for each step in the process, continuous manufacturing allows all steps of vaccine production to continue without interruption as the materials flow through the system.

Source: GAO.

Single-use systems may help vaccine manufacturers increase the flexibility of their vaccine manufacturing facilities. For example, manufacturers can replace traditional stainless-steel vessels—known as bioreactors—that are used to grow the cells that produce antigens with disposable plastic bioreactor bags. Single-use systems eliminate the need for cleaning and sterilizing fixed equipment between vaccine manufacturing runs—also known as batches—resulting in shorter turn-around times and increased efficiency. Since they are discarded after each use, single-use systems may also reduce the potential for contamination caused by inadequate cleaning or sterilization.

Modular bioprocessing systems may also help vaccine manufacturers increase the flexibility of their facilities. For example, a vaccine facility's fixed infrastructure, such as rooms and areas dedicated to specific bioprocessing steps, can be replaced with modular components. These modular components can allow manufacturers to rapidly switch from manufacturing one vaccine to another, scale up manufacturing, or add a new facility in a new location more quickly, according to an expert we spoke to. Modular bioprocessing systems also allow vaccine manufacturers to

continuously customize and reconfigure their equipment to accommodate new vaccines or processes more quickly—changes that are traditionally difficult and costly. This ability to customize bioprocesses could allow vaccine manufacturers to establish smaller vaccine manufacturing facilities at sites closer to infectious disease outbreaks.

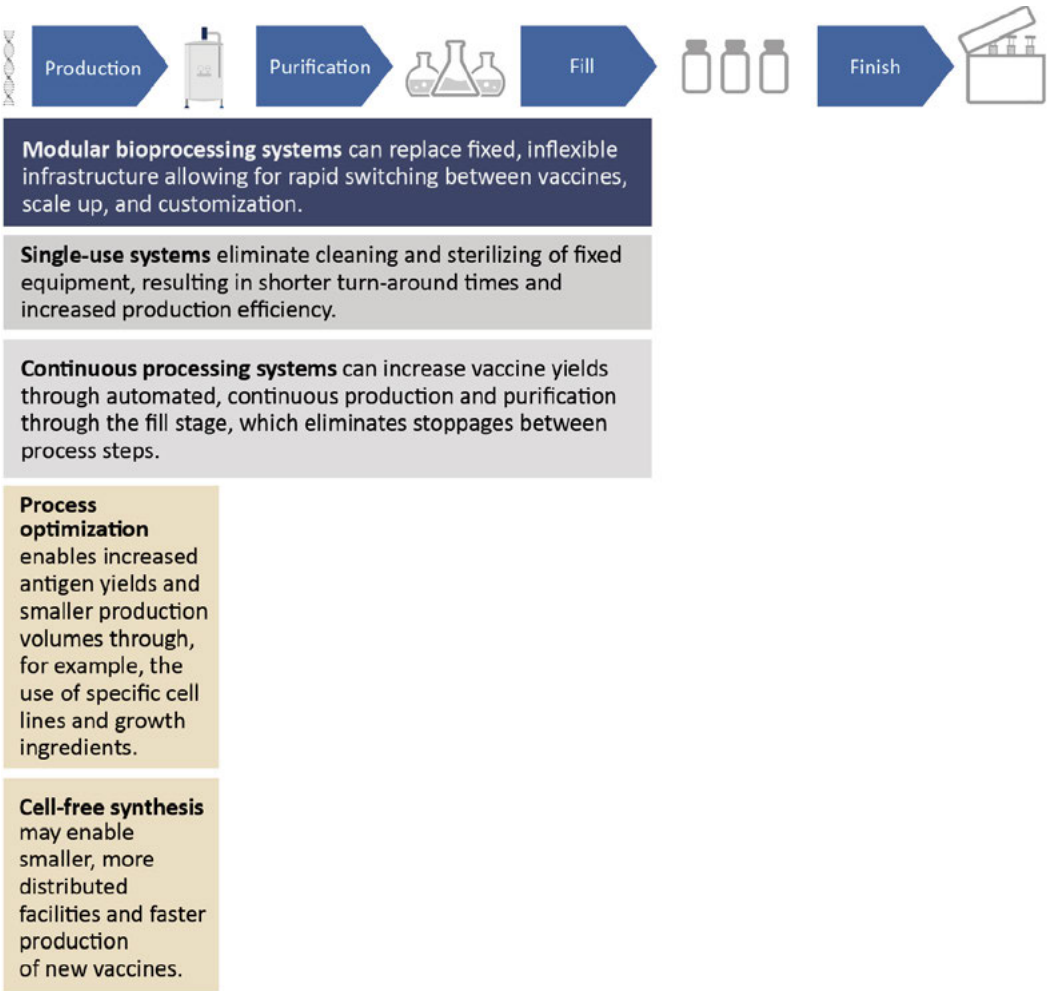


Figure 45.14 Selected applications of vaccine manufacturing technologies and approaches. Source: GAO analysis of scientific literature and rashadashurov/tutti_frutti/StockBURIN/divstock/stock.adobe.com.

For some vaccines, cell-free synthesis may eliminate the need to grow living cells to produce the antigen of interest, potentially resulting in smaller, more distributed facilities, and faster manufacture of new vaccines in existing facilities. Cell-free synthesis combines purified biological molecules to produce antigens,

which are then purified and formulated into vaccines.[24] Additionally, while the first step in mRNA vaccine manufacturing involves traditional growth of cells to generate a key component—DNA—needed to make the vaccine, the subsequent step to manufacture the mRNA uses cell-free synthesis. The flexibility of cell-free synthesis reduces the single point of failure risk that may be associated with centrally located, dedicated product facilities. Further, cell-free synthesis may allow for the simultaneous manufacture of different vaccine antigens within the same facility, which cannot be done in most existing facilities.

Process optimization allows manufacturers to increase antigen yields through, for example, the use of cells specifically developed to increase antigen productivity, known as optimized cell lines, matched with specific growth ingredients, known as growth medium. Antigen productivity can be increased by growing cells at higher densities.[25] High-cell density can be achieved by selecting for or artificially modifying cells that grow to high densities or by changing how the cells are grown. For example, cells that freely grow in liquid medium—called suspension cultures, typically result in higher cell densities and antigen yields than cells that have been adapted to grow attached to a surface, called adherent cultures.

Continuous manufacturing systems may also increase vaccine yields through automated, continuous antigen production and purification. Continuous manufacturing systems may run for weeks or even months, reducing, for example, the requirement to start new cell cultures for antigen production, stoppages between production and purification steps, and the potential for contamination. For example, one manufacturer we spoke with has tested its closed-production systems with continuous downstream processing technology to produce vaccines for clinical trials.[26]

45.10 Challenges Related to Vaccine Manufacturing: Policy Options

Two options for policymakers that may help address challenges related to the adoption of technologies and the improvement of vaccine manufacturing capacity and operational readiness (Table 45.8; next page).

[24]Cell-free synthesis uses a number of purified biological molecules, including enzymes and nucleic acids.

[25]Cell density describes the number of cells in a specific volume of growth medium.

[26]A closed-production system uses equipment designed and operated in such a way that the product is not exposed to the room environment. Materials may be introduced to a closed system, but exposure of the product to the room environment must be avoided.

Table 45.8 Policy options that may help address challenges related to vaccine manufacturing

Policy option	Opportunities	Considerations
Assess vaccine manufacturing capacity and operational readiness *Policymakers could routinely assess U.S. manufacturing capacity and operational readiness.* For example manufacturing capabilities could be pressure tested to determine the nation's overall capability to manufacture current vaccines and meet pandemic surge demands.[a] This could help address the challenges identified related to meeting new demands without negatively impacting manufacturers' ability to produce current vaccines.	Determining U.S. vaccine manufacturing capacity and operational readiness and routinely pressure testing it can help identify gaps as well as key technologies and approaches to address them.	Vaccine manufacturing capacity requirements may change based on the specific infectious disease and vaccine platforms being pressure tested.
Improve preparedness *Policymakers could provide support and coordination for public-private partnerships to strategically develop manufacturing capacity to respond to surge requirements.* To maintain this capacity, partnerships could manufacture prototype vaccine candidates against high-priority pathogens.[b] For example, manufacturing, testing through phase 1-2 clinical trials, and stockpiling prototype vaccine candidates against prioritized classes of pathogens could decrease the amount of time needed to validate and scale up manufacturing processes if a pathogen from those classes does emerge.[c] This could help address the challenges identified related to the ability of the federal government to ensure that the manufacturing capacity to respond to pandemics is available and operational.	Manufacturing, testing and stockpiling vaccine candidates could be mobilized in an emergency and more rapidly mitigate future pandemics. By leveraging strategic partnerships, policymakers could take steps to increase the availability of vaccines to more quickly address threats to the U.S. population.	May require new resources or reallocation of resources from other efforts. There may be a risk that the vaccines manufactured, tested, and stockpiled against prioritized pathogen classes miss certain pandemic pathogens. The stockpiled vaccines would need to be regularly replenished prior to expiration.

Source: GAO.

[a]Operational readiness includes having available, well maintained equipment and facilities as well as enough trained personnel. Further, operational readiness includes maintaining "warm" manufacturing capabilities—for example, by operating at least one shift daily—so that equipment remains operational and personnel retain manufacturing competency.

[b]If able to be developed and manufactured early in a pandemic, monoclonal antibody candidates may also provide some initial benefit.

[c]One expert estimated the cost to produce and test a vaccine through phase 2 clinical trials would be approximately $50 million, with one-third of that cost required for manufacturing the vaccine.

45.11 Vaccine Development: Economics and Role of Incentives

Vaccines confer significant public health and economic benefits. However, economists we spoke with stated that the benefits that vaccines provide are not necessarily commensurate with the return on investment from developing or manufacturing them. Experts attribute the low rate of vaccine investment to market failures (i.e., market interactions that fall short of what would have been socially beneficial), challenging markets for some vaccines, high costs, and risks of development. Experts also stated that uncertainty as to whether a vaccine, once developed, would be recommended for universal use—for example, for all children as opposed to a subset of individuals with certain risk factors—is an additional risk and negatively affects incentives to develop them as it reduces the number of people recommended to be vaccinated.[27] Policymakers have a number of mechanisms to encourage investment in vaccine development. Some of these mechanisms have been used by HHS and the Department of Defense (DOD), including funding for clinical trials, and offering a financial incentive for the successful development of vaccines for COVID-19. However, it is unclear whether policymakers have systematically examined how various tools can be used to incentivize vaccine investment.[28]

As discussed earlier, vaccines have far-reaching, positive effects on public health. In addition to health benefits, such as reducing death and preventing infectious diseases and certain types of cancer, vaccines also produce economic and social benefits. For example, a July 2020 report found that vaccination enhances economic growth due to improved health as well as productivity gains from better physical and cognitive performance.[29]

Vaccines also produce social benefits including improving equity in healthcare, increased life expectancy, and strengthening healthcare resources (Fig. 45.15). The July 2020 report also found that when infrastructure is developed to administer

[27]One expert stated that vaccine entrants who enter a market first will have a larger market share. A 2014 report from McKinsey & Company found that first-to-market entrants into drug markets had a 6 percent market share advantage over later entrants. See M. Cha and F. Yu, *Pharma's First To Market Advantage*, McKinsey & Company, Sept. 2014.

[28]Several entities have reported on large profits earned by pharmaceutical companies for COVID-19 vaccines. Selected economists stated that from a benefit-to-cost perspective, it makes sense for policymakers to invest significant funds in vaccine development, manufacturing capacity, and supply chain development to respond to an active pandemic to meet immediate needs and reduce loss of life. However, investing in vaccine technologies and manufacturing capacity in preparation for future pandemics has the potential to reduce costs for vaccines because these capabilities will not have to be developed rapidly or all at once in response to a pandemic event. Similarly, having vaccine candidates under development for pathogens similar to a potential future pandemic pathogen may further reduce development costs as well as the overall price for vaccines. According to one economist, investment undertaken at a more measured pace can be cheaper because it does not stretch scarce (and thus expensive) inputs. The economist noted that procuring vaccines under less urgency facilitates entry of multiple competitors and allows a competitive tender process to be organized that favors low prices over speed.

[29]C.M., Rodrigues and S.A., Plotkin, "Impact of Vaccines: Health, Economic and Social Perspectives", *Frontiers in Microbiology*, vol. 11, article 1526 (2020): 1–15.

vaccines, it provides a basis for the provision of other health and social care services, particularly improving maternal and infant mortality in developing regions. Although making projections about the economic and social benefit of vaccines is complex, a 2005 economic article reported that current childhood vaccinations against tetanus, polio, measles, mumps, rubella, hepatitis B, and others, when considered together, create significant economic and social benefits.[30]

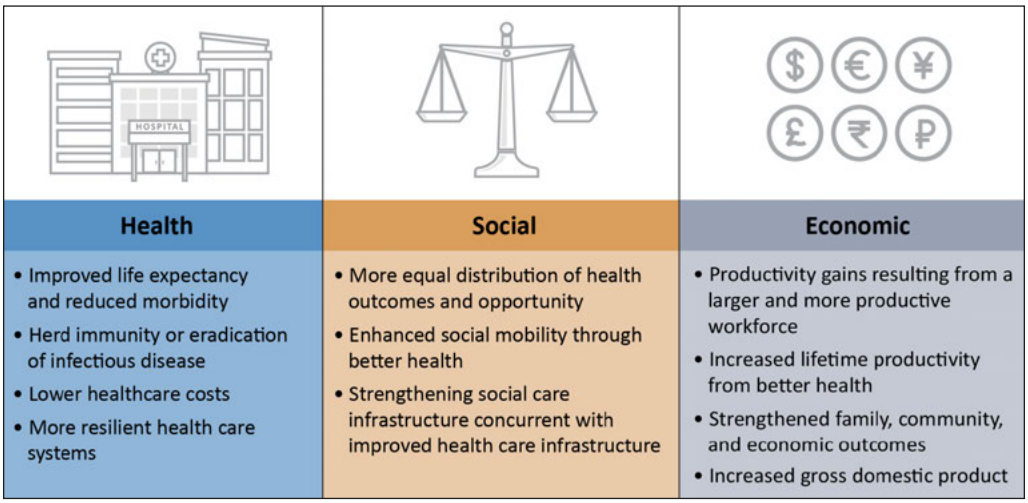


Figure 45.15 Types of benefits vaccines provide. Source: GAO analysis and literature; Vectorfair.com/RealVector/dlyastokiv/stock.adobe.com.

Several mechanisms can potentially be used to incentivize additional investment in vaccines. These mechanisms either subsidize some portion of the development process or provide rewards for successful development. Given the market failure associated with vaccines, it is unlikely private investments will sufficiently support the development of all socially beneficial vaccines. Policymakers could fill this gap by taking actions to incentivize investment in new vaccines to better address infectious disease; improve overall societal, health, and economic outcomes; and prepare for future pandemics. To do so, policymakers need a combination of tools to incentivize investment. HHS has leveraged some mechanisms to incentivize vaccine investment, most recently to respond to the COVID-19 pandemic.

Several mechanisms with the potential to incentivize vaccine investment are possible. According to experts, policymakers need access to a wide range of mechanisms, because different mechanisms are better for some infectious disease scenarios than for others. Policymaker interventions can be broadly classified into push and pull incentives (Table 45.9). Push incentives subsidize the costs of developing a product or general research in vaccines by providing

[30]Current childhood vaccines against diphtheria, tetanus, pertussis, Hib, polio, measles, mumps, rubella (MMR), and hepatitis B, when considered together, were estimated to have a benefit cost ration of more than five to one for direct costs and seventeen to one for societal costs. T.A. Lieu, et al., "Overcoming Economic Barriers to the Optimal Use of Vaccines," *Health Affairs*, vol. 24, no. 3 (2005): 667.

Table 45.9 Potential mechanisms to incentivize vaccine development

Mechanism	Definition	Opportunities	Challenges
Grants (push)	Financial assistance that may cover some or all of the costs associated with vaccine R&D	• Support basic research that cannot be incentivized with pull funding	• Do not guarantee development of a product
Tax incentives (push)	Reductions in tax liabilities to defray some of the costs of R&D	• Support basic research that cannot be incentivized with pull funding	• Do not guarantee development of a product • Can be very costly • Beneficial only if the developer has a tax liability
Advanced purchase commitments (pull)	Agreements to purchase vaccines in the future, after they are fully developed	• Product can be stockpiled for future use or used to vaccinate people immediately, or both • In many cases, paid only if a product is developed and receives emergency use authorization or licensure • Developers can be incentivized to select the most promising products	• Can be expensive, especially if several products are incentivized
Subsidizing manu-facturing capacity (push)	Allowing excess manufacturing capacity to be used to manufacture vaccines for clinical trials at low or no cost	• Developers can be incentivized to take vaccines to clinical trials due to lower manufacturing costs • Excess capacity can be used to respond to future infectious disease outbreaks	• May be expensive to maintain facilities for manufacturing • May not ensure sufficient capacity to respond to all potential infectious diseases scenarios
Prizes (pull)	Reward for receiving authorization or licensure of a vaccine product	• Product can be stockpiled for future use, used to vaccinate people immediately, or both • In many cases, paid only if a product is developed and receives emergency use authorization or licensure	• Do not reduce the cost of R&D • Would have to be sufficiently large to induce investment

Mechanism	Definition	Opportunities	Challenges
Patent extensions (pull)	An extension on a patent that exceeds the usual time limits	• Greater protection from competition and a longer period to benefit from higher prices may encourage greater innovation	• Patents can result in higher prices, making the vaccine too expensive for some patients or governments
Priority review voucher (pull)	Award for the development of drugs and biologics, including vaccines, for tropical diseases, rare pediatric diseases, and material threat medical countermeasures, which can be sold or redeemed for faster review of a future application.	• Potential for additional revenue could provide an incentive to develop vaccines for tropical diseases, rare pediatric diseases, and medical countermeasures	• The financial reward—that is, the amount of revenue earned from sale of a priority review voucher—could decline if more vouchers are awarded and available for sale

Source: GAO.

funding for grants to academic institutions, tax credits for R&D, and low or no cost manufacturing. Pull incentives increase revenue once a vaccine receives authorization or licensure. This can be done, for example, by guaranteeing to purchase a certain quantity or promising a cash prize for successful authorization or licensure. Other examples of pull incentives include patent extensions and priority review vouchers (which can be sold for revenue or used for faster review on a future drug or biologic application).

45.12 Economic Challenges to Vaccine Development: Policy Options

Three policy options (Table 45.10) for policymakers that may help address economic challenges with incentivizing vaccine development are identified. Policymakers could conduct a systematic assessment of the various mechanisms to incentivize vaccine development to determine which incentives could work best for infectious diseases identified as high priority. Policymakers could also examine the authorities necessary to use these mechanisms and, to the extent that agencies lack such authority, take steps to obtain or provide it.

Table 45.10 Policy options that may help address economic challenges related to vaccine development

Policy option	Opportunities	Considerations
Evaluate factors that discourage vaccine investment *Policymakers could collaborate across sectors, such as government, academia and industry, to conduct a systematic evaluation of factors that discourage developers from investing in new vaccines.* This could help address the challenges we identified related to market failures, challenging markets, high costs, and low probability of success.	A clear understanding of the range of factors discouraging vaccine investment would provide the basis for effectively addressing those factors.	Collaboration between policymakers and other stakeholders to obtain all relevant viewpoints can be time-consuming and it may be hard to reach a consensus.
Evaluate mechanisms for increasing vaccine investment *Policymakers could consider conducting a systematic evaluation of the effectiveness of different mechanisms to incentivize vaccine investment and determine what circumstances or time frames may make some mechanisms more or less useful.* For example, policymakers could evaluate the effectiveness of mechanisms used to incentivize COVID-19 vaccine development. This could help identify when mechanisms to incentivize vaccine development is likely to be most successful.	Economic and societal costs from infectious diseases could be reduced.	Evidence on some mechanisms for incentivizing vaccine investment may not be available or sufficient to make determinations on the effectiveness of some mechanisms.
Evaluate authority *Policymakers could consider determining whether HHS, DOD, or other relevant agencies have the authority to use these mechanisms to incentivize vaccine development. For any identified gaps in authority, policymakers could consider seeking or providing such authority.* This could help address the challenges we identified related to incentivizing vaccine development.	Identifying and addressing any gaps in authority could allow policymakers to more effectively address the economic challenges to vaccine development.	Granting additional authority may not result in increased use of these mechanisms to address economic challenges. Even if incentives are used more widely, additional vaccines may not be produced due to technical and other challenges. Expanded use of incentives is likely to require more resources or a shifting of resources from other areas.

Source: GAO.

Chapter 46

Cannabis Products Containing Delta-8 Tetrahydrocannabinol (delta-8 THC): Increased Availability and Reports of Adverse Events[1]

Centers for Disease Control and Prevention, Atlanta, Georgia, USA
PCDeditor@cdc.gov

> How many murders, suicides, robberies, criminal assaults, holdups, burglaries and deeds of maniacal insanity it causes each year, especially among the young, can only be conjectured...No one knows, when he places a marijuana cigarette to his lips, whether he will become a joyous reveller in a musical heaven, a mad insensate, a calm philosopher, or a murderer...
>
> —Harry J. Anslinger, Commissioner of the US Bureau of Narcotics, 1930–1962

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[1]This chapter has been compiled, updated and edited by the Series Editor, Raj Bawa, PhD, MD, based on information and materials provided by the Centers for Disease Control and Prevention (CDC). The CDC is the nation's leading science-based, data-driven, service organization that protects the public's health. All footnotes have been inserted by Dr. Bawa. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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46.1 Introduction

The purpose of this chapter (i.e., a Health Alert Network (HAN) Health Advisory) is to alert public health departments, healthcare professionals, first responders, poison control centers, laboratories, and the public to the increased availability of cannabis products containing delta-8 tetrahydrocannabinol (delta-8 THC) and the potential for adverse events due to insufficient labeling of products containing THC and cannabidiol (CBD).

46.2 Background

The cultivation, legalization, and patterns of individual use of cannabis in the United States is continually evolving. Additional states continue to enact medical and non-medical adult cannabis[2] use legislation. Further, the potency of tetrahydrocannabinol (THC) in cannabis continues to increase and new strains of cannabis with varying levels of THC and cannabidiol (CBD) are being introduced into the marketplace. The number of persons who use cannabis is also increasing, including those reporting frequent cannabis use in the United States.

Marijuana, which can also be called weed, pot, or dope, refers to all parts of the plant *Cannabis sativa L.*, including flower, seeds, and extracts with more than 0.3% tetrahydrocannabinol (THC) by dry weight (Figs. 46.1 and 46.2).[3] Any part of the cannabis plant containing 0.3% or less THC by dry weight is defined as hemp [1]. Hemp has 0.3% or less THC; CBD oil is typically extracted from the hemp plant. The cannabis plant contains more than 100 cannabinoids, including THC, which is psychoactive (i.e., impairing, or mind-altering) and causes a "high" [2]. CBD is another active cannabinoid[4] found in the cannabis plant that is not psychoactive and does not cause a "high".

[2]Cannabis, a genus of flowering plants, contains hundreds of compounds (phytocannabinoids) that have a wide range of effects on the body and brain. Cannabis includes both marijuana (with higher concentrations of THC and lower concentrations of CBD, primarily used to achieve a "high") and hemp (with higher concentrations of CBD and lower concentrations of THC, primarily used for presumed medical purposes). Hemp is a form of cannabis that is not a controlled substance but is regulated by the U.S. Department of Agriculture. CBD and THC have the same chemical formula — 21 carbon atoms, 30 hydrogen atoms, and two oxygen atoms. The difference lies in the way the atoms are arranged. That gives CBD and THC different chemical properties, and they affect your body differently. Both CBD and THC work with receptors that release neurotransmitters in your brain. They can affect pain, mood, sleep, and memory.

[3]The words "cannabis" and "marijuana," are often used interchangeably. Cannabis refers to all products derived from the plant *Cannabis sativa* while marijuana refers to any parts of or products from the plant *Cannabis sativa* that contain substantial amounts of tetrahydrocannabinol (THC). The word "cannabinoid" refers to every chemical substance, regardless of structure or origin, that joins the cannabinoid receptors of the body and brain and that have similar effects to those produced by the Cannabis plant.

[4]*Cannabis sativa* contains approximately 550 compounds, including terpenes and more than 100 cannabinoids, is both herbaceous and diecious, and is grown each year from the seeds produced the previous years.

Figure 46.1 Female *Cannabis sativa* plant.

Figure 46.2 *Cannabis sativa*, scientific drawing from 1900. Franz Eugen Köhler's Medizinal-Pflantzen. Published in 1887 by Gera-Untermhaus.

The term THC most often refers to the delta-9 THC isomer, which is the most prominently occurring THC isomer in cannabis. However, THC has several other isomers that occur in the cannabis plant, including delta-8 THC. Delta-8 THC exists naturally in the cannabis plant in only small quantities and is estimated to be about 50–75% as psychoactive as delta-9 THC [3, 4].

CBD can be synthetically converted into delta-8 THC, as well as delta-9 THC and other THC isomers, with a solvent, acid, and heat to produce higher concentrations of delta-8 THC than those found naturally in the cannabis plant [5]. This conversion process, used to produce some marketed products, may create harmful by-products that presently are not well-characterized.

Delta-8 THC products are increasingly appearing in both marijuana and hemp marketplaces, some of which operate legally under state, territorial, or tribal laws [6]. Most states and territories permit full or restricted hemp marketplaces that sell hemp and hemp-derived CBD products [7]. Products sold as concentrated delta-8 THC are also available online. Delta-8 THC products are sometimes marketed as "weed light" or "diet weed."

The health effects of delta-8 THC have not yet been researched extensively and are not well-understood. However, delta-8 THC is psychoactive and may have similar risks of impairment as delta-9 THC [4]. As such, products that contain delta-8 THC but are labeled with only delta-9 THC content rather than with total THC content likely underestimate the psychoactive potential of these products for consumers. In addition, the sale of delta-8 THC products is not limited to regulated marijuana dispensaries in states, territories, or tribal nations where marketplaces operate under law. Rather, delta-8 THC products are sold by a wide range of businesses that sell hemp. As a result, delta-8 THC products may also have the potential to be confused with hemp or CBD products that are not intoxicating. Consumers who use these products may therefore experience unexpected or increased THC intoxication.

A wide variety of delta-8 THC-containing products have entered the marketplace, including, but not limited to, vapes, smokable hemp sprayed with delta-8 THC extract, distillates, tinctures, gummies, chocolates, and infused beverages. In addition, because testing methods for products like synthetically derived delta-8 THC are still being developed, delta-8 THC products may not be tested systematically for contaminants such as heavy metals, solvents, or pesticides that may have adverse health effects [8].

46.2.1 Recent increases in Delta-8 THC-Involved Adverse Events

In March 2021, the West Virginia Poison Control Center [9] reported two cases of adverse events related to use of delta-8 THC products in adults. In both instances, individuals mistook the products containing delta-8 THC for CBD-like products. These exposures led to symptoms consistent with cannabis intoxication. The Michigan Poison Control Center [10] also reported two cases of severe adverse events to delta-8 THC in two children who ingested a parent's delta-8 THC-infused gummies purchased from a vape shop. Both children experienced deep sedation and slowed breathing with initial increased heart rate progressing to slowed heart rate and decreased blood pressure. The children were admitted to the intensive care unit for further monitoring and oxygen supplementation.

In 2021, The American Association of Poison Control Centers (AAPCC) introduced a product code specific to delta-8 THC into its National Poison Data System (NPDS), allowing for the monitoring of delta-8 THC adverse events.

(From January 1 to July 31, 2021, 660 delta-8 THC exposures were recorded with the new product code, and one additional case was recorded as a delta-8 THC exposure from October 2020. Eighteen percent of exposures (119 of 661 cases) required hospitalization, and 39% (258 of 661 cases) involved pediatric patients less than 18 years of age.)

Syndromic surveillance data from emergency departments participating in the CDC's National Syndromic Surveillance Program (NSSP) show an increase in visits with a mention of delta-8 THC or some variation in the chief complaint text in recent months. More than 4,400 active emergency facilities that represent portions of 49 states and Washington, DC contribute data to NSSP, accounting for approximately 71% of all U.S. non-federal emergency departments. The first suspected visit associated with delta-8 THC in NSSP was observed in September 2020, with three additional visits observed through the end of 2020. Suspected visits have generally increased monthly in 2021 (three suspected visits were observed in January; six in February; 16 in March; 11 in April; 29 in May; 32 in June; and 48 in July 2021). Most of these visits (73%, 109 of 149 visits) occurred in the Department of Health and Human Services' Regions 4 and 6, which are composed primarily of Southern states that have not passed state laws to allow non-medical adult cannabis use [11]. These numbers are likely an underestimate due to the potential for inaccurate and incomplete information about products used by consumers.

Several factors can influence both the type and severity of cannabis-related adverse events, including the type of cannabinoid ingested, concentration, route of exposure, and the individual characteristics of the person who consumed the cannabinoid such as their age, weight, and sex. Delta-8 THC intoxication can cause adverse effects like those observed during delta-9 THC intoxication [10, 12], and may include the following:

- Lethargy
- Uncoordinated movements and decreased psychomotor activity
- Slurred speech
- Increased heart rate progressing to slowed heart rate
- Low blood pressure
- Difficulty breathing
- Sedation
- Coma

46.2.2 Summary

The rise in delta-8 THC products in marijuana and hemp marketplaces has increased the availability of psychoactive cannabis products, even in U.S. states, territories, and tribal nations where non-medical adult cannabis use is not permitted under law. Variations in product content, manufacturing practices, labeling, and potential misunderstanding of the psychoactive properties of delta-8 THC may lead to unexpected effects among consumers. Adverse event reports involving

products that contain delta-8 THC that resulted in consumers' hospital or emergency department treatment have been described. Increased reports of adverse events related to delta-8 THC, as well as preliminary reports of the emergence of other similarly produced products derived from cannabis warrant the continued monitoring and tracking of adverse events related to THC.

46.3 Recommendations for the Public and Consumers

- Consumers should be aware of possible limitations in the labeling of products containing THC and CBD even from approved marijuana and hemp retailers. Products reporting only delta-9 THC concentration, but not total THC may underestimate the psychoactive potential for consumers.
- Consumers should be aware that products labeled as hemp or CBD may contain delta-8 THC, and that products containing delta-8 THC can result in psychoactive effects. Delta-8 THC products are currently being sold in many states, territories, and tribal nations where non-medical adult cannabis use is not permitted by law. In addition, retailers may sell products outside of regulated dispensaries in states, territories, and tribal nations where cannabis use is permitted by law. This may provide consumers with a false sense of safety, as delta-8 THC products may be labeled as hemp or CBD, which consumers may not associate with psychoactive ingredients.
- Parents who consume edibles and other products that contain THC and CBD should store them safely away from children. Children may mistake some edibles that contain THC and CBD (e.g., fruit-flavored gummies containing delta-8 THC) as candy.
- If consumers experience adverse effects of THC- or CBD-containing products that are an immediate danger to their health, they should call their local or regional poison control center at 1-800-222-1222 or 911 or seek medical attention at their local emergency room and report the ingredients of ingested products to healthcare providers. Consumers are also encouraged to report adverse events to MedWatch (https://www.fda.gov/safety/medwatch-fda-safety-information-and-adverse-event-reporting-program) [13].
- Consumers should be aware that the cannabis marketplace continues to evolve. Other cannabis-derived products of potential concern have emerged recently, such as those containing delta-10 tetrahydrocannabinol (delta-10 THC) and THC-O acetate. More research is needed to understand the health effects of products containing these compounds.

46.4 Recommendations for Public Health Departments and Poison Control Centers, Including Those in Locations Where Laws Only Permit Hemp Marketplaces

- Release information to healthcare providers and the public about the psychoactive qualities and the potential health implications of using products

containing delta-8 THC and those products labeled as hemp or CBD may contain delta-8 THC.

- Poison control centers have a new code available to identify delta-8 THC exposures. For patients or providers reporting delta-8 THC consumption, poison control centers should use the American Association of Poison Control Centers code 310146 or product code 8297130 to indicate delta-8 THC exposure and aid in the continued surveillance of these exposures.
- U.S. states, territories, and tribal nations that have passed laws allowing non-medical use of adult cannabis or that may allow such use in the future may consider requiring the reporting of total THC content, including ingredients like delta-8 THC and other compounds that may be synthetically produced, on product labeling.
- Community-based organizations, such as Drug-Free Communities coalitions, can use information from this report to raise awareness in their communities about the potential negative health effects associated with use of delta-8 THC-containing products, as well as the emergence of other cannabis-derived products of potential concern.

46.5 Recommendations for Retailers Selling Cannabis Products

- Retailers selling cannabis products should provide information to consumers about the psychoactive qualities of delta-8 THC.
- Retailers selling cannabis products should report total THC content on product labeling, including ingredients like delta-8 THC that may be synthetically produced to create a psychoactive effect.

46.6 Recommendations for Healthcare Providers

- Healthcare providers should be vigilant in observing patients presenting with THC-like intoxication symptoms who do not report an exposure to marijuana or history of use. Symptomatic patients should be questioned about their use of CBD or delta-8 THC products.
- There is no specific antidote for THC intoxication. Treatment is largely symptomatic and supportive care. The ability to detect delta-8 THC with laboratory tests that hospitals use to detect delta-9 THC currently is not fully characterized. Consult with your hospital's medical toxicologist or local poison control center for toxicology consultations on treatment.

46.7 For More Information

- CDC Marijuana homepage: "Marijuana and Public Health" [14]

- FDA Delta-8 THC Consumer Update: "5 Things to Know about Delta-8 Tetrahydrocannabinol" [15]
- Visit CDC-INFO [16] or call CDC-INFO at 1-800-232-4636
- CDC 24/7 Emergency Operations Center (EOC) 770-488-7100

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**Provided by the series editor, Raj Bawa, PhD, MD.

Appendix

Marijuana and Public Health: Frequently Asked Questions

What is marijuana?

Marijuana—which can also be called cannabis, weed, pot, or dope—refers to the dried flowers, leaves, stems, and seeds of the cannabis plant. The cannabis plant contains more than 100 compounds (or cannabinoids). These compounds include tetrahydrocannabinol (THC), which is impairing or mind-altering, as well as other active compounds, such as cannabidiol (CBD). CBD is not impairing, meaning it does not cause a "high" [1].

Is cannabidiol (CBD) the same thing as marijuana? Is it legal in the United States?

Marijuana is different from cannabidiol (CBD). Marijuana refers to the dried flowers, leaves, stems, and seeds of the cannabis plant. CBD is one of the many compounds, along with THC (tetrahydrocannabinol), that can be present in the cannabis plant. CBD does not cause a "high" by itself. CBD can be derived from hemp, which is defined as any part of a cannabis sativa plant with no more than 0.3% of THC, or non-hemp plants. In 2018, the U.S. Congress passed and signed into law (The Agriculture Improvement Act of 2018 or the 2018 Farm Bill) [2]. This law removed hemp from the federal Controlled Substances Act, effectively legalizing CBD if it comes from hemp. However, a few states have not removed hemp from their state's controlled substances acts, so legality of CBD products differs across states.

Is cannabidiol (CBD) medicine?

Scientists are still learning about how CBD affects the body. The U.S. Food and Drug Administration (FDA) approved Epidiolex, a medicine that contains purified CBD from cannabis plants, to help treat seizure disorders. The FDA has concluded that this drug is safe and effective for the intended use. However, other marketed uses of CBD may not be FDA approved.

How is marijuana used?

Marijuana can be used in a number of ways [3, 4]. Marijuana can be smoked in joints (like a cigarette), in blunts (cigars or cigar wrappers that have been partly or completely refilled with marijuana), or in bongs (pipes or water pipes). Marijuana also can be mixed or infused into foods like cookies, cakes, or brownies (called edibles) and can be infused in drinks. It can be vaped using electronic vaporizing devices (i.e., e-cigarettes or vape pens) or other vaporizers. Compounds (or cannabinoids) in marijuana can also be extracted to make oils and concentrates that can be vaped or inhaled. Smoking oils, concentrates, and extracts from the

marijuana plant, known as "dabbing," is on the rise. Health and safety risks exist for each of the different ways of using marijuana.

Are some ways of using marijuana safer than others?

Marijuana products that contain tetrahydrocannabinol (THC) can have health risks regardless of how they are used because THC is impairing and can affect memory, attention, decision-making, and risk-taking [1]. Health and safety risks exist for each of the different ways of consuming marijuana, and scientists do not have enough evidence to say that consuming marijuana in one way is safer than another. For example, smoking marijuana can expose you and those around you to harmful chemicals [5]. Oils and concentrates used in vaping and dabbing (which is a specific method of inhaling THC concentrates) often have highly concentrated forms of THC and may contain additives or be contaminated with other substances [6]. The effects of using these more concentrated forms of THC are not well understood but may include higher risk of developing cannabis use disorder [1, 7]. Vaping has also been linked to lung injury. In 2019, a national outbreak of lung injury associated with vaping occurred. Data from patient reports and product testing showed THC-containing vaping products that also contained vitamin E acetate were linked to most cases. This outbreak resulted in over 2,800 emergency department visits and 68 confirmed deaths.

What determines how marijuana affects a person?

How marijuana affects a person depends on several factors, including:

- Previous experience with marijuana or other drugs
- Biology (e.g., genes)
- Sex (e.g., women may experience more dizziness after using marijuana compared to men [9])
- How the drug is taken (e.g., consuming edibles or products with high tetrahydrocannabinol (THC) concentration can have delayed or unpredictable effects and increases the risk of overdose or poisoning [1])
- How much of the drug is used
- How often it is used
- If it is used in combination with other substances (using marijuana with alcohol or other drugs could lead to increased risk of harm, especially with unknown drug-to-drug interactions [1])

Is marijuana medicine?

The marijuana plant has compounds that may help symptoms for some health problems [1]. While more states are making it legal to use the plant as medicine for certain conditions, scientists are still learning the ways that marijuana may help or harm people. For example, smoked marijuana may damage your lungs and respiratory system [1]. Certain compounds in marijuana products may affect your brain or body in harmful ways. In addition, no federal standards have been

implemented for the quality and safety of marijuana products sold in state-based medical marijuana dispensaries. These products are not approved by the FDA.

Research on the medical use of marijuana is still in early stages, and much remains unknown about the plant and how it interacts with the body. Currently, the FDA has approved one plant-based marijuana drug (Epidiolex), which contains purified cannabidiol (CBD) from the marijuana plant. The drug is approved for treating seizures associated with two rare and severe forms of epilepsy (Lennox-Gastaut syndrome and Dravet syndrome) as well as seizures associated with tuberous sclerosis complex, a rare genetic disorder that causes benign tumors to form in many parts of the body.

The FDA has also approved two medicines (dronabinol [brand names: Marinol and Syndros] and nabilone [brand name: Cesamet]) made from a synthetic or lab-made chemical that mimics tetrahydrocannabinol (THC). These medicines are used to treat nausea in patients with cancer who are having chemotherapy treatment and to increase appetite in individuals with AIDS who do not feel like eating (wasting syndrome).

Is it possible for someone to become addicted to marijuana?

Yes. Research suggests that 3 in 10 people who use marijuana may have some form of marijuana use disorder [10], meaning they are unable to stop using marijuana even though it is causing health and social problems in their lives [11, 12]. For people who begin using marijuana before the age of 18 and who use marijuana often (daily/near daily), the risk of developing marijuana use disorder is even greater [12]. In addition, the concentration or strength of tetrahydrocannabinol (THC) in marijuana products is increasing [6, 13], and daily or near daily use of marijuana is increasing [14], both of which could make addiction and other health consequences more likely.

How do I know if I am addicted to marijuana?

The signs that someone might have marijuana use disorder [15] are:

- Using more marijuana than intended
- Trying but failing to quit using marijuana
- Spending a lot of time using marijuana
- Craving marijuana
- Using marijuana even when it causes problems at home, school, or work
- Continuing to use marijuana despite social or relationship problems
- Giving up important activities with friends and family in favor of using marijuana
- Using marijuana in high-risk situations, such as driving a car
- Continuing to use marijuana despite physical or psychological problems
- Needing to use more marijuana to get the same high
- Experiencing symptoms when stopping marijuana use

People with marijuana use disorder, compared to those who use marijuana but do not have marijuana use disorder, are at a greater risk for negative consequences, such as problems with attention, memory, and learning.

What are the health risks of using marijuana?

For more on the health risks and effects of marijuana, visit CDC's web page on marijuana and health effects: https://www.cdc.gov/marijuana/health-effects/index.html

Is it possible to "overdose" or have a "bad reaction" to marijuana?

While a fatal overdose caused solely by marijuana is unlikely, marijuana is not harmless. The signs of using too much marijuana are similar to the typical effects of using marijuana but more severe. These signs may include:

- extreme confusion
- anxiety
- paranoia
- panic
- fast heart rate
- delusions or hallucinations
- increased blood pressure
- severe nausea or vomiting

In some cases, these effects can lead to unintentional injury, such as a motor vehicle crash, fall, or poisoning. Overconsumption of marijuana can happen especially when using marijuana-infused products like edibles and beverages, since it can take up to 2 hours to feel the effects from the drug [1]. Infants or young children who accidentally ingest marijuana are more likely to require hospital admission compared to older children who ingest similar concentrations due to their smaller size and weight and increased severity of symptoms [16]. Marijuana may be laced with other substances, either known or unknown to the consumer. Using marijuana in combination with other substances may result in greater impairment than when using marijuana alone and may increase the risk of overdose.

What are the effects of mixing marijuana with alcohol, tobacco, or prescription drugs?

Using alcohol and marijuana at the same time is likely to result in greater impairment than when using either one alone [17]. Greater impairment can result in greater risk of physical harm. Using marijuana and tobacco at the same time may also lead to increased exposure to harmful chemicals that could cause greater risks to the lungs and the cardiovascular system (heart and blood vessels) [18]. Also, marijuana may change how prescription drugs work [19]. Always talk with your doctor about any medications you are taking or thinking about taking and the possible side effects when mixed with other things, such as marijuana.

How harmful is K2/Spice (also called synthetic cannabinoids)?

Synthetic cannabinoids (called spice, K2, and other names) are man-made chemicals and, despite the name, are not marijuana or cannabinoid medicines [20]. Synthetic cannabinoids are often sprayed onto dried plant material that can then be smoked or sold as liquids to use in vaping devices. Synthetic cannabinoids are part of a group of unregulated, mind-altering drugs that attempt to produce effects similar to illicit drugs. Their effects are not fully understood and can cause dangerous and unpredictable health effects because of their unpredictable chemical contents [21]. Once these products are identified in the illegal marketplace, they are added to the list of schedule I substances by the Drug Enforcement Administration. Schedule I substances are illegal throughout the United States and are defined as having no medical use and high potential for abuse.

Synthetic cannabinoids can affect the brain much more powerfully than marijuana, creating unpredictable and, in some cases, life-threatening effects, including:

- nausea
- anxiety
- paranoia
- brain swelling
- seizures
- hallucinations
- aggression
- heart palpitations
- chest pains

Cases of severe injury and death from use of synthetic cannabinoids have been reported, along with regional outbreaks when a contaminated batch enters a specific community [22].

Is it safe for a breastfeeding person to use marijuana?

Breastfeeding persons are encouraged to avoid using marijuana [23]. The health effects of a breastfeeding person's use of marijuana on her infant are not yet fully known, and the available data are limited and conflicting. However, we know that chemicals from marijuana can be passed to a baby through breast milk. In addition, tetrahydrocannabinol (THC) is stored in body fat and is slowly released over time, meaning that a baby could still be exposed even after a breastfeeding person stops using marijuana.

Can secondhand marijuana smoke affect nonsmokers?

More research about the effects of secondhand marijuana smoke is still needed. The known risks of secondhand exposure to tobacco smoke—including risks to the heart or lungs [24]—raise questions about whether secondhand exposure to marijuana smoke causes similar health risks. Secondhand marijuana smoke

contains many of the same toxic and cancer-causing chemicals found in tobacco smoke and contains some of those chemicals in higher amounts [5].

More research is needed to understand how secondhand marijuana exposure may affect children. Secondhand marijuana smoke contains tetrahydrocannabinol (THC), the chemical responsible for most of marijuana's psychological effects (or the "high"). THC can be passed to infants and children through secondhand smoke, and people exposed to secondhand marijuana smoke can experience psychoactive effects, such as feeling high [25, 26]. Recent studies have found strong associations between reports of having someone in the home who uses marijuana (e.g., a parent, relative, or caretaker) and the child having detectable levels of THC [27, 28]. Children exposed to THC are potentially at risk for negative health effects. Other research shows that marijuana use during adolescence can impact the developing teenage brain and cause problems with attention, motivation, and memory, suggesting that secondhand smoke exposure could lead to similar negative health effects in children [29].

Marijuana is legal in many states. Does that mean it's safe?

The fact that marijuana is legal in some states for medical or nonmedical adult use does not mean that it is safe. Using marijuana at any age can lead to negative health consequences:

- Using marijuana heavily (daily or near-daily) can damage your memory, attention, and learning ability. This can last a week or more after the last time marijuana was used [1].
- Using marijuana during pregnancy or while breastfeeding may harm the baby [1, 29].
- Marijuana use has been linked to social anxiety, depression, suicide, and schizophrenia. Scientists don't yet know whether marijuana use directly causes these health issues, but it may make symptoms more severe.
- Smoking any product, including marijuana, can damage your lungs and cardiovascular system (heart and blood vessels) [5]. Eating or drinking foods with marijuana can take longer to have an effect and may increase the chance of consuming too much [1]. Vaping marijuana has led to lung injury and even death [8]. Use of concentrates in vaping or dabbing devices may increase a number of health risks because of the concentration or strength of marijuana being used [6, 30].

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Chapter 47

Natural Doesn't Necessarily Mean Safer, or Better[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA

info@nccih.nih.gov

Keywords: arsenic, aspirin, chlorine, cognitive decline, dementia, dietary supplements, echinacea, *Echinacea purpurea*, Ephedra, ephedrine alkaloids, ginkgo, *Ginkgo biloba*, herbal supplements, isopropyl alcohol, kava, mercury, morphine, *Papaver somniferum*, ricin, *Salix alba*, snake venom, synthetic drugs

A lot of people believe that when it comes to medicine, "natural" is better, healthier, and safer than "unnatural" or synthetic drugs.

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at NIH, the federal focal point for medical research in the US. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented herein is not intended to be a substitute for the medical expertise and advice of your health care provider(s). The NCCIH encourages you to discuss any decisions about treatment or care with your health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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In fact, researchers have looked at some of the reasons why people have that belief. What they found is that a person's preference for natural things involves a range of ideas, including the belief that nature is pure and inherently superior to humans. Researchers have also found that these beliefs, or biases, affect the decisions people make about their health.

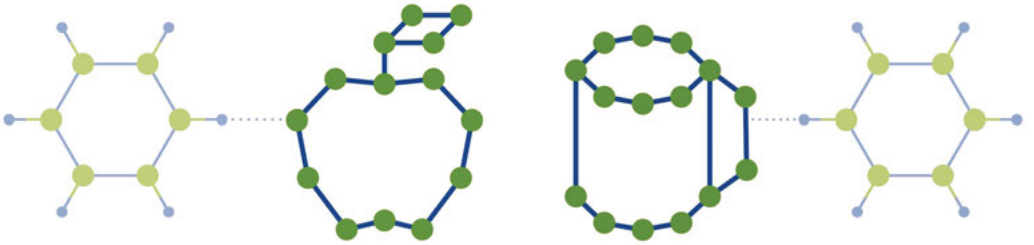
But not all products from nature have been shown to be effective.

Some dietary and herbal supplements have failed to show a benefit when scientists have studied them. For example, several major studies of the herb Echinacea did not find evidence of benefit against the common cold. Studies of ginkgo, including a large study that enrolled more than 3,000 older adults, found that ginkgo supplements don't help prevent or slow dementia or cognitive decline.

For example, kava, a plant native to the islands of the South Pacific, and often used as a dietary supplement for anxiety, may be associated with severe liver damage. Ephedra, an evergreen shrub-like plant native to central Asia and

Mongolia that has been used for centuries for colds, fever, and other conditions, is associated with heart problems and risk of death. In 2004, the US Food and Drug Administration banned the sale of dietary supplements containing ephedrine alkaloids for safety reasons.

Some people also believe that "natural" products are safe because they believe these medicines are free of chemicals.



Some chemicals are harmful. Toxic chemicals are substances that may be harmful to the environment or hazardous to your health if inhaled, ingested, or absorbed through the skin. Chlorine and isopropyl alcohol are examples of some of the toxic chemicals commonly used in certain industries. But there are many toxic chemicals that occur in nature as well—mercury, snake venom, arsenic, and ricin from castor beans. Some chemicals, like iron and oxygen, are necessary for us to live, but at high doses are toxic and can even cause death. Natural medicines such as herbal and dietary supplements are made up of chemicals, too, just like everything else.

But it's important to understand that although many herbal or dietary supplements (and some prescription drugs) come from natural sources, "natural" does not always mean that it's a safer or better option for your health. An herbal supplement may contain dozens of chemical compounds, and all of its ingredients may not be known. Scientists are studying many of these products to identify what ingredients may be active and to better understand their effects in the body.

Chapter 48

How Medications and Supplements Can Interact[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA
info@nccih.nih.gov

Keywords: anticoagulants, aspirin, black cohosh, carbamazepine, chiropractor, complementary health practitioner, cyclosporine, dietary supplements, digitalis, digoxin, drug-supplement, drug-supplement interactions, echinacea, fexofenadine, finasteride, goldenseal, ivabradine, levothyroxine, methadone, narrow therapeutic range, naturopathic practitioner, nifedipine, omeprazol, over-the-counter (OTC), phenytoin, pseudoephedrine, saw palmetto, schisandra, St. John's wort, statins, talinolol, theophylline, verapamil, voriconazole, warfarin

Many Americans take both dietary supplements and prescription or over-the-counter drugs. Sometimes, these drugs and supplements may interact in harmful ways. It's important to tell all your health care providers about all dietary supplements and drugs you take. That way, they can help you avoid harmful interactions.

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48.1 Test Your Knowledge: Are These Statements True or False?

1. **Some supplements can decrease the effects of drugs.**
   True. For example, St. John's wort can decrease the effectiveness of birth control pills, leading to breakthrough bleeding and an increased risk of unintended pregnancy.
2. **Some supplements can increase the effects—including unwanted side effects—of drugs.**
   True. For example, herbs that decrease blood sugar may interact with anti-diabetes drugs to cause blood sugar to drop too far.
3. **Some interactions between supplements and drugs are very dangerous.**
   True. For example, interactions can decrease the effectiveness of critically important drugs—such as drugs that prevent transplanted organs from being rejected.
4. **Scientists know a great deal about drug-supplement interactions**
   False.

48.2 Talk With Your Health Care Providers

It's important to tell your health care providers about all the drugs and supplements you take.

Often, when you visit a health care provider for the first time, you fill out a form that asks you to list all the drugs and supplements you take. Be sure to update this information every time you visit the provider's office.

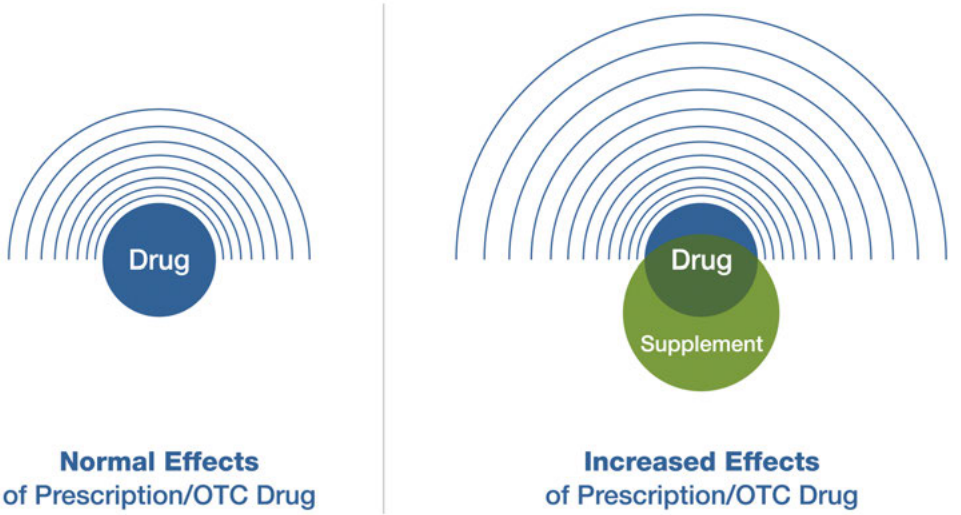
Besides your primary health care provider, which of these people need to know about all the drugs and supplements you're taking?

1. Your dentist
2. Any medical specialist you see, such as a cardiologist or dermatologist
3. Your eye care professional
4. A health care professional who treats you in an emergency room or urgent care facility
5. Any complementary health practitioner you see, such as a chiropractor or naturopathic practitioner
6. All of the above

Answer: All of the above: Yes. They all need this information so that they can help you avoid harmful interactions.

48.3 Some Supplements May Increase the Effects—and Side Effects—of Drugs

Sometimes, taking a drug and a supplement together may increase the drug's effects. The drug's effects may become too strong, and unwanted side effects may increase.



For example, the herb schisandra may slow down the processes in your body that change drugs into inactive substances. So if you take this herb while you're also taking a drug, the amount of the drug in your body may increase. As a result, the drug's effects may be too strong.

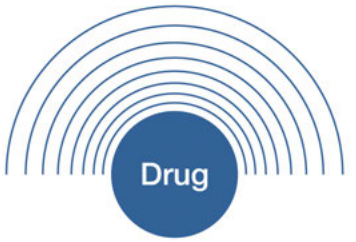
Would you like another example?

Like schisandra, goldenseal may slow down the processes in your body that change drugs into inactive substances, leading to increases in drug levels in your body.

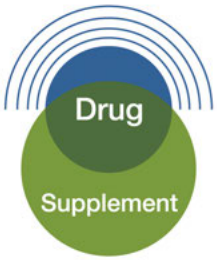
48.4 Some Supplements May Decrease the Effects of Drugs

Sometimes, taking a drug and a supplement together may decrease the drug's effects. This means that you aren't getting the full benefit from the drug that your health care provider wants you to have.

One popular herbal supplement is especially well known for decreasing the effects of drugs. It does this by speeding up the processes in your body that change drugs into inactive substances. *This herb may decrease the effectiveness of more than 70 percent of all drugs.*



Normal Effects
of Prescription/OTC Drug



Decreased Effects
of Prescription/OTC Drug

Which of these herbs is the one that decreases the effects of many drugs?

1. Black cohosh
2. Echinacea
3. Saw palmetto
4. St. John's wort

Answer: St. John's wort

48.5 More about St. John's Wort

St. John's wort interacts with many types of drugs. In most instances, it speeds up the processes that change the drug into inactive substances, leading to a decrease in drug levels in your body.

However, St. John's wort interacts with some drugs in other ways. For example, taking St. John's wort with certain types of antidepressants can cause harmful side effects.

Some drugs that interact with St. John's wort
Anesthetics
Anti-anxiety drugs
Anticoagulants (blood thinners)
Antidepressants
Cancer drugs
Cholesterol-lowering drugs (statins)
Diabetes drugs

Some drugs that interact with St. John's wort
Digoxin (digitalis), a drug used to treat heart problems
Drugs that suppress the immune system (used to prevent rejection of transplanted organs)
Drugs used to prevent seizures
Drugs used to treat HIV infection
Fexofenadine (an antihistamine)
Finasteride (a drug used for prostate problems)
Ivabradine (a drug used to treat angina)
Methadone
Nifedipine and verapamil (used to treat high blood pressure or heart problems)
Omeprazole (an acid reducer used to treat digestive tract problems)
Oral contraceptives (birth control pills)
Talinolol (a beta-blocker used for high blood pressure and heart problems)
Theophylline (an asthma drug)
Voriconazole (a drug used to treat fungal infections)

48.6 Interactions with Over-the-Counter (OTC) Drugs

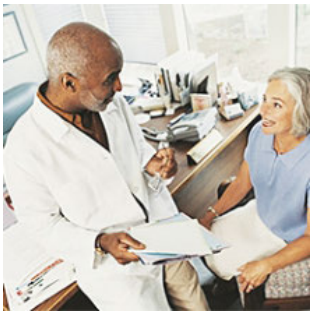
When people think about drug interactions, they often think about prescription drugs. But some drugs that are available over the counter without a prescription can interact with supplements, too. If you're considering taking both an over-the-counter drug and a dietary supplement, it's a good idea to talk with your health care provider or a pharmacist about possible interactions.

Which of these over-the-counter drugs may interact with herbal supplements?

1. Aspirin
2. Pseudoephedrine (a decongestant)
3. Fexofenadine (Allegra)
4. All of them

Answer: All of them. A variety of herbs may interact with aspirin to increase the risk of bleeding. Concentrated green tea supplements interact with pseudoephedrine. St. John's wort interacts with fexofenadine (Allegra).

48.7 When Drug-Supplement Interactions Are Especially Important



Next you'll learn about two situations when drug-supplement interactions can be especially important:

- When you're taking a drug that has what health care providers call a "narrow therapeutic range"
- When you're going to have surgery.

In these situations, it's particularly important to talk with all of your health care providers about the dietary supplements you're taking now and any you may be considering taking in the future.

48.8 Drugs with a Narrow Therapeutic Range

Having the right amount of certain drugs in your body is crucial. If the amount of the drug is even a little too low or too high, it can cause big problems. Drugs like these are said to have a "narrow therapeutic range" or "narrow therapeutic index." Interactions are of special concern for drugs with a narrow therapeutic range.

Examples of Drugs With a Narrow Therapeutic Range
Carbamazepine (used to prevent seizures)
Cyclosporine (used to prevent organ transplant rejection)
Digoxin (used to treat heart problems)
Levothyroxine (used to treat thyroid problems)
Phenytoin (used to prevent seizures)
Warfarin (an anticoagulant—also called a blood thinner)

If you're taking a dietary supplement, and your health care provider prescribes a drug with a narrow therapeutic range, what should you do?

Tell your health care provider that you're taking the supplement and ask the provider what you should do.

48.9 If You're Going to Have Surgery

If you're going to have surgery, talk to your health care providers as far in advance of the operation as possible and tell them about all dietary supplements that you're taking.

Some dietary supplements may cause problems during surgery because:

- They may affect your response to anesthetics or to other medicines that you may be given before, during, or after the operation
- They may increase your risk of bleeding.

Some health care providers will ask patients to discontinue all herbal supplements several weeks before having elective surgery (surgery that can be scheduled in advance).

If you're having an emergency operation, you won't have a chance to stop taking supplements ahead of time. But it's still important for you or a family member to tell your surgeon and anesthesia provider about all dietary supplements that you're taking so that they can be prepared for any problems that might occur.

48.10 Tips on Reading Supplement Labels

Sometimes it isn't obvious what's in the bottle of a dietary supplement.

To find out what's in a supplement, look for the Supplement Facts panel on the product label. The manufacturer is required to list all of the supplement's ingredients on this panel, either in the Supplement Facts chart or in the list of other ingredients below it.

Supplement Facts

Serving Size 1 Tablet

	Amount Per Serving	% Daily Value
Vitamin A (as retinyl acetate and 50% as beta-carotene)	5000 IU	100%
Vitamin C (as ascorbic acid)	60 mg	100%
Vitamin D (as cholecalciferol)	400 IU	100%
Vitamin E (as dl-alpha tocopheryl acetate)	30 IU	100%
Thiamin (as thiamin mononitrate)	1.5 mg	100%
Riboflavin	1.7 mg	100%
Niacin (as niacinamide)	20 mg	100%
Vitamin B_6 (as pyridoxine hydrochloride)	2.0 mg	100%
Folate (as folic acid)	400 mcg	100%
Vitamin B_{12} (as cyanocobalamin)	6 mcg	100%
Biotin	30 mcg	10%
Pantothenic Acid (as calcium pantothenate)	10 mg	100%

Other ingredients: Gelatin, lactose, magnesium stearate, microcrystalline cellulose, FD&C Yellow No. 6, propylene glycol, propylparaben, and sodium benzoate.

Let's say that you're taking the supplement with the label shown here. Does this supplement contain vitamin E? Does it contain vitamin K?

1. It contains both of them.
2. It contains vitamin E but not vitamin K.
3. It contains vitamin K but not vitamin E.
4. It doesn't contain either of them.

Answer: It contains vitamin E but not vitamin K. Vitamin E is listed on the Supplement Facts panel but vitamin K is not.

48.11 Here's a Hint for Your Next Visit to a Health Care Provider

When you visit a health care provider, it's a good idea to bring a written list of

- All the drugs and supplements you take
- How often you take them
- The doses you take.

But there's something else you may also want to do, especially if you take any products that have multiple ingredients.

Bring the bottles of the products to the health care provider's office.

If you have the labeled bottles, any questions about what's in your dietary supplements can be answered right away.

Further Information

To find more information about supplement-drug interactions and other aspects of the safety of dietary supplements, try these resources:

- Your health care providers and your pharmacist
- The National Center for Complementary and Integrative Health's online resources on dietary supplements (https://www.nccih.nih.gov/health/supplements) and herbs (https://www.nccih.nih.gov/health/herbsataglance)
- The Office of Dietary Supplements (https://ods.od.nih.gov/) at the National Institutes of Health
- The US Food and Drug Administration (https://www.fda.gov/)
- MedlinePlus® (https://www.nlm.nih.gov/medlineplus/dietarysupplements.html), a resource provided by the National Library of Medicine.

Chapter 49

Antioxidants: Current Issues and Future Trends[1]

National Center for Complementary and Integrative Health, National Institutes of Health, Bethesda, Maryland, USA
info@nccih.nih.gov

Keywords: age related macular degeneration (AMD), age-related eye disease study (AREDS), age-related eye disease study 2 (AREDS2), Alzheimer's disease, anthocyanins, anticoagulant drugs, antioxidant supplements, antioxidants, beta-carotene, cataracts, complementary and integrative health, free radicals, lutein, lycopene, observational studies, omega-3 fatty acids, oxidative stress, Parkinson's disease, prostate cancer, Research Portfolio Online Reporting Tools Expenditures & Results (RePORTER), Selenium and Vitamin E Cancer Prevention Trial (SELECT), zeaxanthin

49.1 Introduction

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage. Diets high in vegetables and fruits, which are good sources of antioxidants, have been found to be healthy. However, research has not shown antioxidant supplements to be beneficial in preventing diseases.

[1]This chapter has been organized and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the National Center for Complementary and Integrative Health (NCCIH), National Institutes of Health (NIH), Bethesda, MD. NCCIH is 1 of 27 institutes and centers at NIH, the federal focal point for medical research in the U.S. NCCIH is dedicated to exploring complementary health products and practices in the context of rigorous science, training complementary health researchers, and disseminating authoritative information to the public and professionals. The information presented in this chapter is not intended to be a substitute for the medical expertise and advice of a health care provider(s). NCCIH encourages discussion on any decisions about treatment or care with a health care provider. The mention of any product, service, or therapy is not an endorsement by NCCIH. NCCIH thanks Ock Chun, PhD, MPH, University of Connecticut; Balz Frei, Ph.D., Oregon State University; Christopher Gardner, PhD, Stanford University; and D. Lee Alekel, PhD, and John (Jack) Killen, Jr., MD, NCCIH; for their technical expertise and review of the update of this chapter. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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Examples of antioxidants include vitamins C and E, selenium, and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin. This chapter provides basic information about antioxidants, summarizes what the science says about antioxidants and health, and suggests sources for additional information.

49.2 Key Points

- Vegetables and fruits are rich sources of antioxidants. There is good evidence that eating a diet that includes plenty of vegetables and fruits is healthy, and official U.S. Government policy urges people to eat more of these foods. Research has shown that people who eat more vegetables and fruits have lower risks of several diseases. However, it is not clear whether these results are related to the amount of antioxidants in vegetables and fruits, to other components of these foods, to other factors in people's diets, or to other lifestyle choices.
- Rigorous scientific studies involving more than 100,000 people combined have tested whether antioxidant supplements can help prevent chronic diseases, such as cardiovascular diseases, cancer, and cataracts. In most instances, antioxidants *did not* reduce the risks of developing these diseases.
- Concerns have not been raised about the safety of antioxidants in food. However, high-dose supplements of antioxidants may be linked to health risks in some cases. Supplementing with high doses of beta-carotene may *increase* the risk of lung cancer in smokers. Supplementing with high doses of vitamin E may *increase* risks of prostate cancer and one type of stroke.
- Antioxidant supplements may interact with some medicines.
- Tell all of your health care providers about any complementary and integrative health approaches you use. Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

49.3 About Free Radicals, Oxidative Stress, and Antioxidants

Free radicals are highly unstable molecules that are naturally formed when you exercise and when your body converts food into energy. Your body can also

be exposed to free radicals from a variety of environmental sources, such as cigarette smoke, air pollution, and sunlight. Free radicals can cause "oxidative stress," a process that can trigger cell damage. Oxidative stress is thought to play a role in a variety of diseases including cancer, cardiovascular diseases, diabetes, Alzheimer's disease, Parkinson's disease, and eye diseases such as cataracts and age-related macular degeneration (AMD). Antioxidant molecules have been shown to counteract oxidative stress in laboratory experiments (for example, in cells or animal studies). However, there is debate as to whether consuming large amounts of antioxidants in supplement form actually benefits health. There is also some concern that consuming antioxidant supplements in excessive doses may be harmful. Vegetables and fruits are healthy foods and rich sources of antioxidants. Official U.S. Government policy urges people to eat more vegetables and fruits. *Concerns have not been raised about the safety of any amounts of antioxidants in food*. For more information on antioxidants in foods, visit the U.S. Department of Agriculture webpage on antioxidants and phytonutrients (https://www.nutrition.gov/topics/whats-food/phytonutrients).

49.4 Use of Antioxidant Supplements in the United States

A 2009 analysis using data from the National Health and Nutrition Examination Survey (1999–2000 and 2001–2002) estimated the amounts of antioxidants adults in the United States get from foods and supplements. Supplements accounted for 54 percent of vitamin C, 64 percent of vitamin E, 14 percent of alpha- and beta-carotene, and 11 percent of selenium intake.

49.5 Safety

- High-dose antioxidant supplements may be harmful in some cases. For example, the results of some studies have linked the use of high-dose beta-carotene supplements to an increased risk of lung cancer in smokers and use of high-dose vitamin E supplements to an increased risk of hemorrhagic stroke (a type of stroke caused by bleeding in the brain) and prostate cancer.
- Like some other dietary supplements, antioxidant supplements may interact with certain medications. For example, vitamin E supplements may increase the risk of bleeding in people who are taking anticoagulant drugs ("blood thinners"). There is conflicting evidence on the effects of taking antioxidant supplements during cancer treatment; some studies suggest that this may be beneficial, but others suggest that it may be harmful. The National Cancer Institute recommends that people who are being treated for cancer talk with their health care provider before taking supplements.
- For more information about the safety of dietary supplements, see the National Center for Complementary and Integrative Health (NCCIH) fact sheet titled Using Dietary Supplements Wisely (https://www.nccih.nih.gov/health/using-dietary-supplements-wisely).

49.6 What the Science Says

Several decades of dietary research findings suggested that consuming greater amounts of antioxidant-rich foods might help to protect against diseases. Because of these results, there has been a lot of research on antioxidant supplements. Rigorous trials of antioxidant supplements in large numbers of people have not found that high doses of antioxidant supplements prevent disease. This section describes the preliminary research findings, the results of the clinical trials, and possible explanations for the differences in study results.

49.6.1 Observational and Laboratory Studies

Observational studies on the typical eating habits, lifestyles, and health histories of large groups of people have shown that those who ate more vegetables and fruits had lower risks of several diseases, including cardiovascular disease, stroke, cancer, and cataracts. Observational studies can provide ideas about possible relationships between dietary or lifestyle factors and disease risk, but they cannot show that one factor causes another because they cannot account for other factors that may be involved. For example, people who eat more antioxidant-rich foods might also be more likely to exercise and less likely to smoke. It may be that these factors, rather than antioxidants, account for their lower disease risk. Researchers have also studied antioxidants in laboratory experiments. These experiments showed that antioxidants interacted with free radicals and stabilized them, thus preventing the free radicals from causing cell damage.

49.6.2 Clinical Trials of Antioxidants

Because the results of such research seemed very promising, large, long-term studies—many of which were funded by the National Institutes of Health (NIH)—were conducted to test whether antioxidant supplements, when taken for periods of at least a few years, could help prevent diseases such as cardiovascular diseases and cancer in people. In these studies, volunteers were randomly assigned to take either an antioxidant or a placebo (an identical-looking product that did not contain the antioxidant). The research was conducted in a double-blind manner (neither the study participants nor the investigators knew which product was being taken). Studies of this type—called clinical trials—are designed to provide clear answers to specific questions about how a substance affects people's health.

Among the earliest of these studies were three large NIH-sponsored trials of high-dose supplements of beta-carotene, alone or in combination with other nutrients. These trials, completed in the mid-1990s, all showed that beta-carotene did not protect against cancer or cardiovascular disease. In one trial, beta-carotene supplements increased the risk of lung cancer in smokers, and in another trial, supplements containing both beta-carotene and vitamin A had the same effect.

More recent studies have also found that in most instances antioxidant supplements did not help to prevent disease. For example:

- The Women's Health Study, which included almost 40,000 healthy women at least 45 years of age, found that vitamin E supplements did not reduce the risk of heart attack, stroke, cancer, age-related macular degeneration, or cataracts. Although vitamin E supplements were associated with fewer deaths from cardiovascular causes, they did not reduce the overall death rate of study participants.
- The Women's Antioxidant Cardiovascular Study found no beneficial effects of vitamin C, vitamin E, or beta-carotene supplements on cardiovascular events (heart attack, stroke, or death from cardiovascular diseases) or the likelihood of developing diabetes or cancer in more than 8,000 female health professionals, aged 40 years or older, who were at high risk for cardiovascular disease. Antioxidant supplements also did not slow changes in cognitive function among women in this study who were aged 65 or older.
- The Physicians' Health Study II, which included more than 14,000 male physicians aged 50 or older, found that neither vitamin E nor vitamin C supplements reduced the risk of major cardiovascular events (heart attack, stroke, or death from cardiovascular disease), cancer, or cataracts. In fact, vitamin E supplements were associated with an increased risk of hemorrhagic stroke in this study.
- The Selenium and Vitamin E Cancer Prevention Trial (SELECT)—a study of more than 35,000 men aged 50 or older—found that selenium and vitamin E supplements, taken alone or together, did not prevent prostate cancer. A 2011 updated analysis from this trial, based on a longer follow-up period of study participants, concluded that vitamin E supplements increased the occurrence of prostate cancer by 17 percent in men who received the vitamin E supplement alone compared with those who received placebo. There was no increase in prostate cancer when vitamin E and selenium were taken together.

Unlike the studies described above, the Age-Related Eye Disease Study (AREDS), led by the National Eye Institute and cosponsored by other components of NIH, including NCCIH, found a beneficial effect of antioxidant supplements. This study showed that a combination of antioxidants (vitamin C, vitamin E, and beta-carotene) and zinc reduced the risk of developing the advanced stage of age-related macular degeneration by 25 percent in people who had the intermediate stage of this disease or who had the advanced stage in only one eye. Antioxidant supplements used alone reduced the risk by about 17 percent. In the same study, however, antioxidants did not help to prevent cataracts or slow their progression.

- A follow-up study, AREDS2, found that adding omega-3 fatty acids (fish oil) to the combination of supplements did not improve its effectiveness. However, adding lutein and zeaxanthin (two carotenoids found in the eye) improved the supplement's effectiveness in people who were not taking

beta-carotene and those who consumed only small amounts of lutein and zeaxanthin in foods.

49.6.3 Why Don't Antioxidant Supplements Work?

Most clinical studies of antioxidant supplements have not found them to provide substantial health benefits. Researchers have suggested several reasons for this, including the following:

- The beneficial health effects of a diet high in vegetables and fruits or other antioxidant-rich foods may actually be caused by other substances present in the same foods, other dietary factors, or other lifestyle choices rather than antioxidants.
- The effects of the large doses of antioxidants used in supplementation studies may be different from those of the smaller amounts of antioxidants consumed in foods.
- Differences in the chemical composition of antioxidants in foods versus those in supplements may influence their effects. For example, eight chemical forms of vitamin E are present in foods. Vitamin E supplements, on the other hand, typically include only one of these forms—alpha-tocopherol. Alpha-tocopherol also has been used in almost all research studies on vitamin E.
- For some diseases, specific antioxidants might be more effective than the ones that have been tested. For example, to prevent eye diseases, antioxidants that are present in the eye, such as lutein, might be more beneficial than those that are not found in the eye, such as beta-carotene.
- The relationship between free radicals and health may be more complex than has previously been thought. Under some circumstances, free radicals actually may be beneficial rather than harmful, and removing them may be undesirable.
- The antioxidant supplements may not have been given for a long enough time to prevent chronic diseases, such as cardiovascular diseases or cancer, which develop over decades.
- The participants in the clinical trials discussed above were either members of the general population or people who were at high risk for particular diseases. They were not necessarily under increased oxidative stress. Antioxidants might help to prevent diseases in people who are under increased oxidative stress even if they don't prevent them in other people.

49.7 If You Are Considering Antioxidant Supplements

- Do not use antioxidant supplements to replace a healthy diet or conventional medical care, or as a reason to postpone seeing a health care provider about a medical problem.

- If you have age-related macular degeneration, consult your health care providers to determine whether supplements of the type used in the AREDS trial are appropriate for you.
- If you are considering a dietary supplement, first get information on it from reliable sources. Keep in mind that dietary supplements may interact with medications or other supplements and may contain ingredients not listed on the label. Your health care provider can advise you. If you are pregnant or nursing a child, or if you are considering giving a child a dietary supplement, it is especially important to consult your (or your child's) health care provider.
- Tell all of your health care providers about any complementary health approaches you use. Give them a full picture of what you do to manage your health. This will help ensure coordinated and safe care.

49.8 NCCIH- and NIH-Funded Research

Researchers supported by NCCIH and other components of NIH are conducting a variety of studies using antioxidant supplements.

Topics of recent NCCIH research on antioxidants include:

- The ways in which two chemical forms of vitamin E affect inflammation
- The biological effects of selenium on immune function
- The effects of a range of doses of alpha-lipoic acid on oxidative stress
- The effects of alpha-lipoic acid and acetyl-L-carnitine on inflammation in people with sickle cell disease.

NCCIH also funds a center of excellence for research on antioxidant therapies, which is conducting studies on the effects of antioxidants on various diseases and on aging.

Other components of NIH are also sponsoring research on antioxidants. Recent topics include:

- The effects of antioxidant therapy in Alzheimer's disease
- The roles of oxidation and antioxidants in breast cancer risk
- Whether antioxidants from pomegranate can help prevent or treat prostate cancer
- Whether anthocyanins (a group of antioxidants from berries) can help prevent esophageal cancer.

49.9 For More Information

NCCIH Clearinghouse

The NCCIH Clearinghouse provides information on NCCIH and complementary and integrative health approaches, including publications and searches of Federal

databases of scientific and medical literature. The Clearinghouse does not provide medical advice, treatment recommendations, or referrals to practitioners.

Toll-free in the U.S.: 1-888-644-6226
TTY (for deaf and hard-of-hearing callers):
1-866-464-3615
Website: https://nccih.nih.gov/
Email: info@nccih.nih.gov (link sends email)

PubMed®

A service of the National Library of Medicine, PubMed® contains publication information and (in most cases) brief summaries of articles from scientific and medical journals. For guidance from NCCIH on using PubMed, see How To Find Information About Complementary Health Approaches on PubMed.
Website: https://pubmed.ncbi.nlm.nih.gov/

NIH Clinical Research Trials and You

The National Institutes of Health (NIH) has created a website, NIH Clinical Research Trials and You, to help people learn about clinical trials, why they matter, and how to participate. The site includes questions and answers about clinical trials, guidance on how to find clinical trials through ClinicalTrials.gov and other resources, and stories about the personal experiences of clinical trial participants. Clinical trials are necessary to find better ways to prevent, diagnose, and treat diseases.
Website: https://www.nih.gov/health-information/nih-clinical-research-trials-you

Research Portfolio Online Reporting Tools Expenditures & Results (RePORTER)

RePORTER is a database of information on federally funded scientific and medical research projects being conducted at research institutions.
Website: https://reporter.nih.gov

MedlinePlus®

To provide resources that help answer health questions, MedlinePlus (a service of the National Library of Medicine) brings together authoritative information from the National Institutes of Health as well as other Government agencies and health-related organizations.
Website: https://www.medlineplus.gov/

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Chapter 50

Hearing Loss and Hearing Aids: Current Issues

Raj Bawa, PhD, MD

Patent Law Department, Bawa Biotech LLC, Ashburn, Virginia, USA
Guanine Inc., Rensselaer, New York, USA
Teva Pharmaceutical Industries Ltd., Israel

bawa@bawabiotech.com

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Medicine is an ever-changing science and this chapter is no substitute for individual patient assessment based on health care professionals' examination of each patient and consideration of, among other things, age, weight, gender, current or prior medical conditions, medication history, laboratory data, and other factors unique to the patient. The author does not provide medical advice or guidance, and this work is merely a reference tool. Given continuous, rapid advances in medical science and health information, independent professional verification of medical diagnoses, indications, appropriate pharmaceutical selections and dosages, and treatment options should be made, and health care professionals should consult a variety of sources. Accordingly, this chapter is provided "as is," and the author disclaims any and all warranties, express or implied.

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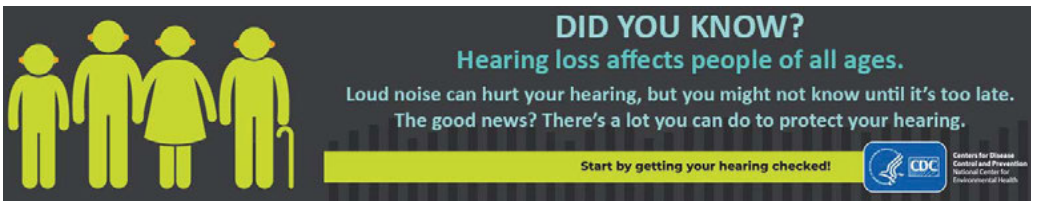
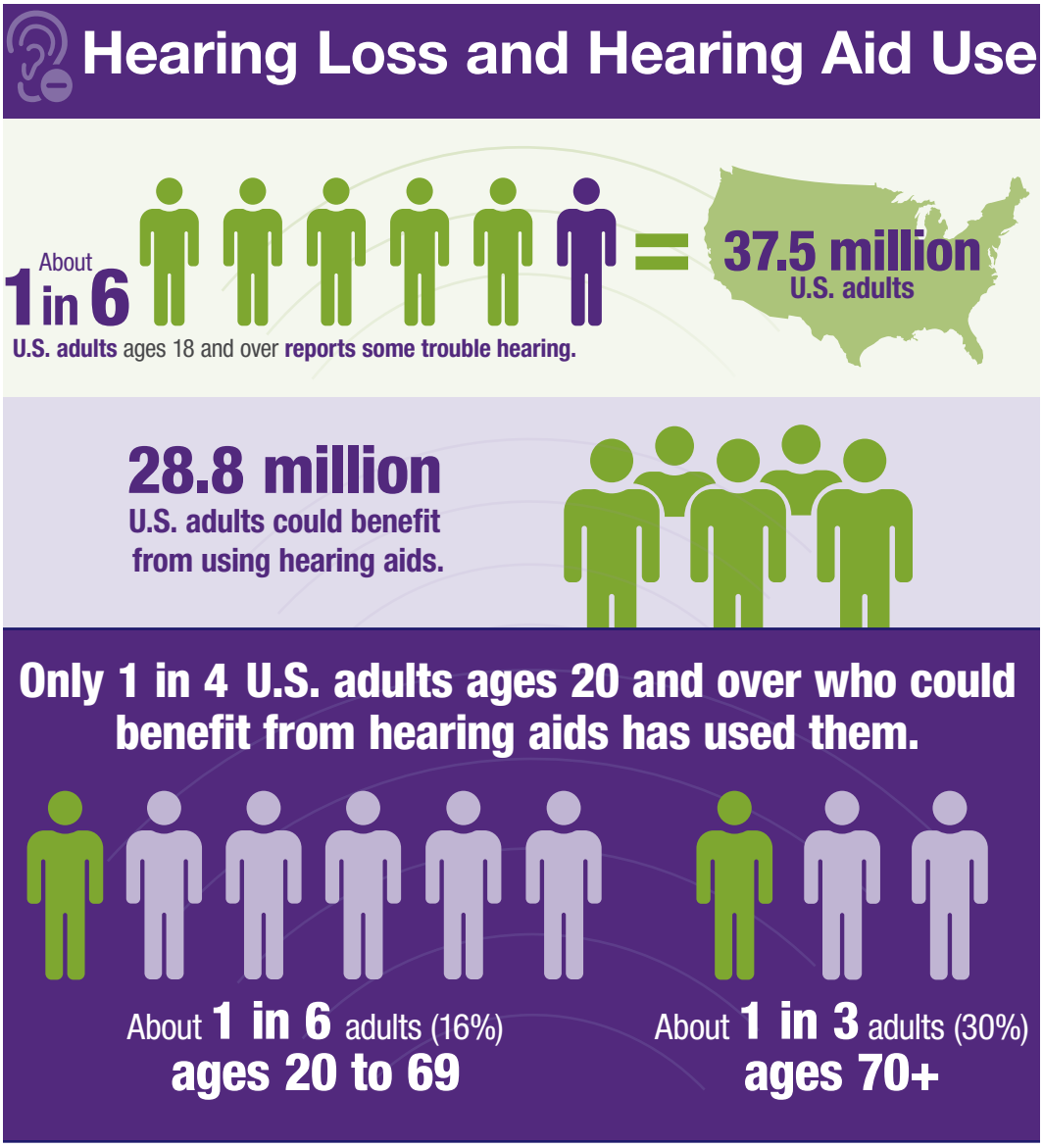
50.1 What Is Hearing Loss?



Hearing loss is a sudden or gradual decrease in how well you can hear. Hearing loss is the third most common chronic health condition in the US. Almost twice as many people report hearing loss as report diabetes or cancer. Noise exposure away from your job can damage your hearing just as much as working in a noisy place. Being around too much loud noise—like using a leaf blower or going to loud concerts—can cause permanent hearing loss. And once it's gone, you can't get it back! You can have hearing loss before you even notice you're having problems. Noise is measured in what are called decibels (dB) (discussed further). Over time, listening to loud sounds at high dB levels can cause hearing loss—or other hearing problems like a ringing sound in your ear that won't go away. The louder a sound is, and the longer you are exposed to it, the more likely it will damage your hearing. The more often you are exposed to loud sounds over time, the more damage occurs. It's important for healthcare providers to ask about hearing and to screen those who are at risk.

Everyday sounds typically do not damage your hearing. However, many people participate in activities that produce harmful sound levels, such as attending loud sporting events and music concerts, and using power tools, which repeated over time will cause hearing loss. Loud sound (noise) can damage sensitive parts of the ear, causing hearing loss, ringing or buzzing in the ear (tinnitus), and increased sensitivity to sound (hyperacusis). Repeated exposure to loud noise over the years affects how well you hear later in life and how quickly you develop hearing problems, even after exposure has stopped.

Perhaps you've heard yourself say those words more often than you'd like to admit. Or maybe you, a family member, or a friend is consistently turning up the TV volume or can't follow simple conversations in restaurants. If you are age 70 or older, this is common. Mild-to-moderate hearing loss affects more than 60 percent of 70-year-olds, and more than 80 percent of 80-year-olds have hearing loss. Hearing health care affordability and accessibility is an urgent public health problem. About 40 million US adults aged 20-69 years have noise-induced hearing loss. More than 1 in 2 US adults with hearing damage from noise do not have noisy jobs while about 1 in 4 US adults who report excellent to good hearing already have hearing damage. This number is rising as the number of senior citizens increases.



Having trouble hearing can make it hard to understand and follow a doctor's advice, to respond to warnings, and to hear doorbells and alarms. It can also make it hard to enjoy talking with friends and family. All of this can be frustrating, embarrassing, and even dangerous. It can have a significant impact on communication, social participation, and overall health and quality of life. Despite the high prevalence and public health impact of hearing loss, only about one-fourth of people who could benefit from a hearing aid seek intervention. The use of hearing aids has been linked to, among other health benefits, reductions in the incidence or severity of cognitive decline, depression, and other health problems in older adults. Additionally, benefits of hearing aid use can include improved social participation and a better quality of life. Besides health benefits for individuals, more-widespread adoption of hearing aids could have broader effects. By increasing social participation, hearing aids could help to improve inclusion of individuals in family, economic, civic, and religious life. Thus, reducing barriers to hearing aid access might contribute to such improvements. This could be particularly true for people of color, rural Americans, low-income individuals, and others for whom barriers to hearing aid access may be especially burdensome. Several barriers likely impede the use of hearing aids in hearing-impaired individuals such as high cost, stigma of being perceived as old or debilitated, and value (perceived hearing benefit relative to price).

50.2 What Noises Cause Hearing Loss?

Loud Noise Can Cause Hearing Loss Quickly or Over Time

Hearing loss can result from a single loud sound (like firecrackers) near your ear. Or, more often, hearing loss can result over time from damage caused by repeated exposures to loud sounds. The louder the sound, the shorter the amount of time it takes for hearing loss to occur. The longer the exposure, the greater the risk for hearing loss (especially when hearing protection is not used or there is not enough time for the ears to rest between exposures).

Common Sources of Noise and Decibel Levels

A whisper is about 30 dB, normal conversation is about 60 dB, and a motorcycle engine running is about 95 dB. Noise above 70 dB over a prolonged period of time may start to damage your hearing. Loud noise above 120 dB can cause immediate harm to your ears. Some sources of loud noise are listed in the table on the next page. If you are repeatedly exposed to them over time, they can cause hearing loss.

Sounds May Be Louder Than What You Hear

How *loud* something sounds to you is not the same as the actual *intensity* of that sound. Sound intensity is the amount of sound energy in a confined space. As stated above, sound is measured in decibels (dB). The decibel scale is logarithmic, which means that loudness is not directly proportional to sound intensity.

Everyday Sounds and Noises	Average Sound Level (measured in decibels)	Typical Response (after routine or repeated exposure)
Softest sound that can be heard	0	
Normal breathing	10	
Ticking watch	20	Sounds at these dB levels typically don't cause any hearing damage.
Soft whisper	30	
Refrigerator hum	40	
Normal conversation, air conditioner	60	
Washing machine, dishwasher	70	You may feel annoyed by the noise
City traffic (inside the car)	80–85	You may feel very annoyed
Gas-powered lawnmowers and leaf blowers	80-85	Damage to hearing possible after 2 hours of exposure
Motorcycle	95	Damage to hearing possible after about 50 minutes of exposure
Approaching subway train, car horn at 16 feet (5 meters), and sporting events (such as hockey playoffs and football games)	100	Hearing loss possible after 15 minutes
The maximum volume level for personal listening devices; a very loud radio, stereo, or television; and loud entertainment venues (such as nightclubs, bars, and rock concerts)	105–110	Hearing loss possible in less than 5 minutes
Shouting or barking in the ear	110	Hearing loss possible in less than 2 minutes
Standing beside or near sirens	120	Pain and ear injury
Firecrackers	140–150	Pain and ear injury

Instead, the intensity of a sound grows very fast. This means that a sound at 20 dB is 10 times more intense than a sound at 10 dB. Also, the intensity of a sound at 100 dB is one billion times more powerful compared to a sound at 10 dB. Two sounds that have equal intensity are not necessarily equally loud. Loudness refers to how you perceive audible sounds. A sound that seems loud in a quiet room might not be noticeable when you are on a street corner with heavy traffic, even though the sound intensity is the same. In general, to measure loudness, a sound must be increased by 10 dB to be perceived as twice as loud. For example, ten violins would sound only twice as loud as one violin. The risk of damaging your hearing from noise increases with the sound intensity, not the loudness of the sound. If you need to raise your voice to be heard at an arm's length, the noise level in the environment is likely above 85 dB in sound intensity and could damage your hearing over time.

How Do I Know the Sound Level is Safe?

The effect of lower noise levels over long periods is the same as louder noise levels over a shorter period. You can use a sound level meter (SLM) to measure noise around you. Free SLMs developed as smartphone apps are available. Some of these apps can predict your maximum allowable daily noise dose, like the NIOSH SLM app developed for iOS devices to help promote better hearing health and prevention efforts. The U.S. Environmental Protection Agency (EPA) and the World Health Organization (WHO) recommend maintaining environmental noises below 70 dBA over 24-hours (75 dBA over 8-hours) to prevent noise-induced hearing loss. The EPA also specified limits for speech interference and annoyance at 55 dBA for outdoors activities and 45 dBA for indoor activities. More details about this topic can be found on the NIOSH Science Blog—Understanding Noise Exposure Limits: Occupational vs. General Environmental Noise (https://blogs.cdc.gov/niosh-science-blog/2016/02/08/noise/).

Preventing Hearing Loss Caused by Chemical (Ototoxicity) Exposure

Millions of workers are exposed to noise in the workplace every day and when uncontrolled, noise exposure may cause permanent hearing loss. Research demonstrates exposure to certain chemicals, called ototoxicants, may cause hearing loss or balance problems, regardless of noise exposure. Substances including certain pesticides, solvents, and pharmaceuticals that contain ototoxicants can negatively affect how the ear functions, causing hearing loss, and/or affect balance. The risk of hearing loss is increased when workers are exposed to these chemicals while working around elevated noise levels. This combination often results in hearing loss that can be temporary or permanent, depending on the level of noise, the dose of the chemical, and the duration of the exposure. This hearing impairment affects many occupations and industries, from machinists to firefighters. Harmful exposure to ototoxicants may occur through inhalation, ingestion, or skin absorption. Health effects caused by ototoxic chemicals vary based on exposure frequency, intensity, duration, workplace exposure to other hazards, and individual factors such as age. Effects may be temporary or permanent, can affect hearing sensitivity and result in a standard threshold shift. Since chemicals can affect central portions of the auditory system (e.g., nerves or nuclei in the central nervous system, the pathways to the brain or in the brain itself), not only do sounds need to be louder to be detected, but also they lose clarity. Specifically, speech discrimination dysfunction, the ability to hear voices separately from background noise, may occur and involve:

- Compressed loudness: sound distortion.
- Frequency resolution: the inability to differentiate two sounds with similar frequency.
- Temporal resolution: the inability to detect time gaps between sounds.
- Spatial resolution: the inability to localize sound.

50.3 How Does Loud Noise Cause Hearing Loss?

Noise can damage hair cells, membranes, nerves, or other parts of your ear. This can cause temporary or permanent hearing loss. Learn how this happens so that you can prevent hearing loss.

Hearing Loss Can Be Temporary or Permanent

Hearing loss is a decrease in your ability to hear or understand speech and sounds around you. Hearing loss can happen when any part of the ear or the nerves that carry information on sounds to your brain do not work in the usual way. In some cases, hearing loss can be temporary. However, it can become permanent when vital parts of the ear have been damaged beyond repair. Damage to any part of the ear can lead to hearing loss.

Loud noise is particularly harmful to the inner ear (cochlea). A one-time exposure to extreme loud sound or listening to loud sounds for a long time can cause hearing loss. Loud noise can damage cells and membranes in the cochlea. Listening to loud noise for a long time can overwork hair cells in the ear, which can cause these cells to die. The hearing loss progresses as long as the exposure continues. Harmful effects might continue even after noise exposure has stopped. Damage to the inner ear or auditory neural system is generally permanent.

Damaged Hair Cells in Your Ears Can Lead to Hearing Loss

The average person is born with about 16,000 hair cells within their cochlea. These cells allow your brain to detect sounds. Up to 30% to 50% of hair cells can be damaged or destroyed before changes in your hearing can be measured by a hearing test. By the time you notice hearing loss, many hair cells have been destroyed and cannot be repaired. After leaving a very loud event, such as a concert or football game, you may notice that you don't hear as well as before. You might not hear whispers, sound might seem muffled, or you may hear ringing in your ears. Normal hearing usually returns within a few hours to a few days. This is because the hair cells, similar to blades of grass, will bend more if the sound is louder. But they will become straight again after a recovery period. However, if loud noise damaged too many of the hair cells, some of them will die. Repeated exposures to loud noises will over time destroy many hair cells. This can gradually reduce your ability to understand speech in noisy places. Eventually, if hearing loss continues, it can become hard to understand speech even in quieter places.

Normal Hair Cells Damaged Hair Cells

Noise Can Also Damage Nerves in Your Ears

In addition to damaging hair cells, noise can also damage the auditory nerve that carries information about sounds to your brain. Early damage may not show up on your hearing test. It can create a "hidden hearing loss" that may make it difficult for you to understand speech in noisy places. The effect of loud noise over time affects how well you might hear later in life. It also affects how quickly you might develop hearing problems, even after exposure has stopped.

50.4 What Is Noise-Induced Hearing Loss (NIHL)?

Every day, we experience sound in our environment, such as the sounds from television and radio, household appliances, and traffic. Normally, these sounds are at safe levels that don't damage our hearing. But sounds can be harmful when they are too loud, even for a brief time, or when they are both loud and long-lasting. These sounds can damage sensitive structures in the inner ear and cause noise-induced hearing loss (NIHL). NIHL can be immediate or it can take a long time to be noticeable. It can be temporary or permanent, and it can affect one ear or both ears. Even if you can't tell that you are damaging your hearing, you could have trouble hearing in the future, such as not being able to understand other people when they talk, especially on the phone or in a noisy room. Regardless of how it might affect you, one thing is certain: noise-induced hearing loss is something you can prevent.

50.4.1 What Causes NIHL?

NIHL can be caused by a one-time exposure to an intense "impulse" sound, such as an explosion, or by continuous exposure to loud sounds over an extended period of time, such as noise generated in a woodworking shop. Recreational activities that can put you at risk for NIHL include target shooting and hunting, snowmobile riding, listening to MP3 players at high volume through earbuds or headphones, playing in a band, and attending loud concerts. Harmful noises at home may come from sources including lawnmowers, leaf blowers, and woodworking tools. Sounds at or below 70 A-weighted decibels (dBA), even after long exposure, are unlikely to cause hearing loss. However, long or repeated exposure to sounds at or above 85 dBA can cause hearing loss. The louder the sound, the shorter the amount of time it takes for NIHL to happen.

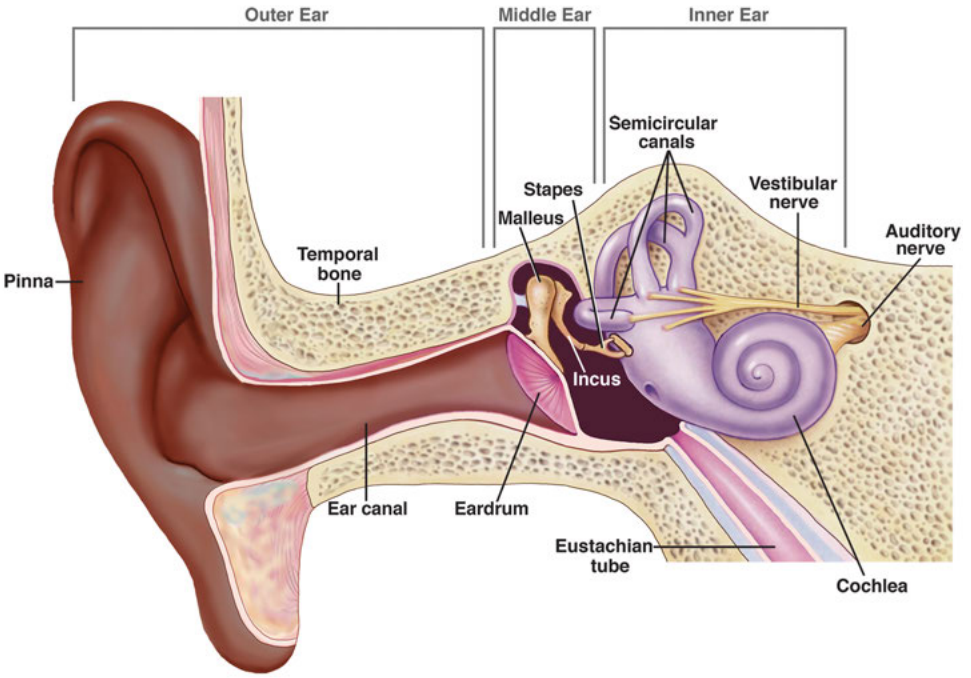
Here are the average decibel ratings of some familiar sounds:

- Normal conversation: 60–70 dBA
- Movie theater: 74–104 dBA
- Motorcyles and dirt bikes: 80–110 dBA
- Music through headphones at maximum volume, sporting events, and concerts: 94–110 dBA
- Sirens: 110–129 dBA
- Fireworks show: 140–160 dBA

Your distance from the source of the sound and the length of time you are exposed to the sound are also important factors in protecting your hearing. A good rule of thumb is to avoid noises that are too loud, too close, or last too long.

50.4.2 How Can Noise Damage Our Hearing?

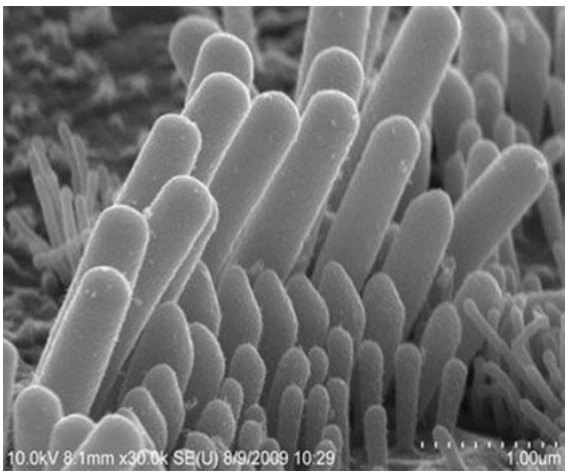
To understand how loud noises can damage our hearing, we have to understand how we hear. Hearing depends on a series of events that change sound waves in the air into electrical signals. Our auditory nerve then carries these signals to the brain through a complex series of steps.



Source: National Institute on Deafness and Other Communication Disorders.

1. Sound waves enter the outer ear and travel through a narrow passageway called the ear canal, which leads to the eardrum.
2. The eardrum vibrates from the incoming sound waves and sends these vibrations to three tiny bones in the middle ear. These bones are called the malleus, incus, and stapes.
3. The bones in the middle ear couple the sound vibrations from the air to fluid vibrations in the cochlea of the inner ear, which is shaped like a snail and filled with fluid. An elastic partition runs from the beginning to the end of the cochlea, splitting it into an upper and lower part. This partition is called the basilar membrane because it serves as the base, or ground floor, on which key hearing structures sit.

4. Once the vibrations cause the fluid inside the cochlea to ripple, a traveling wave forms along the basilar membrane. Hair cells—sensory cells sitting on top of the basilar membrane—ride the wave.
5. As the hair cells move up and down, microscopic hair-like projections (known as stereocilia) that perch on top of the hair cells bump against an overlying structure and bend. Bending causes pore-like channels, which are at the tips of the stereocilia, to open up. When that happens, chemicals rush into the cell, creating an electrical signal.
6. The auditory nerve carries this electrical signal to the brain, which translates it into a sound that we recognize and understand.



Stereocilia perch atop sensory hair cells in the inner ear. Source: Yoshiyuki Kawashima.

Most NIHL is caused by the damage and eventual death of these hair cells. Unlike bird and amphibian hair cells, human hair cells don't grow back. They are gone for good.

50.4.3 What Are the Effects and Signs of NIHL?

When you are exposed to loud noise over a long period of time, you may slowly start to lose your hearing. Because the damage from noise exposure is usually gradual, you might not notice it, or you might ignore the signs of hearing loss until they become more pronounced. Over time, sounds may become distorted or muffled, and you might find it difficult to understand other people when they talk or have to turn up the volume on the television. The damage from NIHL, combined with aging, can lead to hearing loss severe enough that you need hearing aids to magnify the sounds around you to help you hear, communicate, and participate more fully in daily activities. NIHL can also be caused by extremely loud bursts of sound, such as gunshots or explosions, which can rupture the eardrum or damage the bones in the middle ear. This kind of NIHL can be immediate

and permanent. Loud noise exposure can also cause tinnitus (https://www.nidcd.nih.gov/health/tinnitus)—a ringing, buzzing, or roaring in the ears or head. Tinnitus may subside over time, but can sometimes continue constantly or occasionally throughout a person's life. Hearing loss and tinnitus can occur in one or both ears. Sometimes exposure to impulse or continuous loud noise causes a temporary hearing loss that disappears 16 to 48 hours later. Recent research suggests, however, that although the loss of hearing seems to disappear, there may be residual long-term damage to your hearing.

50.5 How Can I Tell If I Have a Hearing Problem?

If you are 18 to 64 years old, the following questions will help you determine if you need to have your hearing tested by a health professional. Answer YES or NO to the following questions:

1. Do you sometimes feel embarrassed when you meet new people because you struggle to hear?
2. Do you feel frustrated when talking to members of your family because you have difficulty hearing them?
3. Do you have difficulty hearing or understanding co-workers, clients, or customers?
4. Do you feel restricted or limited by a hearing problem?
5. Do you have difficulty hearing when visiting friends, relatives, or neighbors?
6. Do you have trouble hearing in the movies or in the theater?
7. Does a hearing problem cause you to argue with family members?
8. Do you have trouble hearing the TV or radio at levels that are loud enough for others?
9. Do you feel that any difficulty with your hearing limits your personal or social life?
10. Do you have trouble hearing family or friends when you are together in a restaurant?

If you answered "yes" to three or more of these questions, you may want to see an otolaryngologist (an ear, nose, and throat specialist) or an audiologist for a hearing evaluation, or learn about over-the-counter hearing aids at https://www.nidcd.nih.gov/health/over-counter-hearing-aids.

Adapted from: Newman, C.W., Weinstein, B.E., Jacobson, G.P., & Hug, G.A. (1990). The Hearing Handicap Inventory for Adults [HHIA]: Psychometric adequacy and audiometric correlates. *Ear Hear,* **11:**430–433.

50.6 Who Can I Turn to for Help with My Hearing Loss?

If you or a family member have questions or concerns about hearing loss, consult a qualified health professional for early and appropriate care. Several types

of professionals can help. Each has a different type of training and expertise, and each can be an important part of your hearing health care.

You may want to start by talking with your primary care provider. They will likely give you a medical exam to see if an infection, injury, or other condition (such as a buildup of ear wax) might be causing your hearing loss. Your primary care provider might then refer you to an otolaryngologist or audiologist for more specific tests and treatment.

Listed below are the types of professionals who can help you with hearing loss.

A *primary care provider* is a physician, nurse practitioner, or physician assistant who provides general health care to patients by identifying and treating common medical conditions. Primary care providers refer patients to medical specialists when necessary. Types of primary care providers include family practitioners or general practitioners, pediatricians, geriatricians, and internists.

An *otolaryngologist* is a physician who provides medical and surgical care, diagnosis, and treatment of the ear, nose, throat, and neck. Sometimes called an ENT, an otolaryngologist will work with you to find out why you're having trouble hearing and offer specific treatment options. They might also refer you to another hearing professional, such as an audiologist, to receive a hearing test and be fitted for a hearing aid.

An *audiologist* has specialized training to test your hearing and identify the type and degree of hearing loss. Audiologists are not physicians. They have a graduate degree focused in audiology (master's degree or doctor of audiology, AuD), which typically requires 4 years to complete after earning a bachelor's degree. They must also pass an exam and complete a clinical fellowship. Audiologists are licensed to fit and dispense hearing aids; they can also work with you and your family to adapt to hearing loss and determine which devices, including hearing aids, would be most helpful.

A *hearing instrument specialist*, also known as a hearing aid specialist, is a state-licensed professional who conducts basic hearing tests, fits and dispenses hearing aids, and educates individuals and their family members about their hearing loss. The licensure requirement varies among states; most states require completing a 2-year apprenticeship.

50.7 Why Am I Losing My Hearing?

Hearing loss happens for different reasons. Many people lose their hearing slowly as they age. This condition is known as presbycusis. Doctors do not know why presbycusis affects some people more than others, but it seems to run in families. Another reason for hearing loss with aging may be years of exposure to loud noise. This condition is known as noise-induced hearing loss. Many construction workers, farmers, musicians, airport workers, yard and tree care workers, and people in the armed forces have hearing problems even in their younger and middle years because of too much exposure to loud noise. (Read the NIDCD fact sheets Age-Related Hearing Loss (https://www.nidcd.nih.gov/health/age-related-

hearing-loss) and Noise-Induced Hearing Loss (https://www.nidcd.nih.gov/health/noise-induced-hearing-loss-0) for more information.) Hearing loss can also be caused by viral or bacterial infections, heart conditions or stroke, head injuries, tumors, and certain medicines.

Problem:

Many people are exposed to noise that damages their hearing.

Hearing gets worse over time the more often people are exposed to loud sounds.

- About 53% of people ages 20-69 who have hearing damage from noise report no on-the-job exposure.
- About 24% of people ages 20-69 who report having excellent hearing have measurable hearing damage.
- About 20% of adults with no job exposure to loud sounds have hearing damage.

Hearing loss often gets worse for years before anyone notices or diagnoses it.

- People may not know that activities away from work can damage hearing just as much as noise on the job.
- People delay reporting hearing loss because they don't know or won't admit they have a problem.
- Less than half (46%) of adults who reported trouble hearing had seen a healthcare provider for their hearing in the past 5 years.

Hearing loss causes many problems.

- Continual exposure to noise can cause stress, anxiety, depression, high blood pressure, heart disease, and many other health problems.
- Some people are at higher risk for hearing loss, including those who:
  - are exposed to loud sounds at home and in the community.
  - work in noisy environments (especially noise of 85 dB or more for 8 hours or longer).
  - take medicines that increase their risk.
  - are male.
  - are age 40 or older.

Hearing loss is costly.

The cost for the first year of hearing loss treatment in older adults is projected to increase more than 500% from $8 billion in 2002 to an **estimated $51 billion in 2030**.

SOURCE: Journal of the American Geriatrics Society, 2010

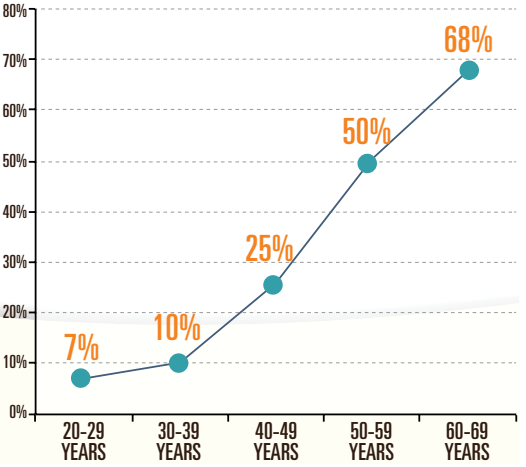
Hearing loss from loud noise can be prevented.

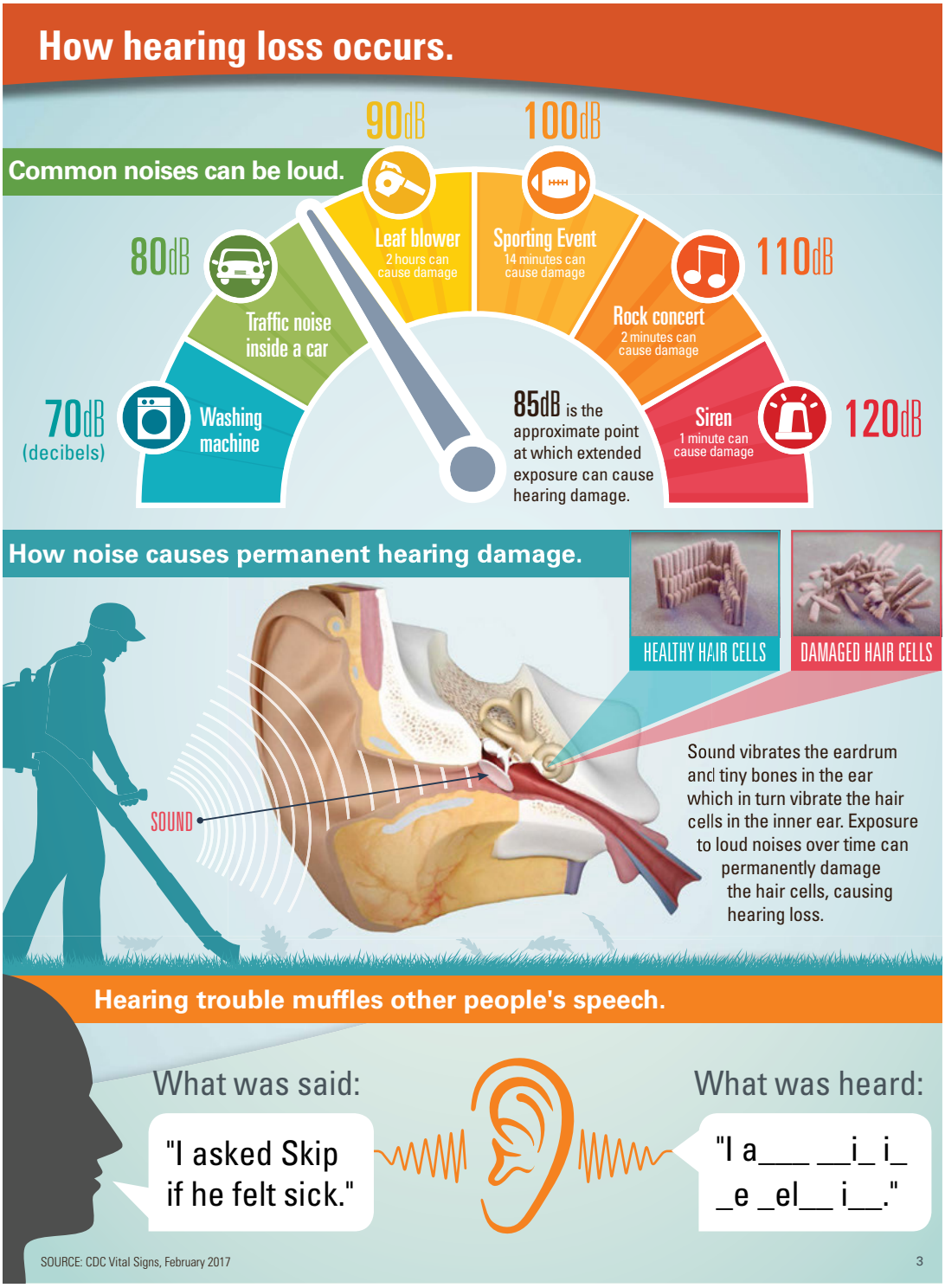
About 70% of people exposed to loud noise never or seldom wear hearing protection.

SOURCE: National Health and Nutrition Examination Survey, 2011-2012

People with hearing loss.

(Not able to hear high-pitched sounds)




How hearing loss occurs.
Common noises can be loud.
70dB
(decibels)
Washing machine
80dB
Traffic noise inside a car
90dB
Leaf blower
2 hours can cause damage
100dB
Sporting Event
14 minutes can cause damage
110dB
Rock concert
2 minutes can cause damage
120dB
Siren
1 minute can cause damage
85dB is the approximate point at which extended exposure can cause hearing damage.
How noise causes permanent hearing damage.
HEALTHY HAIR CELLS
DAMAGED HAIR CELLS
SOUND
Sound vibrates the eardrum and tiny bones in the ear which in turn vibrate the hair cells in the inner ear. Exposure to loud noises over time can permanently damage the hair cells, causing hearing loss.
Hearing trouble muffles other people's speech.
What was said:
"I asked Skip if he felt sick."
What was heard:
"I a___ __i_ i_ _e _el__ i__."
SOURCE: CDC Vital Signs, February 2017
3

50.8 What Is an Audiogram (Hearing Test)?

50.8.1 Audiograms and Hearing threshold

- An audiogram is often called a "hearing test," but there's no pass or fail.
- It is a written record of your hearing levels.
- A series of audiograms can track changes in hearing over time.
- Your hearing threshold levels (the quietest sounds you can hear) are measured in decibels (dB) at different frequencies from low (500 Hz) to high (8000 Hz).

50.8.2 Why Should I Get audiograms?

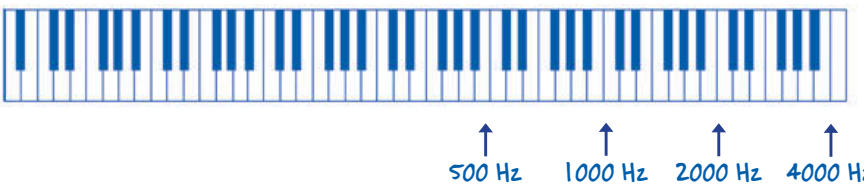
- To measure your hearing ability
- To identify hearing problems
- To monitor success at maintaining your hearing
- To see if noise exposure is affecting your hearing

50.8.3 Do I Have Normal Hearing?

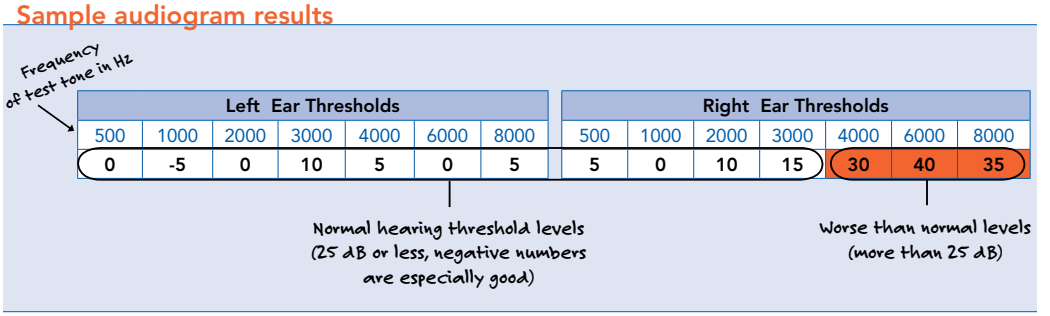
Compare your hearing threshold levels to this scale:

10 – 25 dB Normal hearing
26 – 40 dB Mild loss
41 – 55 dB Moderate loss
56 – 70 dB Moderate/severe loss
71 – 90 dB Severe loss
91 – 100 dB Profound loss

Audiograms test a range of sounds from low to high frequency (pitch). The test frequencies, measured in Hertz (Hz) usually range from 500 Hz (around the middle of a piano's scale) up to 6000 or 8000 Hz (a little above the highest note a piano can play).



Sample audiogram results



Your test results are valuable — don't lose them!

- Keep a copy of your audiogram in a safe place.
- Give a copy to your primary care doctor.
- Give a copy to the administrator of your hearing conservation program.

50.8.4 What Can Cause My Hearing to Get Worse?

Noise is the greatest hearing hazard for most workers, but any of these factors can cause or contribute to hearing loss:

Hazardous noise	Earwax blockage	Medical diseases
Head trauma	Heredity	Frequent ear infections
Aging	Medications	Chemical exposures

See an audiologist or physician for more information about these causes.

50.8.5 Noise Is Everywhere! How Do I Protect Myself?

- If you must shout to be heard over the noise, it's probably too loud!
- Noise doesn't only happen at work. Noisy home and recreational activities can be hazardous.
- Have hearing protectors on hand. Use them on and off the job.

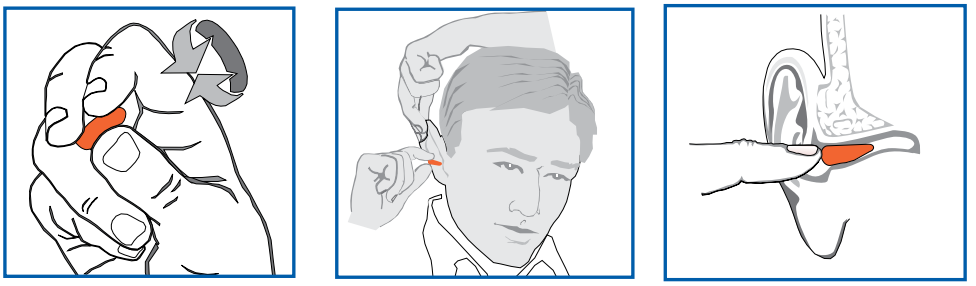
50.8.6 How Do I Select and Use Hearing Protectors?

- Comfort — so you'll wear them.
- Consistency — use them every time, all the time, in hazardous noise.
- Cleanliness — keep plugs and hands as clean as possible.

50.8.7 How Do I Insert a Foam Earplug to Help Protect My Hearing?

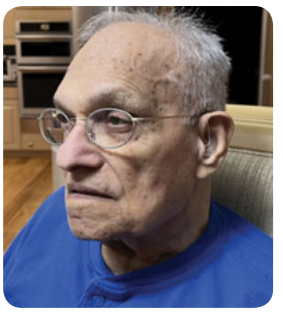
1. Roll the earplug.

2. Pull to open the ear. This step is especially important. You should pull up and away on the top of your ear with the opposite hand so the earplug can slide in easily.
3. Hold the earplug after inserting it.

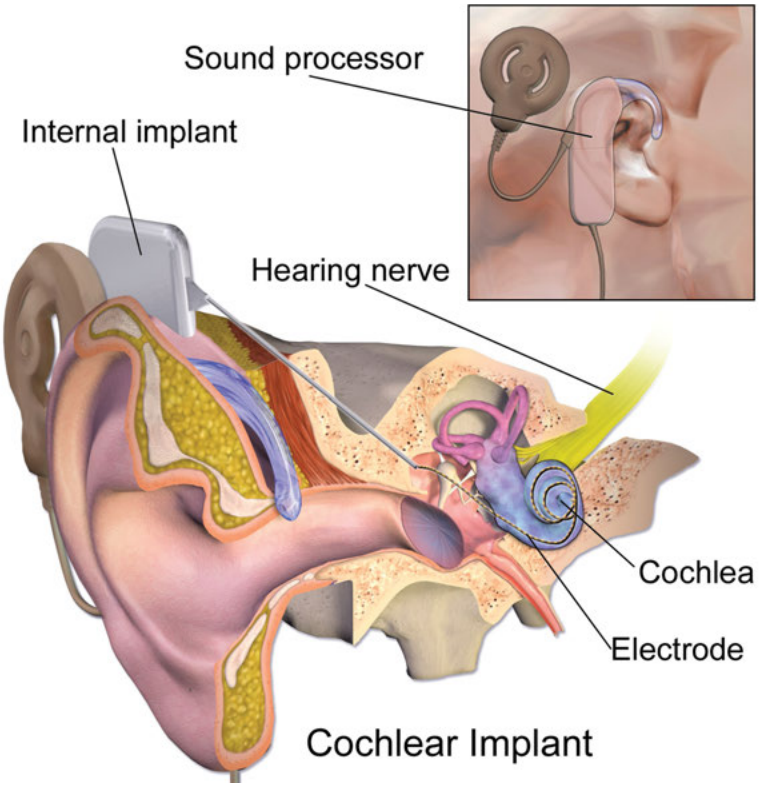


50.9 What Treatments and Devices Can Help?

Your treatment will depend on your hearing loss, so some treatments will work better for you than others. There are a number of devices and aids that can improve hearing loss. Hearing aid technology keeps evolving, which means there's a growing variety of styles and features to consider. The FDA regulates hearing aids to make sure they are safe and effective. There are many types of hearing aids (also known as hearing instruments), which vary in size, power and circuitry. Here are the most common ones:



- **Hearing aids** are electronic instruments you wear in or behind your ear (detailed ahead). They make sounds louder. Things sound different when you wear a hearing aid, but an audiologist or hearing aid specialist can help you get used to it. To find the hearing aid that works best for you, you may have to try more than one. Ask your audiologist or hearing specialist whether you can have a trial period with a few different hearing aids. Both of you can work together until you are comfortable.
- **Cochlear implants** are small electronic devices surgically implanted in the inner ear that help provide a sense of sound to people who are profoundly deaf or hard-of-hearing. If your hearing loss is severe, your doctor may recommend a cochlear implant in one ear or both.
- **Assistive listening devices** include telephone and cell phone amplifying devices, smart phone or tablet "apps," and closed circuit systems (induction coil loops) in places of worship, theaters, and auditoriums.



Cochlear implant. Source: Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". *WikiJournal of Medicine* **1** (2). DOI:10.15347/wjm/2014.010. ISSN 2002-4436.

A symbol commonly used to indicate that an assistive listening system is available. Courtesy of Wikipedia.

- **Lip reading (sometimes called speechreading)** is another option that helps people with hearing problems follow conversational speech. People who use this method pay close attention to others when they talk, by watching how the speaker's mouth and body move. Lip reading is a building block that helps a child with hearing loss understand speech. The child watches

the movements of a speaker's mouth and face, and understands what the speaker is saying. About 40% of the sounds in the English language can be seen on the lips of a speaker in good conditions — such as a well-lit room where the child can see the speaker's face. But some words can't be read. For example: "bop," "mop," and "pop" look exactly alike when spoken. (You can see this for yourself in a mirror). A good speech reader might be able to see only 4 to 5 words in a 12-word sentence. Children and adults often use speech reading in combination with other building blocks — such as auditory training (listening), cued speech, and others. But it can't be successful alone. Babies will naturally begin using this building block if they can see the speaker's mouth and face. But as a child gets older, he or she will still need some training to use this building block. Sometimes, when talking with a person who is deaf or hard-of-hearing, people will exaggerate their mouth movements or talk very loudly. Exaggerated mouth movements and a loud voice can make speech reading very hard. It is important to talk in a normal way and look directly at your child's face and make sure he or she is watching you.

Cochlear implants are different from hearing aids in some respects:

Hearing aids	Cochlear implants
Hearing aids are indicated for individuals with all degrees of hearing loss (from mild to profound).	Cochlear implants are indicated only for individuals with severe-profound hearing loss.
Most hearing aids are not implanted (although some hearing aids have an implanted component).	Cochlear implants are composed of both internal (implanted) and external components. A surgical procedure is needed to place the internal components.
In hearing aids, sound is amplified and conveyed through both the outer and middle ear and finally to the sensory receptor cells (hair cells) in the inner ear. The hair cells convert the sound energy into neural signals that are picked up by the auditory nerve.	Cochlear implants bypass the outer and middle ears, and the damaged hair cells and replace their functions by converting sound energy into electrical energy that directly stimulates the auditory nerve.

50.10 Hearing Aids: More Details

A hearing aid is a small electronic device that you wear in or behind your ear. It makes some sounds louder so that a person with hearing loss can listen, communicate, and participate more fully in daily activities. A hearing aid can help people hear more in both quiet and noisy situations. However, only about one out of five people who would benefit from a hearing aid actually uses one. A hearing aid has three basic parts: a microphone, amplifier, and speaker. The hearing

aid receives sound through a microphone, which converts the sound waves to electrical signals and sends them to an amplifier. The amplifier increases the power of the signals and then sends them to the ear through a speaker. Hearing aids are primarily useful in improving the hearing and speech comprehension of people who have hearing loss that results from damage to the small sensory cells in the inner ear, called hair cells. This type of hearing loss is called sensorineural hearing loss. The damage can occur as a result of disease, aging, or injury from noise or certain medicines.

A hearing aid magnifies sound vibrations entering the ear. Surviving hair cells detect the larger vibrations and convert them into neural signals that are passed along to the brain. The greater the damage to a person's hair cells, the more severe the hearing loss, and the greater the hearing aid amplification needed to make up the difference. However, there are practical limits to the amount of amplification a hearing aid can provide. In addition, if the inner ear is too damaged, even large vibrations will not be converted into neural signals. In this situation, a hearing aid would be ineffective.

50.11 What Are Some Features for Hearing Aids?

Hearing aids have optional features that can be built in to assist in different communication situations. For example:

- *Directional microphone* may help you converse in noisy environments. Specifically, it allows sound coming from a specific direction to be amplified to a greater level compared to sound from other directions. When the directional microphone is activated, sound coming from in front of you (as during a face-to-face conversation) is amplified to a greater level than sound from behind you.
- *T-coil (telephone switch)* allows you to switch from the normal microphone setting to a "T-coil" setting in order to hear better on the telephone. All wired telephones produced today must be hearing aid compatible. In the "T-coil" setting, environmental sounds are eliminated, and sound is picked up from the telephone. This also turns off the microphone on your hearing aid so you can talk without your hearing aid "whistling." The T-coil works well in theaters, auditoriums, houses of worship, and other places that have an induction loop or FM installation. The voice of the speaker, who can be some distance away, is amplified significantly more than any background noise. Some hearing aids have a combination "M" (Microphone)/"T" (Telephone) switch so that, while listening with an induction loop, you can still hear nearby conversation.
- *Direct audio input* allows you to plug in a remote microphone or an FM assistive listening system, connect directly to a TV, or connect to other devices such as your computer, a CD player, tape player, radio, etc.

- *Feedback suppression* helps suppress squeals when a hearing aid gets too close to the phone or has a loose-fitting earmold.

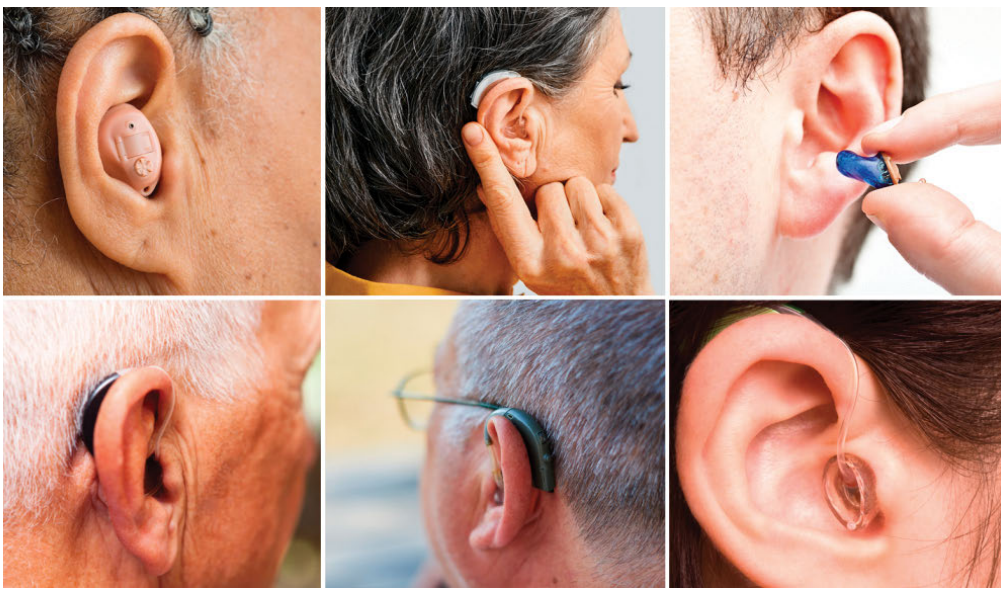
The more complicated features may allow the hearing aids to best meet your particular pattern of hearing loss. They may improve their performance in specific listening situations; however, these sophisticated electronics may significantly add to the cost of the hearing aid as well.

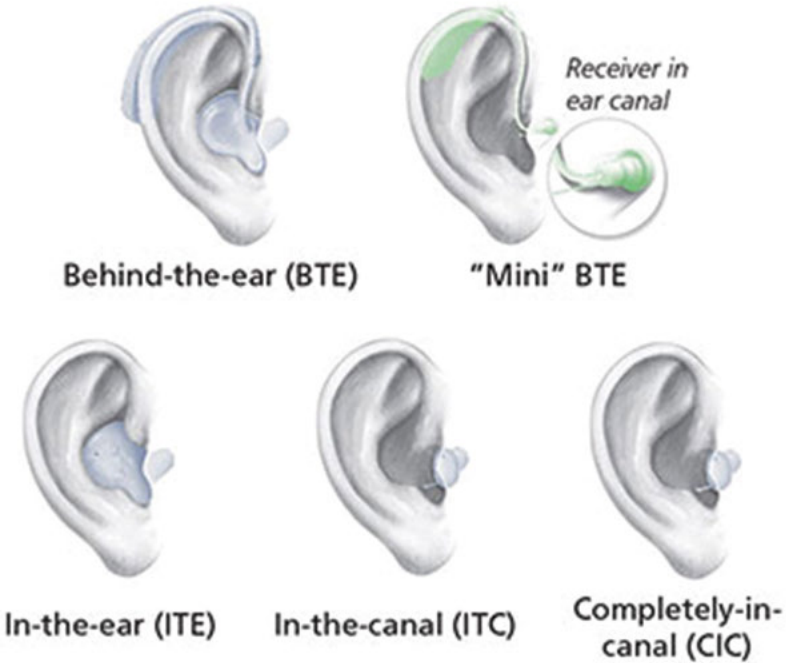
50.12 Hearing Aids and How They Work

People may be born with hearing loss. Or they may develop it later in life—often because the inner ear can wear out as we age or be damaged by years of exposure to loud noises. In some cases, hearing loss is temporary and can be restored with medical help. In other cases, it's permanent but can be improved with hearing aids. Hearing aids (https://www.fda.gov/medical-devices/consumer-products/hearing-aids) are medical devices worn behind or in the ear. They can improve hearing by making sounds louder. However, hearing aids usually won't restore your hearing to normal levels or quality in the way that eyeglasses can often restore vision to 20/20.

50.13 Are There Different Styles of Hearing Aids?

There are three basic styles of hearing aids. The styles differ by size, their placement on or inside the ear, and the degree to which they amplify sound.





- **Behind-the-ear (BTE) hearing aids** consist of a hard plastic case worn behind the ear and connected to a plastic earmold that fits inside the outer ear. The electronic parts are held in the case behind the ear. Sound travels from the hearing aid through the earmold and into the ear. BTE aids are used by people of all ages for mild to profound hearing loss.

  A new kind of BTE aid is an **open-fit hearing aid**. Small, open-fit aids fit behind the ear completely, with only a narrow tube inserted into the ear canal, enabling the canal to remain open. For this reason, open-fit hearing aids may be a good choice for people who experience a buildup of earwax, since this type of aid is less likely to be damaged by such substances. In addition, some people may prefer the open-fit hearing aid because their perception of their voice does not sound "plugged up."

- **In-the-ear (ITE) hearing aids** fit completely inside the outer ear and are used for mild to severe hearing loss. The case holding the electronic components is made of hard plastic. Some ITE aids may have certain added features installed, such as a telecoil. A telecoil is a small magnetic coil that allows users to receive sound through the circuitry of the hearing aid, rather than through its microphone. This makes it easier to hear conversations over the telephone. A telecoil also helps people hear in public facilities that have installed special sound systems, called induction loop systems. Induction loop systems can be found in many churches, schools, airports, and auditoriums. ITE aids usually are not worn by young children because the casings need to be replaced often as the ear grows.

- **Canal aids** fit into the ear canal and are available in two styles. The **in-the-canal (ITC) hearing aid** is made to fit the size and shape of a person's ear canal. A c**ompletely-in-canal (CIC) hearing aid** is nearly hidden in the ear canal. Both types are used for mild to moderately severe hearing loss. Because they are small, canal aids may be difficult for a person to adjust and remove. In addition, canal aids have less space available for batteries and additional devices, such as a telecoil. They usually are not recommended for young children or for people with severe to profound hearing loss because their reduced size limits their power and volume.

50.14 Are New Types of Aids Available?

Although they work differently than the hearing aids described above, implantable hearing aids are designed to help increase the transmission of sound vibrations entering the inner ear. A **middle ear implant (MEI)** is a small device attached to one of the bones of the middle ear. Rather than amplifying the sound traveling to the eardrum, an MEI moves these bones directly. Both techniques have the net result of strengthening sound vibrations entering the inner ear so that they can be detected by individuals with sensorineural hearing loss. A **bone-anchored hearing aid (BAHA)** is a small device that attaches to the bone behind the ear. The device transmits sound vibrations directly to the inner ear through the skull, bypassing the middle ear. BAHAs are generally used by individuals with middle ear problems or deafness in one ear. Because surgery is required to implant either of these devices, many hearing specialists feel that the benefits may not outweigh the risks.

50.15 What Is the Difference between Analog and Digital Hearing Aids?

Analog hearing aids make continuous sound waves louder. These hearing aids essentially amplify all sounds (e.g., speech and noise) in the same way. Some analog hearing aids are programmable. They have a microchip which allows the aid to have settings programmed for different listening environments, such as in a quiet place, like at a library, or in a noisy place like in a restaurant, or in a large area like a soccer field. The analog programmable hearing aids can store multiple programs for the various environments. As the listening environment changes, hearing aid settings may be changed by pushing a button on the hearing aid. Analog hearing aids are becoming less and less common.

Digital hearing aids have all the features of analog programmable aids, but they convert sound waves into digital signals and produce an exact duplication of sound. Computer chips in digital hearing aids analyze speech and other

environmental sounds. The digital hearing aids allow for more complex processing of sound during the amplification process which may improve their performance in certain situations (for example, background noise and whistle reduction). They also have greater flexibility in hearing aid programming so that the sound they transmit can be matched to the needs for a specific pattern of hearing loss. Digital hearing aids also provide multiple program memories. Most individuals who seek hearing help are offered a choice of only digital technology these days.

50.16 Which Hearing Aid Will Work Best for Me?

The hearing aid that will work best for you depends on the kind and severity of your hearing loss. If you have a hearing loss in both of your ears, two hearing aids are generally recommended because two aids provide a more natural signal to the brain. Hearing in both ears also will help you understand speech and locate where the sound is coming from. You and your audiologist should select a hearing aid that best suits your needs and lifestyle. Price is also a key consideration because hearing aids range from hundreds to several thousand dollars. Similar to other equipment purchases, style and features affect cost. However, don't use price alone to determine the best hearing aid for you. Just because one hearing aid is more expensive than another does not necessarily mean that it will better suit your needs. A hearing aid will not restore your normal hearing. With practice, however, a hearing aid will increase your awareness of sounds and their sources. You will want to wear your hearing aid regularly, so select one that is convenient and easy for you to use. Other features to consider include parts or services covered by the warranty, estimated schedule and costs for maintenance and repair, options and upgrade opportunities, and the hearing aid company's reputation for quality and customer service.

50.17 How Do I Get a Hearing Evaluation before Getting Hearing Aids?

Before getting a hearing aid, you should consider having a hearing evaluation to determine the type and amount of your hearing loss. The process can begin with a medical and/or audiological examination.

- *Medical examination.* The medical examination may be performed by any licensed physician including your family doctor or pediatrician, but preferably should be done by an ear, nose, and throat specialist (an otolaryngologist). An examination of your ear, nose, and throat and possibly other testing can be done to rule out any medical reason for your hearing loss, such as infection, injury or deformity, ear wax in the ear canal, and, in rare cases, tumors.
- *Audiological examination.* An audiological exam, or audiogram, involves a hearing evaluation by a hearing health professional that specializes

in evaluation, non-medical treatment, and rehabilitation of hearing loss (an audiologist) to identify the type and amount of your hearing loss, to determine the need for medical/surgical treatment and/or referral to a licensed physician, and to provide rehabilitation of the hearing loss.

50.18 What Is the Difference between Prescription and Over-the-Counter (OTC) Hearing Aids?

Medical evaluation required for children (younger than 18 years of age)

While hearing loss in adults is often caused by aging or noise exposure, the reasons for hearing loss in children are more varied and may be associated with other medical conditions that should be medically evaluated prior to prescribing hearing aids. OTC hearing aids are not intended for and must not be sold to people younger than 18 years of age. Hearing aids intended for people younger than 18 years of age are prescription hearing aids. Prescription hearing aids are sold by audiologists; ear, nose, and throat doctors; or sellers licensed to dispense hearing aids, such as instrument specialists.

Over-the-counter (OTC) hearing aids

Some hearing aids can be legally sold directly to the user over the internet or through mail order if permitted in your state. To broaden access to hearing aids, the FDA in October 2022 created a new category of over-the-counter (OTC) hearing aids that you could buy in the store or online without seeing a physician for an exam or an audiologist for help with fitting.[1] This new category of hearing aids enables consumers to directly buy hearing aids, without visiting a hearing health professional (https://www.nidcd.nih.gov/health/who-can-i-turn-help-my-hearing-loss). Basically, this action enables consumers 18 years of age and older with perceived mild to moderate hearing loss to purchase hearing aids directly from stores or online retailers without the need for a medical exam, prescription, or a fitting adjustment by an audiologist. These devices are intended to help adults with perceived mild to moderate hearing loss. Like prescription hearing aids (https://www.nidcd.nih.gov/health/hearing-aids), OTC hearing aids make sounds louder so that some adults with difficulty hearing are better able to listen, communicate, and participate fully in daily activities. In addition, OTC hearing aids are regulated as medical devices by the U.S. Food and Drug Administration (FDA).

The FDA issued a final rule: Establishing Over-the-Counter Hearing Aids (https://www.federalregister.gov/d/2022-17230) to improve access to safe, effective, and affordable hearing aids for millions of Americans. Concurrently with issuing the final rule, the FDA also issued the final guidance: *Regulatory Requirements*

[1]The FDA Reauthorization Act of 2017 (FDARA) (Pub. L. 115-52) (https://www.govinfo.gov/link/plaw/115/public/52) directs FDA to establish a category of OTC hearing aids through rulemaking, and FDARA sets forth various requirements for OTC hearing aids, including for reasonable assurance of safety and effectiveness, as well as Federal preemption provisions.

for Hearing Aid Devices and Personal Sound Amplification Products (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/regulatory-requirements-hearing-aid-devices-and-personal-sound-amplification-products) to clarify the differences between hearing aids, which are medical devices, and personal sound amplification products, which are not regulated as medical devices, and which help people with normal hearing amplify sounds in certain environments. Read more in the FDA's press release: *FDA Finalizes Historic Rule Enabling Access to Over-the-Counter Hearing Aids for Millions of Americans* (https://www.fda.gov/news-events/press-announcements/fda-finalizes-historic-rule-enabling-access-over-counter-hearing-aids-millions-americans).

OTC hearing aids are an alternative to prescription hearing aids, which are currently only available from hearing health professionals, such as audiologists, otolaryngologists (ear, nose, and throat doctors), and hearing aid specialists. The hearing health professional fits you for the hearing aid, adjusts the device based on your hearing loss, and provides other services.

Today, you can buy OTC hearing aids directly in stores and online, where prescription hearing aids are not available. You fit them yourself, and you may be able to control and adjust the devices in ways that users of prescription hearing aids cannot. Some OTC hearing aids might not look like prescription hearing aids at all.

OTC hearing aids are for adults with perceived mild to moderate hearing loss. They are not meant for children or for adults who have more severe hearing loss or significant difficulty hearing. If you have more severe hearing loss, OTC hearing aids might not be able to amplify sounds at high enough levels to help you.

The FDA has established regulations that manufacturers of OTC hearing aids need to follow. In general, these federal regulations:

- Ensure that the OTC devices are safe and effective for people with perceived mild to moderate hearing loss.
- Set standards for package labels to help buyers understand OTC hearing aids and who might benefit from them. The labels also include warnings

and other information you should know before buying or when using the hearing aid, such as signs that indicate that you should see a doctor.

Personal sound amplification products (PSAPs) are another class of amplifying devices that you can purchase without a prescription or seeing a health care professional. PSAPs are for people without hearing loss. They boost the ability to hear certain sounds in specific situations, such as while bird-watching. While the FDA regulates OTC hearing aids as medical devices for adults for hearing loss, PSAPs are not regulated as medical devices by the FDA. PSAPSs are further discussed in Section 50.24.

	Over-the-Counter (OTC) Hearing Aids	Prescription Hearing Aids (Any hearing aids that do not meet OTC requirements)	Personal Sound Amplification Products
Type of Product	Medical device Electronic product	Medical device Electronic product	Electronic product
Intended Users	• People 18 years and older • For those with perceived mild to moderate hearing loss	• People of any age, including those younger than 18 years • For people with any degree of hearing loss, including severe	• People of any age with normal hearing to amplify sounds in certain environments
Conditions for Sale	• Purchaser must be 18 years or older • No medical exam • No prescription • No fitting by audiologist • No need for licensed seller	• Prescription needed • Must purchase from licensed seller in some states	• No applicable FDA requirements regarding conditions for sale

50.19 Who Are OTC Hearing Aids for?

As indicated above, OTC hearing aids are for adults (18 and older) who believe they have mild to moderate hearing loss, even if they have not had a hearing exam. You might have mild to moderate hearing loss if, for example:

- Speech or other sounds seem muffled.
- You have trouble hearing when you're in a group, in a noisy area, on the phone, or when you can't see who is talking.
- You have to ask others to speak more slowly or clearly, to talk louder, or to repeat what they said.
- You turn up the volume higher than other people prefer when watching TV or listening to the radio or music.

If you have trouble hearing conversations in quiet settings—or have trouble hearing loud sounds, such as cars or trucks, noisy appliances, or loud music—consult a hearing health professional. These are signs that you might have more severe hearing loss and that OTC hearing aids won't work well for you. A hearing health professional can help you determine if a prescription hearing aid or other device can help you hear better.

Some ear problems need medical treatment. If you have any of the following, please see a licensed physician promptly:

- Fluid, pus, or blood coming out of your ear within the previous 6 months.
- Pain or discomfort in your ear.
- A history of excessive ear wax or suspicion that something is in your ear canal.
- Episodes of vertigo (severe dizziness) (https://www.nidcd.nih.gov/health/balance-disorders) with hearing loss.
- Sudden hearing loss or quickly worsening hearing loss (https://www.nidcd.nih.gov/health/sudden-deafness).
- Hearing loss that has gotten more and then less severe within the last 6 months.
- Hearing loss or tinnitus (ringing) (https://www.nidcd.nih.gov/health/tinnitus) in only one ear, or a noticeable difference in how well you can hear in each ear.

50.20 What Questions Should I Ask before Buying a Hearing Aid?

Before you buy a hearing aid, ask your audiologist these important questions:

- What features would be most useful to me?
- What is the total cost of the hearing aid? Do the benefits of newer technologies outweigh the higher costs?
- Is there a trial period to test the hearing aids? (Most manufacturers allow a 30- to 60-day trial period during which aids can be returned for a refund.) What fees are nonrefundable if the aids are returned after the trial period?
- How long is the warranty? Can it be extended? Does the warranty cover future maintenance and repairs?
- Can the audiologist make adjustments and provide servicing and minor repairs? Will loaner aids be provided when repairs are needed?
- What instruction does the audiologist provide?

50.21 How Can I Adjust to My Hearing Aid?

Hearing aids take time and patience to use successfully. Wearing your aids regularly will help you adjust to them.

Become familiar with your hearing aid's features. With your audiologist present, practice putting in and taking out the aid, cleaning it, identifying right and left aids, and replacing the batteries. Ask how to test it in listening environments where you have problems with hearing. Learn to adjust the aid's volume and to program it for sounds that are too loud or too soft. Work with your audiologist until you are comfortable and satisfied.

You may experience some of the following problems as you adjust to wearing your new aid.

- *My hearing aid feels uncomfortable.* Some individuals may find a hearing aid to be slightly uncomfortable at first. Ask your audiologist how long you should wear your hearing aid while you are adjusting to it.
- *My voice sounds too loud.* The "plugged-up" sensation that causes a hearing aid user's voice to sound louder inside the head is called the occlusion effect, and it is very common for new hearing aid users. Check with your audiologist to see if a correction is possible. Most individuals get used to this effect over time.
- *I get feedback from my hearing aid.* A whistling sound can be caused by a hearing aid that does not fit or work well or is clogged by earwax or fluid. See your audiologist for adjustments.
- *I hear background noise.* A hearing aid does not completely separate the sounds you want to hear from the ones you do not want to hear. Sometimes, however, the hearing aid may need to be adjusted. Talk with your audiologist.
- *I hear a buzzing sound when I use my cell phone.* Some people who wear hearing aids or have implanted hearing devices experience problems with the radio frequency interference caused by digital cell phones. Both hearing aids and cell phones are improving, however, so these problems are occurring less often. When you are being fitted for a new hearing aid, take your cell phone with you to see if it will work well with the aid.

50.22 How Can I Care for My Hearing Aid?

Proper maintenance and care will extend the life of your hearing aid. Make it a habit to:

- Keep hearing aids away from heat and moisture.
- Clean hearing aids as instructed. Earwax and ear drainage can damage a hearing aid.
- Avoid using hairspray or other hair care products while wearing hearing aids.
- Turn off hearing aids when they are not in use.

- Replace dead batteries immediately.
- Keep replacement batteries and small aids away from children and pets.

50.23 Can I Obtain Financial Assistance for a Hearing Aid?

Hearing aids are generally not covered by health insurance companies, although some do. For eligible children and young adults ages 21 and under, Medicaid will pay for the diagnosis and treatment of hearing loss, including hearing aids, under the Early and Periodic Screening, Diagnostic, and Treatment (EPSDT) service. Also, children may be covered by their state's early intervention program or State Children's Health Insurance Program.

Medicare does not cover hearing aids for adults; however, diagnostic evaluations are covered if they are ordered by a physician for the purpose of assisting the physician in developing a treatment plan. Since Medicare has declared the BAHA a prosthetic device and not a hearing aid, Medicare will cover the BAHA if other coverage policies are met.

Some nonprofit organizations provide financial assistance for hearing aids, while others may help provide used or refurbished aids. Contact the National Institute on Deafness and Other Communication Disorders (NIDCD) Information Clearinghouse (https://www.nidcd.nih.gov/health/clearinghouse) with questions about organizations that offer financial assistance for hearing aids.

50.24 Hearing Aids vs. Personal Sound Amplification Products (PSAPs)

You may have seen products in stores or online that are known as personal sound amplification products (PSAPs).[2] These are not alternatives to hearing aids and shouldn't be mistaken for hearing aids. PSAPs don't compensate for hearing loss, and buying a PSAP over the counter won't help you hear as well as you did before you experienced hearing loss. While hearing aids and PSAPs both amplify sound for the user, the products have different intended uses. Hearing aids are intended to make up for impaired hearing. PSAPs, in contrast, are intended for people with normal hearing to amplify sounds in certain situations, such as recreational activities like birdwatching or hunting. They are low-cost hearing

[2]PSAPs are not intended to improve hearing for individuals with hearing loss. They are intended to amplify environmental sound for consumers with normal hearing. Examples of situations in which PSAPs typically are used include hunting (listening for prey), bird watching, listening to lectures with a distant speaker, and listening to soft sounds that would be difficult for normal hearing individuals to hear (e.g., distant conversations, performances). Because PSAPs are not intended to diagnose, treat, cure, or mitigate disease and do not alter the structure or function of the body, they are not devices as defined in the Federal Food, Drug, and Cosmetic Act. As such, there is no regulatory classification, product code, or definition for these products. Furthermore, there are no requirements for registration of manufacturers and listing of these products with the U.S. Food and Drug Administration. Source: The American Speech-Language-Hearing Association (ASHA)

devices that can range from just $10 to $500, and can be bought right off the shelf – without so much as a hearing test! They are general sound amplifiers, untailored to an individual's specific hearing loss. Because such PSAPs are regulated as consumer electronics and not medical devices, they may be more variable in terms of product quality compared to hearing aids. The FDA does not regulate such PSAPs for safety and effectiveness like hearing aids.

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- Prevention Research Centers Studies Community-based Prevention of Noise-Induced Hearing Loss: The Oregon Health and Science University PRC (http://www.oregonprc.org/) evaluated the effectiveness of four noise-induced hearing loss prevention programs with primary school students in a tribal community. In 53 fourth-grade classrooms, 95% of students were reported to be at risk for noise-induced hearing loss. While all of the programs were effective, the more effective programs were the interactive programs, such as the two Dangerous Decibels® (http://dangerousdecibels.org/) programs in classrooms. The average improvement across all student survey questions was almost 20% for classroom programs.
- Safe listening (https://en.wikipedia.org/wiki/Safe_listening) is framework for health promotion actions to ensure that sound-related recreational activities (such as concerts, nightclubs, and listening to music, broadcasts, or podcasts) do not pose a risk to hearing.

Additional Resources

- FDA webpage, Hearing Aids. Available at: https://www.fda.gov/medical-devices/consumerproducts/hearing-aids (accessed on November 20, 2022).
- FDA Consumer Update, Cochlear Implants: A Different Kind of 'Hearing.' Available at: https://www.fda.gov/consumers/consumer-updates/cochlear-implants-different-kind-hearing (accessed on November 20, 2022).
- National Institute on Deafness and Other Communications Disorders, Hearing, Ear Infections and Deafness. Available at: https://www.nidcd.nih.gov/health/hearing-ear-infections-deafness (accessed on November 20, 2022).
- Immediately in effect guidance document: conditions for sale for air-conduction hearing aids. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/immediately-effect-guidance-document-conditions-sale-air-conduction-hearing-aids (accessed on November 20, 2022).
- Proposed rule: medical devices; ear, nose, and throat devices; establishing over-the-counter hearing aids. Available at: https://www.federalregister.gov/public-inspection/2021-22473/medical-devices-ear-nose-and-throat-devices-establishing-over-the-counter-hearing-aids (accessed on November 20, 2022).
- Draft Guidance: Regulatory requirements for hearing aid devices and personal sound amplification products. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/regulatory-requirements-hearing-aid-devices-and-personal-sound-amplification-products-0 (accessed on November 20, 2022).

Chapter 51

Bacterial Evolution during Human Infection: Adapt and Live or Adapt and Die

Matthew J. Culyba, MD, PhD,[a,b] and Daria Van Tyne, PhD[a,b]

[a]*Department of Medicine, Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*
[b]*Center for Evolutionary Biology and Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*

vantyne@pitt.edu

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51.1 Introduction

The study of microbial evolution has profound implications for improving public health. Understanding the genetic changes that enable pathogens to adapt and persist in their hosts uncovers fundamental biology and leads to new therapeutic interventions. For example, monitoring antibiotic resistance among circulating bacterial populations enables the rational selection of empiric antibiotic therapy, as well as prioritization of research that aims to develop novel interventions [1]. On an individual level, tracking within-host evolution, or the genetic changes within

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a microbial population infecting a single patient, also leads to critical insights. One prominent example of this, which occurred prior to the advent of modern DNA sequencing, was the development of multidrug regimens to treat tuberculosis to combat the predictable emergence of phenotypic drug resistance during therapy [2]. Now, with greater accessibility of next-generation DNA sequencing technologies and modern analytical tools, our understanding of the evolutionary dynamics underlying these processes is expanding. Within-host evolution can now be studied at high resolution, and, potentially, in real time [3]. This provides a window into the *in vivo* evolutionary dynamics that underlie the emergence of antimicrobial resistance, as well as a wider range of traits important for pathogenesis, persistence, and transmission. With minimal modifications, the same analytical principles applied to *in vitro* microbial evolution experiments can also be employed in *in vivo* studies. Indeed, clinical infections can be viewed as naturally occurring experiments in microbial evolution.

In this review article, we discuss the similarities and differences between studying bacterial evolution *in vivo* versus *in vitro*, as well as the challenges and opportunities presented by studying within-host bacterial evolution during infection in humans. We begin by discussing some of the major differences between studying bacterial evolution *in vitro* versus *in vivo*. Then, we present 2 different ways of thinking about adaptive mutations *in vivo*, which we have termed "adapt-and-live" and "adapt-and-die." Finally, we offer practical considerations for conducting studies of bacterial evolution *in vivo* during human infection. Our goal is to persuade researchers and clinicians that it is feasible to study bacterial evolution *in vivo*, and that much can be learned from conducting such studies.

51.1.1 Evolutionary Dynamics during Experimental Microbial Evolution

In vitro experimental microbial evolution has proven to be a powerful tool to understand the evolutionary dynamics within a bacterial population as it adapts to a new environment [4]. In a typical experiment, a bacterial strain is subjected to serial passaging, where it is exposed to a defined selective pressure, setting the process of evolution in motion. To gain information about the reproducibility of evolution, experiments are conducted with multiple replicates. The bacterial population can be sampled frequently throughout the experiment and, in the present era, subjected to whole genome sequencing (WGS) to track the fates of mutations that rise and fall in frequency through time. Samples of the population can be cryopreserved indefinitely and resuscitated at any time for additional characterization. The classic example of *in vitro* microbial evolution is the *Escherichia coli* long-term evolution experiment (LTEE), which was initiated by Richard Lenski more than 30 years ago [5], continues to this day, and has now exceeded 75,000 bacterial generations. More commonly, however, *in vitro* evolution experiments occur on timescales spanning days to weeks. The technique has also been applied to a growing number of different microbes, including yeasts [6], viruses [7], and bacteriophages [8, 9]. Importantly, experimental conditions can be adjusted to explore specific phenotypes. For example, plastic beads can be used

as a surface for biofilm formation, which enables specific transfer of the biofilm population into fresh media during serial passaging. This method has been used to study pathogens such as *Burkholderia cenocepacia* [10, 11] and *Pseudomonas aeruginosa* [12] to uncover biofilm-specific signatures of evolution.

Evolution is extraordinarily dynamic, even when studied in a static, well-controlled environment. In the LTEE, the glucose-limiting growth medium and the passaging procedure remained constant, yet dramatic changes in bacterial biology still occurred. By 20,000 generations, the bacteria could grow 70% faster in culture [13]. After 30,000 generations, one of the 12 replicate populations gained the ability to import citrate from the culture medium and use it for aerobic growth [14]. Six populations acquired defects in DNA repair, elevating their mutation rates by approximately 100-fold [15]. Finally, fitness measurements from the most recent analysis, after 60,000 generations and more than 25 years, showed that the bacteria continued to enhance their ability to grow in the media [16, 17]. Thus, even in a stable environment, spontaneous mutations provide an ample supply of genetic diversity to continuously fuel the successive evolution and refinement of new traits. Furthermore, because the *E. coli* population chosen for the LTEE is asexual, meaning that the bacteria lack plasmids or bacteriophages that could mediate horizontal gene transfer (HGT), evolution occurs solely by mutation, genetic drift, and natural selection. In the absence of HGT, mutations cannot be shared between different clones. This results in an especially strong linkage disequilibrium of mutations and a high degree of clonal interference. Clonal interference occurs when beneficial mutations that arise in different clones compete against each other, which can cause a beneficial mutation present in one clone to be extinguished from the population as it is outcompeted by a different, more fit clone [18]. In sexual bacterial populations, some mutations can be shared. In this case, the population size and the rate of gene exchange become important factors that determine the adaptive advantage of HGT [19]. Notably, HGT is a pervasive mechanism in the evolution of antibiotic resistance in clinical settings [20].

Experimental conditions greatly influence the timescale of evolution. The identical citrate utilization pathway that evolved after 30,000 generations during the LTEE can be selected for in just 12 to 100 generations if the population is subjected to prolonged selection during nutrient starvation [21]. Similarly, DNA repair defects that elevated the mutation rates in the LTEE can be evolved by chemically mutagenizing an *E. coli* population and immediately applying 2 rounds of different selective antibiotics [22]. The conditions of these experiments differ from the LTEE because they enable the bacterial population to survey much greater genetic diversity, while also being subjected to stronger selective pressure. The effect of population size (N) on evolution is also a critical parameter. First, it determines the minimal effect size of a mutant allele (i.e., the relative fitness advantage conferred) that natural selection can detect, with small effect sizes requiring larger N in order to be selected [23]. Second, due to spontaneous mutations, N greatly contributes to the amount of genetic diversity available for selection, with diversity increasing in proportion to N. Although stochastic

phenomena have a greater influence in small populations and can occasionally lead to big leaps of adaptation across a fitness landscape, the greater sensitivity to selective forces and the more genetic diversity that is present within large populations tends to favor adaptive potential [24]. These basic principles of population genetics are critical considerations for investigating evolutionary phenomena, both *in vitro* and *in vivo*.

51.1.2 Bacterial Evolution *in vitro* versus *in vivo*

Human infections occur in a complex environment. This *in vivo* complexity is evident when considering the numerous tissue microenvironments that can be accessed by a bacterial population within a single individual. Bacteria may colonize different anatomical sites without causing disease (e.g., skin surface and gut lumen), and then invade a variety of different tissue compartments (e.g., soft tissue, blood, bone, joints, and lungs) within an individual host. Each tissue type represents a distinct microenvironment, and a unique fitness landscape, for the infecting bacteria. If an infection spreads within an individual, the bacteria are likely to experience population bottlenecks at tissues interfaces. Bottlenecks also occur during transmission between individuals, since relatively few bacteria initiate infection or colonization of new hosts. Due to the small N of initial founder populations, the statistical phenomenon of genetic drift will have a strong influence on mutant allele frequencies early during infection. As the population expands, however, spontaneous mutations supply genetic diversity for the production of new traits. Mutant alleles associated with beneficial traits can then rise in frequency due to positive selection from new tissue microenvironments, immune responses, and antibiotic treatments.

Although many infecting populations can be expected to evolve rapidly, studying this process can be difficult due to several practical constraints. First, an infection must either be persistent or chronic to allow for serial sampling of the bacterial population over time. Sampling should also start early enough in the infection so that the population can be assessed before substantial selection has occurred. Second, the sampling method must capture sufficient genetic diversity to be able to detect genetic changes through time. Outside of a dedicated clinical trial, however, sampling is usually limited to clinical specimens collected for diagnosis during the routine care of the patient, which may be limiting. A third challenge is that the parameters that govern the evolutionary dynamics of the infecting population, such as population size, rates and sources of mutation, and the nature of the selective pressures, must be expected to produce detectable mutations within the timescale of the infection. Finally, the infection must be common enough to accrue a sufficient number of independent individuals and infections for study, so that evidence for parallel or convergent evolution can be pursued and more general conclusions about evolutionary signals can be made. Due to these constraints, not all bacterial infections are suited to this type of study.

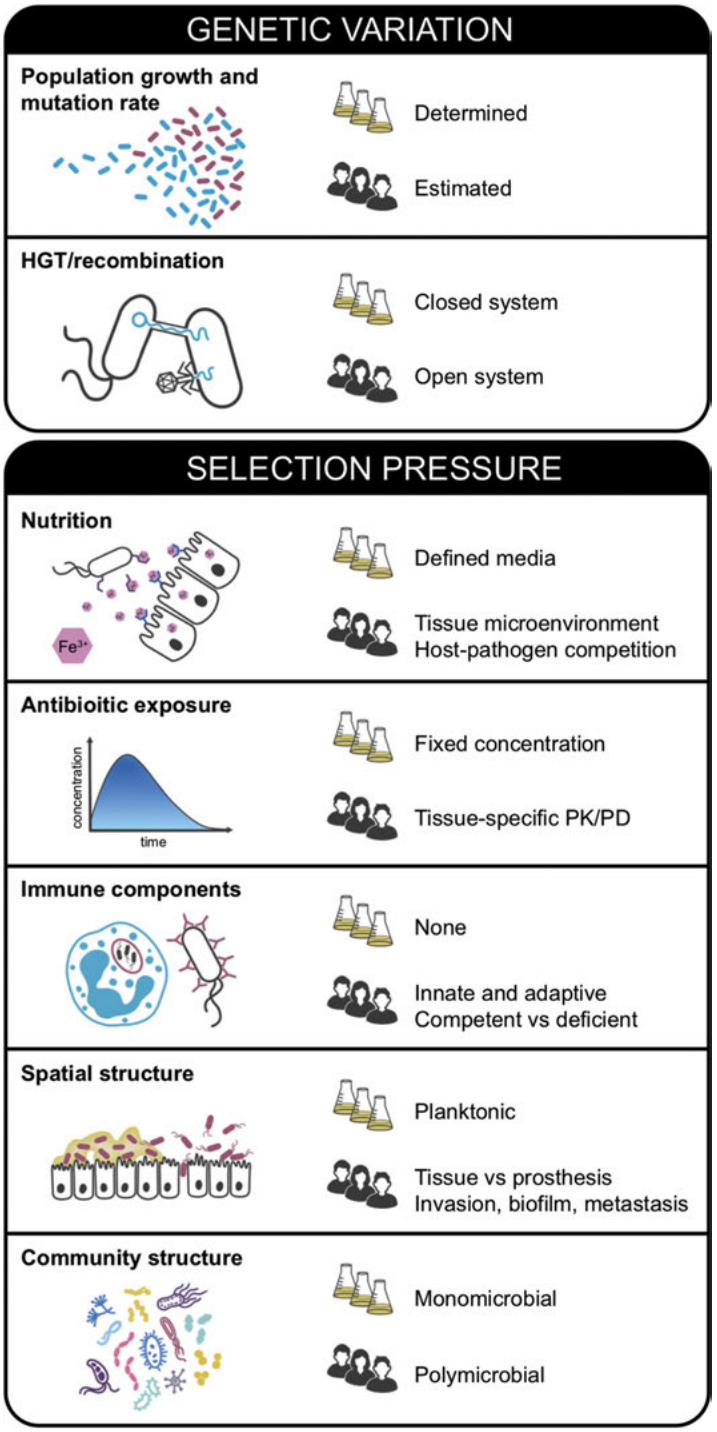

Figure 51.1 *In vitro* versus *in vivo* bacterial evolution. Comparison of parameters that differ between bacterial evolution *in vitro* (flasks) and *in vivo* (people). *Abbreviations*: HGT, horizontal gene transfer; PK/PD, pharmacokinetics/pharmacodynamics.

Beyond the practical limitations, the primary differences between *in vitro* and *in vivo* systems are that (i) the amount of genetic variation and (ii) the nature of the selective pressure(s) are much more dynamic *in vivo* (Fig. 51.1). *In vitro*, bacterial population size, growth rate, mutation rate, and mobile genetic element movement can all be determined. Whereas, *in vivo*, these parameters can often only be estimated, and they can also be variable between patients. Perhaps the most important difference between *in vitro* and *in vivo* settings, however, is the nature of the selective pressures. *In vitro*, environmental conditions are experimentally defined, such as the concentration of an antibiotic, the specifics of the growth medium, and even the spatial structure of the microbial population. For example, a recent *in vitro* study of *Acinetobacter baumannii* compared the evolution of antibiotic resistance between cells growing in planktonic cultures with cells growing in biofilms [25]. Due to the ability to precisely define all other experimental variables, this study was able to conclude that the differences observed in the evolution of antibiotic resistance were due to the spatial structures of the bacterial communities. Such control is not possible *in vivo*, where selection is almost certainly due to a combination of factors (Fig. 51.1). Patient infections occur in a variety of different tissue microenvironments and commonly involve implanted medical devices, such as prosthetic joints, central venous catheters, or cardiac pacemakers. Each site of infection is likely to differ in terms of available nutrients, antibiotic exposures, immune responses, spatial structure, and microbial community structure (Fig. 51.1). Some of these features have been explored by *in vitro* studies, for example, through the use of synthetic media that more accurately mirrors *in vivo* nutrient conditions [26], variable antibiotic selection regimens [27], or *in vitro* studies of polymicrobial interactions [28]. Despite these advances, however, the complexity of many *in vivo* environments is unlikely to ever be perfectly mirrored *in vitro*. Nonetheless, we do not view the many unknowns of the *in vivo* environment described above as a hindrance for discovery. Quite to the contrary, studying bacterial evolution *in vivo* poises investigators to make novel discoveries. We believe that studying within-host evolution can serve as the starting point for a discovery pipeline that produces important insights into the causal mechanisms underlying bacterial pathogenesis, persistence, and transmission.

51.1.3 Adapt-and-Live versus Adapt-and-Die Mutations

Many *in vivo* microbial evolution studies are focused on identifying mutations; more specifically, *de novo* mutations, which confer an adaptive advantage. Because sampling of bacterial populations is nearly always limiting, there is an inherent detection bias toward high-frequency mutations that are long lived. This bias poses a practical barrier to identifying short-lived and low-frequency mutations that are still of great interest. With this in mind, here, we categorize *de novo* adaptive mutations into 2 groups, using the timescale in which a mutation persists after its inception as the distinguishing feature between groups:

1. Adapt-and-live mutations are long lived (months to years) and are associated with chronic infections or asymptomatic colonization. They may or may not be transmitted to other people, but the opportunity for transmission is higher given that they persist for longer timescales. Some mutations may even enhance pathogen transmission.
2. Adapt-and-die mutations are short lived (days to weeks). They arise due to their selective advantage but are then rapidly extinguished. In acute infections, this is often because the infection resolves, or it is at an anatomic site that represents an evolutionary "dead-end" (see below). During chronic infections, the demise of these mutations is primarily due to the evolutionary dynamics of fitness trade-offs, epistatic effects, clonal interference, or stochastic elimination due to a sudden drop in population size. These mutations are much less likely to be transmitted to other hosts, meaning that they arise independently in each newly infected host.

51.1.4 Adapt-and-Live Mutations: Evolution of Beneficial Traits That Persist

When bacteria cause infections in humans, they often encounter environments that are quite different from the natural environmental niches to which they are well adapted. For example, bacteria that naturally reside in soil or water can cause opportunistic infections in humans, where they will experience large differences in temperature and available nutrients, as well as the presence of antibiotic and immune selective pressures. If the bacteria are able to adapt to these new conditions, they will be able to divide more rapidly, persist longer before dying, and survive long enough to find a new home within the same or a different host. As researchers, we want to understand how bacteria evolve during human infections. We frequently do this by identifying and characterizing mutations that occur in bacterial populations during the course of infection. Chronic bacterial infections offer the opportunity to study these adaptations on timescales from months to decades. In this section, we focus on two primary examples of adapt-and-live mutations, in which genetic adaptations that occur during infection prolong bacterial survival, either through transmission to new hosts or through the establishment of chronic infection.

One example of an adapt-and-live scenario is the evolution of antibiotic resistance during active pulmonary infection with *Mycobacterium tuberculosis*. After reactivation of a latent tuberculosis infection, active disease can last for many years; this long timescale, combined with intrinsic tolerance to many antibiotics and poor antibiotic permeability, allows the bacteria to evolve resistance [29]. In contrast to many other bacteria, where antibiotic resistance can be acquired through HGT, *M. tuberculosis* evolves resistance *de novo* through the accumulation of mutations in genes encoding antibiotic targets, regulators of those targets, as well as the up-regulation of efflux pumps [30]. Numerous studies have described the development of antibiotic resistance during *M. tuberculosis*

infection in humans, first using molecular typing and more recently using WGS [31–33]. One of the more troubling features of antibiotic resistance in *M. tuberculosis* is that resistant bacteria can be transmitted from infected to uninfected hosts [34] (Fig. 51.2). While antibiotic resistance mutations frequently carry fitness costs, in *M. tuberculosis*, these costs can be mitigated by compensatory mutations [35], which enable the bacteria to propagate through human populations even in the absence of ongoing antibiotic selection.

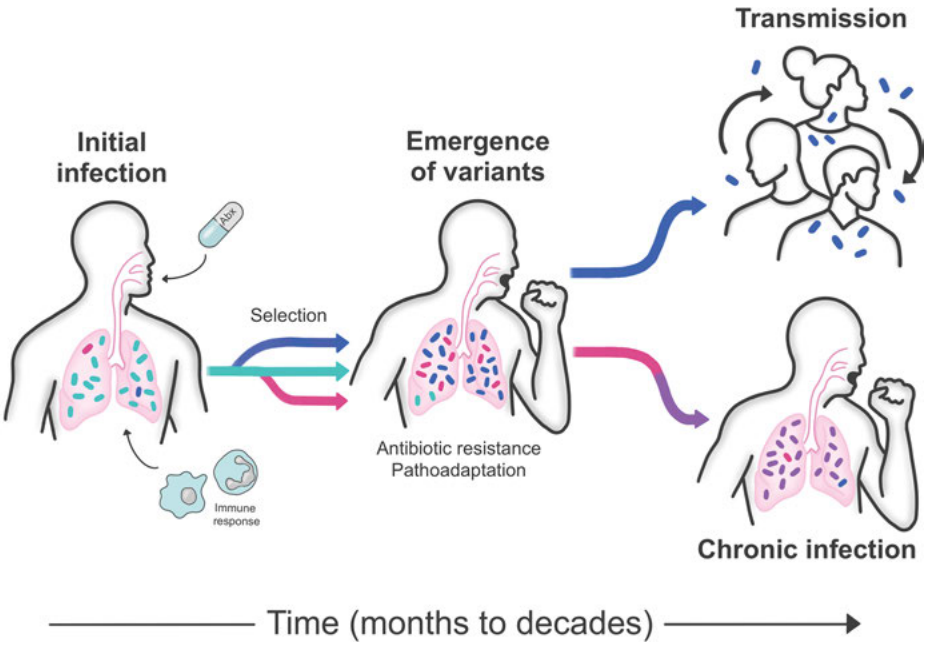

Figure 51.2 Bacterial evolution during chronic infection. Pulmonary infection is shown as an example. Infection starts with a population of bacteria entering a new environment, such as the lung. Antibiotics, immune responses, and other pressures exert selection on the bacterial population, causing the emergence of bacterial variants possessing antibiotic resistance and other adaptive traits in a process referred to as pathoadaptation. Bacteria from the adapted population can be transmitted to other individuals or can continue to adapt and cause chronic infection, lasting months to decades.

A second example of an adapt-and-live scenario is chronic pulmonary infections with *P. aeruginosa* and *Burkholderia* species, which have been most intensely studied in patients with cystic fibrosis (CF). Similar to *M. tuberculosis* infections, patients with CF are often infected for years to decades with a population of bacteria in which genetic and phenotypic diversity arise over time [36–38]. Selection on this population drives a gradual process of pathoadaptation, ultimately resulting in a chronic and antibiotic-resistant infection [39] (Fig. 51.2). WGS offers an opportunity to observe how this process unfolds on both short and long timescales [40, 41]. A recent study of over 400 *P. aeruginosa* isolates sampled longitudinally from 39 young CF patients over 10 years found that

pathoadaptation in the CF lung involves an initial 2- to 3-year period of rapid adaptation, followed by a transition to persistent infection [40]. During the initial period, bacterial growth rate was found to slow and antibiotic resistance increased through accumulation of ciprofloxacin resistance–associated mutations in *gyrA/B* and mutations in the efflux pump repressor *nfxB*. Infections were then able to persist long term via both convergent evolution [42], as well as through the maintenance of a genetically diverse population [43, 44]. These populations remodel their regulatory and metabolic networks (largely through mutations in transcriptional regulators), gain the ability to adhere more proficiently (driven by biofilm-increasing mutations and loss of flagella), develop mucoid and/or hypermutator phenotypes, produce fewer extracellular virulence factors, and acquire antibiotic resistance [45, 46]. These prior studies have shown that even among different patients and genetically diverse *P. aeruginosa* populations, evolution is relatively predictable in this setting.

Many of the above themes are repeated when considering the adaptation of *Burkholderia* to the lungs of CF patients. A landmark 2011 paper described a retrospective study of 112 *Burkholderia dolosa* genomes from 14 individuals with CF that were isolated over 16 years [47]. The authors identified parallel adaptive mutations in antibiotic resistance genes (DNA gyrase subunits *gyrA/B*, ribosomal protein *rpl4*, and others), genes involved in outer membrane synthesis (such as glycosyltransferases *wbaD* and *mtfA*), and the two-component system *fixLJ*, which senses oxygen tension and governs biofilm formation, motility, and persistence within macrophages [48]. A similar study of *Burkholderia pseudomallei* evolution during chronic infection in 7 CF patients identified parallel mutations in genes conferring antibiotic resistance, genes in the type 3 and type 6 secretion systems that were predicted to decrease virulence, and fatty acid biosynthesis genes including *fabF* and *fabG*, which are predicted to impact membrane fluidity and permeability [49]. Genes involved in lipid transport and metabolism were also identified as likely targets of selection in a separate study of long-term evolution of *Burkholderia multivorans* over 20 years in a single CF patient [50]. Over the decades-long timescales documented in several studies, it has also been possible to observe reductive evolution in both *B. cenocepacia* and *B. pseudomallei* [37, 49]. This process of genome "slimming" often happens during long-term adaptation and specialization within a defined host [51]. Finally, a study of 32 *B. cenocepacia* isolates from 8 CF patients identified parallel mutations in the RNA polymerase subunit *rpoB*, catalase *katG*, the copper sensor kinase *cusS*, and the methionine-sulfoxide reductase *yedY* [52]. The functional consequences of these mutations, and how they impact bacterial survival during chronic infection in CF patients, remain to be determined.

In all of the above examples, the patients that were studied can be considered as single observations within larger *in vivo* evolutionary experiments. Combining the results of these replicates across studies reveals several common themes of bacterial evolution in the adapt-and-live scenario. First, evolution of antibiotic resistance is clearly important in the setting of chronic bacterial infections. This is likely because antibiotic treatment imposes strong selective pressure, and

resistance-conferring mutations have a large effect size [53, 54]. Second, a highly variable suite of mutations occur that impact the ways that the bacteria interact with their host. These include mutations that modulate bacterial virulence, often by down-regulating acute virulence in favor of strategies that promote persistence in the face of innate and adaptive immune pressures [38, 55–57]. Third, as was also observed in the *in vitro* LTEE, hypermutators frequently arise. Rapid evolution of hypermutators *in vitro* is facilitated by serial exposure to strong and varying selection pressures [22]. This observation likely explains the frequent emergence of hypermutators in the CF lung, where bacterial populations are able to persist in the face of variable antibiotic treatments and a dynamic host immune response [49, 58, 59]. Emergence of hypermutator strains is clinically concerning, as it portends rapid acquisition of antibiotic resistance [60], and may be a marker of disease progression in CF [61]. While these appear to be some of the common "rules" that govern the evolution of *M. tuberculosis*, *P. aeruginosa*, and *Burkholderia* during chronic infection, it is important to note that genotypic and phenotypic heterogeneity are frequently found across studies [44, 62]. This highlights the dynamic nature of bacterial evolution and points to a need for deep sampling of many patients in order to gain a more complete understanding of bacterial adaptation in the adapt-and-live scenario.

51.1.5 Adapt-and-Die Mutations: Evolution of Beneficial Traits That Do Not Persist

In contrast to adapt-and-live mutations, adapt-and-die mutations are ultimately extinguished from the infecting population, despite being beneficial. The death of a beneficial mutation can occur through a variety of mechanisms. For example, if a mutation is conditionally beneficial and offers a relative fitness advantage in only one type of environment within the host, and perhaps confers a severe fitness cost under other conditions, these conditions will hinder survival of the mutant. Alternatively, as observed in the LTEE, some beneficial mutations may simply be outcompeted by fitter clones due to clonal interference. Also, despite their relative advantage, clones that harbor beneficial mutations may still ultimately be eliminated by host immunity or antibiotic treatment. Finally, some mutations may confer a fitness advantage only in a tissue compartment within the host that does not permit transmission to a new host, thus creating an evolutionary "dead-end" [63, 64]. Due to their relatively transient nature, these adapt-and-die mutations may escape detection, even though the information they encode is still valuable for understanding bacterial adaptation and mechanisms of pathogenesis. In this section, we argue that adapt-and-die mutations represent an untapped resource for information about bacterial evolution during human infection.

The evolution of vancomycin-resistant *Staphylococcus aureus* (VRSA) is a notable example of an adapt-and-die scenario that generates a fitness cost restricting transmission to other hosts. *S. aureus* is a common colonizer of the CF airway, and several recent studies have compared *S. aureus* adaptation in CF

patients to the adaptation of *P. aeruginosa* in the same environment [65–67]. *S. aureus* also frequently causes skin and soft tissue infections, bone and joint infections, and bacteremia. Vancomycin is the primary therapy for severe methicillin-resistant *S. aureus* (MRSA) infections, and its evolution to VRSA is concerning. Worldwide, there have been at least 52 VRSA isolates described since the first report in 2002 [68], yet epidemic spread has not occurred. VRSA is believed to evolve by conjugative transfer of an Inc18-like *vanA*-encoding plasmid from vancomycin-resistant enterococci (VRE) to MRSA. Detailed studies of a subset of cases have revealed that successful *vanA* plasmid transfer requires MRSA to harbor a pSK41-like conjugative plasmid [69]. The evolutionary jump from MRSA to VRSA can take place when VRE co-colonize the same anatomical site as a pSK41-positive precursor MRSA strain (Fig. 51.3A). The clinical features of VRSA cases suggest that this most often occurs within a polymicrobial biofilm that is present, for example, on a skin wound or indwelling medical device [70]. Because the recipient MRSA strain has a low prevalence [71], *vanA* plasmid transfer between VRE and MRSA is a relatively rare event. Moreover, in the absence of vancomycin pressure, the *vanA* gene cluster causes a large growth defect in *S. aureus* [72]. Transmission of *S. aureus* from person to person occurs by direct contact and colonization of the skin. To date, no such VRSA transmission events have been reported, presumably due to the fitness cost imposed by the *vanA* operon (Fig. 51.3A). Although rare, VRSA cases continue to be reported, thus future opportunities for the acquisition of compensatory mutations that might permit epidemic transmission remain a dangerous possibility.

A second notable example of the adapt-and-die scenario is when beneficial mutations are selected within tissue compartments that do not permit their transmission to new hosts (Fig. 51.3B). Generally, bacterial transmission occurs via the respiratory route, fecal-to-oral route, or by direct skin contact. Although tissue compartments outside of a pathogen's main transmission route, such as the bloodstream or central nervous system, are commonly invaded during infection, bacteria are generally unable to be transmitted directly from these compartments to other individuals. Furthermore, invasive infections are often caused by bacteria that colonize a different body site, such as the skin [73], nasopharynx [74], or gastrointestinal tract [75]. Due to the process of niche adaptation, these bacteria are often well adapted to reside at anatomical sites of colonization, rather than sites of infection. Because transmission does not happen between the bacterial populations at these more invasive sites of infection, beneficial traits must re-evolve within each new patient. This process of evolution "on repeat" can be studied by sequencing pathogen genomes sampled from sites of colonization and infection within the same patient [76] or by sequencing the genomes of bacteria from outbreaks of a particular kind of infection [77, 78]. One example of this is the enterococci, which are stable colonizers of the gastrointestinal tracts of land mammals [79]. In the antibiotic era, select enterococcal lineages have emerged that now cause invasive infections among immunocompromised and hospitalized patients [80]. One recent study of an outbreak of bacteremia caused by multidrug-

resistant *Enterococcus faecalis* among 53 patients at a single hospital identified repeated adaptations in genes affecting cell surface polysaccharide production, including the enterococcal polysaccharide antigen (epa) and lipoteichoic acid [77]. In the latter case, independent mutations in the same transcriptional repressor were observed in 21 different patients. The mutated repressor was shown to cause overexpression of an enzyme that altered the abundance and structure of lipoteichoic acid; these changes conferred increased resistance to antibiotic treatment and innate immune stresses, which likely drove their occurrence [77]. Identical repressor mutations were observed in very few patients, strongly suggesting that these mutations were selected during bacterial growth in the human bloodstream and that they frequently died out instead of being transmitted to other patients (Fig. 51.3B).

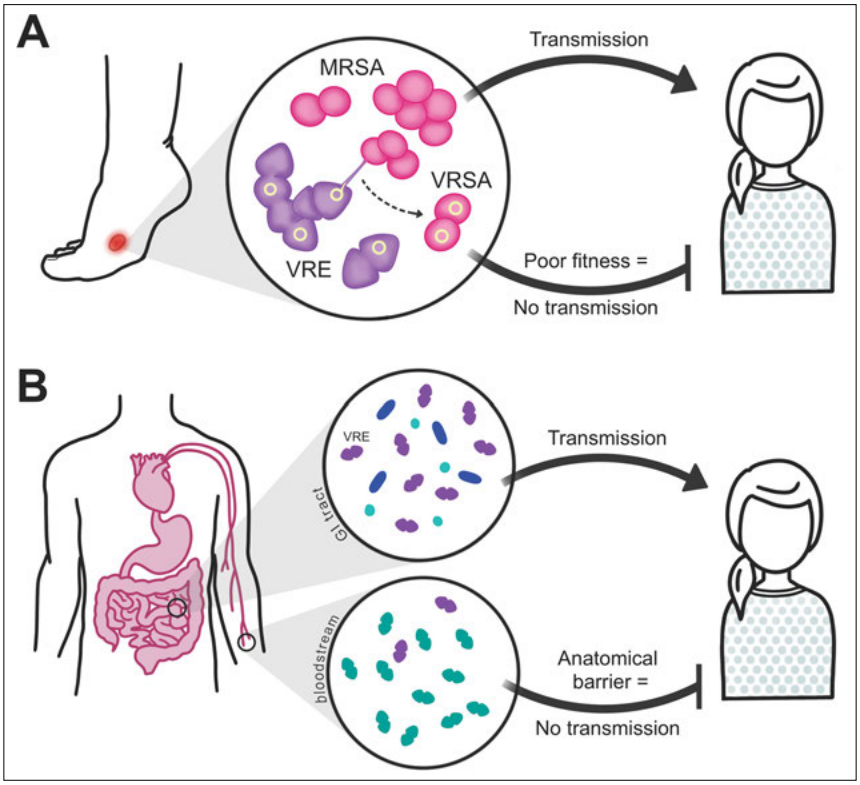

Figure 51.3 Bacterial evolution during acute infection. (A) Evolution of VRSA during coinfection with MRSA and VRE. While MRSA can be easily transmitted to other patients, VRSA have poor fitness and cannot be transmitted. (B) Different VRE populations in the GI tract and bloodstream of an infected patient. While VRE from the GI tract can be transmitted to other patients, VRE infecting the bloodstream are not transmitted. Conditionally beneficial mutations (shown in green) that are selected during growth in the bloodstream will similarly not be transmitted to other patients. Because anatomical barriers prevent conditionally beneficial adaptations from being transmitted, body sites such as the bloodstream can be considered evolutionary "dead-ends." *Abbreviations*: GI, gastrointestinal; MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant *Enterococcus*; VRSA, vancomycin-resistant *S. aureus*.

Similar to enterococci, *S. aureus* also adapt during the transition from skin colonization to invasive infection. Young and colleagues sequenced 1,163 *S. aureus* genomes from 105 patients with nose colonization that developed invasive infections of the bloodstream, soft tissues, or bones and joints [81]. Five different colonies were sampled per cultured site for each patient. This enhanced sampling was critical and enabled the discovery of over 1,000 *de novo* mutations with many examples of convergent evolution. Significant signatures of adaptation were observed in genes responding to the bacterial regulators *rsp* and *agr*, as well as genes that protect against host-derived antimicrobial peptides. *rsp* regulates the expression of surface antigens and toxins, while *agr* is involved in quorum sensing, toxin production, and abscess formation. Adaptive mutations associated with pathogenesis were more likely to occur in bacteria isolated from sites of infection compared to colonizing bacteria, and these mutations did not occur in *S. aureus* sampled from healthy individuals. Mutations associated with invasive infection of the bloodstream or joints in this study were likely of the adapt-and-die category, given that these tissue compartments are likely evolutionary "dead-ends." In contrast, skin and soft tissue infections aid transmission, and there is evidence that adapt-and-live mutations arising at these sites can prime community outbreaks of *S. aureus* [78].

51.1.6 Convergent Evolution across Species

Convergent evolution has been observed across different bacterial species, which demonstrates the generalizability of some *in vivo* adaptations. For example, mutations in *relA* that cause constitutive activation of the bacterial stringent response have been identified in persistent bloodstream infections caused by both *Enterococcus faecium* [82, 83] and *S. aureus* [82–84]. Activation of the stringent response results in metabolic quiescence and antibiotic tolerance, thus promoting persistent infection [85]. Because strains carrying *relA* mutations all evolve in the bloodstream, exhibit growth defects, and show no evidence of transmission between patients, we would assign these mutations to the adapt-and-die category. Separately, a large analysis of published bacterial genomes recently uncovered several examples of common adaptive strategies found across different bacterial pathogens [54]. This dataset consisted of bacterial genomes from studies where the infecting pathogen was isolated and sequenced from at least 2 different time points from the same patient. It included over 7,000 isolates from 1,421 patients and encompassed 29 different bacterial species. Convergent adaptive changes across species often included common antigens recognized by the immune system, such as the flagellar filament, or those involved in antibiotic resistance and tolerance, such as mutations in RNA polymerase. By contrast, changes in virulence factors tended to be species specific. Overall, these findings suggest that the fate of an adaptive mutation is likely to depend on where and when it arises, as well as the species and type of infection in which it arises.

51.1.7 Practical Considerations for Studying Bacterial Evolution *in vivo* in Humans

As researchers, we are interested in studying how bacteria evolve *in vivo* during human infection in order to learn more about the basic biology of these organisms and to identify possible targets for therapeutic intervention. There are several limitations that currently hinder our ability to study *in vivo* bacterial evolution in sufficient depth to draw confident conclusions. In this section, we present what we consider to be practical considerations for studying bacterial evolution *in vivo* during human infection.

One major challenge to conducting rigorous studies of bacterial evolution *in vivo* is a lack of access to high-quality samples or the inability to collect the right sample at the right time. Studying bacterial evolution *in vivo* in humans requires the sampling of bacteria directly from human infections, which presents a formidable obstacle. While much can be learned from studying bacterial isolates collected during routine clinical care (which are usually considered discarded specimens and exempt from informed consent), such sample sets of convenience are likely to be sparse, incomplete, and/or biased in ways that limit their utility for conducting rigorous and well-controlled analyses. The choice of infection and sampling strategy should ideally be tailored to a set of research questions established at the outset of the study. Many researchers, however, are unable to consent and enroll patients in a clinical or interventional study, which leaves them to rely on the collection of whatever samples happen to be available for a particular organism or infection of interest. We propose that physicians and researchers should work together to collect samples from patients that (1) meet defined sets of inclusion criteria; (2) provide informed consent; and (3) can be sampled systematically and routinely to yield high-quality data for these studies.

A second major challenge that currently limits many studies of bacterial evolution *in vivo* is the reliance on sequencing and analysis of single clones of bacteria taken from an infection-derived clinical specimen. While the use of WGS to compare bacteria sampled from infected patients is a powerful approach for studying pathogen adaptation [3, 86, 87], the sampling of bacteria from infected patients nearly always involves the isolation of a single “representative” clone from the population of bacteria within the patient. This standard approach, while efficient and cost-effective, is based on the false assumption that infections are caused by clonal populations. The end result is a dramatic undersampling of bacterial genetic diversity within infected patients, which can lead to incorrect inferences about bacterial transmission and overlook low frequency variants that might be clinically relevant, such as antibiotic-resistant minority clones [87, 88]. When bacterial clonal diversity within patients has been studied, only a small number of patients have been sampled [89, 90]. This limits the conclusions that can be drawn from such studies and represents a critical barrier to progress in the field. Additionally, sampling the same patient longitudinally, even if over a short time period, adds another important dimension to these studies. We propose

that sampling strategies should be tailored according to well-defined study questions. When it is possible and appropriate for the study, bacterial populations should also be collected longitudinally and should be sequenced alongside representative clones, as doing so is likely to yield additional insights beyond what can be learned from studying single bacterial isolates sampled from single time points.

A final challenge to conducting rigorous and well-controlled studies of bacterial evolution *in vivo* in humans is that appropriate tools for comparative and functional genomic analyses still need to be developed. A number of analysis tools have been developed for studying bacterial evolution *in vitro* and conducting genome-wide association studies in microbial pathogens [91, 92], and many of these can be adapted to account for additional complexities that exist in human infections. One big hurdle that still remains is how to account for the movement of mobile genetic elements as well as recombination in these systems. *In vitro* experimental systems are largely genetically "closed," meaning that new genetic material cannot enter the system and recombination is not a source of additional genetic variation in these settings (Fig. 51.1). *In vivo*, however, we know that mobile element movement and recombination can play major roles in pathogen evolution [93, 94], and these changes should ideally be analyzed alongside *de novo* mutations. Current approaches largely focus on genetic changes occurring within well-conserved genomic regions and are insufficient to detect genomic regions under selection that may not be highly conserved. Finally, comparative genomics approaches can only identify genes that appear to be under selection or mutations that appear to be beneficial for a particular organism. Functional studies are needed in order to test the hypotheses generated by comparative genomics analyses and to establish molecular mechanisms underlying beneficial adaptations. We propose that existing analysis tools should be adapted and new tools should be developed that can account for the added complexity of *in vivo* settings, for example, by incorporating pangenome analyses, using *in vivo* observations to develop computational models of infection, and refining these models to be able to predict the likely impact of observed mutations within a given infection context. The results of these analyses should then be considered as hypotheses that should be formally tested with functional follow-up approaches.

51.2 Conclusions

We conclude this chapter with a few key points. First, microbes are constantly recording valuable information into their genomes in the form of mutations due to natural selection, and obtaining this information from clinical samples is now accessible to a growing body of researchers. Second, *in vivo* evolutionary dynamics during bacterial infections in humans are more complicated than *in vitro* experiments of microbial evolution, but uncovering signals of adaptation in this setting can result in important biological insights and uncover entirely

new areas for investigation. Third, beneficial traits that arise during infection, but are short lived, are more challenging to identify, yet also provide critical insight into how bacteria adapt to new environments. And, finally, through this review, we hope to inspire clinicians and researchers alike to consider ways that they can move their research closer to the study of bacterial evolution *in vivo* in humans. Such studies certainly present numerous challenges compared to well-controlled *in vitro* evolution experiments. However, we believe that the potential benefits of *in vivo* studies of bacterial evolution during human infection are both highly impactful and directly translatable to improving the treatment and care of infected patients.

Disclosures and Conflict of Interest

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Chapter 52

Roadblocks in Chagas Disease Care in Endemic and Nonendemic Countries: Argentina, Colombia, Spain, and the United States. The NET-Heart Project

Andres F. Miranda-Arboleda, MD,[a,b] Ezequiel Jose Zaidel, MD,[c] Rachel Marcus, MD,[d,e] Maria Jesus Pinazo, MD, PhD,[f] Luis Eduardo Echeverria, MD,[g] Clara Saldarriaga, MD,[h] Alvaro Sosa Liprandi, MD,[c] and Adrian Baranchuk, MD,[b] on behalf of the Neglected Tropical Diseases and other Infectious Diseases affecting the Heart (NET-Heart) project

[a]*Cardiology Department, Pablo Tobón Uribe Hospital, Medellín, Colombia*
[b]*Division of Cardiology, Kingston Health Science Centre, Queen's University, Kingston, Ontario, Canada*
[c]*Cardiology Department, Sanatorio Güemes, and School of Medicine, University of Buenos Aires, Buenos Aires, Argentina*
[d]*LASOCHA, Washington, DC, USA*
[e]*Medstar Union Memorial Hospital, Baltimore, Maryland, USA*
[f]*ISGlobal, Hospital Clinic, University of Barcelona, Barcelona, Spain*
[g]*Cardiology Department, Cardiovascular Foundation of Colombia, Floridablanca, Colombia*
[h]*Cardiology Service, Clínica CardioVID, Universidad de Antioquia, Medellín, Colombia*

ezaidel@fsg.edu.ar

Keywords: antitrypanosomal drugs, benznidazole, Chagas disease (CD), Coronavirus Disease 2019 (COVID-19), immunosuppressed patient, neglected tropical disease (NTD), nifurtimox, peridomestic, polymerase chain reaction (PCR), SARS-CoV-2 pandemic, *Trypanosoma cruzi*, *Trypanosoma cruzi I, Trypanosoma cruzi II*, vectoral transmission

52.1 Introduction

Chagas disease (CD) is both preventable and treatable; however, the number of infected individuals worldwide remains approximately 6 to 7 million. Numerous

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barriers to care are responsible for this impressive figure (Fig. 52.1) [1]. In a recent document, the Inter American Society of Cardiology (SIAC) and the World Heart Federation (WHF) delineated the obstacles to providing effective care for this neglected tropical disease (NTD) [2]. That document functioned as a frame of reference and was not intended to address country-specific barriers.

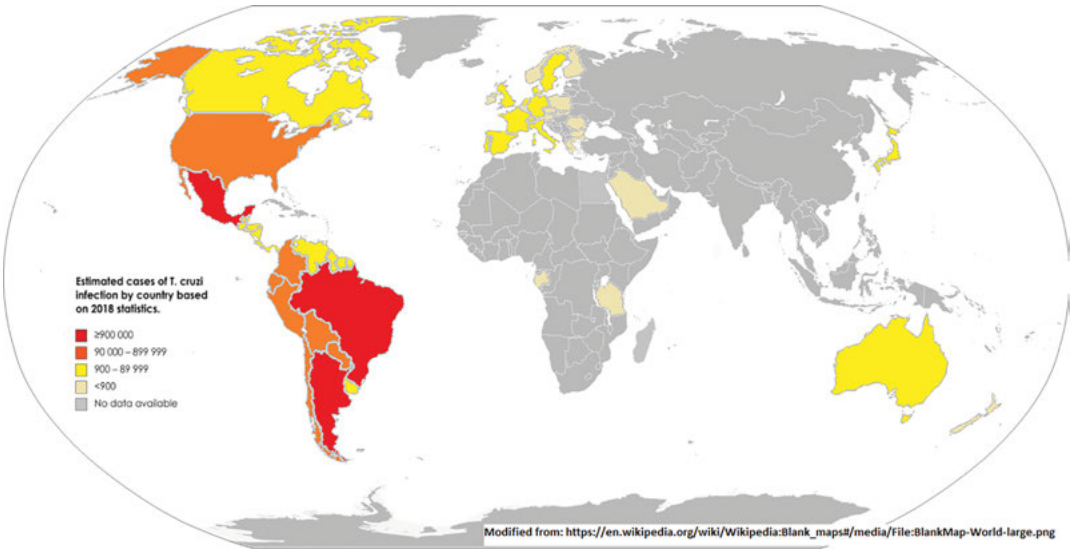

Figure 52.1 Global epidemiology of Chagas disease. Data from World Health Organization Control of Tropical Neglected Disease. Modified from: https://en.wikipedia.org/wiki/Wikipedia:Blank_maps#/media/File:BlankMap-World-large.png.

Argentina and Colombia are 2 upper-middle-income countries that have had resources and programs for the eradication of CD for decades. Spain is a nonendemic country, but large-scale immigration from endemic countries in Latin America has introduced CD and transmission by nonvectorial routes. Finally, there has been a growing understanding that CD poses an important problem in the United States (US), chiefly as a disease in immigrants, but also with an as-of-yet incompletely understood burden of domestically acquired disease [3].

The SIAC's NET-Heart project is a program that evaluates current evidence about NTDs and other infectious diseases with cardiac manifestations [4]. Gathering experts from the SIAC-WHF roadmap and NET-Heart project, we aimed to analyze and classify current barriers for optimal care for CD in endemic and nonendemic countries.

52.2 Methods

We performed a nonsystematic review of literature published in indexed journals from 1955 to 2021 and a selection of gray literature including abstracts, local

guidelines issued by health entities, blogs, media, and local policies. Lists of references were used to complete the search. Experts representing SIAC NET-Heart project [4] and the WHF Roadmap [2], from 2 endemic countries (Argentina and Colombia) as well as 2 nonendemic countries (Spain and the US) performed a detailed analysis of national barriers based on data identified, knowledge of the disease, and local context. Barriers were classified, and potential solutions were proposed.

52.3 Results

52.3.1 Situation Analysis of CD in Specific Countries

The SIAC-WHF roadmap classified roadblocks in 4 main clinical areas: prevention, diagnosis, treatment of CD, and diagnosis and treatment of disease complications. An analysis of these barriers by country is tabulated below (Fig. 52.2 and Table 52.1).

52.3.2 Current Situation of CD in Argentina

In Argentina, a marked decrease in vectorial transmission has been achieved since the early 1990s via the international collaborative project INCOSUR [7]. Despite this, Argentina is the country where most CD patients live worldwide, with approximately 1 case of CD for every 27 Argentinians [2]. Health personnel are chiefly located in cities where there are not vectors ("vinchucas"), and have little awareness of CD, so it remains undiagnosed for decades [2].

Rural-to-urban migration and the reduction of vectorial transmission have made vertical transmission the main route of infection in the country. In local estimates, the proportion of pregnant women with positive Chagas serology was significantly reduced over time, but the proportion of infected neonates remained stable [8].

Diagnostic lab tests have variable accuracy, even in blood banks, where CD screening is mandated and must then communicated to a seropositive donor [9]. However, there is no oversight for this screening process, and positive cases may not receive either confirmation or access to treatment.

Currently, in Argentina, both adult dose and pediatric dose benznidazole is produced domestically and is widely available; it is also exported internationally. Nifurtimox, however, is imported, and its supply is interrupted intermittently. A pediatric dosage is under development in the country [10].

Recently, the Ministry of Health published updated guidelines for the diagnosis and management of CD [11]. Unfortunately, these recommendations have not been fully disseminated or implemented, mainly due to fragmented provincial legislation and a concomitant shift in focus away from Chagas due to the Coronavirus Disease 2019 (COVID-19) pandemic.

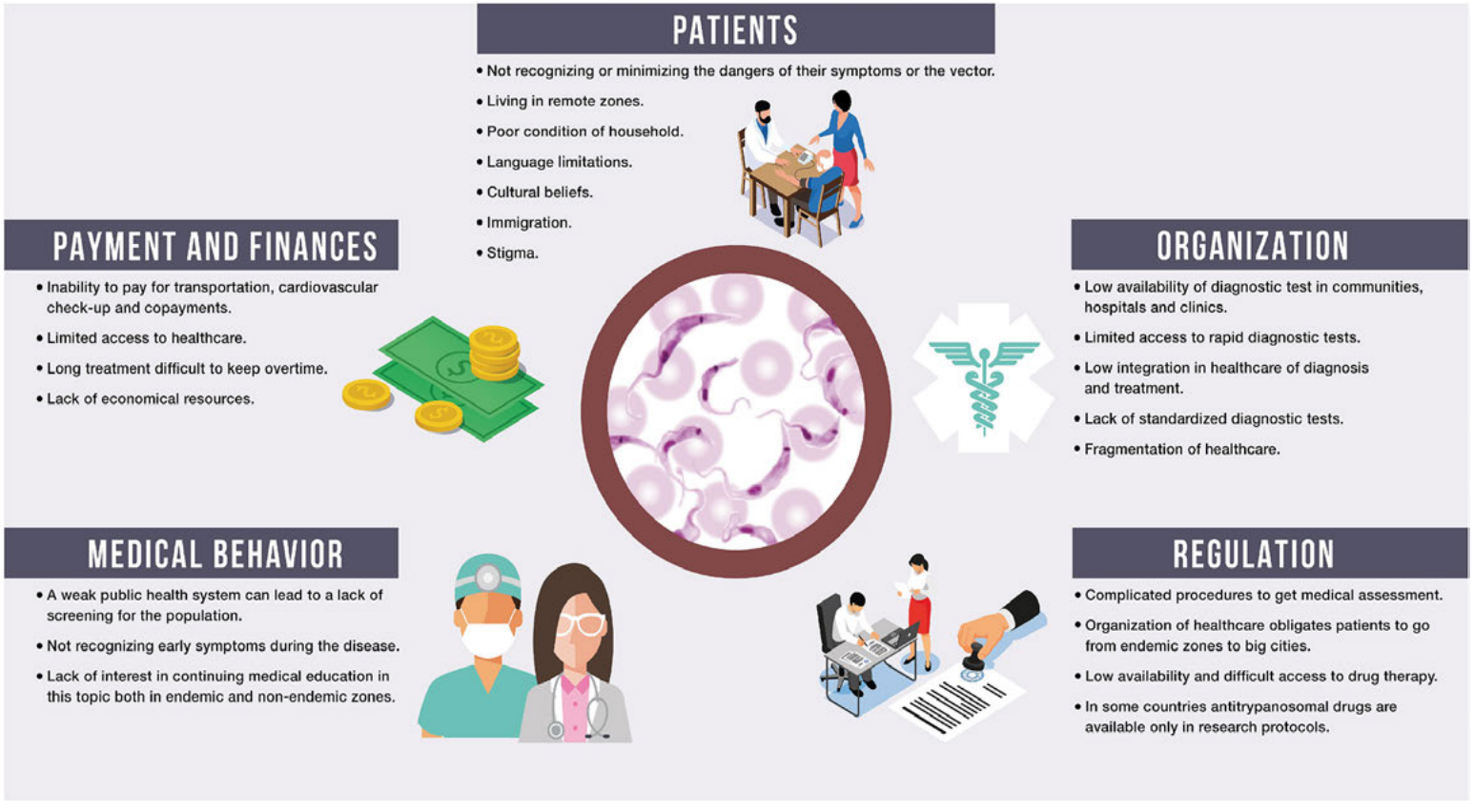

Figure 52.2 Barriers and roadblocks limiting Chagas disease care.

Table 52.1 Specific roadblocks by country to access optimal care of CD integrated with SIAC-WHF roadmap

Barriers and potential solutions	Argentina	Colombia	Spain	USA
Prevention	Insufficient screening, no prioritization even in endemic provinces. Lack of implementation of home spraying programs.	Insufficient serologic testing to screen asymptomatic patients.	Insufficient awareness and lack of national guidelines for prenatal testing. Ignorance or denial of the disease by patients.	Limited vector surveillance. No oral route detected. Incomplete testing in organ donors.
Diagnosis	Lack of availability of diagnostic tests in rural areas. Analysis in noncertified laboratories, heterogeneous cutoff points for tests. PCR is not available.	Any serologic test for CD or its complications is available in endemic areas. Lack of classification and availability of rapid tests. Patients' copayments. Highly specialized care is remote from rural communities.	Serologic testing for *T. cruzi* is carried out in expert centers only. Despite having health insurance, many undocumented immigrants do not access the health system.	Diverse immigrant population with testing well validated only for *T. cruzi* II. Difficult to access confirmatory testing. Cost of testing is prohibitive. Undocumented individuals have very limited access to care.
Treatment	Intermittent availability of Nifurtimox. Difficulties in accessing Benznidazole.	Delays in importation of trypanocidal drugs, treatment reserves are sometimes scarce. Delivery of drugs to patients requires a laborious and bureaucratic process.	Imported drugs, bureaucratic procedures that make immediate access to drugs difficult.	Recent approval of Benznidazole and Nifurtimox. Lack of familiarity with medications among providers.
Treatment of complications	Disconnection between primary care and rural physicians with specialists in treating complications. Insufficient access to medication, implantable heart devices, and heart transplantation.	Complications are usually treated and followed in tertiary care hospitals, far away from endemic areas. Lack of awareness of complications symptomatology and clinical findings.	Lack of knowledge of progression of cardiac and digestive complications by specialists, as well as the efficacy of treatments.	Lack of awareness among cardiologists of CD and its treatment. Undocumented individuals have varying but generally poor access to care.

Abbreviations: CD, Chagas disease; PCR, polymerase chain reaction.
Note: References used to create the table are as follows: [2, 5, 6].

Gastrointestinal complications are infrequent in Argentina, but simultaneously underrecognized. Cardiovascular complications are widely recognized in both endemic areas and in urban centers. Nevertheless, because of the lack of a strong Chagas-specific evidence base, uncertainty about the efficacy of pharmacologic and device therapy for Chagas heart disease compared to other heart diseases means a lack of a "standard of care."

One of the main determinants of the persistence of CD is that it is neglected by most policy makers. The budget for CD was less than 3% of what is allocated to political publicity campaigns and governmental events, and of what is allocated, less than 50% is implemented. CD requires long-term health policies that are difficult to implement in a rapidly changing political scenario [12].

52.3.3 Current Situation of CD in Colombia

In Colombia, it is estimated that 2% of the population has CD, with regional rates as high as 7%. The most affected age group are working-aged adults. Universal screening for CD in blood donors has been mandated by law since 1995, and seroprevalence based on blood bank data is approximately 0.5% [13].

Zones of higher prevalence are characterized by domestic and peridomestic cycles of *T. cruzi*, higher rates of natural infection in triatomines, and nonhuman mammal species that serve as natural reservoirs [13].

As an endemic country, vectorial transmission continues to be a problem, with rates up to 23.1 cases/1,000 habitants in high-endemicity locales. Appreciation of the importance of oral transmission has increased in urban and rural areas; outbreaks around the country have been attributed to ingestion of beverages or food contaminated with *T. cruzi*. Oral infection causes a more severe acute presentation, with rapid evolution to complications such as myocarditis, and is associated with increased mortality [14].

Since the inception of the national CD control program in 1996, Colombia has continuously made efforts to control CD: Program goals are to clarify the national epidemiological status; identify the distribution of vectors, index of domiciliary infection, rates of infection in school children, and conditions of housing; and prioritize efforts to control the disease. In 2007, the Ministry of Social Protection updated the National Guidelines for the diagnosis and management of patients with CD [15].

Eradication of CD has been an area of interest for the Colombian government; the country is part of the Andean Initiative to Control Vectorial and Transfusional Transmission of CD and Medical Care of CD (IPA). Due to this initiative, by 2018, Colombia achieved interruption of vectorial transmission in more than 23 municipalities and 4 departments [16, 17].

Colombia is currently implementing the Strategy for the Integrated Management of Health Promotion, Prevention, and Control of Vector-Transmitted Diseases for 2012–2021, which aims for a reduction of 40% of social, clinical, and economic consequences of vector-borne diseases including CD [18].

52.3.4 Current Situation of CD in Spain

According to the Spanish National Institute of Statistics, the number of foreign nationals living in Spain exceeded 5 million in 2020 [19, 20]. Over the last 20 years, there has been an exponential increase in the number of migrants from Latin America, and many diagnoses of CD have been reported in an area considered to be nonendemic, which challenges the concept of endemicity itself.

Spain is currently the European country with the greatest number of estimated cases of CD in absolute numbers (between 48,000 and 86,000 people) [21] and in percentage (between 2.7% and 4.9% of the Latin American population) [22].

Prior analyses of barriers to care in CD have highlighted a lack of knowledge among health professionals, but there are few studies evaluating awareness of CD among healthcare professionals. Based on the study by Ramos-Rincón and colleagues, the level of knowledge of CD among healthcare professionals and students in a specific territory in Spain was adequate but could be improved [23].

One of the main challenges in Spain is the significant underdiagnosis of the disease. Despite evidence suggesting the cost-effectiveness of screening [24], it is voluntary and is not widespread in Spain. National regulation regarding screening in pregnancy to prevent congenital disease has been established, and in autonomous regions like Catalonia and Valencia, control measures for *T. cruzi* infection in pregnant women at risk and control programs of newborns have already been approved [25]. Spain's ministry of health requires screening for *T. cruzi* infection before organ donation from immigrants as well as children born to mothers from endemic countries [26].

Clinical guidelines and consensus documents have been published to provide protocols for the care of chronic Chagas cardiovascular and digestive disease [27–30]. Recommendations about the management of CD in primary care [31] and in the immunosuppressed patient have also been published [32, 33].

The 2 antiparasitic drugs used currently to treat *T. cruzi* infection (benznidazole and nifurtimox) are available in Spain. However, because they are imported, there is limited access due to (1) requirement that a hospital-based pharmacy disburse the medication; and (2) bureaucratic procedures that delay the immediate start of treatment [34].

Several studies focus on the social dimension of CD and describe the complex psychosocial barriers [35]. CD diagnosis has caused social and labor discrimination in endemic countries, and at-risk individuals postpone diagnosis/treatment due to lack of economic resources, fear, and even the lack of interest of some toward a disease that manifests mainly in the long term [35].

In nonendemic countries, migrants at risk for CD face similar concerns [36, 37]. People residing legally in Spain have access to universal healthcare through the National Health System, theoretically facilitating treatment, but barriers such as work schedules, legal issues, language barriers, unfamiliarity with rights, social exclusion, and direct and indirect discrimination hinder their care [38].

During the last decade, as these barriers have been identified, patient advocacy organizations have organized to demand easier access to care for individuals with CD [39, 40].

52.3.5 Current Situation of CD in the US

Despite its ability to provide state-of-the-art medical treatment, the US remains a difficult place in which to receive appropriate care for CD. There are approximately 300,000 patients with CD in the US [41]. There are numerous barriers to care that can be grouped into 4 categories:

Lack of awareness of CD is profound in the US, both in the at-risk community and in the medical community from whom it receives care. While immigrants from regions in countries in which there are a very high prevalence of CD may recognize the disease name and pathology, immigrants from other countries, notably Mexico and El Salvador, frequently have not heard of the disease [42], which complicates their ability to advocate for screening and treatment. Medical schools in the US do not recognize CD as a diagnosis that is likely to be encountered domestically, and poor knowledge of the disease promotes resistance to screening efforts and recognition of the disease when it is manifest. Recently, American Heart Association (AHA) launched a scientific statement regarding all aspects of the management of cardiac complications of CD [43], but for busy clinicians who do not think that they will see patients with this disease, this resource is likely underutilized.

Individuals with CD in the US are chiefly foreign-born immigrants; while both reduviid bugs and *T. cruzi* are found within the lower half of the US, transmission appears to be very rare. Individuals with CD are frequently non-English speakers, indigent, uninsured, and undocumented. The medical communities that care for them are also frequently low-resource settings, in which the ability to address a complicated disease is difficult and potentially cost-prohibitive [6].

The recent approval of both benznidazole and nifurtimox have eased access to therapeutics, but the complexity of this disease and the lack of robust data to support either cure or reduction in cardiac events for most infected individuals is a barrier to the adoption of widespread screening. Testing for CD in the diverse immigrant population in the US, infected with both *T. cruzi* I and *T. cruzi* II sp., highlights the lack of quality diagnostics that have been proven to perform accurately in patients infected with either parasite strain [44].

Finally, there are structural problems that complicate the appropriate delivery of care in the US. Successful models of screening and treatment programs exist, but in states that have beneficent healthcare programs for immigrants, even those without documentation. These cannot be easily extrapolated to states where this is not the case [5]. Furthermore, the state public health labs frequently play a role in testing, particularly as the Centers for Disease Control and Prevention (CDC) is currently the only institution that can provide confirmatory testing. Each state, and often each county, may have a different policy about how to deal with these

labs. Few states mandate reporting of CD. Medical care in the US is also guideline-driven, and without US-based professional societies endorsing screening, it is very difficult to effectively promote CD screening programs [45].

52.4 Discussion

We have found diverse barriers that make CD difficult to manage, many of which are found in both endemic and nonendemic environments. The main barriers identified through this review were organization of and access to the healthcare system, medical behavior, lack of awareness, and sociocultural aspects. Our analysis, nevertheless, showed that while similar, roadblocks manifest differently in different countries and require regional strategies for better control of CD. Regardless, increased awareness of the disease and its manifestations, coupled with appropriate guidelines about screening and treatment, could synergistically overcome those barriers globally.

With regard to financing, CD received 0.67% of the total financing of all NTD during 10 years of assessment, so it earns its description as "the most neglected of the neglected" NTD [46]. CD causes a loss of 752,000 days of work every year due to premature death, with an average cost of 1.2 billion dollars per year in southern countries of Latin America [47].

Since the arrival of the SARS-CoV-2 pandemic in late 2019, many vector eradication, screening, and diagnostic programs, as well as trypanocidal treatments, have been downscaled. A recently published document covers the possible interactions between COVID-19 and CD, as well as the potential negative impact on all aspects of CD caused directly or indirectly by the pandemic. If CD was always considered a neglected disease, in the context of a pandemic, it is even more so [48].

In this chapter, our goal was to search for globally relevant barriers, then align corrective measures for them with the specific needs of endemic and nonendemic countries using 2 different countries as models for every case. We then integrated our findings with strategies proposed by the SIAC-WHF roadmap pointing to reduce the global impact of CD, along with the WHO objectives for 2030 to improve access to trypanocidal treatment, interrupt vectorial transmission and vertical transmission, and eliminate transmission related to blood transfusions and tissue transplantation in targeted countries [1].

To improve the care of CD patients, different policies must be implemented in endemic and nonendemic countries. Improved access to screening and diagnosis in populations at risk, specifically serological testing in remote areas, implementation of screening in at-risk pregnant women, blood banks, and tissue donors will improve the possibility of appropriate intervention to mitigate disease. Better integration between community and rural hospitals and the tertiary care institutions required to care for patients with significant cardiovascular or gastrointestinal complications will improve outcomes. Ongoing educational

programming will help health professionals involved in the care of CD need to be able to recognize its risk factors, clinical presentation, complications, screening indications, and treatment. Economic barriers are an important hindrance to the optimal care of CD. Reducing copayments, strengthening local programs to avoid the need of patients traveling to main cities, and guaranteeing access to "essential" medication will ameliorate the economic barriers to care.

Finally, a collaborative effort by policy makers, healthcare providers, and patients should strive to eradicate sociocultural barriers. Educational programs in endemic regions focusing on prevention, recognition of the vector and its link with CD, improved/upgraded housing construction, and integration of indigenous communities are all factors that must be taken into account. In nonendemic countries, ameliorating language barriers will reduce stigma generated by this condition. The WHO's work to establish World Chagas Disease Day (April 14th) is a positive step toward raising awareness of the issues described in this chapter, but the current barriers to care for those affected by CD have prompted the Chagas coalition [49], the WHF, and SIAC to make a strong call for immediate action.

52.4.1 Limitations

Access to care for individuals with CD is evolving, and some of the identified barriers may change in a short period of time. Some of the local policies and strategies to address CD care are only published by local governmental health entities of each country. This issue may impact the ability to find the sources of information.

This chapter did not include any authors from Brazil, which is one of the most affected countries in South America.

52.5 Conclusions

Despite its discovery and comprehensive description more than 100 years ago, multiple barriers continue to adversely impact the prognosis and global burden of CD. Identification of these roadblocks globally and regionally is necessary to improve the implementation of policies and strategies for both prevention and treatment of this potentially catastrophic disease.

Key Learning Points

- Chagas disease (CD) is endemic in Latin America, but it has become a problem of global magnitude.
- The clinical outcomes of CD are influenced by a complex interplay of political, social, cultural, economic, and environmental factors. At each of these levels, there are barriers that limit healthcare for patients with this condition.

- The main barriers are payment and financing, organization and regulation of the health system, medical behavior, and sociocultural aspects.
- Recognizing national and global barriers will allow the development of adjustable strategies to mitigate the impact of this condition.
- Optimal care and control of CD is multimodality and multidisciplinary and involves all the actors of the system to overcome each of the barriers detected.

Top Five Papers

1. Echavarría NG, Echeverría LE, Stewart M, Gallego C, Saldarriaga C. Chagas Disease: Chronic Chagas Cardiomyopathy. *Curr Probl Cardiol*. 2021 Mar; 46(3): 100507.
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Disclosures and Conflict of Interest

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Appendix

CHAGAS DISEASE

Neglected Parasitic Infections in the United States

Accessible version: www.cdc.gov/parasites/chagas/resources/npi_chagas.html

Triatomine bug, which can carry the parasite that causes Chagas disease.

Chagas disease is a preventable infection caused by the parasite *Trypanosoma cruzi* and spread by infected insects called triatomine bugs, also known as "kissing bugs." The initial infection usually does not cause severe symptoms and is often not even diagnosed. After years of chronic infection, some people develop heart diseases such as abnormal heart rhythms, heart failure, and an increased risk of sudden death. Chagas disease can also cause gastrointestinal problems, such as severe constipation and difficulty swallowing.

Infection is typically spread by contact with the triatomine bug, most commonly found in rural parts of Mexico, Central America, or South America. However, the disease can also be transmitted from mother to baby (congenital transmission), through organ transplants, or through blood transfusion. Chagas disease is one of several parasitic diseases that results in significant illness among those who are infected and is often poorly understood by healthcare providers.

CHAGAS DISEASE IN THE UNITED STATES

An estimated 300,000 infected people are living in the United States, nearly all of whom were originally infected in endemic areas. These persons often do not know they are infected and are at risk for severe cardiac or gastrointestinal problems from the disease. Diagnosis and treatment can reduce this risk.

Donor screening to detect *T. cruzi* infection in the blood supply began in early 2007. As of December 2019, **more than 2,460 confirmed positive infections among blood donors were** reported to AABB (formerly American Association of Blood Banks) by blood centers. While these efforts have likely reduced the risk of getting Chagas disease from blood products, the large number of positive donors identified indicates that many people with Chagas disease do not know they are infected and could benefit from diagnosis and treatment.

Infected triatomine bugs and wild animals that harbor *T. cruzi* infection have been found in the United States for decades. There are some reports of vector-borne (spread by contact with the bug) infection originating in the United States.

CDC IS CURRENTLY WORKING TO ADDRESS CHAGAS DISEASE BY

Partnering with state and local health departments to educate and advise health professionals to help them better care for patients with Chagas disease

Supporting physicians and patients in the United States with confirmatory diagnostic testing and answering questions regarding management of Chagas disease

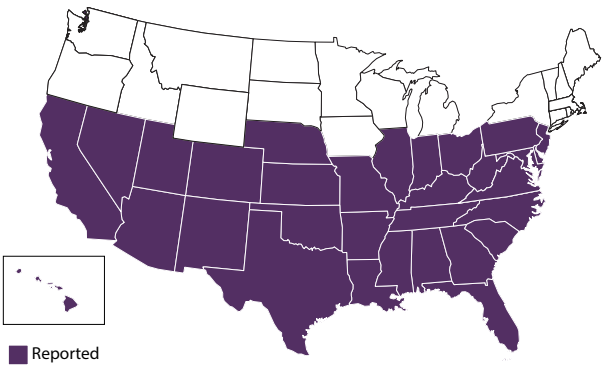
Increasing awareness of Chagas disease among healthcare providers, including publishing free, Web-based Continuing Medical Education (CME) and Continuing Nursing Education (CNE) programs

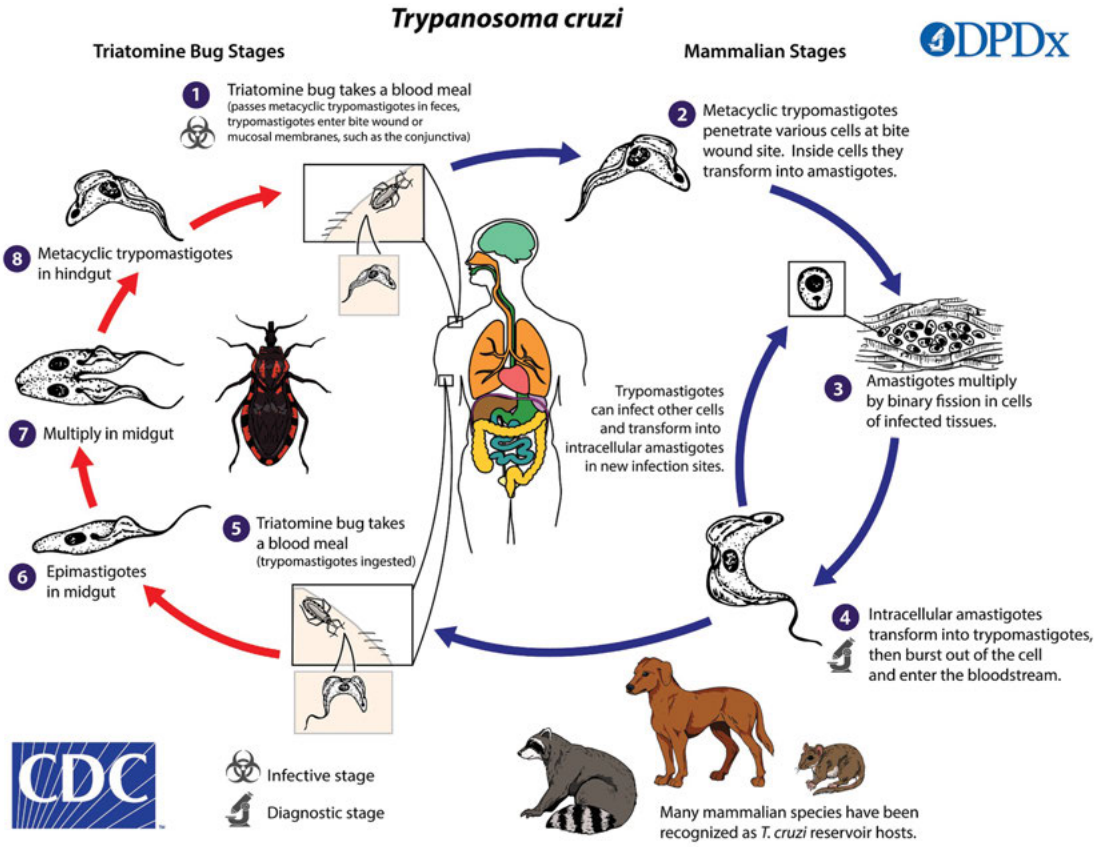
Studying perception, awareness, and understanding of Chagas disease among patients and healthcare providers to help direct outreach efforts and address barriers to care for Chagas disease patients

ADDITIONAL WORK NEEDED INCLUDES

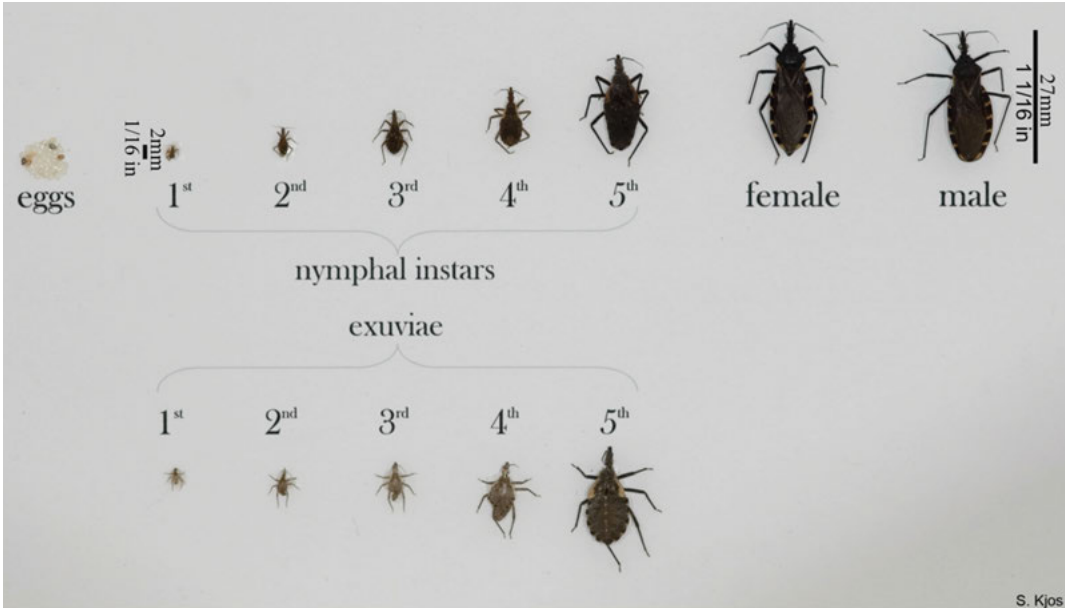
- Improve outreach to healthcare providers so they can better care for patients with Chagas disease
- Determine the risk of transmission of *T. cruzi* in the United from mothers with Chagas disease to their unborn babies
- Quantify the number of people with heart disease that was caused by Chagas disease
- Identify ways to prevent new infections from infected bugs in the United States

Triatomine Bug Occurrence by State





An infected triatomine insect vector (or "kissing" bug) takes a blood meal and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host through the bite wound or intact mucosal membranes, such as the conjunctiva 1. Inside the host, the trypomastigotes invade cells near the site of inoculation, where they differentiate into intracellular amastigotes 2. The amastigotes multiply by binary fission 3 and differentiate into trypomastigotes, and then are released into the circulation as bloodstream trypomastigotes 4. Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector. The "kissing" bug becomes infected by feeding on human or animal blood that contains circulating parasites 5. The ingested trypomastigotes transform into epimastigotes in the vector's midgut 6. The parasites multiply and differentiate in the midgut 7 and differentiate into infective metacyclic trypomastigotes in the hindgut 8. Other less common routes of transmission include blood transfusions, organ transplantation, transplacental transmission, and foodborne transmission (via food/drink contaminated with the vector and/or its feces).



Various triatomine bugs in all life stages, from eggs to nymphs to fully grown adults. A variety of bug species, that share similar traits, are pictured.

American Trypanosomiasis

Trypanosoma cruzi in thick blood smears stained with Giemsa

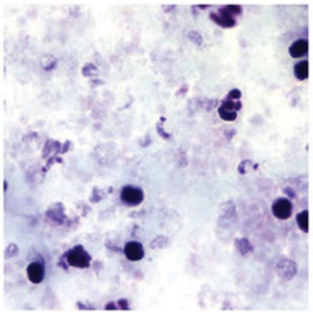


Figure A: *T. cruzi* trypomastigotes in a thick blood smear stained with Giemsa.

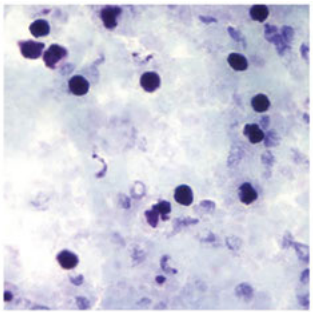


Figure B: *T. cruzi* trypomastigotes in a thick blood smear stained with Giemsa.

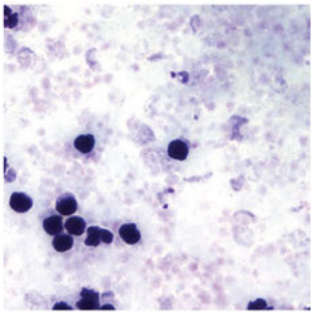


Figure C: *T. cruzi* trypomastigotes in a thick blood smear stained with Giemsa.

Trypansoma cruzi trypomastigotes are the only stage found in the blood of an infected person. Motile circulating trypomastigotes are readily seen on slides of fresh anticoagulated blood in acute infection but are rarely detectable by microscopy in chronic *T. cruzi* infection. A typical trypomastigote has a large, subterminal or terminal kinetoplast, a centrally located nucleus, an undulating membrane, and a flagellum running along the undulating membrane, leaving

the body at the anterior end. Trypanosomes measure from 12 to 30 μm in length. Trypomastigotes may be seen in cerebrospinal fluid (CSF) in central nervous system infections; also the amastigote stage parasite may be seen in histopathology specimens from affected organs.

T. cruzi in thin blood smears stained with Giemsa

Trypansoma cruzi trypomastigotes are the only stage found in the blood of an infected person. Motile circulating trypomastigotes are readily seen on slides of fresh anticoagulated blood in acute infection but are rarely detectable by microscopy in chronic *T. cruzi* infection. A typical trypomastigote has a large, subterminal or terminal kinetoplast, a centrally located nucleus, an undulating membrane, and a flagellum running along the undulating membrane, leaving the body at the anterior end. Trypanosomes measure from 12 to 30 μm in length. Trypomastigotes may be seen in cerebrospinal fluid (CSF) in central nervous system infections; also the amastigote stage parasite may be seen in histopathology specimens from affected organs.

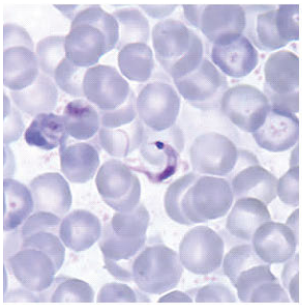


Figure A: *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa. Note the typical C-shape of the trypomastigote that characterizes *T. cruzi* in fixed blood smears.

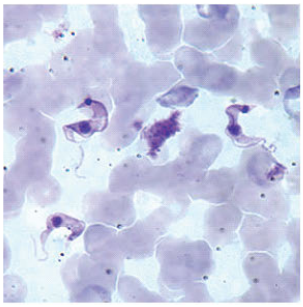


Figure B: Three *T. cruzi* trypomastigotes in a thin blood smear stained with Giemsa.

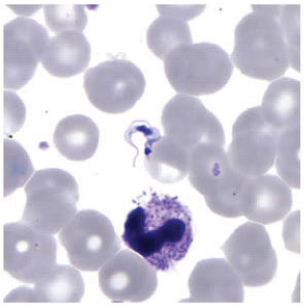


Figure C: *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa.

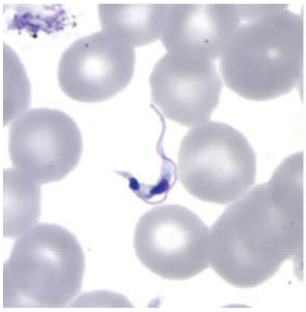


Figure D: Higher magnification of Figure C, *T. cruzi*.

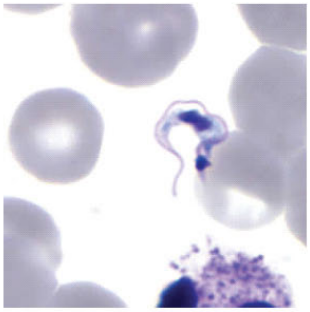


Figure E: Higher magnification of Figure C, *T. cruzi*.

T. cruzi in thin blood smears stained with Giemsa

Trypansoma cruzi trypomastigotes are the only stage found in the blood of an infected person. Motile circulating trypomastigotes are readily seen on slides of fresh anticoagulated blood in acute infection but are rarely detectable by microscopy in chronic *T. cruzi* infection. A typical trypomastigote has a large, subterminal or terminal kinetoplast, a centrally located nucleus, an undulating membrane, and a flagellum running along the undulating membrane, leaving the body at the anterior end. Trypanosomes measure from 12 to 30 μm in length. Trypomastigotes may be seen in cerebrospinal fluid (CSF) in central nervous system infections; also the amastigote stage parasite may be seen in histopathology specimens from affected organs.

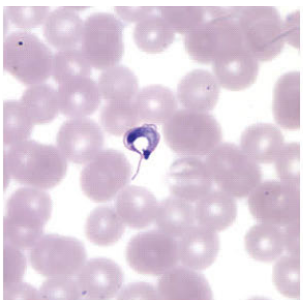


Figure A: *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa.

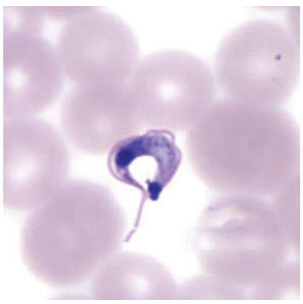


Figure B: Higher magnification of Figure A, *T. cruzi*.

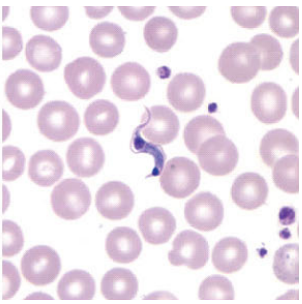


Figure C: *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa. Note the more anterior location of the nucleus.

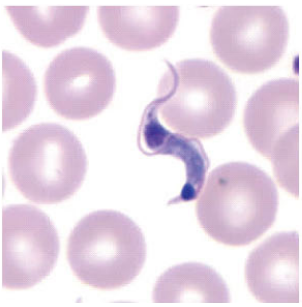


Figure D: Higher magnification of Figure C, *T. cruzi*.

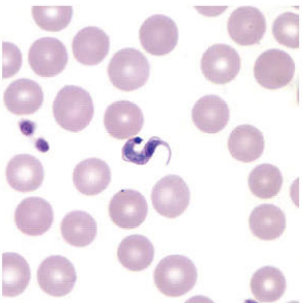


Figure E: *T. cruzi* trypomastigote in a thin blood smear stained with Giemsa.

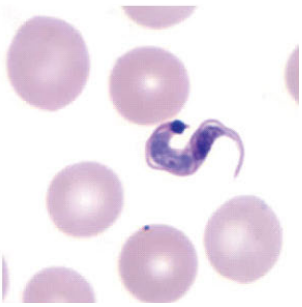


Figure F: Higher magnification of Figure E, *T. cruzi*.

T. cruzi in cerebrospinal fluid (CSF) stained with Giemsa

Trypansoma cruzi trypomastigotes are the only stage found in the blood of an infected person. Motile circulating trypomastigotes are readily seen on slides of fresh anticoagulated blood in acute infection but are rarely detectable by microscopy in chronic *T. cruzi* infection. A typical trypomastigote has a large, subterminal or

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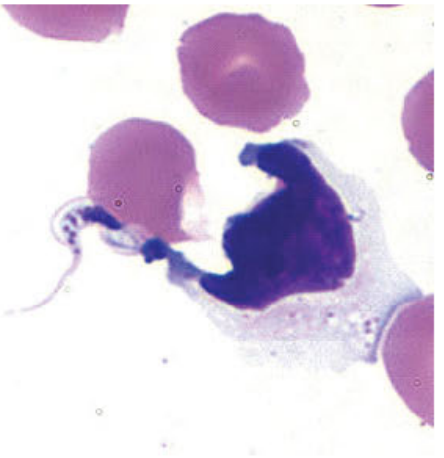

Figure A: *Trypanosoma cruzi* trypomastigote in cerebrospinal fluid (CSF) stained with Giemsa.

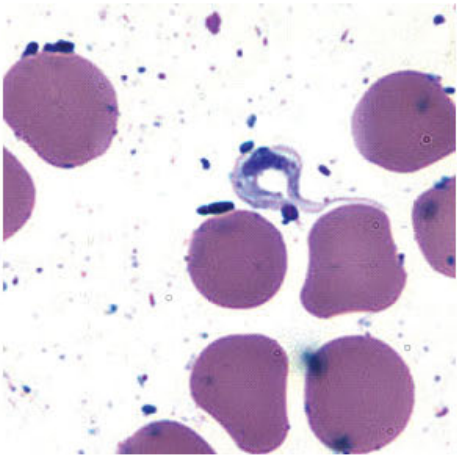

Figure B: *Trypanosoma cruzi* trypomastigote in cerebrospinal fluid (CSF) stained with Giemsa.

T. cruzi amastigotes in heart tissue

Trypomastigotes of *T. cruzi* are the only stage found circulating in human blood or CSF. In tissue, the parasite forms amastigotes characterized by a single nucleus and kinetoplast. The amastigotes of *T. cruzi* are morphologically indistinguishable from those of *Leishmania* spp.

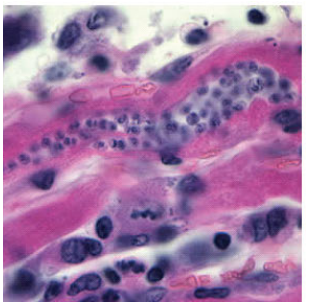

Figure A: *Trypanosoma cruzi* amastigotes in heart tissue. The section is stained with hematoxylin and eosin (H&E).

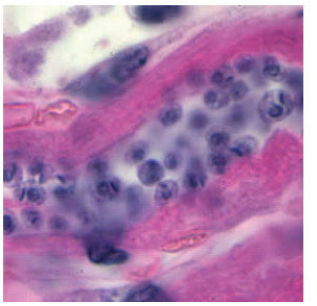

Figure B: Higher magnification of Figure A.

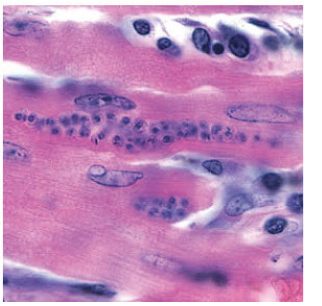

Figure C: *Trypanosoma cruzi* amastigotes in heart tissue. The section is stained with H&E.

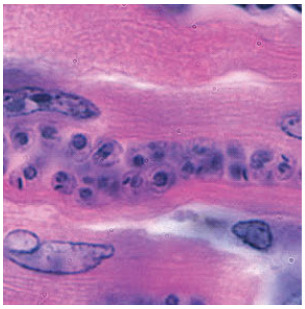

Figure D: Higher magnification of Figure C.

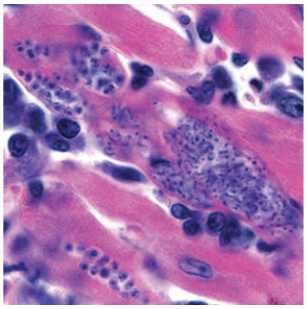

Figure E: *Trypanosoma cruzi* amastigotes in heart tissue. The section is stained with H&E.

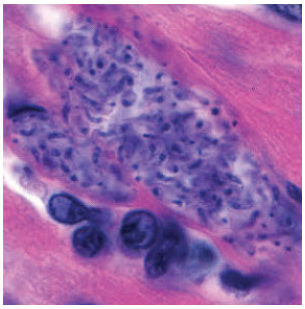

Figure F: Higher magnification of Figure E. The amastigotes in this image appear to be transforming into trypomastigotes.

T. cruzi epimastigotes, from culture

The epimastigote stage is not seen in humans but can be found in the midgut of triatomines that have ingested trypomastigotes from an infected host.

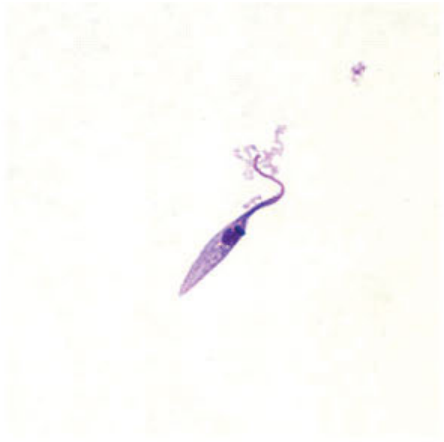

Figure A: *Trypanosoma cruzi* epimastigote from culture. Note the location of the kinetoplast anterior to the nucleus.

Figure B: *Trypanosoma cruzi* epimastigotes from culture. Note the location of the kinetoplast anterior to the nucleus.

Triatomine bug, the *T. cruzi* vector

Trypanosoma cruzi is transmitted by kissing bugs (Hemiptera: Reduviidae). The most common genera responsible for transmission of the disease are *Triatoma*, *Rhodnius*, and *Panstrongylus*. Infection usually occurs after bugs defecate on the bite site and are rubbed into the wound by the host scratching.

Figure A: Triatomine bug, *Trypanosoma cruzi* vector.

Figure B: Triatomine bugs, the vector of *Trypanosoma cruzi* vectors.

Figure C: Triatomine bug, *Trypanosoma cruzi* vector, defecating on the wound after taking a blood meal.

Chapter 53

Pathogens Infecting the Central Nervous System

Yohann Le Govic, PharmD, PhD,[a,b] Baptiste Demey,[a,c] Julien Cassereau, PhD,[d,e] Yong-Sun Bahn, PhD,[f] and Nicolas Papon, PhD[g]

[a]*Infectious Agents, Resistance and Chemotherapy (AGIR), University of Picardy Jules Verne, Amiens France*
[b]*Parasitology-Mycology Department, Center for Human Biology, University Hospital of Amiens- Picardie, Amiens, France*
[c]*Virology Department, Center for Human Biology, University Hospital of Amiens- Picardie, Amiens, France*
[d]*Department of Neurology, Angers University Hospital, Angers, France*
[e]*Univ Angers, Inserm, CNRS, MITOVASC, SFR ICAT, Angers, France*
[f]*Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea*
[g]*Univ Angers, Univ Brest, IRF, SFR ICAT, Angers, France*

ysbahn@yonsei.ac.kr, nicolas.papon@univ-angers.fr

Keywords: adherens junctions (AJs), *Blastomyces*, blood–brain barrier (BBB), brain microvascular endothelial cells (BMECs), *Candida albicans*, central nervous system (CNS), cerebrospinal fluid (CSF), *Cladophialophora bantiana*, *Coccidioides*, *Cryptococcus neoformans*, enterovirus (EV), Epstein–Barr virus (EBV), *Escherichia coli*, *Exophiala dermatitidis*, *Haemophilus influenzae* type B (Hib), hematogenous spread, herpes simplex virus (HSV), herpes simplex virus 2 (HSV-2), *Histoplasma*, human immunodeficiency virus (HIV), human neurovascular unit (hNVU), human parechoviruses (HPeVs), hyaluronic acid (HA), interferon gamma (IFNγ), interleukin (IL), interleukin-1β (IL-1β), Japanese encephalitis virus (JEV), John Cunningham virus (JCV), *Listeria monocytogenes*, meningitis, necrosis factor alpha (TNFα), *Neisseria meningitidis*, *Paracoccidioides*, Phaeohyphomycetes, progressive multifocal leukoencephalopathy (PML), *Rhinocladiella mackenziei*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Taenia solium*, tick-borne encephalitis virus (TBEV), tight junctions (TJs), *Toxoplasma gondii*, *Treponema pallidum*, *Trypanosoma brucei*, trypanosomes, tumor necrosis factor alpha (TNFα), varicella-zoster virus (VZV), West Nile virus (WNV)

53.1 Introduction

Infections of the central nervous system (CNS) are among the most devastating infectious diseases worldwide and often result in medical emergencies that require

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prompt management. Pathogens may access the CNS by crossing the blood–brain barrier (BBB), which normally protects the CNS from microbial invasion, or via transneuronal routes that bypass the BBB. A broad array of infectious agents can cause CNS infections in the meningeal or parenchymal compartments (Fig. 53.1, Table 53.1). Infection of the cerebrospinal fluid (CSF) and its surrounding meninges, termed meningitis, is accompanied with the acute onset of fever, headache, and neck stiffness. Infection of the CNS parenchyma leads to encephalitis, which clinically involves fever, neuropsychological impairment, and seizures. By contrast, CNS infection confined to small areas of focal lesions or abscesses are more likely to occur in immunocompromised individuals. Here, we summarize the etiologies of these potentially vaccine-preventable infections, their transmission routes, and the recent advances in understanding the mechanisms of CNS invasion by different neurotropic pathogens.

53.1.1 Bacterial Infections

The CNS may be infected by a wide variety of bacteria (Fig. 53.1, Table 53.1). The spectrum of these infections varies from focal infections, such as brain abscesses, to generalized entities such as meningoencephalitis. Contiguous spread from the upper airways, hematogenous spread from another primary site, and direct inoculation through trauma or surgery can contribute to the development of a CNS bacterial infection. The etiology of bacterial meningitis varies according to age group and immune status. The most frequent infective agents affecting newborns in the first week, *Streptococcus agalactiae* and *Escherichia coli*, are replaced by *Streptococcus pneumoniae* and *Neisseria meningitidis* by the sixth week [1]. Subsequently, *S. pneumoniae* remains the most common bacterial agent, followed by *N. meningitidis* and *Listeria monocytogenes* [1, 2]. *Haemophilus influenzae* type B (Hib) used to be a leading cause of pediatric meningitis in the pre-Hib vaccination era [3]. Similarly, the introduction of pneumococcal and meningococcal conjugate vaccines has substantially reduced the burden of bacterial meningitis. Gram-negative bacilli and *Staphylococcus* spp. are the most common causes of nosocomial CNS infections.

Atypical bacteria can also reach the brain. *Mycobacterium tuberculosis* causes tuberculous meningitis, which originates from a pulmonary focus that spreads via the lymphatic system but also intracranial tuberculomas. The local inflammatory response leads to nerve palsy and alterations in the CSF and cerebral blood flow. The mortality rate in treated cases remains high, ranging from 20% to 67% [4]. Spirochetes are also responsible for CNS diseases that are mediated by local inflammatory responses. *Treponema pallidum*, a sexually transmitted pathogen, usually cause neurosyphilis in the late stage of infection [5]. Pathogenic Leptospira spp. cause leptospirosis, an acute febrile disease mainly transmitted by brown rats, eliciting neurological complications or meningoencephalitis [6]. *Borrelia burgdorferi*, an agent of a tick-borne Lyme borreliosis, causes encephalitis or meningitis associated with arthropathies [7].

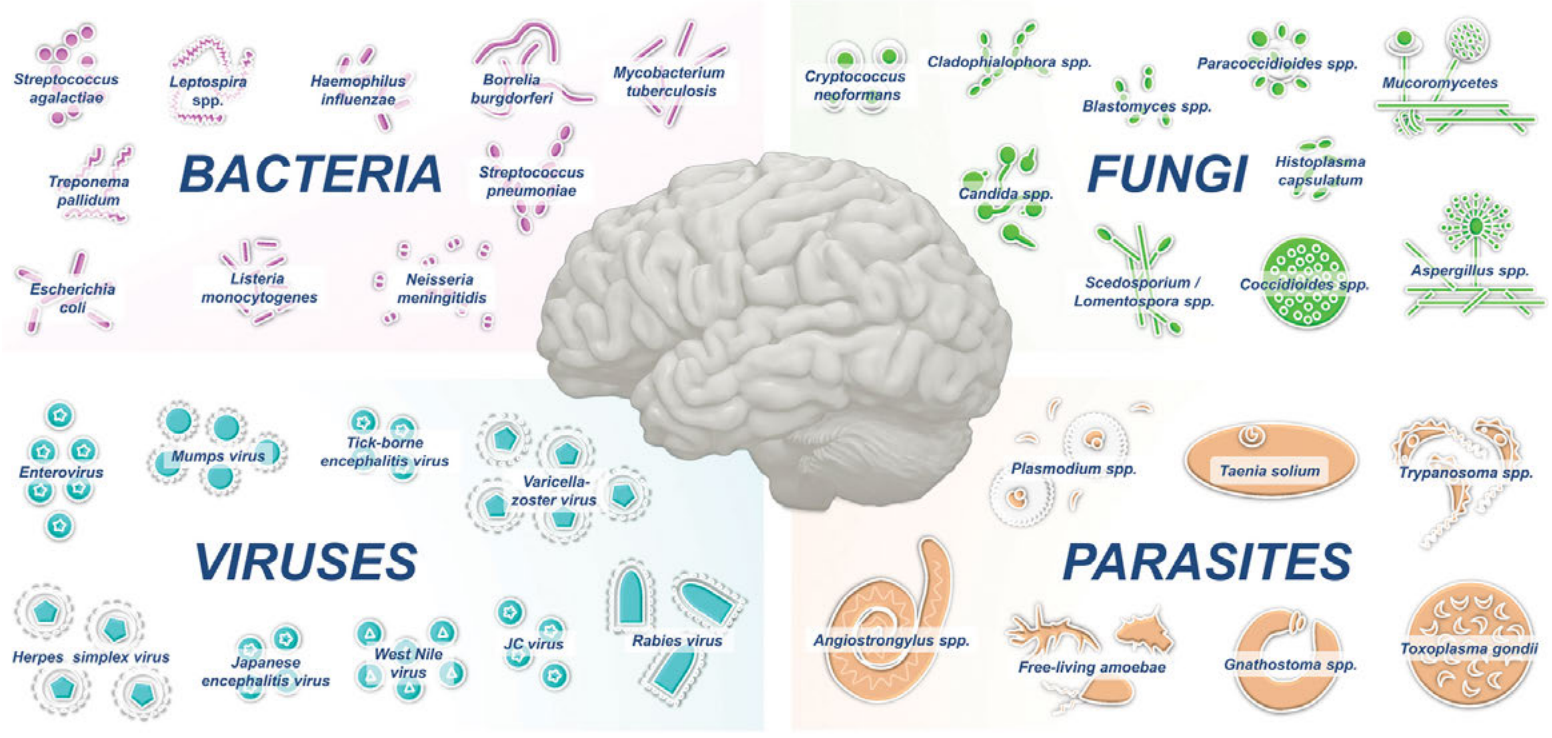

Figure 53.1 Compilation of prominent bacteria, fungi, viruses, and parasites that infect the CNS. *Abbreviation*: CNS, central nervous system.

Table 53.1 Etiology and epidemiology of CNS infections

Pathogen	Geographic distribution	Transmission route	Demographics	Clinical presentation
Bacteria				
Streptococcus agalactiae	Worldwide	Vertical transmission (mother to child) through the birth canal	Neonates	Meningitis
Escherichia coli				
Neisseria meningitidis		Inhalation (droplets produced by coughing or sneezing)	Children and adults	
Streptococcus pneumoniae				
Haemophilus influenzae			Elderly and immunocompromised individuals	
Listeria monocytogenes		Transplacental	Elderly, immunocompromised, (neonates)	Meningitis, rhombencephalitis
Mycobacterium tuberculosis	Worldwide; vast majority in Africa/Asia	Inhalation (Flugge droplets)	Children and adults Immunocompromised (HIV infected patients)	Meningitis, cerebral tuberculomas
Treponema pallidum (neurosyphilis)	Worldwide	Direct contact (sexual)	Adults	Meningoencephalitis; General paresis; Tabes dorsalis
Leptospira spp.	Worldwide	Contact with infected mammals (rodents)	Children and adults	Meningitis; Meningoencephalitis; Myelitis
Borrelia burgdorferi (neuroborreliosis)	North America and Eurasia	Arthropod borne (tick: *Ixodes* spp.)	Children and adults	Encephalitis; Meningitis; Encephalopathy
Viruses				
EV	Worldwide	Fecal/oral Inhalation (EV-D68)	Children and adults	Meningitis Encephalitis (rare)

Pathogen	Geographic distribution	Transmission route	Demographics	Clinical presentation
HSV	Worldwide	Skin/mucosa	Children (mainly HSV1) and adults (mainly HSV2) Immunocompromised	Meningitis Encephalitis
VZV	Worldwide	Skin/mucosa Inhalation	Adults (mostly immunocompromised)	Encephalitis; Meningitis (rare); Myelitis
Mumps virus	Worldwide	Inhalation	Children and adults (mostly unvaccinated)	Meningitis; Myelitis;
Rabies virus	Worldwide; vast majority in Africa/Asia	Contact with infected mammal (dogs)	Children and adults	Encephalitis
WNV	Worldwide	Arthropod borne (mosquito: *Culex* spp.)	Children and adults (mostly elderly population)	Meningitis; Encephalitis
JEV	Asia, Australia, and western Pacific	Arthropod borne (mosquito: *Culex* spp.)	Children and adults (mostly pediatric population)	Encephalitis
TBEV	Central and northern Europe	Arthropod borne (tick: *Ixodes ricinus*)	Children and adults	Encephalitis
JCV	Worldwide	Inhalation	Adults with severe immune deficiency	PML
Fungi				
Candida spp. (neurocandidiasis)	Worldwide (human commensal)	Nosocomial (neurosurgery, CNS devices)	Preterm neonates, children, and adults	Meningoencephalitis; brain abscesses
Cryptococcus neoformans (neurocryptococcosis)	Worldwide; frequent in Europe	Inhalation (bird droppings)	Immunocompromised (especially for $CD4^+$ T-cell counts <100/ mm^3)	Meningoencephalitis
Aspergillus spp.	Worldwide	Inhalation	Immunocompromised	Brain abscesses (frequently secondary to lung infections)

(*Continued*)

Table 53.1 (*Continued*)

Pathogen	Geographic distribution	Transmission route	Demographics	Clinical presentation
Scedosporium spp. *Lomentospora* spp.	Worldwide	Inhalation	Children and adults (mostly immunocompromised)	Brain abscesses (secondary to lung infections or near-drowning)
Mucoromycetes (*Rhizopus*, *Lichtheimia*, *Mucor*...)	Worldwide	Inhalation	Immunocompromised	Brain abscesses (frequently secondary to sinus infections)
Histoplasma capsulatum (histoplasmosis)	Central and eastern United States (var. capsulatum); Africa (var. duboisii)	Inhalation (bird or bat droppings)	Children and adults (immunocompetent)	Meningitis, meningoencephalitis, and brain abscesses
Blastomyces spp. (blastomycosis)	North America	Inhalation (soil dust)	Children and adults (immunocompetent)	Meningitis, meningoencephalitis, and brain abscesses
Coccidioides spp. (coccidioidomycosis)	Southwest US, Mexico, and South America	Inhalation (soil dust)	Children and adults (immunocompetent)	Meningitis, meningoencephalitis, and brain abscesses
Paracoccidioides spp. (paracoccidioidomycosis)	Central and South America	Inhalation (soil dust)	Children and adults (immunocompetent)	Brain abscesses and meningitis
Dematiaceous molds (phaeohyphomycosis)	Worldwide	Inhalation	Children and adults (immunocompetent)	Brain abscesses
Parasites				
Toxoplasma gondii	Worldwide	Ingestion of tissue cysts or sporulated oocysts Organ transplant Blood transfusion	Immunocompromised (especially for $CD4^+$ T-cell counts <200/ mm^3)	Encephalitis and brain abscesses

Pathogen	Geographic distribution	Transmission route	Demographics	Clinical presentation
Trypanosoma brucei (sleeping disease)	Africa	Arthropod borne (Tsetse flies, Glossinidae)	Children and adults	Mental and behavioral disorders, sleep and sleep–wake cycles disturbances
Plasmodium spp. (cerebral malaria)	Tropical and subtropical regions	Arthropod borne (Mosquito: *Anopheles* spp.)	Children and adults	Impaired consciousness and coma
Taenia solium (neurocysticercosis)	Africa, Asia, and Latin America	Fecal–oral	Children and adults (mostly from resource-limited countries)	Intracerebral cysts (epilepsy)
Angiostrongylus spp. (neuroangiostrongyliasis)	Southeast Asia, Oceania, and the Americas	Ingestion of terrestrial mollusks (snails/slugs)	Children and adults	Eosinophilic meningitis; Intracranial hemorrhage
Gnathostoma spp. (gnathostomiasis)	Southeast Asia, Japan, Korea, and Latin America	Food borne (mostly raw freshwater fish, amphibians, and reptiles)	Children and adults	Eosinophilic meningitis; Intracranial hemorrhage
Free-living amoebae (*Acanthamoeba*, *Balamuthia* and *Naegleria*)	Worldwide	Inhalation (contaminated water)	Immunocompromised (*Acanthamoeba*, *Balamuthia*) Immunocompetent (*Naegleria*)	Granulomatous amebic encephalitis (*Acanthamoeba*, *Balamuthia*) Primary amebic meningoencephalitis (*Naegleria*)

Abbreviations: CNS, central nervous system; EV, enterovirus; HSV, herpes simplex virus; JCV, John Cunningham virus; JEV, Japanese encephalitis virus; PML, progressive multifocal leukoencephalopathy; TBEV, tick-borne encephalitis virus; VZV, varicella-zoster virus; WNV, West Nile virus.

53.1.2 Viral Infections

Viral meningitis is by far the most frequent clinical presentation of CNS infections (Fig. 53.1, Table 53.1). Enteroviruses (EVs), members of the Picornaviridae family, are involved in approximately 90% of cases [8]. EV-A71, EV-D68, and Coxsackievirus B are most frequently detected in patients with aseptic meningitis. Human parechoviruses (HPeVs), especially HPeV-3, are other Picornaviridae that are commonly responsible for meningitis. EVs can replicate in the upper respiratory and intestinal epithelial cells and then disseminate into the bloodstream and CNS through infected immune cells [9]. EV meningitis is usually a benign, self-limiting condition.

The Herpesviridae family is the second leading cause of viral meningitis. Herpes simplex virus 2 (HSV-2) is the predominant causal agent, but HSV-1, varicella-zoster virus (VZV), and Epstein–Barr virus (EBV) also cause meningitis. HSV-2 meningitis may occur as a result of primary infection or reactivation [10]. Mumps virus, causing parotitis and hearing loss, is one of the main causes of viral meningitis in unvaccinated populations [11].

In addition to meningitis, most of the aforementioned viruses are also capable of causing encephalitis. HSV-1 can cause necrotic acute encephalitis during reactivation in the adult population. Similarly, VZV encephalitis is common after reactivation of the virus (zoster). Other viruses are typically characterized as causal agents of encephalitis. Historically, rabies is the most popular viral brain infection and caused by various *Lyssavirus* species that are transmitted by dog bites in approximately 99% of human cases [12]. Animal control and vaccination programs aim to prevent infections that occur in approximately 150 countries and affect approximately 3 billion people.

Arthropod-borne viruses (arboviruses) are responsible for encephalitis in endemic areas. Over the years, however, climatic and ecological changes have altered the geographical distribution of arboviral infections. The West Nile virus (WNV) rapidly emerged in Northern America and Southern Europe during the 21st century. This *Flavivirus* is transmitted by mosquitoes and often cause encephalitis especially in elderly patients. The Japanese encephalitis virus (JEV) is another mosquito-borne *Flavivirus* and causes viral encephalitis in Asia. Tick-borne encephalitis virus (TBEV) is the third most common encephalitis-causing arbovirus. This *Flavivirus* is primarily transmitted to humans by the widespread hard tick species *Ixodes ricinus* [13]. Although the mortality rate is low, up to 30% of patients with TBEV encephalitis develop neurological sequelae. CNS diseases may be caused by other neurotropic viruses such as human immunodeficiency virus (HIV), cytomegalovirus, human herpesvirus 6, influenza virus, or measles virus [12].

Finally, John Cunningham virus (JCV), a polyomavirus that commonly establishes asymptomatic infection in the general population, is responsible for progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the CNS, in patients with severe immune deficiency. The development of new

immunomodulatory and immunosuppressive drugs expanded the spectrum of conditions associated with PML [14].

53.1.3 Fungal Infections

Unlike bacteria, fungi are eukaryotic (mostly saprophytic) organisms with membrane-bound nuclei that obtain nutrients from organic matter. Fungal infections of the CNS are commonly opportunistic, resulting from hematogenous dissemination in immunocompromised hosts. However, immunocompetent individuals are increasingly being reported as possible hosts for such infections. The fungal infections often originate from direct inoculation (e.g., trauma or surgery) of fungal spores.

Medically important fungi that invade the CNS include yeasts, molds (filamentous fungi), and dimorphic fungi (Fig. 53.1, Table 53.1). CNS-infecting yeasts include a number of ubiquitous species, such as *Candida* spp. and *Cryptococcus neoformans*, the latter showing strong neurotropism. CNS-infecting molds include hyalohyphomycetes with septate hyphae (e.g., *Aspergillus* and *Scedosporium/Lomentospora* species) and the mucormycetes with nonseptate or sparsely septate hyphae (e.g., *Mucor*, *Rhizopus*, and *Lichtheimia* species). These fungi are distributed worldwide. Phaeohyphomycetes (dark molds) represent a third group of ubiquitous neurotropic molds (e.g., *Cladophialophora bantiana*, *Exophiala dermatitidis*, and *Rhinocladiella mackenziei*). Dimorphic fungi, such as *Histoplasma*, *Blastomyces*, *Coccidioides*, and *Paracoccidioides*, have a confined geographical distribution in the American continents and often infect the CNS [15].

Importantly, the morphology of the fungus influences the pathogenesis of CNS lesions. Fungi that develop as budding yeasts *in vivo* primarily cause meningitis (dimorphic fungi) or meningoencephalitis (*Cryptococcus* and *Candida* species). Those that exhibit yeast-to-hyphae transition (e.g., *Candida albicans*) can be more invasive, leading to necrosis and brain abscesses. Those that grow large hyphae (i.e., filamentous fungi) have a propensity for macrovascular invasion, causing hemorrhagic stroke, aneurysms, and cerebral abscesses. Cryptococcal meningoencephalitis is the most frequent fungal infection of the CNS, whereas candidiasis is the most common nosocomial infection. Aspergillosis and mucormycosis are relatively rare but devastating in immunosuppressed patients, while cerebral phaeohyphomycoses mainly occur in immunocompetent individuals [16]. Besides immunological disorders, some environmental, iatrogenic, and host-related factors may predispose an individual to the fungal CNS infection.

53.1.4 Parasitic Infections

Parasitic diseases involving the CNS are major threats, especially in low- and middle-income countries. The causative agents include miscellaneous unicellular and multicellular organisms such as protozoa and worms, respectively (Fig. 53.1, Table 53.1). Certain parasitic agents are highly dreaded in specific contexts, such

as cerebral malaria in travelers returning from endemic regions with fever and any neurological symptoms or cerebral toxoplasmosis in patients infected with HIV. Parasitic infections of the CNS may be suspected in patients with nonspecific manifestations, such as meningitis, encephalitis, ventriculitis, myelitis, or brain abscess, with fever and headaches as chief complaints. The clinical presentation depends on the localization and size of the lesions, but distinct parasites may lead to the same symptomatology, making diagnosis challenging. Although a number of CNS parasitic infections are endemic in tropical countries, they are now spreading globally due to international migration and travel [17].

Nematode infections are the main cause of eosinophilic meningoencephalitis (especially *Angiostrongylus* and *Gnathostoma* species). In addition, neurocysticercosis, caused by larval cysts of the tapeworm *Taenia solium*, is the most common cause of epileptic seizures in low-income countries. In this respect, extraparenchymal forms (i.e., outside the brain tissue) result in high morbidity and mortality. In a pathophysiological perspective, the inflammatory response toward the larva is the hallmark of the disease and is supposed to contribute to BBB breakdown. Some protozoan species are also known for infecting the human CNS. This is notably the case of the flagellate *Trypanosoma brucei*, the etiological agent of African trypanosomiasis (sleeping sickness), which induces life-threatening meningoencephalitis. Although much rarer, the pathogenic free-living amoebae (*Acanthamoeba*, *Balamuthia*, and *Naegleria* species) are noteworthy causal agents due to their high case-fatality rates.

53.1.5 BBB Crossing Mechanisms of CNS-Infecting Pathogens

The human BBB is a neurovascular unit composed of brain microvascular endothelial cells (BMECs), pericytes, astrocytic end feet, microglia, and neurons. The presence of tight junctions (TJs) and adherens junctions (AJs) makes paracellular movements of even a small molecule extremely difficult [18]. Nevertheless, neurotropic pathogens can cross the BBB via (i) transcellular migration; (ii) paracellular migration; and/or (iii) a Trojan horse mechanism. In the transcellular mechanism, a pathogen binds to BMECs, then is taken up by BMECs through receptor-mediated endocytosis, is transported within a vacuole without fusion with lysosomes, and is finally released to the brain tissues. In the paracellular mechanism, a pathogen can traverse between BMECs by disrupting TJs and/or AJs, which can be facilitated by the induced expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, and interferon gamma (IFNγ), in BMECs, pericytes, and astrocytes. In the Trojan horse mechanism, phagocytes infected with a pathogen cross the BBB paracellularly.

Several CNS-infecting bacteria, including *E. coli*, group B *Streptococcus*, *S. pneumoniae*, *N. meningitidis*, *L. monocytogenes*, and *M. tuberculosis*, cross the BBB transcellularly (see [19, 20]). The latter 2 pathogens also cross the BBB via a Trojan horse mechanism. Bacterial surface adhesins are generally required for

BBB crossing. These include CD48-interacting type I fimbrial adhesin FimH and gp96-interacting outer-membrane protein A in *E. coli*, a platelet-activating factor receptor-interacting cell wall phosphorylcholine in *S. pneumoniae*, laminin-binding protein, the fibrinogen-binding protein FbsA and invasion-associated gene A in *S. agalactiae*, and type IV pili PilC in *N. meningitidis*. In addition, several invasion proteins modulate host cytoskeleton regulation to promote transcellular traversal of bacterial pathogens.

Viruses can infect the CNS by either directly traversing the BBB through one of the mechanisms described above or by taking nonhematogenous routes, including retrograde axonal transport from peripheral nerves to the CNS and the nasal olfactory epithelium and neurons. In particular, CNS-infecting viruses can stimulate the production of pro-inflammatory cytokines and matrix metalloproteases in BMECs, astrocytes, and pericytes, which can destabilize TJs by activating the RhoA kinase pathway and promoting BBB crossing of neurotropic viruses [21, 22].

Among the various neuroinfectious fungal pathogens, 2 pathogenic yeasts, *C. albicans* and *C. neoformans*, traverse the BBB transcellularly [23]. Inositol, which is abundantly present in the brain, is taken up by *C. neoformans* through inositol transporters Itr1a and Itr3c and induces the expression of hyaluronic acid (HA) synthase gene *CPS1* in *C. neoformans*. The HA produced enhances the binding of the fungal pathogen to the CD44 glycoprotein in blood endothelial cells [24, 25]. *C. neoformans*–derived extracellular microvesicles and the metalloprotease Mpr1 also contribute to the BBB crossing process of *C. neoformans* [26, 27]. Recent systematic BBB crossing analyses of signature-tagged mutants and CRISPR/Cas-9–based gene deletion mutants in *C. neoformans* revealed that a variety of proteins involved in diverse biological functions are involved in BBB crossing and pathogen survival in the brain parenchyma [28]. In addition, *C. neoformans* can cross the BBB via Trojan horse mechanism [29]. *C. albicans* can cross the BBB by utilizing fungal invasins, Als3 and Ssa1, the former of which can interact with the gp96 receptor on BMECs [30].

The BBB crossing mechanisms of parasites are relatively well studied in *Toxoplasma gondii* and trypanosomes [31]. *T. brucei* can cross the BBB by expressing the cysteine protease cathepsin L (brucipain) that interacts with G-protein coupled receptors on host endothelial cells. *T. gondii* can cross the BBB in a Trojan horse mechanism. In this process, *T. gondii* secretes cyclophilin 18, which interacts with the chemokine receptor CCR5 present on phagocytic cells.

53.1.6 Toward New Therapeutic Approaches for Treating CNS Infections

Therapeutic options for CNS infections are highly limited, because the delivery of antimicrobial agents to the affected brain compartments is challenged by the structural complexity and tightness of the human BBB. To study the molecular

mechanisms of microbial CNS invasion and expedite screening drugs for CNS infections and disorders, intensive efforts have been made to construct *in vitro* BBB models in the past years. The simplest but most widely used *in vitro* BBB model is a transwell system containing a monolayer of human BMECs with or without astrocytes [32]. Microfluidic devices have recently been developed to better reflect complex three-dimensional BBB structures [33]. Most recently, the human neurovascular unit (hNVU) chip, which contains all the necessary cellular and extracellular brain components, was used to examine the neurotropism and BBB penetration of *C. neoformans* [34]. Although screening and development of antimicrobial agents with a good BBB permeability are important, the application of BBB-penetrating conjugates could be a promising approach. In particular, a number of peptide-based BBB shuttles have been developed in the past decades [35–36]. These BBB shuttles could be applied to a variety of currently available antimicrobial agents in future.

In conclusion, recent advances in *ex*/*in vivo* and *in vitro* BBB and CNS models will not only facilitate understanding of the CNS infection pathophysiology, but also support the screening of novel antimicrobial agents for the treatment of microbial meningitis. Better treatment strategy targeting CNS infections is an essential prerequisite to improving the global management of these life-threatening microbial infections.

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Chapter 54

Current Issues in Vaccines[1]

Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Keywords: 2-phenoxyethanol, alum, aluminum hydroxide, aluminum phosphate, autism, avian influenza, cephalosporins, Cervarix, chickenpox, compulsory vaccination, D,L-alpha-tocopherol, diphtheria, diphtheria-tetanus-acellular pertussis (DTaP) ethylmercury, fetal bovine serum, flu vaccine, Fluad, formaldehyde, *Haemophilus influenzae* type B (Hib), HepA vaccine, hepatitis A, hepatitis B, HepB vaccine, Heplisav-B, human serum albumin (HSA), inactivated influenza vaccine (IIV), influenza, injection site reactions (ISR), measles, measles-mumps-rubella (MMR), measles-mumps-rubella-varicella (MMRV), methylmercury, monophosphoryl lipid A (MPL), mumps, neuropsychological, penicillins, pertussis, pneumococcal 23-valent polysaccharide (PPSV23), pneumococcus, polio, polio virus, *Quillaja saponaria*, recombinant zoster vaccine (RZV), rotavirus, rubella, Shingrix, smallpox vaccine, squalene, statistically significant, sulfa drugs, tetanus, thimerosal, Vaccine Adverse Event Reporting System (VAERS), varicella vaccine, whooping cough

54.1 History of Vaccines

54.1.1 Overview

The Centers for Disease Control and Prevention (CDC) recommends getting 29 doses of 9 vaccines (https://www.britannica.com/science/vaccine) (plus a yearly flu shot after six months old) for kids aged 0 to six [121]. No US federal laws mandate vaccination, but all 50 states require certain vaccinations for children entering public schools. Most states offer medical and religious exemptions; and some states allow philosophical exemptions [1].

[1]This chapter has been compiled and edited by the Series Editor, Raj Bawa, PhD, MD. It is based on information and materials kindly provided to Dr. Bawa by the Centers for Disease Control and Prevention. This chapter is not copyrighted and is in the public domain. Duplication is encouraged.

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Go to the following link for an *Encyclopaedia Britannica* video about how vaccines work: https://www.britannica.com/video/185623/strategies-vaccines-use-pathogens-human-immune-system.

Proponents say that vaccination is safe and one of the greatest health developments of the 20th century. They point out that illnesses, including rubella, diphtheria, smallpox, polio, and whooping cough, are now prevented by vaccination and millions of children's lives are saved. They contend adverse reactions to vaccines are extremely rare. Opponents say that children's immune systems can deal with most infections naturally, and that injecting questionable vaccine ingredients into a child may cause side effects, including seizures, paralysis, and death. They contend that numerous studies prove that vaccines may trigger problems like ADHD and diabetes.

54.1.2 Early History of Vaccines

1802 painting of smallpox vaccine inventor Dr. Edward Jenner vaccinating a room full of people who then sprout cows from their bodies. The painting illustrates popular 17th century fears about vaccination. The caption reads "The Cow Pock – or – the Wonderful Effects of the New Inoculation". Kindly provided by the National Library of Medicine History of Medicine Collection.

The Chinese used inoculation techniques against smallpox as early as 1000 AD and similar techniques were also used in ancient Africa and Turkey [2]. The first instance of vaccine promotion in the US was in 1721 when Cotton Mather (https://www.britannica.com/biography/Cotton-Mather), a Puritan minister, encouraged smallpox vaccination in response to an outbreak [3]. Vaccination as

practiced today came into being when Edward Jenner (https://www.britannica.com/biography/Edward-Jenner), English physician and scientist, created the first smallpox vaccine using cowpox (a disease similar to smallpox that infects cows) and vaccinating an eight-year-old boy in 1796 [2, 3]. Jenner's innovation was used for 200 years, with updates, and eradicated smallpox [2].

In 1801 Benjamin Waterhouse (https://www.britannica.com/biography/Benjamin-Waterhouse), physician and co-founder and President of Harvard Medical School, began using the "Cowpox Vaccine," leading to Massachusetts becoming the first US state to promote the use of vaccination [3]. In 1809 the town of Milton, Massachusetts became the first US town to offer free smallpox vaccinations, which was followed by a state law that same year requiring the smallpox vaccination [3, 4].

Later, on February 27, 1813, US President James Madison (https://www.britannica.com/biography/James-Madison) signed into law *An Act to Encourage Vaccination*, which created the National Vaccine Agency (now part of the US Department of Health and Human Services) [5].

"Death the Vaccinator" published in the late 1800s by the London Society for the Abolition of Compulsory Vaccination. Kindly provided by the College of Physicians of Philadelphia, www.historyofvaccines.org.

In 1855 Massachusetts passed the first US state law mandating vaccinations for schoolchildren [5], followed by New York (1862), Connecticut (1872), Indiana (1881), Arkansas (1882), Illinois (1882), Virginia (1882), Wisconsin (1882), California (1888), Iowa (1889), and Pennsylvania (1895) [6]. By 1963, 20 states would require immunization to attend public schools; and 29 states by 1970 [4].

In response to immunization laws, in 1878, the National Anti-Compulsory Vaccination Reporter stated that "the dangerous illnesses following the vaccine process are… on the whole… a greater evil to humanity than small-pox itself!" [7]. The Anti-Vaccination Society of America was founded in 1879 in response to the states enacting vaccination mandates and with the belief that it "is undignified" to mandate vaccinations and that the "efficacy of vaccination as a disease preventative is a matter of individual opinion" [8]. In 1882 the New England Anti-Compulsory Vaccination League was founded and in 1885 the Anti-Vaccination League of New York City was created [7]. With their influence, the anti-vaccination groups began getting vaccine mandates repealed in California, Illinois, Indiana, Minnesota, Utah, West Virginia, and Wisconsin [7].

The first laboratory-created vaccine was for avian cholera (which most commonly infects chickens), developed by Louis Pasteur (https://www.britannica.com/biography/Louis-Pasteur), French chemist and microbiologist, in 1879 [2]. In 1885, Pasteur created the rabies vaccine, beginning an active period of vaccine development for human illnesses through the 1930s that saw vaccines developed for typhoid (1899), cholera (1911), diphtheria (1914), tuberculosis (1921), and tetanus (1924), among others [2]. Vaccines for polio (1955), measles (1963), mumps (1967), and rubella (1969) followed in the mid-twentieth century [2]. During the active period of vaccine development, in 1901 the first Nobel Prize in Physiology or Medicine was awarded to Emil von Behring (https://www.britannica.com/biography/Emil-von-Behring), a German physiologist, for his work developing serum therapy in connection to a diphtheria vaccination [10]. On July 1, 1902, Congress passed *An Act to Regulate the Sale of Viruses, Serums, Toxins, and Analogous Products* (also referred to as the *Biologics Control Act*), which was the first legislation to control the quality of drugs, specifically the quality of vaccines [2]. Later, on Feb. 20, 1905, mandatory vaccination was upheld by the US Supreme Court in *Jacobson v. Massachusetts* (7–2) [9]. In the aftermath of the ruling more states across the country began to implement mandatory child vaccination as a condition of public school attendance.

On Nov. 13, 1922, the constitutionality of mandatory vaccination of school children was once again challenged and upheld in the *Zucht v. King*; the US Supreme Court declined to hear the case, stating that it was "within the police power of a state to provide for compulsory vaccination" [11] [5]. In 1951, Jonas Salk, MD, and his team developed a method to cultivate the polio virus in monkey kidney tissue in order to be able to produce large amounts of the vaccine [12]. On Apr. 12, 1955 the results of the Salk vaccine trials showed the vaccine was 80–90% effective and the US government licensed the IPV polio vaccine the same day [12].

The vaccination program was suspended on May 8, 1955 to investigate paralysis resulting from the vaccine injection; changes to the production method were made and vaccination resumed on May 27, 1955 [12]. The number of paralytic polio cases decreased from 28,985 in 1955 to 72 in 1965 [13]. The last case of the disease in the United States was reported in 1993, and polio was declared eliminated in the western hemisphere on Sep. 29, 1994 by the Pan American Health Organization [27, 12].

1975 World Health Organization poster promoting vaccination. Kindly provided by the National Library of Medicine History of Medicine Collection titled *Immunize and Protect Your Child.*

54.1.3 *National Childhood Vaccine Injury Act* and Vaccine Adverse Event Reporting System

In 1986 the *National Childhood Vaccine Injury Act* [14] was passed in response to a large number of lawsuits filed claiming vaccines were causing adverse reactions including brain damage and death [15]. The act shielded medical professionals and vaccine manufacturers from liability if an individual suffered injury from receiving vaccines. The act mandated that vaccine injury claims be filed with the

US Court of Federal Claims rather than filed directly against physicians or vaccine manufacturers in civil court. Unlike civil court, people filing injury claims are not required to prove negligence or failure to warn; they only need to prove that a vaccine caused injury [16].

On Oct. 1, 1988, the National Vaccine Injury Compensation Program (VICP) was created under the *National Childhood Vaccine Injury Act* [17]. The VICP was "established to ensure an adequate supply of vaccines, stabilize vaccine costs, and establish and maintain an accessible and efficient forum for individuals found to be injured by certain vaccines" [17]. Between 1989 and July 1, 2014, 3,645 compensation awards have been made (amounting to over $2.7 billion in awards and $113.2 million to cover legal costs) and 9,786 claims have been dismissed (amounting to $62.8 million paid to 4,925 dismissed claimants to cover legal costs) [17].

Subsequently, in 1990 the CDC and FDA created the Vaccine Adverse Event Reporting System (VAERS). VAERS collects information about adverse events via reports filed by anyone, including medical professionals and family members [18]. VAERS receives about 30,000 reports each year [18]. 85–90% of VAERS reports are for "mild adverse events such as fever, local reactions [such as redness at the injection site], and episodes or crying or mild irritability" [18]. The other 10–15% of VAERS reports is for "serious adverse events involving life-threatening conditions, hospitalization, permanent disability, or death, which may or may not have been caused by a vaccine" [18].

1920s school children getting diphtheria vaccination. Kindly provided by the College of Physicians of Philadelphia, www.historyofvaccines.org.

In 1993, the US Congress passed the *Comprehensive Childhood Immunization Act of 1993* [19] that created the Vaccines for Children (VFC) program to provide vaccinations free of charge to children in need in order to increase the number of vaccinated children.

54.1.4 Andrew Wakefield and the Autism Controversy

In Feb. 1998 Lancet published an article by Andrew Wakefield, MD, titled "Ileal-Lymphoid-Nodular Hyperplasia, Non-Specific Colitis, and Pervasive Developmental Disorder in Children" [20]. The article claimed "Rubella virus is associated with autism and the combined measles, mumps, and rubella [MMR] vaccine… has also been implicated" [20]. Anti-vaccination groups and parents began using Wakefield's article as rationale to opt-out of vaccinating their children. Between 2003 and 2012, Brian Deer, an investigative reporter, examined the story and published 36 articles which accused Wakefield of "falsifying medical histories of children and essentially concocting a picture, which was the picture he was contracted to find by lawyers hoping to sue vaccine manufacturers and to create a vaccine scare" [21]. On Mar. 3, 2004 ten of the twelve co-authors of Wakefield's article released a "Retraction of an Interpretation" in Lancet, stating "We wish to make it clear that in this paper no causal link was established between MMR vaccine and autism as the data were insufficient" [22]. Lancet retracted Wakefield's article on Feb. 2, 2010, stating "it has become clear that several elements of the 1998 paper by Wakefield et al are incorrect" [23]. On Jan. 5, 2011, the British Journal of Medicine published an article stating that Wakefield received over $674,000 from lawyers and that, of 12 children examined, five had developmental problems before being vaccinated and three never had autism [21, 24]. As a result, on May 24, 2011, Britain stripped Wakefield of his medical license, stating Wakefield had "abused his position of trust" and "brought the medical profession into disrepute" [21]. Wakefield contends that the investigation of his work is part of a conspiracy to "discredit and silence his research" in order to "shield the government from exposure on the vaccine scandal" [25].

54.1.5 Thimerosal and Autism

On July 9, 1999, in response to growing concern over a link between vaccination and autism, the American Academy of Pediatrics (AAP) and the US Public Health Service (PHS) recommended that thimerosal be removed from vaccines "as soon as possible". However, they also stated, "there are no data or evidence of any harm caused by the level of exposure that some children may have encountered in following the existing immunization schedule" and that "the large risks of not vaccinating children far outweigh the unknown and probably much smaller risk, if any, of cumulative exposure to thimerosal-containing vaccines over the first 6 months of life" [26]. In May 2003, Representative Dan Burton (R-IN) released a report titled "Mercury in Medicine – Taking Unnecessary Risks" in which

he requested that the FDA remove thimerosal from the flu vaccine and recommended independent research on the link between thimerosal in vaccines and autism [27].

1977 Star Wars vaccination promotion poster. Kindly provided by the National Library of Medicine History of Medicine Collection titled *Parents of Earth, 1977.*

In 2005, Robert F. Kennedy Jr. wrote an article co-published by Salon.com (June 16) and *Rolling Stone* (July 14) titled "Deadly Immunity," arguing that the 2000 Simpsonwood CDC Conference was spent "discussing how to cover up the damaging data" that there were a "staggering number of earlier studies that indicate a link between thimerosal and speech delays, attention-deficit disorder, hyperactivity, and autism" [28]. The article was corrected multiple times within days of publication, and was retracted and deleted by Salon.com and *Rolling Stone* on Jan. 16, 2011 [28]. [112] The controversy resulted in an 18-month investigation by the US Senate Committee on Health, Education, Labor and Pensions, which

concluded that Kennedy's allegation was unsubstantiated and "thimerosal was [being] voluntarily removed from childhood vaccines distributed in the United States as a precaution," prompted by a joint request by the American Academy of Pediatrics and the US Public Health Service [29]. As of 2007, vaccines for children 6 years old and younger contain no thimerosal or only trace amounts, except for inactivated flu vaccines, which are available in both thimerosal-containing and preservative-free versions. By Nov. 30, 2009, the mercury-based preservative thimerosal had been phased out of all vaccines in the United States with the exception of certain influenza, meningococcal, and tetanus vaccines [30].

On Aug. 27, 2010 the US Court of Appeals for the Federal Circuit ruled (3-0) that there is no link between vaccination and autism in the case of Cedillo v. Secretary of Health and Human Services [31]. The decision upheld two earlier rulings: a 2007 ruling by the United States Court of Federal Claims Office of Special Masters and an affirmation of that ruling by the Court of Federal Claims. On Feb. 22, 2011, the US Supreme Court ruled (6-2) in the case of *Bruesewitz v. Wyeth* [32] that vaccine injury claims must continue to be filed with the US Court of Federal Claims set up under the *National Childhood Vaccine Injury Act of 1986*, and cannot be filed directly against physicians or vaccine manufacturers in civil court.

On Aug. 25, 2011 the Institute of Medicine (IOM) issued a report, "Adverse Effects of Vaccines: Evidence and Causality" [33]. The report brief stated that "evidence favors rejection of a causal relationship" between the Measles-Mumps-Rubella (MMR) vaccine and autism [34]. The Cochrane Collaboration, in a Feb. 15, 2012 independent investigation of studies on vaccines and autism concluded, "We could assess no significant association between MMR immunization and the following conditions: autism, asthma, leukanemia, hay fever, type 1 diabetes, gait disturbance, Crohn's disease, demyelinating diseases, or bacterial or viral infections" [35]. A study published in the *Proceedings of the National Academy of Sciences of the United States of America* (PNAS) on Oct. 6, 2015 found that infant rhesus macaques, whose physiology closely resembles human infants, injected with human childhood vaccines from the early 1990s (that contained thimerosal) and from 2008 (after thimerosal was phased out of childhood vaccines) exhibited no behavioral or neuropathological changes that could be linked to autism [114, 115]. A Mar. 5, 2019 study published in the *Annals of Internal Medicine*, studied 657,461 children over ten years in Denmark, and concluded, "The study strongly supports that MMR vaccination does not increase the risk for autism, does not trigger autism in susceptible children, and is not associated with clustering of autism cases after vaccination" [122].

54.1.6 Potential Consequences for Unvaccinated Children and Their Parents

State laws in North Carolina, Ohio, and New York allow the public school system to suspend children who are not vaccinated [36–38]. Approximately 2,000 seventh- to twelfth-grade children not vaccinated against pertussis (whooping cough) were

barred from attending classes in San Francisco in 2011 [39]. On June 22, 2014, federal Judge William Kuntz upheld New York state law barring unvaccinated children from public school when other children have chickenpox [38, 40]. Many pediatricians will not treat children who have not been vaccinated [41]. Some legal experts believe that parents who do not vaccinate their children should be subject to criminal prosecution (including criminally negligent homicide and monetary damages) if their unvaccinated children infect and harm other children who are too young or immunocompromised to receive vaccines [42].

54.1.7 Eradication and Elimination of Disease

Elimination means that the disease is not present in a region, while eradication means that the disease does not exist anywhere globally. Smallpox was declared globally eradicated in 1980, the first and only disease to be eradicated thus far. Polio was declared eliminated in the United States in 1979 and in the Western Hemisphere in 1994 [120]. Rubella was declared eliminated in the Americas on Apr. 29, 2015, and measles on Sep. 27, 2016 [116, 119]. The World Health Organization (https://www.britannica.com/topic/World-Health-Organization) states that eradication and elimination is the product of vaccination programs that promote high rates of inoculation, while those opposed to vaccination state that better sanitation and clean water led to the elimination of the diseases [117, 118].

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Vaccine-Preventable Diseases and the Vaccines that Prevent Them

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Chickenpox	**Varicella vaccine** protects against chickenpox.	Air, direct contact	Rash, tiredness, headache, fever	Infected blisters, bleeding disorders, encephalitis (brain swelling), pneumonia (infection in the lungs), death
Diphtheria	**DTaP*** vaccine protects against diphtheria.	Air, direct contact	Sore throat, mild fever, weakness, swollen glands in neck	Swelling of the heart muscle, heart failure, coma, paralysis, death
Hib	**Hib** vaccine protects against *Haemophilus influenzae* type b.	Air, direct contact	May be no symptoms unless bacteria enter the blood	Meningitis (infection of the covering around the brain and spinal cord), intellectual disability, epiglottitis (life-threatening infection that can block the windpipe and lead to serious breathing problems), pneumonia (infection in the lungs), death
Hepatitis A	**HepA** vaccine protects against hepatitis A.	Direct contact, contaminated food or water	May be no symptoms, fever, stomach pain, loss of appetite, fatigue, vomiting, jaundice (yellowing of skin and eyes), dark urine	Liver failure, arthralgia (joint pain), kidney, pancreatic, and blood disorders, death
Hepatitis B	**HepB** vaccine protects against hepatitis B.	Contact with blood or body fluids	May be no symptoms, fever, headache, weakness, vomiting, jaundice (yellowing of skin and eyes), joint pain	Chronic liver infection, liver failure, liver cancer, death
Influenza (Flu)	**Flu vaccine** protects against influenza.	Air, direct contact	Fever, muscle pain, sore throat, cough, extreme fatigue	Pneumonia (infection in the lungs), bronchitis, sinus infections, ear infections, death
Measles	**MMR**** vaccine protects against measles.	Air, direct contact	Rash, fever, cough, runny nose, pink eye	Encephalitis (brain swelling), pneumonia (infection in the lungs), death

(*Continued*)

(*Continued*)

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Mumps	**MMR**** vaccine protects against mumps.	Air, direct contact	Swollen salivary glands (under the jaw), fever, headache, tiredness, muscle pain	Meningitis (infection of the covering around the brain and spinal cord), encephalitis (brain swelling), inflammation of testicles or ovaries, deafness, death
Pertussis	**DTaP*** vaccine protects against pertussis (whooping cough).	Air, direct contact	Severe cough, runny nose, apnea (a pause in breathing in infants)	Pneumonia (infection in the lungs), death
Polio	**IPV** vaccine protects against polio.	Air, direct contact, through the mouth	May be no symptoms, sore throat, fever, nausea, headache	Paralysis, death
Pneumo-coccal	**PCV13** vaccine protects against pneumococcus.	Air, direct contact	May be no symptoms, pneumonia (infection in the lungs)	Bacteremia (blood infection), meningitis (infection of the covering around the brain and spinal cord), death
Rotavirus	**RV** vaccine protects against rotavirus.	Through the mouth	Diarrhea, fever, vomiting	Severe diarrhea, dehydration, death
Rubella	**MMR**** vaccine protects against rubella.	Air, direct contact	Sometimes rash, fever, swollen lymph nodes	Very serious in pregnant women—can lead to mis-carriage, stillbirth, premature delivery, birth defects
Tetanus	**DTaP*** vaccine protects against tetanus.	Exposure through cuts in skin	Stiffness in neck and abdominal muscles, difficulty swallowing, muscle spasms, fever	Broken bones, breathing difficulty, death

*DTaP combines protection against diphtheria, tetanus, and pertussis.

**MMR combines protection against measles, mumps, and rubella.

This schedule is recommended by the Advisory Committee on Immunization Practices (ACIP; https://www.cdc.gov/vaccines/acip/index.html) and approved by the Centers for Disease Control and Prevention (CDC; https://www.cdc.gov/index.htm), American Academy of Pediatrics (AAP; http://www.aap.org/), and American Academy of Family Physicians (AAFP; http://www.aafp.org/).

54.2 Common Ingredients in U.S. Licensed Vaccines

The vast majority of the over one billion doses of vaccines manufactured worldwide each year are given to healthy babies, children and adults. Thus, it is critical that vaccines be demonstrated to be safe and effective. FDA requires that vaccines undergo a rigorous and extensive development program in the laboratory, as well as in animal studies and human clinical trials, to determine their safety and effectiveness. Highly trained FDA scientists and clinicians carefully evaluate all of the information in a marketing application and make a determination whether to license (approve) a vaccine before it can be used in the United States. Prior to licensure, as part of FDA's evaluation, FDA takes all of the ingredients of a vaccine into account, including the active ingredients as well as other substances. After FDA approves a vaccine, FDA continuously monitors its safety.

54.2.1 Why Is Aluminum in Some Vaccines?

Aluminum salts are incorporated into some vaccine formulations as an adjuvant. An adjuvant is a substance added to some vaccines to enhance the immune response of vaccinated individuals. The aluminum salts in some U.S. licensed vaccines are aluminum hydroxide, aluminum phosphate, alum (potassium aluminum sulfate), or mixed aluminum salts. For example: aluminum salts are used in DTaP vaccines, the pneumococcal conjugate vaccine, and hepatitis B vaccines.

Aluminum adjuvant containing vaccines have a demonstrated safety profile of over six decades of use and have only uncommonly been associated with severe local reactions. A study conducted by FDA (https://wayback.archive-it.org/7993/20170405003134/https:/www.fda.gov/BiologicsBloodVaccines/ScienceResearch/ucm284520.htm) determined that the risk to infants posed by the total aluminum exposure received from the entire recommended series of childhood vaccines over the first year of life is extremely low. This study provided additional scientific information confirming that the benefits of aluminum-containing vaccines administered during the first year of life outweigh any theoretical concerns about the potential effect of aluminum on infants. Of note, the most common source of exposure to aluminum is from eating food or drinking water.

54.2.2 Are Other Adjuvants Used in FDA-Approved Vaccines?

Yes. Cervarix, a vaccine to prevent cervical cancer caused by human papillomavirus types 16 and 18, includes AS04 in its formulation. AS04 is a combination of aluminum hydroxide and monophosphoryl lipid A (MPL). MPL is a purified fat-like substance. The manufacturer no longer markets Cervarix in the United States.

One vaccine for the prevention of H5N1 influenza, commonly referred to as avian influenza or "bird flu," contains the adjuvant AS03, an oil-in-water emulsion. The AS03 adjuvant is made up of the oily compounds, D,L-alpha-tocopherol (vitamin E) and squalene (https://www.fda.gov/vaccines-blood-biologics/vaccine-safety-availability/influenza-h5n1-virus-monovalent-vaccine-adjuvanted-manufactured-id-biomedical-corporation-questions#squalene), and an emulsifier, polysorbate

80, which helps ingredients mix together and keep them from separating, and water containing small amounts of salts. The vaccine is not commercially available, but included within the U.S. government's National Stockpile if public health officials determine it is needed.

Fluad, a vaccine for the prevention of seasonal influenza in adults 65 years of age and older, includes MF59, also an oil-in-water emulsion of squalene (https://www.fda.gov/vaccines-blood-biologics/vaccine-safety-availability/influenza-h5n1-virus-monovalent-vaccine-adjuvanted-manufactured-id-biomedical-corporation-questions#squalene) oil.

Heplisav-B, a vaccine for the prevention of infection caused hepatitis B virus in adults 18 years of age and older, includes CpG 1018, an adjuvant based on synthetic DNA sequences.

Shingrix, a vaccine for the prevention of shingles in adults 50 years of age and older, includes AS01B. AS01B is made of up MPL, a purified fat-like substance, and QS-21 which is purified from the bark of the *Quillaja saponaria* (soap bark) evergreen tree native to central Chile.

54.2.3 How Does FDA Evaluate Adjuvants for Safety and Efficacy?

When evaluating a vaccine for safety and efficacy, FDA considers adjuvants as a component of the vaccine; they are not licensed separately.

54.2.4 Why Are Antibiotics in Some Vaccines?

Certain antibiotics may be used in some vaccine production to help prevent bacterial contamination during manufacturing. As a result, small amounts of antibiotics may be present in some vaccines. Because some antibiotics can cause severe allergic reactions in those children allergic to them (such as hives, swelling at the back of the throat, and low blood pressure), some parents are concerned that antibiotics contained in vaccines might be harmful. However, antibiotics most likely to cause severe allergic reactions (e.g., penicillins, cephalosporins and sulfa drugs) are not used in vaccine production, and therefore are not contained in vaccines.

Examples of antibiotics used during vaccine manufacture include neomycin, polymyxin B, streptomycin and gentamicin. Some antibiotics used in vaccine production are present in the vaccine, either in very small amounts or they are undetectable. For example, antibiotics are used in some production methods for making inactivated influenza virus vaccines. They are used to reduce bacterial growth in eggs during processing steps, because eggs are not sterile products. The antibiotics that are used are reduced to very small or undetectable amounts during subsequent purification steps. The very small amounts of antibiotics contained in vaccines have not been clearly associated with severe allergic reactions.

54.2.5 Why Is Formaldehyde in Some Vaccines?

Formaldehyde has a long history of safe use in the manufacture of certain viral and bacterial vaccines. It is used to inactivate viruses so that they don't cause

disease (e.g., polio virus used to make polio vaccine) and to detoxify bacterial toxins, such as the toxin used to make diphtheria vaccine. Formaldehyde is diluted during the vaccine manufacturing process, but residual quantities of formaldehyde may be found in some current vaccines. The amount of formaldehyde present in some vaccines is so small compared to the concentration that occurs naturally in the body that it does not pose a safety concern.

Formaldehyde is also produced naturally in the human body as a part of normal functions of the body to produce energy and build the basic materials needed for important life processes. This includes making amino acids, which are the building blocks of proteins that the body needs.

Formaldehyde is also found in the environment and is present in different ways. It is used in building materials, as a preservative in labs and to produce many household products.

The body continuously processes formaldehyde, both from what it makes on its own and from what it has been exposed to in the environment. When the body breaks down formaldehyde, it does not distinguish between formaldehyde from vaccines and that which is naturally produced or environmental. The amount of formaldehyde in a person's body depends on their weight; babies have lower amounts than adults. Studies have shown that for a newborn of average weight of 6–8 pounds, the amount of formaldehyde in their body is 50–70 times higher than the upper amount that they could receive from a single dose of a vaccine or from vaccines administered over time.

Excessive exposure to formaldehyde may cause cancer, but the latest research has shown that the highest risk is from the air when formaldehyde is inhaled from breathing, and occurs more frequently in people who routinely use formaldehyde in their jobs. There is no evidence linking cancer to infrequent exposure to tiny amounts of formaldehyde via injection as occurs with vaccines.

54.2.6 Why Are Sugars, Amino Acids, and Proteins Added to Some Vaccines?

These substances may be added as stabilizers. They help protect the vaccine from adverse conditions such as the freeze-drying process, for those vaccines that are freeze dried. Stabilizers added to vaccines include sugars such as sucrose and lactose, amino acids such as glycine or the monosodium salt of glutamic acid and proteins such as human serum albumin or gelatin. Sugars, amino acids and proteins are not unique to vaccines and are encountered in everyday life in the diet and are components that are in the body naturally.

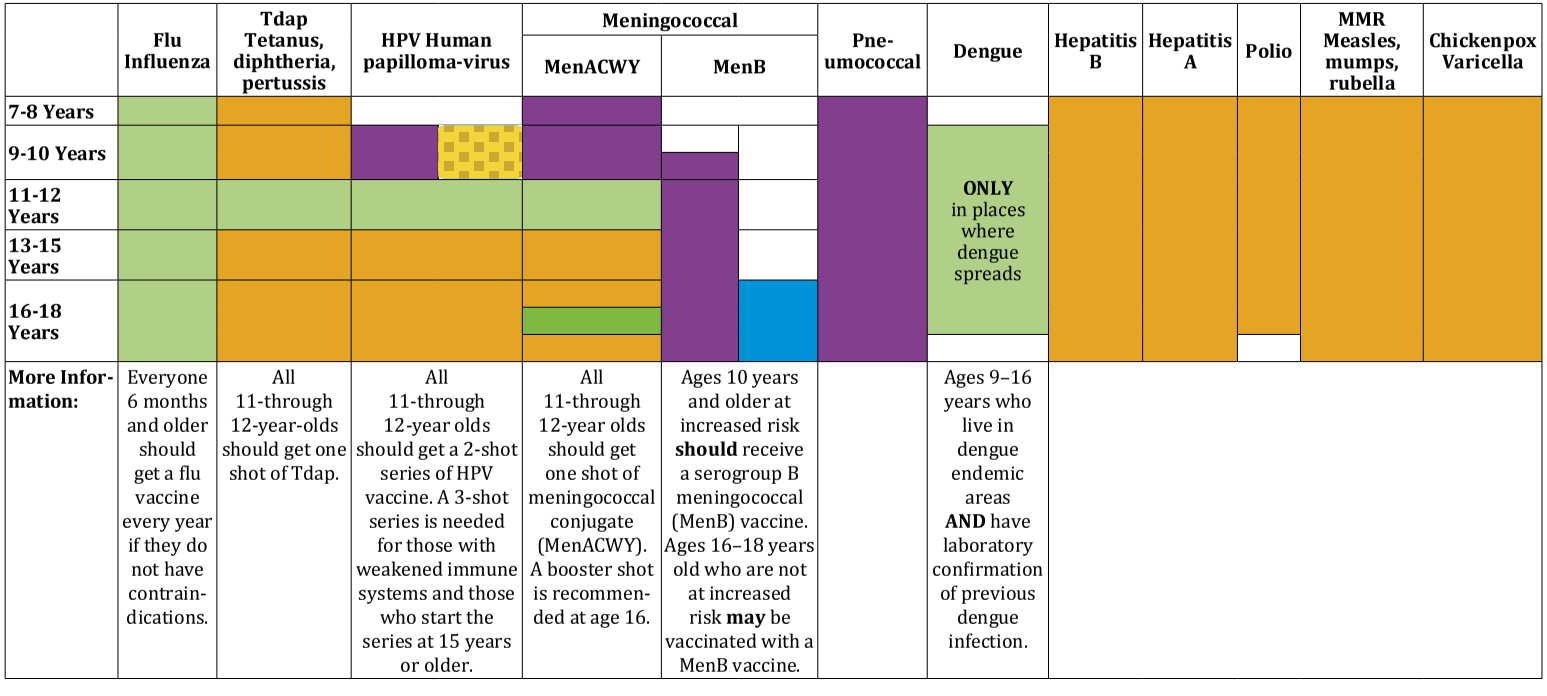
54.2.7 Why Are There Preservatives in Some Vaccines?

Preservatives are added to some vaccine formulations to prevent the growth of bacteria or fungi that may be introduced into the vaccine during its use, e.g., repeated puncture of a multi-dose vaccine vial with a needle.

54.2.8 Why Is Fetal Calf/Bovine Serum in Some Vaccines?

In the manufacture of viral vaccines, the virus may be grown in cells. These cells need a source of nutrition, which in some instances may be provided by fetal bovine serum.

Recommended Vaccinations for Children 7 to 18 Years Old, Parent-Friendly Version



Legend

- These shaded boxes indicate when the vaccine is recommended for all children unless your doctor tells you that your child cannot safely receive the vaccine.
- These shaded boxes indicate the vaccine **SHOULD** be given if a child is catching up on missed vaccines.
- These shaded boxes indicate the vaccine is recommended for children with certain health or lifestyle conditions that put them at an increased risk for serious diseases. See vaccine-specific recommendations at www.cdc.gov/vaccines/hcp/acip-recs/index.html.
- This shaded box indicates children not at increased risk **MAY** get the vaccine if they wish after speaking to a provider.
- This shade box indicates vaccination may begin in this age group.

Talk to your child's doctor or nurse about the vaccines recommended for their age. COVID-19 vaccination is recommended for ages 5 years and older.

Vaccine-Preventable Diseases and the Vaccines that Prevent Them

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Chickenpox	Varicella vaccine protects against chickenpox.	Air, direct contact	Rash, tiredness, headache, fever	Infected blisters, bleeding disorders, encephalitis (brain swelling), pneumonia (infection in the lungs), death
Dengue	Dengue vaccine protects against dengue	Bite from infected mosquito	May be no symptom, fever, headache, pain behind the eyes, rash, joint pain, body ache, nausea, loss of appetite feeling tired, abdominal pain	Severe bleeding, seizures, shock, damage to liver, heart, and lungs, death
Diphtheria	Tdap* and Td** vaccines protect against diphtheria.	Air, direct contact	Sore throat, mild fever, weakness, swollen glands in neck	Swelling of the heart muscle, heart failure, coma, paralysis, death
Hepatitis A	HepA vaccine protects against hepatitis A.	Direct contact, contaminated food or water	May be no symptoms, fever, stomach pain, loss of appetite, fatigue, vomiting, jaundice (yellowing of skin and eyes), dark urine	Liver failure, arthralgia (joint pain), kidney, pancreatic and blood disorders, death
Hepatitis B	HepB vaccine protects against hepatitis B.	Contact with blood or body fluids	May be no symptoms, fever, headache, weakness, vomiting, jaundice (yellowing of skin and eyes), joint pain	Chronic liver infection, liver failure, liver cancer, death
Human Papillomavirus	HPV vaccine protects against human papillomavirus.	Direct skin contact	May be no symptoms, genital warts	Cervical, vaginal, vulvar, penile, anal, oropharyngeal cancers
Influenza (Flu)	Flu vaccine protects against influenza.	Air, direct contact	Fever, muscle pain, sore throat, cough, extreme fatigue	Pneumonia (infection in the lungs), bronchitis, sinus infections, ear infections, death
Measles	MMR*** vaccine protects against measles.	Air, direct contact	Rash, fever, cough, runny nose, pink eye	Encephalitis (brain swelling), pneumonia (infection in the lungs), death

(*Continued*)

(*Continued*)

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Meningococcal Disease	MenACWY and MenB vaccines protect against meningococcal disease.	Air, direct contact	Sudden onset of fever, headache, and stiff neck, dark purple rash	Loss of limb, deafness, nervous system disorders, developmental disabilities, seizure disorder, stroke, death
Mumps	MMR*** vaccine protects against mumps.	Air, direct contact	Swollen salivary glands (under the jaw), fever, headache, tiredness, muscle pain	Meningitis (infection of the covering around the brain and spinal cord), encephalitis (brain swelling), inflammation of testicles or ovaries, deafness, death
Pertussis	Tdap* vaccine protects against pertussis.	Air, direct contact	Severe cough, runny nose, apnea (a pause in breathing in infants)	Pneumonia (infection in the lungs), death
Polio	Polio vaccine protects against polio.	Air, direct contact, through the mouth	May be no symptoms, sore throat, fever, nausea, headache	Paralysis, death
Pneumococcal Disease	Pneumococcal vaccine protects against pneumococcal disease.	Air, direct contact	May be no symptoms, pneumonia (infection in the lungs)	Bacteremia (blood infection), meningitis (infection of the covering around the brain and spinal cord), death
Rubella	MMR*** vaccine protects against rubella.	Air, direct contact	Sometimes rash, fever, swollen lymph nodes	Very serious in pregnant women—can lead to miscarriage, stillbirth, premature delivery, birth defects
Tetanus	Tdap* and Td** vaccines protect against tetanus.	Exposure through cuts on skin	Stiffness in neck and abdominal muscles, difficulty swallowing, muscle spasms, fever	Broken bones, breathing difficulty, death

*Tdap combines protection against diphtheria, tetanus, and pertussis.

**Td combines protection against diphtheria and tetanus.

***MMR combines protection against measles, mumps, and rubella.

This schedule is recommended by the Advisory Committee on Immunization Practices (ACIP; https://www.cdc.gov/vaccines/acip/index.html) and approved by the Centers for Disease Control and Prevention (CDC; https://www.cdc.gov/index.htm), American Academy of Pediatrics (AAP; http://www.aap.org/), and American Academy of Family Physicians (AAFP; http://www.aafp.org/).

54.3 Understanding the Vaccine Adverse Event Reporting System (VAERS)

- The Vaccine Adverse Event Reporting System (VAERS) is one component of the United States' comprehensive vaccine safety monitoring system.
- VAERS reports are monitored carefully by the Centers for Disease Control and Prevention (CDC) and the Food and Drug Administration (FDA).
- Reports of adverse events (possible side effects) after vaccination do not mean that the reported problem was caused by a vaccine. Reports are signals that alert scientists of possible cause-and- effect relationships that need to be investigated.
- Anyone can submit a report to VAERS including health care professionals, vaccine manufacturers, vaccine recipients, and parents or family members of people who have received a vaccine.

What Is VAERS?

VAERS is a national vaccine safety surveillance program overseen by CDC and FDA. VAERS collects and analyzes reports of adverse events that happen after vaccination. Each year, VAERS receives around 30,000 reports. Most of these reports describe known, mild side effects such as fever. Scientists at CDC and FDA monitor VAERS reports closely to identify reported adverse events that need to be studied further. Sometimes, it is only after a vaccine has been approved and used broadly that rare side effects can be detected by monitoring systems such as VAERS.

How Are the VAERS Data Used?

VAERS scientists look for unusually high numbers of reports of an adverse event after a particular vaccine or a new pattern of adverse events. If scientists see either of these situations, focused studies in other systems are done to determine if the adverse event is or is not a side effect of the vaccine. Information from VAERS and vaccine safety studies is shared with the public. Throughout the process of monitoring VAERS, conducting studies, and sharing findings, appropriate actions are taken to protect the public's health.

For example, if VAERS identifies a mild adverse event that is verified as a side effect in a focused study, this information is reviewed by CDC, FDA, and vaccine policy makers. In this situation, the vaccine may continue to be recommended if the disease-prevention benefits from vaccination outweigh the risks of a newly found side effect.

Information about newly found side effects is added to the vaccine's package insert that lists safety information. Newly found side effects also are added to the Vaccine Information Statement (VIS) for that vaccine. If serious side effects are found, and if the risks of the vaccine side effect outweigh the benefits, the recommendation to use the vaccine is withdrawn.

Vaccine Information Statements (VISs) are information sheets produced by the Centers for Disease Control and Prevention (CDC) that explain to vaccine recipients, their parents, or their legal representatives both the benefits and risks of a vaccine. Federal law requires that VISs be handed out whenever (before each dose) certain vaccinations are given.

Adverse events reported to VAERS are not necessarily side effects caused by vaccination. An *adverse event* is a health problem that happens after vaccination that may or may not be caused by a vaccine. These events may require further investigation. By definition, a *side effect* has been shown to be linked to a vaccine by scientific studies.

Before the FDA licenses (approves) a vaccine for use, the vaccine must be tested with volunteers during clinical trials to make sure it is safe and effective. Sometimes side effects show up in clinical trials. Most often side effects found in clinical trials are minor, such as possible pain at the injection site, and the vaccine is licensed because the disease-prevention benefits outweigh the risk of getting the side effect.

As part of the United States' comprehensive vaccine safety monitoring system, VAERS detects rare vaccine adverse events, signaling to scientists that focused studies are needed to determine whether the adverse event is a side effect or if there is no medical link.

Vaccines Are Tested before They Are Used, So Why Are There Possible Unknown Side Effects?

When vaccines are ready for tests in humans, they are tested on thou sands to tens of thousands of volunteers. However, even this large number is not always enough to find rare side effects, such as a one-in-a-million side effect. So, VAERS is needed to constantly look for possible side effects that might not have been detected previously.

Are All Events Reported to VAERS Caused by Vaccinations?

VAERS data alone usually cannot be used to answer the question, "Does a certain vaccine cause a certain side effect?" This is mainly because adverse events reported to VAERS may or may not be caused by vaccines. There are reports in VAERS of common conditions that are found shortly after vaccination, often related by chance alone, and investigations find no medical link between vaccination and the condition.

To know if a vaccine causes a side effect, scientists must know whether the adverse event is occurring after vaccination with a particular vaccine more often

than would be expected without vaccination. They also need to consider whether the association between the vaccine and the adverse event is consistent with existing medical knowledge about how vaccines work in the body.

Who Can Report to VAERS?

Anyone can submit a report to VAERS including parents, patients, and health care professionals. Vaccine manufacturers who receive reports of adverse events also report the information to VAERS. FDA and CDC encourage anybody who experiences any adverse event after vaccination to report to VAERS. Individuals completing a report can work with a health care professional to make sure they fill out the report form completely. By working together, health care profession als and patients/parents can provide FDA and CDC with data that will be most useful and accurate for examining possible trends.

Why Should I Report to VAERS?

Reporting to VAERS gives valuable information that helps CDC and FDA ensure that vaccines are very safe. If a previously unknown adverse event does come up, timely reports will help scientists find it and determine how to best address the issue.

How Do I Report to VAERS?

Reports can be submitted online, by fax, or by mail. To report to VAERS online, go to https://vaers.hhs.gov/esub/step1 and follow the 5 steps. Or, to print out the form to return it by fax or mail, go to https://vaers.hhs.gov/resources/vaers_form.pdf. To request a form by phone, call 1-800-822-7967. Forms may be returned by fax to 1-877-721-0366 or mailed to VAERS, P.O. Box 1100, Rockville, MD 20849-1100. VAERS staff may call for more information.

What Events Should I Report to VAERS?

VAERS encourages the reporting of all adverse events that occur after administration of any vaccine licensed in the United States.

How Do I Find Out If a Vaccine Adverse Event Has Been Reported to VAERS?

VAERS data is available to the public for download at http://vaers.hhs.gov/data/index. You may also request information about adverse events reported to VAERS by sending a fax to 301-443-1726, by call ing 301-827-6500, or by writing to: Food and Drug Administration, Freedom of Information Staff (HFI-35), 5600 Fishers Lane, Rockville, MD 20857.

Remember, just because an adverse event or condition has been reported does not prove that the adverse event is caused by vaccination. Parents who are concerned about vaccine side effects should talk to their child's health care professional.

54.4 Science Summary: CDC Studies on Thimerosal in Vaccines

The evidence is clear: thimerosal is not a toxin in vaccines, but merely a preservative, preventing contamination, that has been used in vaccines for decades. This fact sheet provides a summary of thimerosal-related studies that were conducted by CDC or with CDC's involvement.

Study	Summary and citation
Brain function, behavior, language, coordination and thimerosal Thimerosal exposure in early life and neuropsychological outcomes 7–10 years later	This study assessed whether prenatal thimerosal exposure or thimerosal exposure between birth and 7 months of age was associated with seven specific neuropsychological outcomes in children ages 7–10 years. The study found no associations with thimerosal and general intellectual functioning, verbal memory, fine motor coordination, executive functioning, behavior regulation and language. There was a small association between early thimerosal exposure and the presence of tics in boys, but no association among girls. It is necessary to perform additional studies examining the association between thimerosal and tics using more reliable and valid measures of tics. • Barile JP, Kuperminc GP, Weintraub ES, Mink JW, Thompson WW. Thimerosal exposure in early life and neuropsychological outcomes 7–10 years later. *J Pediatric Psy chol*. 2012 January/February; 37(1): 106–118
Thimerosal exposure in the womb and in infancy: Prenatal and infant exposure to thimerosal from vaccines and immunoglobulins and risk of autism	This study compared children with Autism to those without, and looked at prenatal and infant exposure to thimerosal from vaccines. This study found no difference in exposure to thimerosal between children with and without Autism. • Price CS, Thompson WW, Goodson B, Weintraub ES, Croen LA, et al. Prenatal and infant exposure to thimerosal from vaccines and immunoglobulins and risk of autism. *Pediatrics*. Epub 2010 Sep 13.
Long-term results of thimerosal exposure Neuropsychological performance 10 years after immunization in infancy with thimerosal-containing vaccines	CDC funded this follow-up study in Italy that compared neuropsychological outcomes of children who were randomly assigned to receive one of two forms of diphtheria-tetanus-acellular pertussis vaccine (DTaP) in the first year of life: one containing thimerosal and the other containing 2-phenoxyethanol. Ten years after vaccination, the two groups were tested on 24 neuropsychological outcomes. Results show that thimerosal in vaccines is not harmful to children. • Tozzi AE, Bisiacchi P, Tarantino V, De Mei B, D'Elia L, et al. Neuropsychological performance 10 years after immunization in infancy with thimerosal-containing vaccines. *Pediatrics*. 2009; 123(2): 475–482.

Study	Summary and citation
Thimerosal in US, UK, and Denmark Thimerosal-containing vaccines: evidence versus public apprehension	Three large epidemiological studies that analyzed data from US health maintenance organizations, the UK General Practice Research Database, and the entire country of Denmark failed to find an association between exposure to thimerosal-containing vaccines and autism. • DeStefano F. Thimerosal-containing vaccines: evidence versus public apprehension. *Exp Opin Drug Safety*. 2009; 8(2): 1–4.
Thimerosal and children's flu shots: Inactivated influenza vaccine (IIV) in children <2 years of age: Examination of selected adverse events reported to the Vaccine Adverse Event Reporting System (VAERS) after thimerosal-free or thimerosal-containing vaccine	This study measured the proportion of injection site reactions (ISR), rash, and infections reported to the Vaccine Adverse Event Reporting System (VAERS) after testing three versions of an inactivated influenza vaccine (IIV) in children less than 2 years of age. The three versions of IIV included thimerosal-free, thimerosal-including, and ones in which the presence of thimerosal could not be determined. The study found no difference between the proportion of ISR, rash, or infections in all three versions of IIV. • McMahon AW, Iskander JK, Haber P, Braun MM, Ball R. Inactivated influenza vaccine (IIV) in children <2 years of age: examination of selected adverse events reported to the Vaccine Adverse Event Reporting System (VAERS) after thimerosal-free or thimerosal-containing vaccine. *Vaccine*. 2008 Jan; 26(3): 427–429.
Thimerosal and neurodevelopmental disorders Early Thimerosal Exposure and Neuropsychological Outcomes at 7 to 10 Years	This study measured neurodevelopmental disorders in children. The study found only a few statistically significant associations between exposure from thimerosal and neuropsychological functioning. Results of this study show no link between thimerosal-containing vaccines and neurodevelopmental disorders in children. • Thompson WW, Price C, Goodson B, Shay DK, Benson P, et al. Early thimerosal exposure and neuropsychological outcomes at 7 to 10 Years. *N Engl J Med*. 2007; 357: 1281–1292.
Thimerosal and health outcomes Safety of Thimerosal-containing vaccines: a two-phased study of computerized health maintenance organization databases	This study looked for possible links between thimerosal-containing vaccines and a variety of health problems. CDC and research partners found statistically significant associations between thimerosal and language delays and tics. However, the associations were weak and were not consistent between study populations. The study found no link between thimerosal and Autism. • Verstraeten T, Davis RL, DeStefano F, Lieu TA, Rhodes PH, et al. Safety of thimerosal-containing vaccines: a two-phased study of computerized health maintenance organization databases. *Pediatrics*. 2003 Nov; 112(5): 1039–48.

Study	Summary and citation
Effects of removing thimerosal Autism and thimerosal-containing vaccines: lack of consistent evidence for an association. Effects of removing thimerosal	This study compared the prevalence and incidence of autism in California, Sweden, and Denmark with average exposures to thimerosal-containing vaccines between the mid-1980s and the late-1990s. In California, thimerosal in vaccines increased throughout the 1990s. In contrast, Sweden and Denmark decreased thimerosal in the late 1980s and eliminated thimerosal in the early 1990s. In all three countries, the incidence and prevalence of autism increased significantly between the mid-1980s and the late 1990s. These findings indicate that increased exposure to thimerosal-containing vaccines is not responsible for increased rates of autism. • Stehr-Green P, Tull P, Stellfeld M, Mortenson PB, Simpson D. Autism and thimerosal-containing vaccines: lack of consistent evidence for an association. *Am J Prev Med*. 2003 Aug; 25(2): 101–106.
Comparing outcomes with and without thimerosal Autism and thimerosal-containing vaccines: lack of consistent evidence for an association	In 1992, Denmark and Sweden stopped using thimerosal in vaccines. This study compared the rate of Autism in these countries before and after thimerosal was removed. In both countries, Autism rates increased between 1987 and 1999. If thimerosal exposure was related to Autism, one would expect that Autism rates would decrease after 1992 when children were no longer being exposed. • Stehr-Green P, Tull P, Stellfeld M, Mortenson PB, Simpson D. Autism and thimerosal-containing vaccines: lack of consistent evidence for an association. *Am J Prev Med*. 2003 Aug; 25(2): 101–6.

54.5 Pregnancy and Vaccination

You probably know that when you are pregnant, you share everything with your baby. That means when you get vaccines, you aren't just protecting yourself—you are giving your baby some early protection too. You should get a flu shot and

whooping cough vaccine (also called Tdap) during each pregnancy to help protect yourself and your baby.

54.5.1 Whooping Cough Vaccine

Whooping cough (or pertussis) can be serious for anyone, but for your newborn, it can be life- threatening. Up to 20 babies die each year in the United States due to whooping cough. About half of babies younger than 1 year old who get whooping cough need treatment in the hospital. The younger the baby is when he or she gets whooping cough, the more likely he or she will need to be treated in a hospital. It may be hard for you to know if your baby has whooping cough because many babies with this disease don't cough at all. Instead, it can cause them to stop breathing and turn blue.

*When you get the whooping cough vaccine during your pregnancy, your body will create protective antibodies and pass some of them to your baby before birth***.** These antibodies will provide your baby some short-term, early protection against whooping cough.

Learn more at www.cdc.gov/pertussis/pregnant/.

54.5.2 Flu Vaccine

Changes in your immune, heart, and lung functions during pregnancy make you more likely to get seriously ill from the flu. Catching the flu also increases your chances for serious problems for your developing baby, including premature labor and delivery. *Get the flu shot if you are pregnant during flu season—it's the best way to protect yourself and your baby for several months after birth from flu- related complications.*

Flu seasons vary in their timing from season to season, but CDC recommends getting vaccinated by the end of October, if possible. This timing helps protect you before flu activity begins to increase.

Find more on how to prevent the flu by visiting www.cdc.gov/flu/.

54.5.3 Keep Protecting Your Baby after Pregnancy

Your ob-gyn or midwife may recommend you receive some vaccines right after giving birth. Postpartum vaccination will help protect you from getting sick and you will pass some antibodies to your baby through your breastmilk. Vaccination after pregnancy is especially important if you did not receive certain vaccines before or during your pregnancy.

Keep in mind that many diseases rarely seen in the United States are still common in other parts of the world. Talk to your ob-gyn or midwife about vaccines if you are planning international travel during your pregnancy. More information is available at www.cdc.gov/travel/.

Your baby will also start to get his or her own vaccines to protect against serious childhood diseases. You can learn more about CDC's recommended immunization schedule for children and the diseases vaccines can prevent at www.cdc.gov/vaccines/parents/.

Even before becoming pregnant, make sure you are up to date on all your vaccines. This will help protect you and your child from serious diseases. For example, rubella is a contagious disease that can be very dangerous if you get it while you are pregnant. In fact, it can cause a miscarriage or serious birth defects. The best protection against rubella is MMR (measles-mumps-rubella) vaccine, but if you aren't up to date, you'll need it before you get pregnant.

54.6 Understanding Thimerosal, Mercury, and Vaccine Safety

- Thimerosal is a mercury-containing compound that prevents the growth of dangerous bacteria and fungus. It is used as a preservative for flu vaccines in multi-dose vials, to keep the vaccine free from contamination. Thimerosal is also used during the manufacturing process for some vaccines to prevent the growth of microbes.
- In 1999, as a precautionary measure, the U.S. Public Health Service recommended removing thimerosal as a preservative from vaccines to reduce mercury exposure among infants as much as possible.
- Today, except for some flu vaccines in multi-dose vials, no recommended childhood vaccines contain thimerosal as a preservative.
- In all other recommended childhood vaccines, no thimerosal is present, or the amount of thimerosal is close to zero.
- No reputable scientific studies have found an association between thimerosal in vaccines and autism.
- There are two different compounds that contain mercury: ethylmercury and methylmercury. The low levels of ethylmercury in vaccines are broken down by the body differently and clear out of the blood more quickly than methylmercury

54.6.1 What Is Thimerosal? Is It the Same as Mercury?

Thimerosal is a compound that contains mercury. Mercury is a metal found naturally in the environment.

54.6.2 Why Is Thimerosal Used in Some Vaccines?

Because it prevents the growth of dangerous microbes, thimerosal is used as a preservative in multi-dose vials of flu vaccines, and in two other childhood vaccines, it is used in the manufacturing process. When each new needle is inserted into the multi-dose vial, it is possible for microbes to get into the vial. The preservative, thimerosal, prevents contamination in the multi-dose vial when individual doses are drawn from it. Receiving a vaccine contaminated with bacteria can be deadly.

For two childhood vaccines, thimerosal is used to prevent the growth of microbes during the manufacturing process. When thimerosal is used this way, it is removed later in the process. Only trace (very tiny) amounts remain. The only childhood vaccines today that have trace amounts of thimerosal are one DTaP and one DTaP-Hib combination vaccine.

54.6.3 Why Was Thimerosal Removed from Vaccines Given to Children?

In 1999, the Food and Drug Administration (FDA) was required by law to assess the amount of mercury in all the products the agency oversees, not just vaccines. The U.S. Public Health Service decided that as much mercury as possible should be removed from vaccines, and thimerosal was the only source of mercury in vaccines. Even though there was no evidence that thimerosal in vaccines was dangerous, the decision to remove it was a made as a precautionary measure to decrease overall exposure to mercury among young infants. This decision was possible because childhood vaccines could be reformulated to leave out thimerosal without threatening their safety, effectiveness, and purity.

Today, no childhood vaccine used in the U.S.—except some formulations of flu vaccine in multi-dose vials—uses thimerosal as a preservative.

54.6.4 Why Is Thimerosal Still in Some Flu Vaccines That Children May Receive?

To produce enough flu vaccine for the entire country, some of it must be put into multi-dose vials. When each individual vaccine dose is drawn from the vial with a fresh needle, it is possible for microbes to get into the vial. So, this preservative is needed to prevent contamination of the vial when individual doses are drawn from it. Children can safely receive flu vaccine that contains thimerosal. Flu vaccine in single-dose vials that does not contain thimerosal also is available.

54.6.5 Was Thimerosal in Vaccines a Cause of Autism?

Reputable scientific studies have shown that mercury in vaccines given to young children is not a cause of autism.

June 30, 1999
Food and Drug Administration (FDA) announces results of its review of total amount of ethylmercury from thimerosal preservative in childhood vaccines.
July 9, 1999
Public Health Service (PHS) and American Academy of Pediatrics (AAP) recommend removing thimerosal as a preservative from childhood vaccines as a precautionary measure.
1999
Manufacturers begin to phase out use of thimerosal as a preservative in childhood vaccines.
2001
Manufacturing of childhood vaccines with thimerosal as a preservative ceases.
2003
All childhood vaccines with thimerosal as a preservative have passed their expiration date and are no longer available in the U.S. The amount of mercury in vaccines recommended for children is close to zero.
2004
Influenza vaccination recommended for U.S. children ages 6 through 23 months old. Thimerosal is used as a preservative in multi-dose vials to prevent contamination of the vial when individual vaccine doses are drawn from it. Single-dose vials without thimerosal are available.
2008
Influenza vaccination recommended for all U.S. children ages 6 months through 18 years.

The studies used different methods. Some examined rates of autism in a state or a country, comparing autism rates before and after thimerosal was removed as a preservative from vaccines. In the United States and other countries, the number of children diagnosed with autism has not gone down since thimerosal was removed from vaccines.

54.6.6 What Keeps Today's Childhood Vaccines from Becoming Contaminated If They Do Not Contain Thimerosal as a Preservative?

The childhood vaccines that used to contain thimerosal as a preservative are now put into single-dose vials, so no preservative is needed. In the past, the vaccines were put into multi-dose vials, which could become contaminated when new needles were used to get vaccine out of the vial for each dose.

What is the difference between ethylmercury and methylmercury?

When learning about thimerosal and mercury it is important to understand the difference between two different compounds that contain mercury: ethylmercury and methylmercury. They are totally different materials. Methylmercury is formed in the environment when mercury metal is present. If this material is found in the body, it is usually the result of eating some types of fish or other food. High amounts of methylmercury can harm the nervous system. This has been found in studies of some populations that have long-term exposure to methylmercury in foods at levels that are far higher than the U.S. population. In the United States, federal guidelines keep as much methylmercury as possible out of the environment and food, but over a lifetime, everyone is exposed to some methylmercury. Ethylmercury is formed when the body breaks down thimerosal. Low-level ethylmercury exposures from vaccines are very different from long-term methylmercury exposures because ethylmercury is broken down by the body differently and clears out of the blood more quickly.

54.6.7 Was Thimerosal Used in All Childhood Vaccines?

No. A few vaccines contained other preservatives, and they still do. Some other vaccines, including the measles-mumps-rubella (MMR) vaccine, never contained any preservative or any mercury.

The science

The studies below are examples of some of the different methods that researchers have used to examine thimerosal safety in vaccines. Researchers have looked at very large groups, such as all children born in a six-year period in Denmark, as well as smaller, defined groups, such as children diagnosed with autism in California. In some of the studies, researchers compared rates of autism among those who were vaccinated with thimerosal-containing vaccines and those who were not. Researchers consistently found that children who received thimerosal in vaccines were not more likely to have autism than those who did not.

Thimerosal Exposure in Infants and Developmental Disorders: A Retrospective Cohort Study in the United Kingdom Does Not Support a Causal Association by Nick Andrews et al. *Pediatrics*. September 2004. Vol 114: pages 584–591.
http://pediatrics.aappublications.org/cgi/content/full/114/3/584

Pervasive Developmental Disorders in Montreal, Quebec, Canada: Prevalence and Links with Immunizations by Eric Frombonne et al. *Pediatriacs*. July 2006. Vol 118: e139–e150.
http://pediatrics.aappublications.org/cgi/content/full/118/1/e139

Association between Thimerosal-Containing Vaccine and Autism by Anders Hviid et al. *Journal of the American Medical Association*. October 2003. Vol 290: pages 1763–1766.
http://jama.ama-assn.org/cgi/content/full/290/13/1763

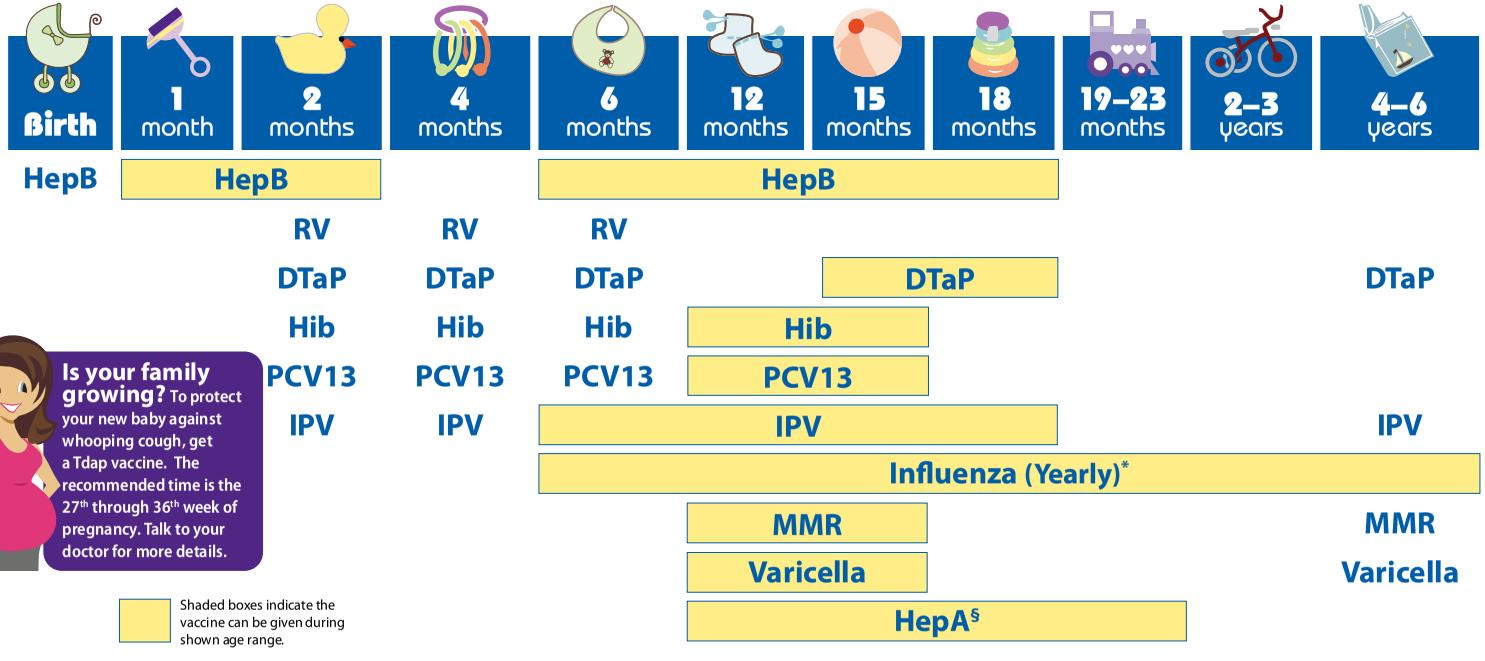
Immunization Safety Review: Vaccines and Autism. Institute of Medicine. The National Academies Press: 2004.
http://www.iom.edu/Reports/2004/Immunization-SafetyReview-Vaccines-and-Autism.aspx

Prenatal and Infant Exposure to Thimerosal from Vaccines and Immunoglobulins and Risk of Autism by Cristofer Price et al. *Pediatrics.* September 2010. Vol 126: pages 656–664.
http://pediatrics.aappublications.org/cgi/reprint/peds.20100309v1

Continuing Increases in Autism Reported to California's Developmental Services System by Robert Schechter et al. *Archives of General Psychiatry*. January 2008. Vol 65: pages 19–24.
http://archpsyc.ama-assn.org/cgi/content/full/65/1/19

Early Thimerosal Exposure and Neuropsychological Outcomes at 7 to 10 Years by William Thompson et al. *The New England Journal of Medicine*. September 2007. Vol 357: pages 1281–1292.
http://www.nejm.org/doi/pdf/10.1056/NEJMoa071434

54.7 2022 Recommended Immunizations for Children from Birth through 6 Years Old



COVID-19 VACCINATION IS RECOMMENDED FOR AGES 5 YEARS AND OLDER.

NOTE:
If your child misses a shot, you don't need to start over. Just go back to your child's doctor for the next shot. Talk with your child's doctor if you have questions about vaccines.

FOOTNOTES:

* Two doses given at least four weeks apart are recommended for children age 6 months through 8 years of age who are getting an influenza (flu) vaccine for the first time and for some other children in this age group.

§ Two doses of HepA vaccine are needed for lasting protection. The first dose of HepA vaccine should be given between 12 months and 23 months of age. The second dose should be given 6 months after the first dose. All children and adolescents over 24 months of age who have not been vaccinated should also receive 2 doses of HepA vaccine.

If your child has any medical conditions that put him at risk for infection or is traveling outside the United States, talk to your child's doctor about additional vaccines that he or she may need.

See back page for more information on vaccine-preventable diseases and the vaccines that prevent them.

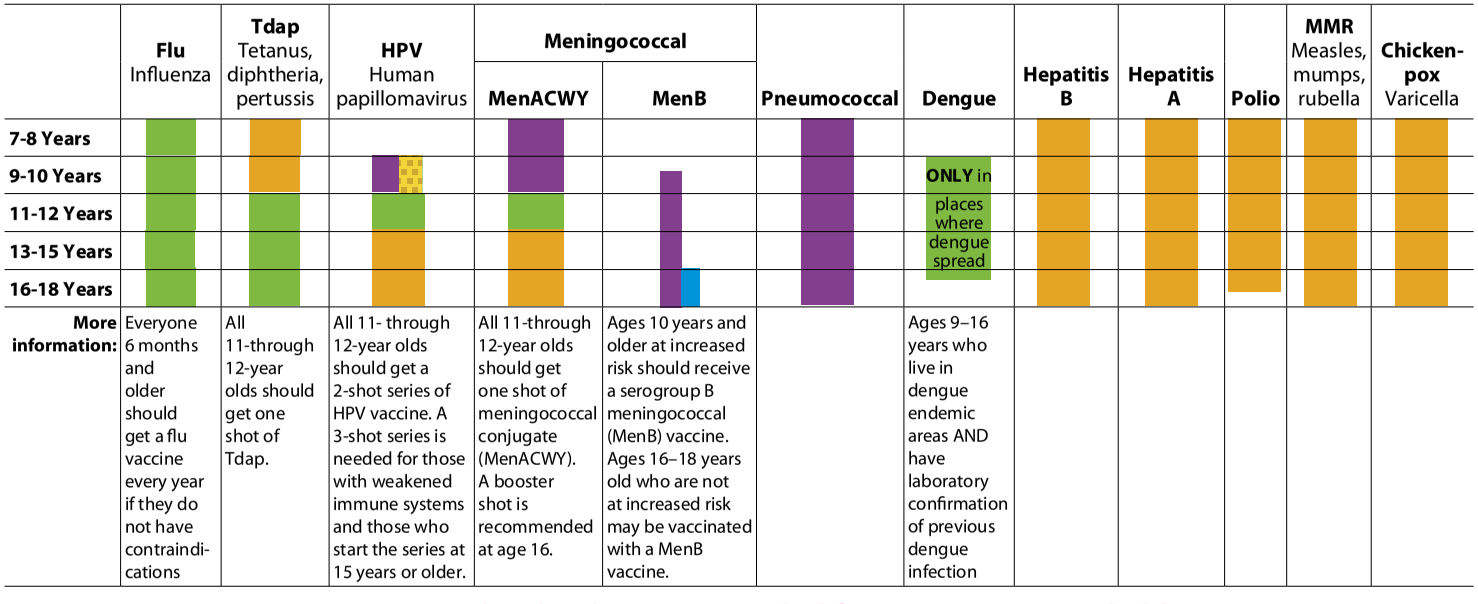
Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Chickenpox	Varicella vaccine protects against chickenpox.	Air, direct contact	Rash, tiredness, headache, fever	Infected blisters, bleeding disorders, encephalitis (brain swelling), pneumonia (infection in the lungs), death
Diphtheria	DTaP* vaccine protects against diphtheria.	Air, direct contact	Sore throat, mild fever, weakness, swollen glands in neck	Swelling of the heart muscle, heart failure, coma, paralysis, death
Hib	Hib vaccine protects against *Haemophilus influenzae* type b.	Air, direct contact	May be no symptoms unless bacteria enter the blood	Meningitis (infection of the covering around the brain and spinal cord), intellectual disability, epiglottitis (life-threatening infection that can block the windpipe and lead to serious breathing problems), pneumonia (infection in the lungs), death
Hepatitis A	HepA vaccine protects against hepatitis A.	Direct contact, contaminated food or water	May be no symptoms, fever, stomach pain, loss of appetite, fatigue, vomiting, jaundice (yellowing of skin and eyes), dark urine	Liver failure, arthralgia (joint pain), kidney, pancreatic and blood disorders, death
Hepatitis B	HepB vaccine protects against hepatitis B.	Contact with blood or body fluids	May be no symptoms, fever, headache, weakness, vomiting, jaundice (yellowing of skin and eyes), joint pain	Chronic liver infection, liver failure, liver cancer, death
Influenza (Flu)	Flu vaccine protects against influenza.	Air, direct contact	Fever, muscle pain, sore throat, cough, extreme fatigue	Pneumonia (infection in the lungs), bronchitis, sinus infections, ear infections, death
Measles	MMR** vaccine protects against measles.	Air, direct contact	Rash, fever, cough, runny nose, pink eye	Encephalitis (brain swelling), pneumonia (infection in the lungs), death

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Mumps	MMR** vaccine protects against mumps.	Air, direct contact	Swollen salivary glands (under the jaw), fever, headache, tiredness, muscle pain	Meningitis (infection of the covering around the brain and spinal cord), encephalitis (brain swelling), inflammation of testicles or ovaries, deafness, death
Pertussis	DTaP* vaccine protects against pertussis (whooping cough).	Air, direct contact	Severe cough, runny nose, apnea (a pause in breathing in infants)	Pneumonia (infection in the lungs), death
Polio	IPV vaccine protects against polio.	Air, direct contact, through the mouth	May be no symptoms, sore throat, fever, nausea, headache	Paralysis, death
Pneumococcal	PCV13 vaccine protects against pneumococcus.	Air, direct contact	May be no symptoms, pneumonia (infection in the lungs)	Bacteremia (blood infection), meningitis (infection of the covering around the brain and spinal cord), death
Rotavirus	RV vaccine protects against rotavirus.	Through the mouth	Diarrhea, fever, vomiting	Severe diarrhea, dehydration, death
Rubella	MMR** vaccine protects against rubella.	Air, direct contact	Sometimes rash, fever, swollen lymph nodes	Very serious in pregnant women—can lead to miscarriage, stillbirth, premature delivery, birth defects
Tetanus	DTaP* vaccine protects against tetanus.	Exposure through cuts in skin	Stiffness in neck and abdominal muscles, difficulty swallowing, muscle spasms, fever	Broken bones, breathing difficulty, death

*DTaP combines protection against diphtheria, tetanus, and pertussis.
**MMR combines protection against measles, mumps, and rubella.

54.8 2022 Recommended Immunizations for Children 7–18 Years

INFORMATION FOR PARENTS | 2022 Recommended Immunizations for Children 7–18 Years Old



COVID-19 vaccination is recommended for ages 5 years and older.
Talk to your child's doctor or nurse about the vaccines recommended for their age.

These shaded boxes indicate when the vaccine is recommended for all children unless your doctor tells you that your child cannot safely receive the vaccine.

These shaded boxes indicate the vaccine is recommended for children with certain health or lifestyle conditions that put them at an increased risk for serious diseases. See vaccine-specific recommendations at www.cdc.gov/vaccines/hcp/acip-recs/.

These shaded boxes indicate the vaccine **SHOULD** be given if a child is catching up on missed vaccines.

This shaded box indicates children not at increased risk **MAY** get the vaccine if they wish after speaking to a provider.

This shaded box indicates children not at increased risk may get the vaccine if they wish after speaking to a provider.

Vaccine-Preventable Diseases and the Vaccines that Prevent Them

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Chickenpox	Varicella vaccine protects against chickenpox.	Air, direct contact	Rash, tiredness, headache, fever	Infected blisters, bleeding disorders, encephalitis (brain swelling), pneumonia (infection in the lungs), death
Dengue	Dengue vaccine protects against dengue.	Bite from infected mosquito	May be no symptom, fever, headache, pain behind the eyes, rash, joint pain, body ache, nausea, loss of appetite feeling tired, abdominal pain	Severe bleeding, seizures, shock, damage to liver, heart, and lungs, death
Diphtheria	Tdap* and Td** vaccines protect against diphtheria.	Air, direct contact	Sore throat, mild fever, weakness, swollen glands in neck	Swelling of the heart muscle, heart failure, coma, paralysis, death
Hepatitis A	HepA vaccine protects against hepatitis A.	Direct contact, contaminated food or water	May be no symptoms, fever, stomach pain, loss of appetite, fatigue, vomiting, jaundice (yellowing of skin and eyes), dark urine	Liver failure, arthralgia (joint pain), kidney, pancreatic and blood disorders, death
Hepatitis B	HepB vaccine protects against hepatitis B.	Contact with blood or body fluids	May be no symptoms, fever, headache, weakness, vomiting, jaundice (yellowing of skin and eyes), joint pain	Chronic liver infection, liver failure, liver cancer, death
Human Papillomavirus	HPV vaccine protects against human papillomavirus.	Direct skin contact	May be no symptoms, genital warts	Cervical, vaginal, vulvar, penile, anal, oropharyngeal cancers
Influenza (Flu)	Flu vaccine protects against influenza.	Air, direct contact	Fever, muscle pain, sore throat, cough, extreme fatigue	Pneumonia (infection in the lungs), bronchitis, sinus infections, ear infections, death
Measles	MMR*** vaccine protects against measles.	Air, direct contact	Rash, fever, cough, runny nose, pink eye	Encephalitis (brain swelling), pneumonia (infection in the lungs), death

(*Continued*)

(*Continued*)

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Meningococcal Disease	MenACWY and MenB vaccines protect against meningococcal disease.	Air, direct contact	Sudden onset of fever, headache, and stiff neck, dark purple rash	Loss of limb, deafness, nervous system disorders, developmental disabilities, seizure disorder, stroke, death
Mumps	MMR*** vaccine protects against mumps.	Air, direct contact	Swollen salivary glands (under the jaw), fever, headache, tiredness, muscle pain	Meningitis (infection of the covering around the brain and spinal cord), encephalitis (brain swelling), inflammation of testicles or ovaries, deafness, death
Pertussis	Tdap* vaccine protects against pertussis.	Air, direct contact	Severe cough, runny nose, apnea (a pause in breathing in infants)	Pneumonia (infection in the lungs), death
Pneumococcal Disease	Pneumococcal vaccine protects against pneumococcal disease.	Air, direct contact	May be no symptoms, pneumonia (infection in the lungs)	Bacteremia (blood infection), meningitis (infection of the covering around the brain and spinal cord), death
Polio	Polio vaccine protects against polio.	Air, direct contact, through the mouth	May be no symptoms, sore throat, fever, nausea, headache	Paralysis, death
Rubella	MMR*** vaccine protects against rubella.	Air, direct contact	Sometimes rash, fever, swollen lymph nodes	Very serious in pregnant women—can lead to miscarriage, stillbirth, premature delivery, birth defects
Tetanus	Tdap* and Td *** vaccines protect against tetanus.	Exposure through cuts on skin	Stiffness in neck and abdominal muscles, difficulty swallowing, muscle spasms, fever	Broken bones, breathing difficulty, death

*Tdap combines protection against diphtheria, tetanus, and pertussis.

**Td combines protection against diphtheria and tetanus.

***MMR combines protection against measles, mumps, and rubella.

54.9 Recommended Adult Immunization Schedule for Ages 19 Years or Older (United States, 2022)

How to use the adult immunization schedule

1. Determine recommended vaccinations by age (Table 54.1)
2. Assess need for additional recommended vaccinations by medical condition or other indication (Table 54.2)
3. Review vaccine types, frequencies, intervals, and considerations for special situations (Notes)
4. Review contraindications and precautions for vaccine types (Appendix)

Vaccines in the adult immunization schedule*

Vaccine	Abbreviation(s)	Trade name(s)
Haemophilus influenzae type b vaccine	Hib	ActHIB® Hiberix® PedvaxHIB®
Hepatitis A vaccine	HepA	Havrix® Vaqta®
Hepatitis A and hepatitis B vaccine	HepA-HepB	Twinrix®
Hepatitis B vaccine	HepB	Engerix-B® Recombivax HB® Heplisav-B®
Human papillomavirus vaccine	HPV	Gardasil 9®
Influenza vaccine (inactivated)	IIV4	Many brands
Influenza vaccine (live, attenuated)	LAIV4	FluMist® Quadrivalent
Influenza vaccine (recombinant)	RIV4	Flublok® Quadrivalent
Measles-mumps-rubella (MMR) vaccine	MMR	M-M-R II®
Meningococcal serogroups A, C, W, Y vaccine	MenACWY-D MenACWY-CRM MenACWY-TT	Menactra® Menveo® MenQuadfi®
Meningococcal serogroup B vaccine	MenB-4C MenB-FHbp	Bexsero® Trumenba®
Pneumococcal 15-valent conjugate vaccine	PCV15	Vaxneuvance™
Pneumococcal 20-valent conjugate vaccine	PCV20	Prevnar 20™
Pneumococcal 23-valent polysaccharide vaccine	PPSV23	Pneumovax 23®
Tetanus and diphtheria toxoids	Td	Tenivac® Tdvax™
Tetanus and diphtheria toxoids and acellular pertussis vaccine	Tdap	Adacel® Boostrix®
Varicella vaccine	VAR	Varivax®
Zoster vaccine, recombinant	RZV	Shingrix

*Administer recommended vaccines if vaccination history is incomplete or unknown. Do not restart or add doses to vaccine series if there are extended intervals between doses. The use of trade names is for identification purposes only and does not imply endorsement by the ACIP or CDC.

Report

- Suspected cases of reportable vaccine-preventable diseases or outbreaks to the local or state health department
- Clinically significant postvaccination reactions to the Vaccine Adverse Event Reporting System at www.vaers.hhs.gov or 800-822-7967

Injury claims

All vaccines included in the adult immunization schedule except pneumococcal 23-valent polysaccharide (PPSV23) and zoster (RZV) vaccines are covered by the Vaccine Injury Compensation Program. Information on how to file a vaccine injury claim is available at www.hrsa.gov/vaccinecompensation.

Questions or comments

Contact www.cdc.gov/cdc-info or 800-CDC-INFO (800-232-4636), in English or Spanish, 8 a.m.–8 p.m. ET, Monday through Friday, excluding holidays.

Helpful information

- Advisory Committee on Immunization Practices (ACIP) recommendations: www.cdc.gov/vaccines/hcp/acip-recs/index.html
- General Best Practice Guidelines for Immunization (including contraindications and precautions): www.cdc.gov/vaccines/hcp/acip-recs/general-recs/index.html
- Vaccine information statements: www.cdc.gov/vaccines/hcp/vis/index.html
- Manual for the Surveillance of Vaccine-Preventable Diseases (including case identification and outbreak response): www.cdc.gov/vaccines/pubs/survmanual
- Travel vaccine recommendations: www.cdc.gov/travel
- Recommended Child and Adolescent Immunization Schedule, United States, 2022: www.cdc.gov/vaccines/schedules/hcp/child-adolescent.html
- ACIP Shared Clinical Decision-Making Recommendations: www.cdc.gov/vaccines/acip/acip-scdm-faqs.html

Table 54.1 Recommended adult immunization schedule by age group, United States, 2022

Vaccine	19–26 years	27–49 years	50–64 years	≥65 years
Influenza inactivated (IIV4) or **Influenza recombinant** (RIV4)	1 dose annually			
or	or			
Influenza live, attenuated (LAIV4)	1 dose annually			
Tetanus, diphtheria, pertussis (Tdap or Td)	1 dose Tdap each pregnancy; 1 dose Td/Tdap for wound management (see notes)			
	1 dose Tdap, then Td or Tdap booster every 10 years			
Measles, mumps, rubella (MMR)	1 or 2 doses depending on indication (if born in 1957 or later)			
Varicella (VAR)	2 doses (if born in 1980 or later)		2 doses	
Zoster recombinant (RZV)	2 doses for immunocompromising conditions (see notes)		2 doses	
Human papillomavirus (HPV)	2 or 3 doses depending on age at initial vaccination or condition	27 through 45 years		
Pneumococcal (PCV15, PCV20, PPSV23)	1 dose PCV15 followed by PPSV23 OR 1 dose PCV20 (see notes)			1 dose PCV15 followed by PPSV23 OR 1 dose PCV20
Hepatitis A (HepA)	2 or 3 doses depending on vaccine			
Hepatitis B (HepB)	2, 3, or 4 doses depending on vaccine or condition			
Meningococcal A, C, W, Y (MenACWY)	1 or 2 doses depending on indication, see notes for booster recommendations			
Meningococcal B (MenB)	2 or 3 doses depending on vaccine and indication, see notes for booster recommendations			
	19 through 23 years			
***Haemophilus influenzae* type b** (Hib)	1 or 3 doses depending on indication			

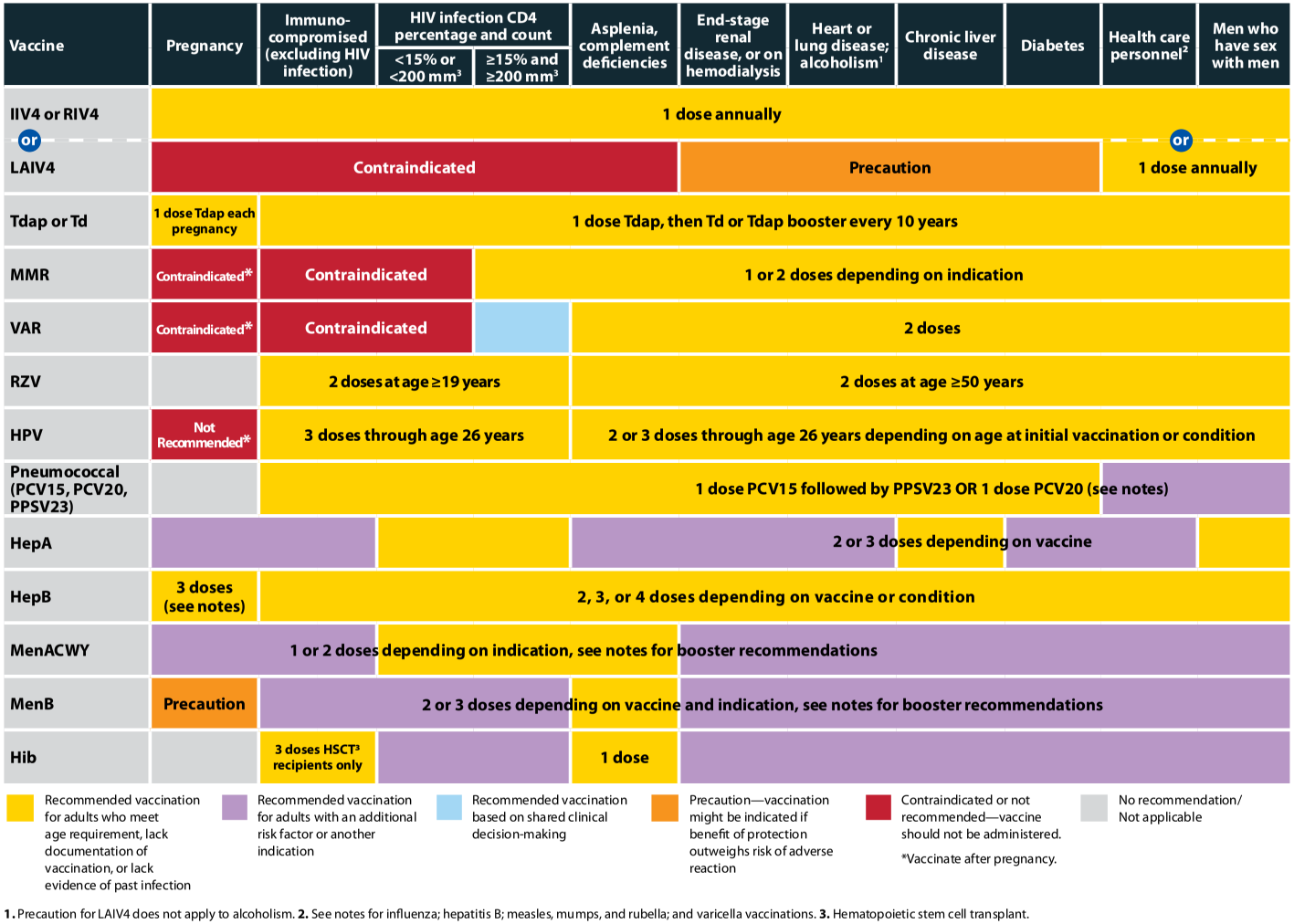
Recommended vaccination for adults who meet age requirement, lack documentation of vaccination, or lack evidence of past infection

Recommended vaccination for adults with an additional risk factor or another indication

Recommended vaccination based on shared clinical decision-making

No recommendation/ Not applicable

Table 54.2 Recommended adult immunization schedule by medical condition or other indication, United States, 2022



1. Precaution for LAIV4 does not apply to alcoholism. **2.** See notes for influenza; hepatitis B; measles, mumps, and rubella; and varicella vaccinations. **3.** Hematopoietic stem cell transplant.

54.9.1 Notes: Recommended Adult Immunization Schedule for ages 19 years or older, United States, 2022

For vaccine recommendations for persons 18 years of age or younger, see the Recommended Child and Adolescent Immunization Schedule.

COVID-19 Vaccination

COVID-19 vaccines are recommended within the scope of the Emergency Use Authorization or Biologics License Application for the particular vaccine. ACIP recommendations for the use of COVID-19 vaccines can be found at www.cdc.gov/vaccines/hcp/acip-recs/vacc-specific/covid-19.html.

CDC's interim clinical considerations for use of COVID-19 vaccines can be found at www.cdc.gov/vaccines/covid-19/clinical-considerations/covid-19-vaccines-us.html.

54.9.1.1 *Haemophilus influenzae* type b vaccination

Special situations

- Anatomical or functional asplenia (including sickle cell disease): 1 dose if previously did not receive Hib; if elective splenectomy, 1 dose, preferably at least 14 days before splenectomy
- Hematopoietic stem cell transplant (HSCT): 3-dose series 4 weeks apart starting 6–12 months after successful transplant, regardless of Hib vaccination history

54.9.1.2 Hepatitis A vaccination

Routine vaccination

- **Not at risk but want protection from hepatitis A** (identification of risk factor not required): 2-dose series HepA (Havrix 6–12 months apart or Vaqta 6–18 months apart [minimum interval: 6 months]) or 3-dose series HepA-HepB (Twinrix at 0, 1, 6 months [minimum intervals: dose 1 to dose 2: 4 weeks / dose 2 to dose 3: 5 months])

Special situations

- **At risk for hepatitis A virus infection:** 2-dose series HepA or 3-dose series HepA-HepB as above
  - **Chronic liver disease** (e.g., persons with hepatitis B, hepatitis C, cirrhosis, fatty liver disease, alcoholic liver disease, autoimmune hepatitis, alanine aminotransferase [ALT] or aspartate aminotransferase [AST] level greater than twice the upper limit of normal)

- **HIV infection**
- **Men who have sex with men**
- **Injection or noninjection drug use**
- **Persons experiencing homelessness**
- **Work with hepatitis A virus** in research laboratory or with nonhuman primates with hepatitis A virus infection
- **Travel in countries with high or intermediate endemic hepatitis A** (HepA-HepB [Twinrix] may be administered on an accelerated schedule of 3 doses at 0, 7, and 21–30 days, followed by a booster dose at 12 months)
- **Close, personal contact with international adoptee** (e.g., household or regular babysitting) in first 60 days after arrival from country with high or intermediate endemic hepatitis A (administer dose 1 as soon as adoption is planned, at least 2 weeks before adoptee's arrival)
- **Pregnancy** if at risk for infection or severe outcome from infection during pregnancy
- **Settings for exposure, including** health care settings targeting services to injection or noninjection drug users or group homes and nonresidential day care facilities for developmentally disabled persons (individual risk factor screening not required)

54.9.1.3 Hepatitis B vaccination

Routine vaccination

- **Age 19 through 59 years**: complete a 2- or 3-, or 4-dose series
  - 2-dose series only applies when 2 doses of Heplisav-B* are used at least 4 weeks apart
  - 3-dose series Engerix-B or Recombivax HB at 0, 1, 6 months [minimum intervals: dose 1 to dose 2: 4 weeks/dose 2 to dose 3: 8 weeks / dose 1 to dose 3: 16 weeks])
  - 3-dose series HepA-HepB (Twinrix at 0, 1, 6 months [minimum intervals: dose 1 to dose 2: 4 weeks/dose 2 to dose 3: 5 months])
  - 4-dose series HepA-HepB (Twinrix) accelerated schedule of 3 doses at 0, 7, and 21–30 days, followed by a booster dose at 12 months
  - 4-dose series Engerix-B at 0, 1, 2, and 6 months for persons on adult hemodialysis (note: each dosage is double that of normal adult dose, i.e., 2 mL instead of 1 mL)

*__Note:__ Heplisav-B not recommended in pregnancy due to lack of safety data in pregnant women

Special situations

- **Age 60 years or older* and at risk for hepatitis B virus infection**: 2-dose (Heplisav-B) or 3-dose (Engerix-B, Recombivax HB) series or 3-dose series HepA-HepB (Twinrix) as above
  - **Chronic liver disease** (e.g., persons with hepatitis C, cirrhosis, fatty liver disease, alcoholic liver disease, autoimmune hepatitis, alanine amino-transferase [ALT] or aspartate aminotransferase [AST] level greater than twice upper limit of normal)
  - **HIV infection**
  - **Sexual exposure risk** (e.g., sex partners of hepatitis B surface antigen [HBsAg]-positive persons; sexually active persons not in mutually monogamous relationships; persons seeking evaluation or treatment for a sexually transmitted infection; men who have sex with men)
  - **Current or recent injection drug use**
  - **Percutaneous or mucosal risk for exposure to blood** (e.g., household contacts of HBsAg-positive persons; residents and staff of facilities for developmentally disabled persons; health care and public safety personnel with reasonably anticipated risk for exposure to blood or blood-contaminated body fluids; hemodialysis, peritoneal dialysis, home dialysis, and predialysis patients; patients with diabetes)
  - **Incarcerated persons**
  - **Travel in countries with high or intermediate endemic hepatitis B**

*__Note:__ Anyone age 60 years or older who does not meet risk-based recommendations may still receive Hepatitis B vaccination.

54.9.1.4 Human papillomavirus vaccination

Routine vaccination

- **HPV vaccination recommended for all persons through age 26 years**: 2- or 3-dose series depending on age at initial vaccination or condition:
  - **Age 15 years or older at initial vaccination**: 3-dose series at 0, 1–2 months, 6 months (minimum intervals: dose 1 to dose 2: 4 weeks/dose 2 to dose 3: 12 weeks/dose 1 to dose 3: 5 months; repeat dose if administered too soon)
  - **Age 9–14 years at initial vaccination and received 1 dose or 2 doses less than 5 months apart**: 1 additional dose
  - **Age 9–14 years at initial vaccination and received 2 doses at least 5 months apart**: HPV vaccination series complete, no additional dose needed

- Interrupted schedules: If vaccination schedule is interrupted, the series does not need to be restarted.
- No additional dose recommended when any HPV vaccine series has been completed using the recommended dosing intervals.

Shared clinical decision-making

- **Some adults age 27–45 years: Based on shared clinical decision-making**, 2- or 3-dose series as above

Special situations

- **Age ranges recommended above for routine and catch-up vaccination or shared clinical decision-making also apply in special situations**
  - **Immunocompromising conditions, including HIV infection**: 3-dose series, even for those who initiate vaccination at age 9 through 14 years.
  - **Pregnancy:** Pregnancy testing is not needed before vaccination; HPV vaccination is not recommended until after pregnancy; no intervention needed if inadvertently vaccinated while pregnant.

54.9.1.5 Influenza vaccination

Routine vaccination

- **Age 19 years or older**: 1 dose any influenza vaccine appropriate for age and health status annually.
- For the 2021–2022 season, see www.cdc.gov/mmwr/volumes/70/rr/rr70-05a1.htm.
- For the 2022–23 season, see the 2022–23 ACIP influenza vaccine recommendations.

Special situations

- **Egg allergy, hives only**: any influenza vaccine appropriate for age and health status annually
- **Egg allergy–any symptom other than hives** (e.g., angioedema, respiratory distress) or required epinephrine or another emergency medical intervention: see Appendix listing contraindications and precautions
- **Severe allergic reaction** (e.g., anaphylaxis) to a vaccine component or a previous dose of any influenza vaccine: see Appendix listing contraindications and precautions
- **History of Guillain-Barré syndrome within 6 weeks after previous dose of influenza vaccine**: Generally, should not be vaccinated unless vaccination benefits outweigh risks for those at higher risk for severe complications from influenza

54.9.1.6 Measles, mumps, and rubella vaccination

Routine vaccination

- **No evidence of immunity to measles, mumps, or rubella**: 1 dose
  - **Evidence of immunity**: Born before 1957 (health care personnel, see below), documentation of receipt of MMR vaccine, laboratory evidence of immunity or disease (diagnosis of disease without laboratory confirmation is not evidence of immunity)

Special situations

- **Pregnancy with no evidence of immunity to rubella**: MMR contraindicated during pregnancy; after pregnancy (before discharge from health care facility), 1 dose
- **Nonpregnant women of childbearing age with no evidence of immunity to rubella**: 1 dose
- **HIV infection with CD4 percentages ≥15% and CD4 count ≥200 cells/mm^3 for at least 6 months and no evidence of immunity to measles, mumps, or rubella**: 2-dose series at least 4 weeks apart; MMR contraindicated for HIV infection with CD4 percentage <15% or CD4 count <200 cells/mm^3
- **Severe immunocompromising conditions**: MMR contraindicated
- **Students in postsecondary educational institutions, international travelers, and household or close, personal contacts of immunocompromised persons with no evidence of immunity to measles, mumps, or rubella**: 2-dose series at least 4 weeks apart if previously did not receive any doses of MMR or 1 dose if previously received 1 dose MMR
- **Health care personnel**
  - **Born before 1957 with no evidence of immunity to measles, mumps, or rubella**: Consider 2-dose series at least 4 weeks apart for measles or mumps or 1 dose for rubella
  - **Born in 1957 or later with no evidence of immunity to measles, mumps, or rubella**: 2-dose series at least 4 weeks apart for measles or mumps or at least 1 dose for rubella

54.9.1.7 Meningococcal vaccination

Special situations for MenACWY

- **Anatomical or functional asplenia (including sickle cell disease), HIV infection, persistent complement component deficiency, complement inhibitor (e.g., eculizumab, ravulizumab) use**: 2-dose series MenACWY-D (Menactra, Menveo, or MenQuadfi) at least 8 weeks apart and revaccinate every 5 years if risk remains

- **Travel in countries with hyperendemic or epidemic meningococcal disease, or microbiologists routinely exposed to *Neisseria meningitidis*:** 1 dose MenACWY (Menactra, Menveo, or MenQuadfi) and revaccinate every 5 years if risk remains
- **First-year college students who live in residential housing (if not previously vaccinated at age 16 years or older) or military recruits**: 1 dose MenACWY (Menactra, Menveo, or MenQuadfi)
- For MenACWY **booster dose recommendations** for groups listed under "Special situations" and in an outbreak setting (e.g., in community or organizational settings and among men who have sex with men) and additional meningococcal vaccination information, see www.cdc.gov/mmwr/volumes/69/rr/rr6909a1.htm

Shared clinical decision-making for MenB

- **Adolescents and young adults age 16–23 years (age 16–18 years preferred) not at increased risk for meningococcal disease**: Based on shared clinical decision-making, 2-dose series MenB-4C (Bexsero) at least 1 month apart or 2-dose series MenB-FHbp (Trumenba) at 0, 6 months (if dose 2 was administered less than 6 months after dose 1, administer dose 3 at least 4 months after dose 2); MenB-4C and MenB-FHbp are not interchangeable (use same product for all doses in series)

Special situations for MenB

Anatomical or functional asplenia (including sickle cell disease), persistent complement component deficiency, complement inhibitor (e.g., eculizumab, ravulizumab) use, or microbiologists routinely exposed to *Neisseria meningitidis*:

- 2-dose primary series MenB-4C (Bexsero) at least 1 month apart or 3-dose primary series MenB-FHbp (Trumenba) at 0, 1–2, 6 months (if dose 2 was administered at least 6 months after dose 1, dose 3 not needed); MenB-4C and MenB-FHbp are not interchangeable (use same product for all doses in series); 1 dose MenB booster 1 year after primary series and revaccinate every 2–3 years if risk remains
- **Pregnancy:** Delay MenB until after pregnancy unless at increased risk and vaccination benefits outweigh potential risks
- For MenB **booster dose recommendations** for groups listed under "Special situations" and in an outbreak setting (e.g., in community or organizational settings and among men who have sex with men) and additional meningococcal vaccination information, see www.cdc.gov/ mmwr/volumes/69/rr/rr6909a1.htm

Note: MenB vaccines may be administered simultaneously with MenACWY vaccines if indicated, but at a different anatomic site, if feasible.

54.9.1.8 Pneumococcal vaccination

Routine vaccination

- **Age 65 years or older** who have not previously received a pneumococcal conjugate vaccine or whose previous vaccination history is unknown: 1 dose PCV15 or 1 dose PCV20. If PCV15 is used, this should be followed by a dose of PPSV23 given at least 1 year after the PCV15 dose. A minimum interval of 8 weeks between PCV15 and PPSV23 can be considered for adults with an immunocompromising condition,* cochlear implant, or cerebrospinal fluid leak to minimize the risk of invasive pneumococcal disease caused by serotypes unique to PPSV23 in these vulnerable groups.
- For guidance for patients who have already received a previous dose of PCV13 and/or PPSV23, see www.cdc.gov/mmwr/volumes/71/wr/mm7104a1.htm.

Special situations

- **Age 19–64 years** with certain underlying medical conditions or other risk factors** who have not previously received a pneumococcal conjugate vaccine or whose previous vaccination history is unknown: 1 dose PCV15 or 1 dose PCV20. If PCV15 is used, this should be followed by a dose of PPSV23 given at least 1 year after the PCV15 dose. A minimum interval of 8 weeks between PCV15 and PPSV23 can be considered for adults with an immunocompromising condition,* cochlear implant, or cerebrospinal fluid leak to minimize the risk of invasive pneumococcal disease caused by serotypes unique to PPSV23 in these vulnerable groups.
- For guidance for patients who have already received a previous dose of PCV13 and/or PPSV23, see www.cdc.gov/mmwr/volumes/71/wr/mm7104a1.htm.

*__Note:__ Immunocompromising conditions include chronic renal failure, nephrotic syndrome, immunodeficiency, iatrogenic immunosuppression, generalized malignancy, human immunodeficiency virus, Hodgkin disease, leukemia, lymphoma, multiple myeloma, solid organ transplants, congenital or acquired asplenia, sickle cell disease, or other hemoglobinopathies.

**__Note:__ Underlying medical conditions or other risk factors include alcoholism, chronic heart/liver/lung disease, chronic renal failure, cigarette smoking, cochlear implant, congenital or acquired asplenia, CSF leak, diabetes mellitus, generalized malignancy, HIV, Hodgkin disease, immunodeficiency, iatrogenic immunosuppression, leukemia, lymphoma, multiple myeloma, nephrotic syndrome, solid organ transplants, or sickle cell disease or other hemoglobinopathies.

54.9.1.9 Tetanus, diphtheria, and pertussis vaccination

Routine vaccination

- **Previously did not receive Tdap at or after age 11 years:** 1 dose Tdap, then Td or Tdap every 10 years

Special situations

- **Previously did not receive primary vaccination series for tetanus, diphtheria, or pertussis:** 1 dose Tdap followed by 1 dose Td or Tdap at least 4 weeks after Tdap and another dose Td or Tdap 6–12 months after last Td or Tdap (Tdap can be substituted for any Td dose, but preferred as first dose), Td or Tdap every 10 years thereafter
- **Pregnancy:** 1 dose Tdap during each pregnancy, preferably in early part of gestational weeks 27–36
- **Wound management:** Persons with 3 or more doses of tetanus-toxoid-containing vaccine: For clean and minor wounds, administer Tdap or Td if more than 10 years since last dose of tetanus-toxoid-containing vaccine; for all other wounds, administer Tdap or Td if more than 5 years since last dose of tetanus-toxoid-containing vaccine. Tdap is preferred for persons who have not previously received Tdap or whose Tdap history is unknown. If a tetanus-toxoid-containing vaccine is indicated for a pregnant woman, use Tdap. For detailed information, see www.cdc.gov/mmwr/volumes/69/wr/mm6903a5.htm

54.9.1.10 Varicella vaccination

Routine vaccination

- **No evidence of immunity to varicella:** 2-dose series 4–8 weeks apart if previously did not receive varicella-containing vaccine (VAR or MMRV [measles-mumps-rubella-varicella vaccine] for children); if previously received 1 dose varicella-containing vaccine, 1 dose at least 4 weeks after first dose
  - **Evidence of immunity**: U.S.-born before 1980 (except for pregnant women and health care personnel [see below]), documentation of 2 doses varicella-containing vaccine at least 4 weeks apart, diagnosis or verification of history of varicella or herpes zoster by a health care provider, laboratory evidence of immunity or disease

Special situations

- **Pregnancy with no evidence of immunity to varicella:** VAR contraindicated during pregnancy; after pregnancy (before discharge from health care facility), 1 dose if previously received 1 dose varicella-containing vaccine or dose 1 of 2-dose series (dose 2: 4–8 weeks later) if previously did not receive any varicella-containing vaccine, regardless of whether U.S.-born before 1980

- **Health care personnel with no evidence of immunity to varicella:** 1 dose if previously received 1 dose varicella-containing vaccine; 2-dose series 4–8 weeks apart if previously did not receive any varicella-containing vaccine, regardless of whether U.S.-born before 1980
- **HIV infection with CD4 percentages ≥15% and CD4 count ≥200 cells/mm^3 with no evidence of immunity:** Vaccination may be considered (2 doses 3 months apart); VAR contraindicated for HIV infection with CD4 percentage <15% or CD4 count <200 cells/mm^3
- **Severe immunocompromising conditions:** VAR contraindicated

54.9.1.11 Zoster vaccination

Routine vaccination

- **Age 50 years or older:** 2-dose series RZV (Shingrix) 2–6 months apart (minimum interval: 4 weeks; repeat dose if administered too soon), regardless of previous herpes zoster or history of zoster vaccine live (ZVL, Zostavax) vaccination (administer RZV at least 2 months after ZVL)

Special situations

- **Pregnancy:** There is currently no ACIP recommendation for RZV use in pregnancy. Consider delaying RZV until after pregnancy.
- **Immunocompromising conditions (including HIV):** RZV recommended for use in persons age 19 years or older who are or will be immunodeficient or immunosuppressed because of disease or therapy. For detailed information, see www.cdc.gov/mmwr/volumes/71/wr/mm7103a2.htm.

54.9.2 Appendix: Guide to Contraindications and Precautions to Commonly Used Vaccines

Adapted from Table 4-1 in Advisory Committee on Immunization Practices (ACIP) General Best Practice Guidelines for Immunization: Contraindication and Precautions available at www.cdc.gov/vaccines/hcp/acip-recs/general-recs/contraindications.html and ACIP's Recommendations for the Prevention and Control of 2021–22 Seasonal Influenza with Vaccines available at www.cdc.gov/mmwr/volumes/70/rr/rr7005a1.htm.

Interim clinical considerations for use of COVID-19 vaccines including contraindications and precautions can be found at www.cdc.gov/vaccines/covid-19/clinical-considerations/covid-19-vaccines-us.html

Vaccine	Contraindications[1]	Precautions[2]
Influenza, egg-based, inactivated injectable (IIV4)	• Severe allergic reaction (e.g., anaphylaxis) after previous dose of any influenza vaccine (i.e., any egg-based IIV, ccIIV, RIV, or LAIV of any valency) • Severe allergic reaction (e.g., anaphylaxis) to any vaccine component[3] (excluding egg)	• Guillain-Barré syndrome (GBS) within 6 weeks after a previous dose of any type of influenza vaccine • Persons with egg allergy with symptoms other than hives (e.g., angioedema, respiratory distress) or required epinephrine or another emergency medical intervention: Any influenza vaccine appropriate for age and health status may be administered. If using egg-based IIV4, administer in medical setting under supervision of health care provider who can recognize and manage severe allergic reactions. May consult an allergist. • Moderate or severe acute illness with or without fever
Influenza, cell culture-based inactivated injectable [(ccIIV4), Flucelvax® Quadrivalent]	• Severe allergic reaction (e.g., anaphylaxis) to any ccIIV of any valency, or to any component[3] of ccIIV4	• Guillain-Barré syndrome (GBS) within 6 weeks after a previous dose of any type of influenza vaccine • Persons with a history of severe allergic reaction (e.g., anaphylaxis) after a previous dose of any egg-based IIV, RIV, or LAIV of any valency. If using ccIV4, administer in medical setting under supervision of health care provider who can recognize and manage severe allergic reactions. May consult an allergist. • Moderate or severe acute illness with or without fever
Influenza, recombinant injectable [(RIV4), Flublok® Quadrivalent]	• Severe allergic reaction (e.g., anaphylaxis) to any RIV of any valency, or to any component[3] of RIV4	• Guillain-Barré syndrome (GBS) within 6 weeks after a previous dose of any type of influenza vaccine • Persons with a history of severe allergic reaction (e.g., anaphylaxis) after a previous dose of any egg-based IIV, ccIIV, or LAIV of any valency. If using RIV4, administer in medical setting under supervision of health care provider who can recognize and manage severe allergic reactions. May consult an allergist. • Moderate or severe acute illness with or without fever

Vaccine	Contraindications[1]	Precautions[2]
Influenza, live attenuated [LAIV4, Flumist® Quadrivalent]	• Severe allergic reaction (e.g., anaphylaxis) after previous dose of any influenza vaccine (i.e., any egg-based IIV, ccIIV, RIV, or LAIV of any valency) • Severe allergic reaction (e.g., anaphylaxis) to any vaccine component[3] (excluding egg) • Adults age 50 years or older • Anatomic or functional asplenia • Immunocompromised due to any cause including, but not limited to, medications and HIV infection • Close contacts or caregivers of severely immunosuppressed persons who require a protected environment • Pregnancy • Cochlear implant • Active communication between the cerebrospinal fluid (CSF) and the oropharynx, nasopharynx, nose, ear, or any other cranial CSF leak • Received influenza antiviral medications oseltamivir or zanamivir within the previous 48 hours, peramivir within the previous 5 days, or baloxavir within the previous 17 days.	• Guillain-Barré syndrome (GBS) within 6 weeks after a previous dose of any type of influenza vaccine • Asthma in persons aged 5 years old or older • Persons with egg allergy with symptoms other than hives (e.g., angioedema, respiratory distress) or required epinephrine or another emergency medical intervention: Any influenza vaccine appropriate for age and health status may be administered. If using LAIV4 (which is egg based), administer in medical setting under supervision of health care provider who can recognize and manage severe allergic reactions. May consult an allergist. • Persons with underlying medical conditions (other than those listed under contraindications) that might predispose to complications after wild-type influenza virus infection [e.g., chronic pulmonary, cardiovascular (except isolated hypertension), renal, hepatic, neurologic, hematologic, or metabolic disorders (including diabetes mellitus)] • Moderate or severe acute illness with or without fever
Haemophilus influenzae type b (Hib)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • For Hiberix, ActHib, and PedvaxHIB only: History of severe allergic reaction to dry natural latex	• Moderate or severe acute illness with or without fever

(*Continued*)

(*Continued*)

Vaccine	Contraindications[1]	Precautions[2]
Hepatitis A (HepA)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] including neomycin	• Moderate or severe acute illness with or without fever
Hepatitis B (HepB)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] including yeast • For Heplisav-B only: Pregnancy	• Moderate or severe acute illness with or without fever
Hepatitis A- Hepatitis B vaccine [HepA-HepB, (Twinrix®)]	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] including neomycin and yeast	• Moderate or severe acute illness with or without fever
Human papillomavirus (HPV)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3]	• Moderate or severe acute illness with or without fever
Measles, mumps, rubella (MMR)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • Severe immunodeficiency (e.g., hematologic and solid tumors, receipt of chemotherapy, congenital immunodeficiency, long-term immunosuppressive therapy or patients with HIV infection who are severely immunocompromised) • Pregnancy • Family history of altered immunocompetence, unless verified clinically or by laboratory testing as immunocompetent	• Recent (≤11 months) receipt of antibody-containing blood product (specific interval depends on product) • History of thrombocytopenia or thrombocytopenic purpura • Need for tuberculin skin testing or interferon-gamma release assay (IGRA) testing • Moderate or severe acute illness with or without fever

Vaccine	Contraindications[1]	Precautions[2]
Meningococcal ACWY (MenACWY) [MenACWY-CRM (Menveo®); MenACWY-D (Menactra®); MenACWY-TT (MenQuadfi®)]	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • For MenACWY-D and Men ACWY-CRM only: severe allergic reaction to any diphtheria toxoid – or CRM197 – containing vaccine • For MenACWY-TT only: severe allergic reaction to a tetanus toxoid – containing vaccine	• Moderate or severe acute illness with or without fever
Meningococcal B (MenB) [MenB-4C (Bexsero); MenB-FHbp (Trumenba)]	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3]	• Pregnancy • For MenB-4C only: Latex sensitivity • Moderate or severe acute illness with or without fever
Pneumococcal conjugate (PCV15)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • Severe allergic reaction (e.g., anaphylaxis) to any diphtheria-toxoid – containing vaccine or to its vaccine component[3]	• Moderate or severe acute illness with or without fever
Pneumococcal conjugate (PCV20)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • Severe allergic reaction (e.g., anaphylaxis) to any diphtheria-toxoid – containing vaccine or to its vaccine component[3]	• Moderate or severe acute illness with or without fever
Pneumococcal polysaccharide (PPSV23)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3]	• Moderate or severe acute illness with or without fever
Tetanus, diphtheria, and acellular pertussis (Tdap) Tetanus, diphtheria (Td)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3]	• Guillain-Barré syndrome (GBS) within 6 weeks after a previous dose of tetanus-toxoid–containing vaccine • History of Arthus-type hypersensitivity reactions after a previous dose of diphtheria-toxoid — containing or tetanus-toxoid– containing vaccine; defer vaccination until at least 10 years have elapsed since the last tetanus-toxoid–containing vaccine

(*Continued*)

(*Continued*)

Vaccine	Contraindications[1]	Precautions[2]
	• For Tdap only: Encephalopathy (e.g., coma, decreased level of consciousness, prolonged seizures), not attributable to another identifiable cause, within 7 days of administration of previous dose of DTP, DTaP, or Tdap	• Moderate or severe acute illness with or without fever • For Tdap only: Progressive or unstable neurological disorder, uncontrolled seizures, or progressive encephalopathy until a treatment regimen has been established and the condition has stabilized
Varicella (VAR)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3] • Severe immunodeficiency (e.g., hematologic and solid tumors, receipt of chemotherapy, congenital immunodeficiency, long- term immunosuppressive therapy or patients with HIV infection who are severely immunocompromised) • Pregnancy • Family history of altered immunocompetence, unless verified clinically or by laboratory testing as immunocompetent	• Recent (≤11 months) receipt of antibody-containing blood product (specific interval depends on product) • Receipt of specific antiviral drugs (acyclovir, famciclovir, or valacyclovir) 24 hours before vaccination (avoid use of these antiviral drugs for 14 days after vaccination) • Use of aspirin or aspirin-containing products • Moderate or severe acute illness with or without fever
Zoster recombinant vaccine (RZV)	• Severe allergic reaction (e.g., anaphylaxis) after a previous dose or to a vaccine component[3]	• Moderate or severe acute illness with or without fever • Current herpes zoster infection

[1]When a contraindication is present, a vaccine should NOT be administered. Kroger A, Bahta L, Hunter P. ACIP General Best Practice Guidelines for Immunization. www.cdc.gov/vaccines/hcp/acip-recs/general-recs/contraindications.html

[2] When a precaution is present, vaccination should generally be deferred but might be indicated if the benefit of protection from the vaccine outweighs the risk for an adverse reaction. Kroger A, Bahta L, Hunter P. ACIP General Best Practice Guidelines for Immunization. www.cdc.gov/vaccines/hcp/acip-recs/general-recs/contraindications.html

[3]Vaccination providers should check FDA-approved prescribing information for the most complete and updated information, including contraindications, warnings, and precautions. Package inserts for U.S.-licensed vaccines are available at www.fda.gov/vaccines-blood-biologics/approved-products/vaccines-licensed-use-united-states.

Chapter 55

What Are Embryonic Stem Cells and How Can They Help Us?

Eran Meshorer, PhD

Department of Genetics and the Edmond and Lily Center for Brain Sciences, Hebrew University of Jerusalem, Israel

meshorer@cc.huji.ac.il

Keywords: 1993 NIH Revitalization Act, blastocyst, cloning, differentiation, embryonic stem cells (ESCs), fertilization, fetal tissue, *in vitro* fertilization (IVF), induced pluripotent stem cells (iPSCs), nuclear transfer embryonic stem cells (NT-ESCs), Parkinson's disease, pluripotent, pluripotent stem cells (PSCs), regenerative medicine, reprogramming, somatic cell nuclear transfer (SCNT), therapeutic cloning, transplanting cells, type 1 diabetes

All living things, including humans, are made of cells. Each tissue and organ of the body contains cells that are specialized to perform specific jobs–the liver contains liver cells, the brain contains neurons, the eyes contain light-detecting cells, and so on. But all human life begins with the encounter between two cells: the sperm cell from the father and the egg cell from the mother. Fertilization occurs when the sperm cell meets the egg cell. The fertilized egg cell divides into two cells. Each cell then divides into two additional cells and so on until, after a few days of cell division, a tiny embryo develops. In the early stages, the microscopic embryo is made up of cells that have the potential to develop into all types of cells. Scientists managed to grow these embryonic cells in the lab and named them embryonic stem cells (ESCs). While ESCs offer promising and exciting opportunities, like the possibility of growing organs in the lab, the production of ESCs requires human embryos, which involves many technical and ethical problems. In 2007, researchers found a way to produce human cells with the abilities of ESCs by reprogramming regular cells so that they become stem cells. Today, scientists can change almost every type of cell into almost every other type of cell!

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55.1 It All Begins in Fertilization, When the Sperm Meets the Egg

Embryonic development begins the moment of fertilization, when the sperm meets the egg (Fig. 55.1). Fertilization brings together the genetic material (DNA) from both parents, half from the egg and half from the sperm, and this combination of genetic material produces the embryo. From the moment of fertilization, the fertilized egg goes through a process of cell division. The fertilized egg first divides into two cells, then each of these divides into two more cells and so on until, a few days later, we have a small ball made up of a few dozen embryonic cells. About a week after fertilization, the developing embryo looks like a hollow ball of cells, which will later become the placenta, and an internal pile of cells, which will become the embryo itself (Fig. 55.1). Since this small number of cells will become a complete baby, these early cells must have the ability to become every cell in the body, like skin cells, muscle cells, liver cells, or brain cells. Because of this ability, these cells are called pluripotent (*pluri*, a lot; *potent,* ability). About a week after fertilization, the embryonic cells gradually lose their pluripotency and gradually become the various tissues and organs. So, there is a relatively narrow window during which pluripotent ESCs exist in the embryo.

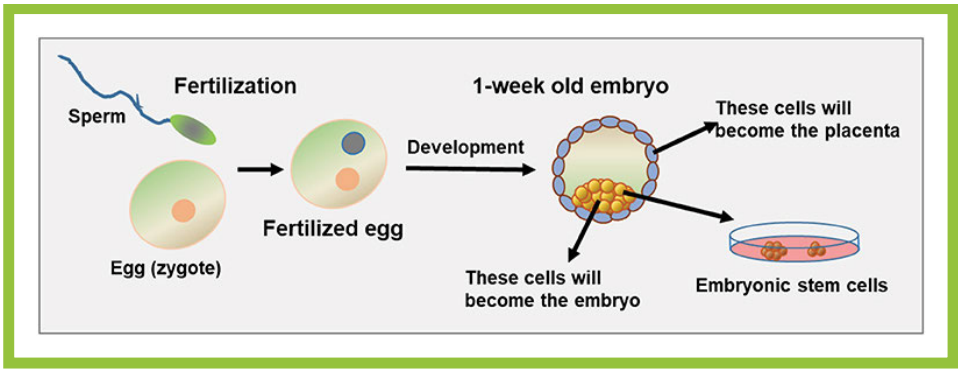


Figure 55.1 Early embryonic development and pluripotent cells. On the left, you can see the egg and the sperm prior to fertilization. Once the two meet, the egg is fertilized and starts to develop. About 1 week after fertilization, cell division has created a tiny embryo containing outer cells that will become the placenta (gray-blue), and inner cells that will become the embryo (yellow). If fertilization happens outside of the body (*in vitro* fertilization), it is possible to remove the internal cells of the embryo at this stage and grow them in the lab. If they successfully grow, they are called embryonic stem cells (ESCs).

In 1998 scientists from the US and Israel first managed to grow pluripotent cells from human embryos in the lab. They worked with embryos that were created by *in vitro* fertilization (IVF), which is a process that allows couples that experience difficulty getting pregnant to have children. At the end of the IVF process, doctors are usually left with many 1-week-old embryos that are no longer needed. These tiny embryos can be used for research, and scientists used

them to figure out how to grow pluripotent cells in the lab (Fig. 55.2). These cells are called embryonic stem cells (ESCs).

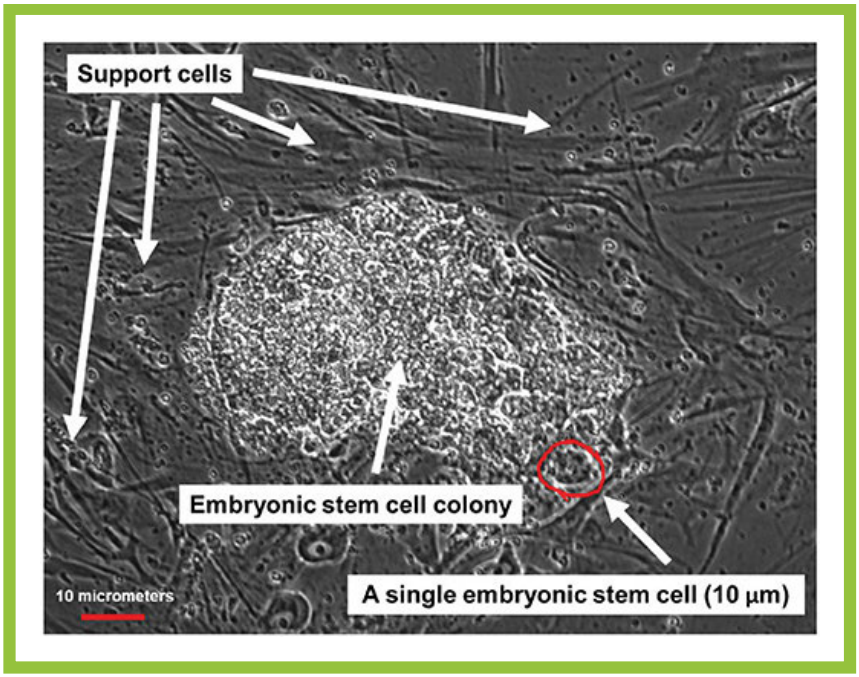


Figure 55.2 Microscope image of human ESCs. ESCs grow as a cluster of cells, which can be seen in the middle of the figure. Around this cluster are darker cells supporting the growth of the ESCs, which are called support cells.

Unlike cells in the embryo itself, ESCs grown in the lab can stay in their pluripotent state if the right growth conditions are present. These cells divide about once a day so, over time, scientists can grow millions or billions of ESCs. If the growth conditions for the ESCs are changed appropriately, scientists can stimulate the ESCs to go through a process, in which the ESCs can develop into any of the different cell types present in the body! Scientists have been working on this amazing project for more than 20 years!

The production of ESCs from human embryos is both technically difficult and ethically complex. Therefore, many efforts have been made to produce pluripotent stem cells from other cells, to avoid the use of actual embryos. The idea is to reprogram mature cells to turn them back into stem cells.

55.2 Cloning

The first attempts to turn mature cells back into pluripotent stem cells involved a process called cloning. In the cloning process, an egg is fertilized in the lab, and right after fertilization, the DNA is removed from the egg. The empty egg is then injected with DNA from another mature cell, such as a skin or blood cell.

Even though the DNA is from a mature cell, the environment of the egg will basically reprogram the genetic material from the mature cell, so that it can create an embryo. If the egg keeps developing, it will develop into a clone of the person or animal from which the mature cell was taken. Human cloning is illegal, but in the early 1960's, English researchers successfully cloned frogs. Frogs have relatively very large eggs, so they are easy to work with. The researchers took a fertilized egg from a frog, removed the DNA, and injected the egg with genetic material from an intestinal cell of another frog. After about 40 days, the egg matured and developed into a tadpole. The tadpole was genetically identical to the frog from which the intestinal cell was taken. Soon, some labs started performing this process in mammals, but the early attempts at this process failed. After 30 years of failed attempts, in 1996, researchers from Scotland successfully cloned a sheep named Dolly (Fig. 55.3A), proving that cloning is also possible in mammals. Since the end of the 1990's, a wide variety of animals has been successfully cloned, including mice, rabbits, cows, pigs, horses, donkeys, camels, and even a type of endangered wolf. The famous singer Barbara Streisand, who felt very sad when her dog died, paid researchers a large amount of money to clone two puppies identical to the original dog! In 2018, macaque monkeys were cloned successfully for the first time (Fig. 55.3B), and in 2019, the Chinese police announced the cloning of a police dog that has since started to take a training course.

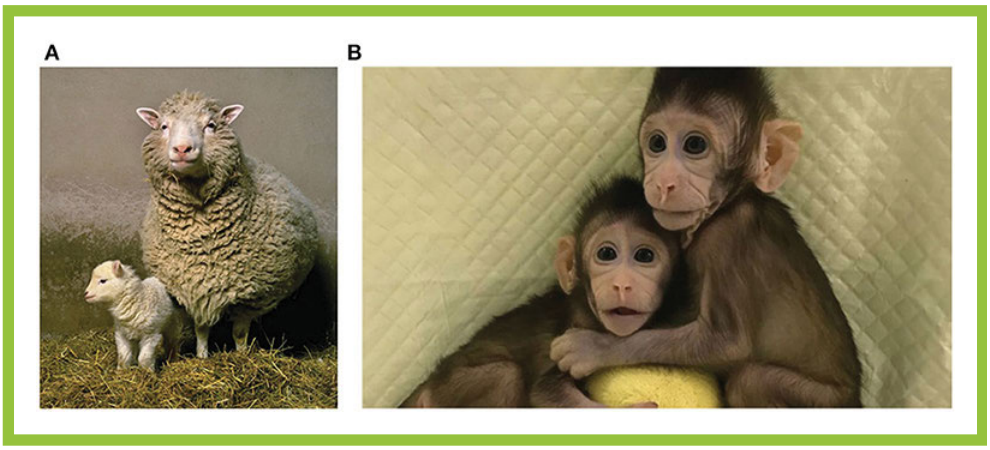


Figure 55.3 (A) Successful cloning of mammals. Dolly the sheep, the first successfully cloned mammal, was cloned in Scotland in 1996. Dolly is shown with her first offspring, Bonnie (left). (B) The first cloned monkeys, Hua Hua and Zhong Zhong, were successfully cloned in China in 2018.

55.3 Therapeutic Cloning

As we mentioned, human cloning is illegal, but a procedure called therapeutic cloning is allowed, meaning cloning for medical purposes. The same procedure described above for frogs is performed on fertilized human eggs, which are

emptied and injected with genetic material from another cell, usually a skin or blood cell. After about a week, pluripotent cells are removed and used to grow ESCs. These ESCs contain the DNA of the person from which the skin or blood cell was taken. In this way, it is possible to produce ESCs from any living person! This means that, in the future, when we know how to produce tissues, such as liver tissue from ESCs, it might be possible to grow new organs to use for transplants. The big advantage in using genetically identical cells for transplants is that the immune system does not see the transplanted organ as foreign and therefore will not attack it the same way it would attack an organ from a genetically different individual. This prevents many problems and complications.

55.4 Reprogramming

Therapeutic cloning might sound like a great thing, but this procedure is very problematic and complex. Moreover, it requires using eggs, which are very hard to get. But, in 2007, Japanese researchers found an amazing way to transform mature cells, like skin or blood cells, directly into stem cells without using eggs! They found a combination of proteins that, if injected into the mature cells, gradually reprogrammed the mature cells into stem cells. This procedure is much simpler than cloning and every lab can quite easily produce stem cells from almost every type of cells. This process is called reprogramming, and the cells produced are called induced pluripotent stem cells (iPSCs).

55.5 Therapy?

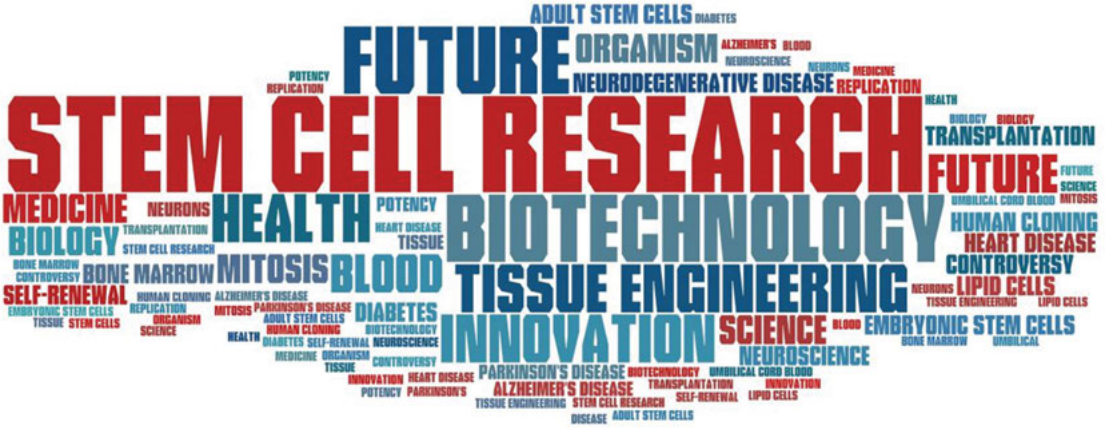
The hope is to be able to use both types of stem cells, both embryonic and induced, to treat diseases that are caused by the death of cells in the body. For instance, in type 1 diabetes, the beta cells in the pancreas die. Beta cells are responsible for producing the hormone insulin, which helps the tissues to absorb sugar from the blood to provide the body with energy. Children who are born with type 1 diabetes eventually lose all of their beta cells and stop producing insulin. Since it is impossible to live without insulin, these children must get insulin injections every day. A few research groups are trying to transform ESCs into beta cells that can be transplanted into diabetes patients, so that the patients can produce insulin again. This is a big dream because, as of today, there is still no successful treatment based on transplanting cells grown from stem cells, even though there are experiments in humans currently going on. In one of these experiments, neurons prepared from ESCs are being transplanted into the brains of patients with Parkinson's disease, in which certain brain cells die. In a second experiment, retina cells are being transplanted to patients suffering blindness caused by a disease that results in the loss of cells in the eye.

Hopefully, in the coming years, researchers will successfully produce more and more types of cells and even organs from embryonic or induced pluripotent stem cells. This will lead to more and more successful trials in humans, so that it

will be possible to treat and even cure a large number of diseases in which cells of the body die, or that require replacement of organs.

55.6 Addendum by the Series Editor, Dr. Raj Bawa: The Future of Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) offer great promise in regenerative medicine because, technically speaking, they are totipotent (i.e., can turn into any cell in the human body), offering the potential to repair and regenerate tissue damaged by a host of diseases (Fig. 55.4). Apart from this, they can also be used to screen drug candidates for toxicity.



Obviously, embryos must be destroyed to generate stem cell lines for regenerative medicine research. Although it is illegal to create embryos specifically for such research, US biomedical scientists can use embryos from *in vitro* fertilization (IVF) that would otherwise be discarded and have been donated for research. IVF is a procedure with a high failure rate and it is routine to select the healthiest embryos for implantation, the rest being discarded. However, opponents continue to argue that even such research is unethical because deriving the stem cells destroys the blastocyst (an unimplanted human embryo at the sixth to eighth day of development). They argue that the blastocyst is a human and the US government should not support the taking of innocent human life. I disagree with this point-of-view and fully support biomedical research involving embryos from IVF. I argue that since most embryos are discarded during IVF, the deliberate use of an embryo in research or to derive embryonic stem cell lines therefrom should not become illegal given that these discarded embryos hold enormous promise for research on crippling diseases. I even broadly support fetal tissue research from aborted/donated fetuses given that the 1993 NIH Revitalization Act permits donated fetal tissue to be used for research. Where and how can a red line be drawn to distinguish fetal tissue from embryonic stem cell research?

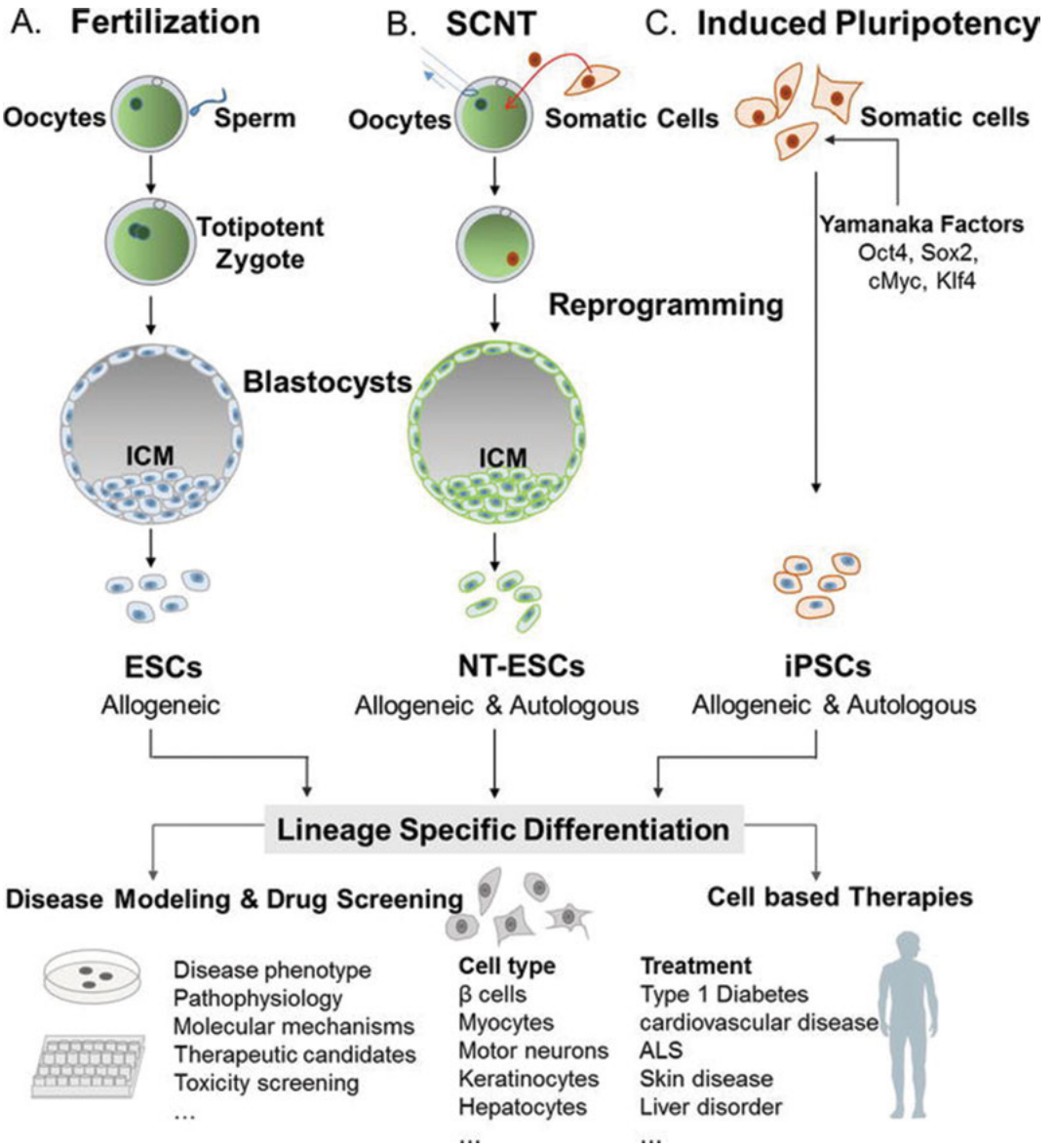


Figure 55.4 Derivation of pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) from fertilization of oocytes and sperm (A), nuclear transfer ESCs (NT-ESCs) from somatic cell nuclear transfer (SCNT) of somatic cell nuclei into denucleated oocytes (B), and induced pluripotent stem cells from forced expression of Yamanaka transcription factors in somatic cells (C). All the three types of PSCs can be propagated extensively *in vitro* and undergo directed differentiation into any cell type of the body, which can be utilized in disease modeling and drug screening and developed as cell-based therapies. As generation of ESCs involve disruption of the embryos, they can only be used as an allogenic source, while NT-ESCs and iPSCs can be developed in both allogenic and autologous settings. Source: Z. Loewy. (2019). *Innovations in Cell Research and Therapy*. Chapter 5. DOI: 10.5772/intechopen.88790, Open Access.

Given this backdrop, I caution that it is possible that biomedical scientists using ESCs or fetal tissue could find their studies at risk in conservative US states aiming to redefine "personhood" following the recent US Supreme Court's rollback of abortion rights.[1] Essentially, some US states would give embryos and fetuses the same rights as people declaring that an embryo is a person from the moment of fertilization. Although the laws primarily are intended to focus on abortions, but they have broader consequences on a range of issues, including regenerative medicine research. In my view, if personhood is granted to embryos without specific exceptions for derivation of stem cells from embryos, then there can be no stem cells on which to do research or develop medicinal products.[2] If *in vitro* fertilization is banned, then harvesting ECSs will not be possible. If a US state defines a person from the moment of conception, then it would effectively ban research using embryos.[3] Furthermore, if that applies to an embryo outside the body, effectively, it means that IVF for fertility purposes itself is now in question.

Glossary

Fertilization: The encounter between the sperm cell and the egg cell. From the moment of fertilization, embryonic development begins. An egg with no sperm is an unfertilized egg.

DNA: The genetic material that is responsible for the characteristics of organisms. For example, a certain area in the DNA is responsible for the color of the eyes, another for the color of the skin, etc.

Pluripotency: A feature of embryonic stem cells that means the ability to transform into every type of cell. "Pluri" means "a lot" and "potent" means "ability," so pluripotent means "very-capable."

***In Vitro* Fertilization (IVF)**: When an egg cell is met with a sperm cell in the lab, outside of the body.

Embryonic Stem Cells (ESCs): Cells taken from a 1-week-old embryo. These cells have two special features: they can divide in an unlimited way under certain conditions, and they can become all types of cells.

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[1]In this context, note that earlier this year in June 2022, the US Supreme Court overruled *Roe* in *Dobbs v. Jackson Women's Health Organization* on the grounds that the substantive right to abortion was not "deeply rooted in this Nation's history or tradition", nor considered a right when the Due Process Clause was ratified in 1868, and was unknown in US law until *Roe*. This view was disputed by some legal historians and criticized by the dissenting opinion, which argued that many other rights—contraception, interracial marriage, and same-sex marriage—did not exist when the Due Process Clause was ratified in 1868, and thus were unconstitutional by the *Dobbs* majority's logic (Source: Wikipedia).

[2]NIH funded more than $2 billion in stem cell research in fiscal 2021, including $309 million in embryonic stem cell research. While there are just seven cell and gene therapies on the US market today, there are more than 1,000 cell and gene therapies currently in the pipeline, with 50–75 therapies pending FDA approval expected around 2030 (Source: Faster Cures).

[3]At this point, I am uncertain as to the exact impact of personhood laws on regenerative medicine research because it depends on the wording of each US state's proposed statute, and whether a "person" is defined from "conception" or "implantation" or a "heartbeat."

Differentiation: A process in which a stem cell transforms into a mature cell.

Cloning: A process in which the DNA of a mature cell is injected into an empty fertilized egg after it has been emptied of its DNA and the DNA of the sperm cell. If this egg is returned into the animal, the baby will be genetically identical to the cell from which the DNA was taken.

Reprogramming: A process in which regular, mature cells are transformed back into pluripotent embryonic stem cells.

Disclosures and Conflict of Interest

This chapter was originally published as: Meshorer, E. (2020) What are embryonic stem cells and how can they help us?. *Front. Young Minds*. **8**, 32, doi: 10.3389/frym.2020.00032, under the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/), and appears here, with edits and updates. The author of this chapter declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 56

The Rise and Rise of Mitochondrial DNA Mutations

Conor Lawless, MEng, PhD, Laura Greaves, PhD, Amy K. Reeve, PhD, Doug M. Turnbull, PhD, MD, and Amy E. Vincent, PhD

Wellcome Centre for Mitochondrial Research, Clinical and Translational Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle, UK

doug.turnbull@ncl.ac.uk, amy.vincent@ncl.ac.uk

Keywords: α-proteobacterium, *Caenorhabditis elegans* (*C. elegans*), clonal expansion, cytochrome *c* oxidase, formalin-fixed paraffin-embedded (FFPE), heteroplasmy, induced pluripotent stem cells (iPSCs), mitochondrial DNA (mtDNA), mutation, oxidative phosphorylation (OXPHOS), Parkinson's disease, perinuclear niche hypothesis, post-mitotic cells, reactive oxygen species (ROS), respiratory chain deficiency

56.1 Evolutionary Aspects of Mitochondrial Genetics

The endosymbiotic origins of mitochondria in eukaryotic cells are apparent in the similarities between mitochondria and bacteria, such as the plasmid-like multi-copy circular genome that resides in the mitochondrial matrix. Mitochondria are thought to be descended from α-proteobacterium due to sequence similarities between the genome and mitochondrial DNA (mtDNA) [1]. In present-day humans the mitochondrial genome is approximately 16.5 kb and encodes only 37 genes. This genome has been greatly reduced over evolutionary time via transfer of genes to the nuclear genome [2] so that it now only encodes 2 rRNAs, 13 oxidative phosphorylation (OXPHOS) subunits and 22 tRNAs [3].

Unlike diploid nuclear DNA, mtDNA exists in a highly polyploid state within each cell. This means that mutations in the mtDNA can exist in a subset of the total cellular mtDNA, a state termed 'heteroplasmy'. mtDNA molecules are replicated during mitosis, in much the same way that nuclear DNA molecules are (defined as strict replication [4]), but mtDNA molecules are also continuously replicated

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independently of the cell cycle (defined as relaxed replication [4]), in a similar fashion to the replication of bacterial genomes. Molecules of mtDNA have been shown to be continuously selected at random for relaxed replication in pulse-chase thymidine analogue experiments in dividing mouse cells [5].

Packaged as nucleoids, mtDNA resides close to the inner mitochondrial membrane and the oxidative phosphorylation (OXPHOS) complexes. The mitochondrial genome is hyper-mutable compared with nuclear DNA and this is thought to be due to damage caused by the high levels of reactive oxygen species (ROS) to which it is exposed [6, 7], as well as the fact that mtDNA is replicated more frequently. Further, the compact nature of mtDNA means that there is very little that is non-coding and therefore mutations arising in mtDNA are much more likely to have pathological impact than mutations arising in the nuclear genome, where a large amount of the DNA is intronic.

Mutations in mtDNA can either be inherited or sporadically acquired throughout life. Inherited mutations are randomly segregated between primary oocytes and between different tissues. Work assessing mutation load in different fetal tissues has demonstrated that mutation load is similar across tissues [8]. Single cell analysis at this stage has not yet been completed, so we have no way of knowing whether the segregation of mutations during tissue formation contributes to the variability of mutation load or whether this is purely due to clonal expansion after development. In individuals who inherit a low mutation load, mitochondrial disease pathology is not likely to arise for a long time, if at all. The timing of the onset of mitochondrial disease is variable and pathology can be heterogeneous, affecting some tissues or parts of tissues or cells, but not others [9, 10].

In comparison, sporadically acquired mutations arise in a single mtDNA molecule in a single cell and occur during healthy ageing, mtDNA maintenance disorders and a range of other diseases [11–13]. This single mutated mtDNA molecule is then either lost from the cell or clonally expands to higher levels. There is a tissue-specific pattern to the clonal expansion of sporadic mtDNA mutations: the accumulation of mtDNA point mutations is more common in mitotic cells whereas the accumulation of mtDNA deletions is more common in post-mitotic cells. Up to three or four different mtDNA deletions have been found to have clonally expanded in single muscle fibres and neurons, respectively [13–15], although 37 mtDNA deletion species have been detected in a single neuron by ultra-deep sequencing [13].

56.2 Clonal Expansion of mtDNA Mutations

The dynamic process by which mtDNA mutations accumulate, which we often term ‘clonal expansion’, is thought to be one of the contributing factors behind the progress of many forms of mitochondrial disease. Furthermore, evidence suggests that the functional consequences of having clonally expanded mtDNA

mutations may contribute to pathogenicity in other age-related diseases such as Parkinson's disease [16]. As such, this has become a very important question for mitochondrial biologists to answer, since it presents a potential therapeutic avenue that can be applied across a range of diseases.

56.2.1 Clonal Expansion Theories

One of the biggest unanswered questions regarding clonal expansion is whether there is any selective advantage or driver for the accumulation of mtDNA mutations. There have been a series of theories developed to explain how clonal expansion of mtDNA mutations occurs, with the random genetic drift theory assuming no selective pressures [17, 18], and 'survival of the smallest' [19], 'survival of the sickest' [2, 20], the negative feedback loop [21, 22] and the 'perinuclear niche' [23] all assuming different possible selective mechanisms (Fig. 56.1). *In silico* predictions have been made from several of these theories using estimations of important biological variables such as mtDNA copy number and mitochondrial turnover. All models have their limits but provide a means to test the degree to which a hypothesis can explain the levels of mitochondrial dysfunction that are observed in human samples.

So far evidence suggests that random genetic drift by relaxed replication is sufficient to explain the clonal expansion of point mutations but not necessarily mtDNA deletions. Random genetic drift suggests that mtDNA molecules are selected randomly for strict or relaxed replication leading to an accumulation of mutated mtDNA by chance. Stochastic, dynamic simulation models of mtDNA population dynamics during relaxed replication were previously developed [17, 18, 24] to explain clonal expansion as a form of random genetic drift [25]. Random genetic drift, which could explain clonal expansion without relying on any selective advantage or feedback at all, has formed a useful null hypothesis for testing theories about clonal expansion for the past 20 years.

In comparison with mtDNA point mutations, the clonal expansion of mtDNA deletions does not seem to be fully explained by random genetic drift, since results from random genetic drift modelling work to date do not accurately predict the levels of mtDNA deletions observed in substantia nigra neurons and muscle fibres (discussed further below). As such alternative hypotheses that include selective pressures have been considered. The first theory suggested to explain how mtDNA deletions expand clonally, suggesting that deleted, and therefore smaller, mitochondrial genomes would be replicated more quickly and so would have a selective advantage over wild-type genomes [19]. An alternative hypothesis suggests that the driving mechanism is a link between mtDNA encoded protein products and mtDNA replication [21, 22]. *MT-ND4*, *MT-ND5* and *MT-ND6* were proposed as possible candidates for such a feedback mechanism with an mtDNA deletion that encompassed these genes leading to a decrease in their proteins and a compensatory increase in mtDNA transcription and replication to replenish these proteins. This increased replication would therefore provide a

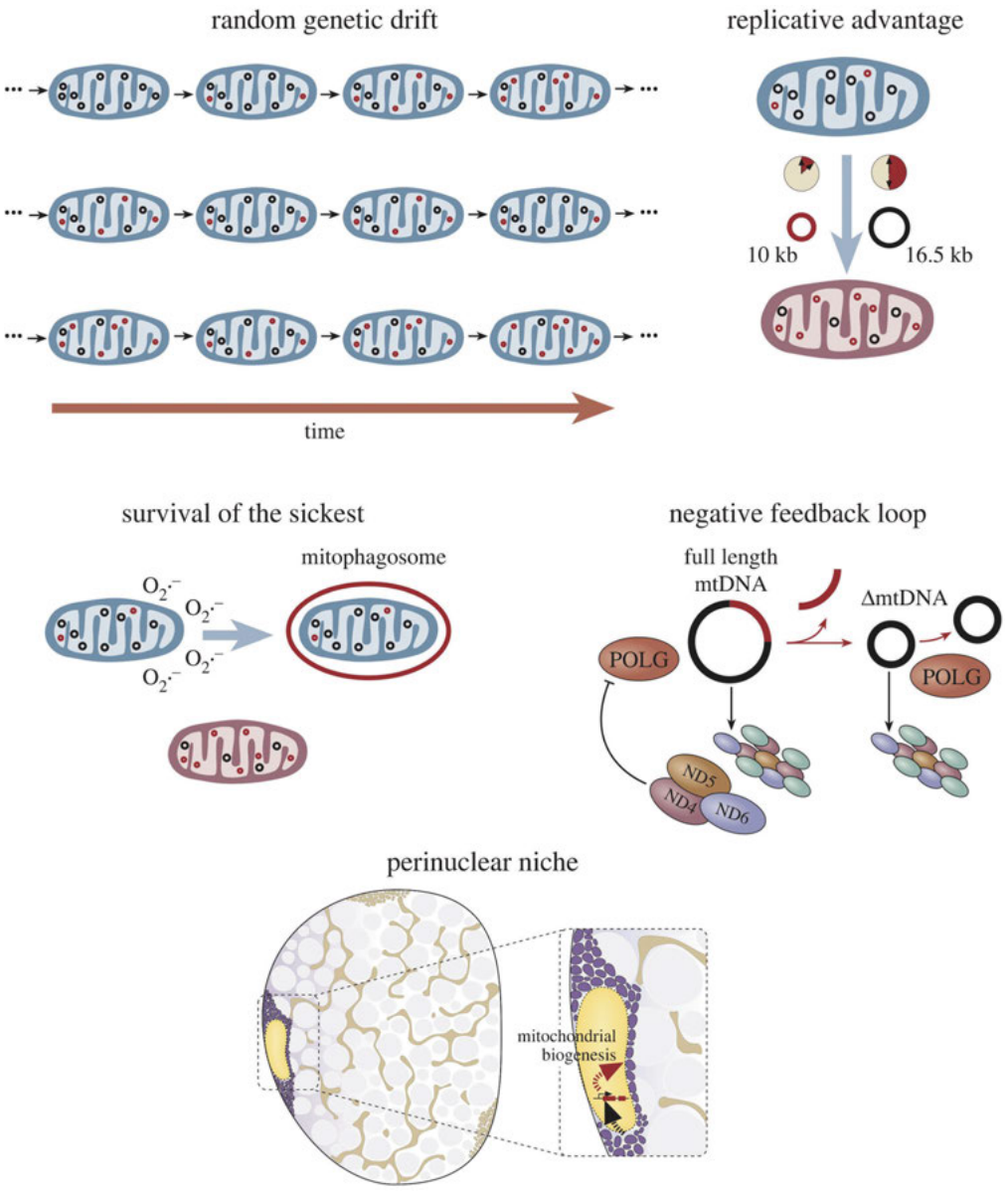

Figure 56.1 Theories of clonal expansion. The mechanism by which clonal expansion happens has been proposed to be explain by several possible theories about the mechanism. Random genetic drift suggests the accumulation happens randomly during relaxed mtDNA replication. A replicative advantage suggests that smaller deleted mtDNA genomes are replicated faster and take over the cell. Survival of the sickest suggests that the most dysfunctional mitochondria survive mitophagy due to a reduced production of ROS and therefore accumulate. A negative feedback look suggests a reduction in a mtDNA encoded protein product from a mutated mtDNA molecule drives further transcription and replication. Finally, the perinuclear niche hypothesis suggests a localized upregulation of mitochondrial biogenesis is triggered by mtDNA mutations accumulating adjacent to the myonuclei in muscle fibres.

possible selective advantage for the deleted mtDNA. Subsequently observations in muscle have suggested that close proximity to nuclei provides a similar feedback mechanism for mtDNA deletions upregulating mtDNA replication, but through retrograde stress signalling for example changes in ATP/ADP or NAD^+/NADH ratios [23]. Both the positive transcriptional feedback loop and perinuclear niche hypothesis could feasibly contribute to clonal expansion of mtDNA point mutations also, however evidence suggests these selective pressures are not needed for clonal expansion. Similarly, it also remains possible that predictions from an adjusted random genetic drift model, fully incorporating uncertainty about parameters such as mtDNA copy number, mtDNA turnover and mitochondrial dynamics, will not be significantly different from observations in human samples.

56.3 Population Dynamics of mtDNA Point Mutations

56.3.1 Formation of Point Mutations

The mechanism by which mtDNA point mutations are generated in somatic tissues is an area of much debate. Initial hypotheses suggested that mtDNA was susceptible to damage induced by ROS due to its close proximity to the respiratory chain in the mitochondrial matrix [26]. ROS induced DNA damage causes base modifications, double and single-strand breaks, sugar damage and abasic sites [27]. The most commonly reported base lesions are thymine glycols and 7,8-dihydro-8-oxo-2′-deoxyguanosine [8-oxo-dG] [28]. 8-oxo-dG is thought to be the most mutagenic lesion, which can cause the mtDNA polymerase to mis-incorporate an A base opposite the oxidized G resulting in a G:C to T:A transversion following a second round of replication [28]. More recent analysis of mtDNA mutational spectra in ageing cells has revealed that it is not consistent with the predicted ROS induced damage pattern, instead the most commonly reported mutations are G:C to A:T transitions [29, 30]. Transitions are more likely to be the result of errors of the Polγ, which is responsible for replication of the mtDNA, and/or spontaneous cytosine deamination to uracil which then mis-pairs with adenine resulting in the G>A transition [31]. These data, alongside analysis of mtDNA point mutation spectra in transgenic animals [32], suggest there is a limited role for ROS-induced mtDNA mutagenesis causing point mutations in somatic cells.

56.3.2 Sporadic mtDNA Point Mutations

Sporadically acquired mtDNA point mutations, are the most common clonally expanded mutations in mitotic tissues. These were first detected by Nekhaeva et al. [33], who sequenced individual cells from normal ageing buccal epithelium and found that a subset of cells contained mtDNA point mutations at high levels. Following this, Taylor et al. showed that a significant proportion of ageing colonic

epithelial crypts (on average 15% by the age of 70) demonstrated histochemical loss of cytochrome *c* oxidase activity, which was caused by somatic, clonally expanded mtDNA mutations [10]. This work was followed up by a number of studies demonstrating an age-related increase in the frequency of cells with OXPHOS defects due to clonally expanded mtDNA mutations in mitotic tissues including the small intestine, stomach, oesophagus, prostate and liver [34–37].

Computational modelling of clonal expansion within mitotic cells has shown that random genetic drift is sufficient to explain the population dynamics of mtDNA point mutations observed experimentally in individual cells over time [24, 38]. Random genetic drift models suggest that clonal expansion of a mutated mtDNA molecule to high levels within a cell is a relatively slow process and that initial mutational events must occur early in life. These early life mutations then either propagate randomly through mtDNA replication and segregation at cell division or are lost. Successive cycles of this stochastic process allow mutated mtDNA molecules to become the dominant species within some cells, resulting in a mosaic pattern of cellular OXPHOS defects which are seen in ageing human mitotic tissues. In addition, most mitotic tissues have a high turnover rate and are maintained by long-lived stem cells. mtDNA mutations which expand clonally within these stem cells will then be propagated in their progeny. In tissues such as the liver, prostate, stomach and colon, this results in large, clonal patches of cells with identical mtDNA mutations and associated OXPHOS defects [34, 36, 38, 39].

Despite numerous studies providing evidence that mtDNA mutations expand clonally causing a mosaic pattern of OXPHOS defects in ageing human tissues (e.g. colon; Fig. 56.2), the functional consequences of these have not yet been fully elucidated. A study by Nooteboom et al. showed that colonic crypts with loss of MTCO1 protein expression had fewer actively proliferating cells and were significantly smaller than those with normal MTCO1 levels, however whether this further effects tissue function is unknown [40]. Further insights have been possible through the development of mouse models. The mtDNA mutator mouse has a D257A amino acid change in the proof-reading domain of Polγ resulting in an error prone polymerase, causing an accelerated acquisition of mtDNA point mutations [41, 42]. These mice show a premature ageing phenotype and significantly reduced lifespan. A mosaic pattern of OXPHOS defects due to clonally expanded mtDNA mutations have been shown in the small intestine [43] and the colon of these mice [44]. In the small intestine these defects have been shown to cause an increase in the frequency of apoptosis, a decrease in cell proliferation, a reduction in dietary fat absorption and a loss of the capacity to form stem cell derived organoids *in vitro* [45]. Analysis of the haematopoietic compartment of the mutator mice revealed no direct effect of mitochondrial dysfunction on the haematopoietic stem cells themselves, but instead defects during early differentiation were noted [46]. These differentiation blocks resulted in abnormal myeloid lineages and caused anaemia and lymphopenia in the mice [47].

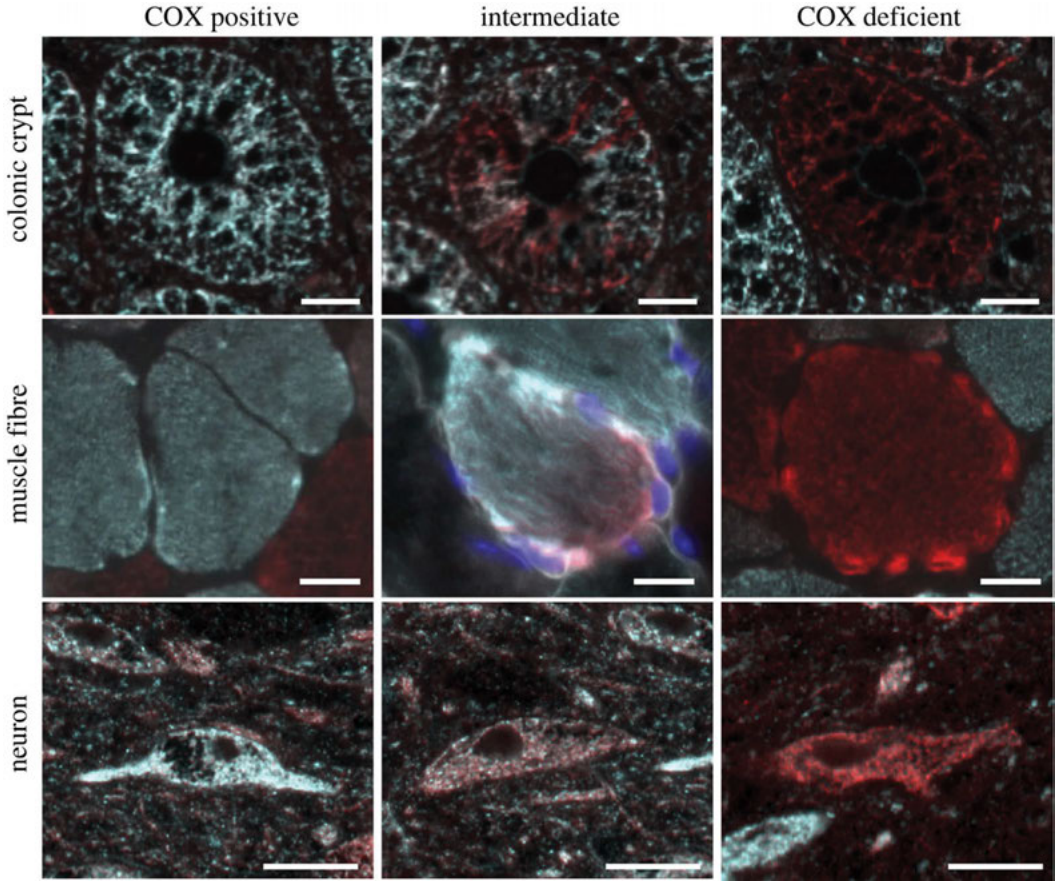

Figure 56.2 Consequences of clonally expanded mitochondrial DNA mutations. Mitochondrial DNA mutations accumulate with age and disease in both mitotic tissues such as the colon, and post-mitotic cells such as muscle fibres and neurons. At low levels the mitochondrial DNA mutation will have little functional impact (COX positive). As the percentage of mitochondrial DNA mutations within a cell accumulate, they will exceed a biochemical threshold causing mitochondrial respiratory chain dysfunction (COX deficient). The transition from COX positive to COX deficient is slightly different in different tissues, with the most noticeable differences being that colonic crypts can be partially COX deficient, muscle fibres can have focal deficiency, whereas neurons are more commonly observed to have low deficiency across the full cell body (although it is never possible to view a full neuron). Scale bar, 25 μm. The images were collected from the authors' own research; see ethics statement at end of chapter for further details.

56.3.3 Inherited mtDNA Point Mutations

In contrast to the accumulation of somatic mtDNA mutations in ageing tissues, some inherited mtDNA point mutations are found to be systematically lost throughout life in rapidly dividing cells including blood, buccal mucosa and colonic epithelium, while remaining stable in post-mitotic tissues [48–52]. However,

it is not known whether the decline in mtDNA mutation load in rapidly dividing cells is also the result of random genetic drift or the result of active selection against functionally deleterious variants (e.g. due to the loss of entire affected cells).

56.4 Population Dynamics of mtDNA Deletions

56.4.1 Deletion Formation

mtDNA deletions have been proposed to form either during replication or repair. Features of mtDNA breakpoints have previously been used to infer the mechanism of formation, with deletions defined as class I if they have direct repeats, class II with indirect repeats and class III if they have no repeats [53]. Initially deletions were proposed to form during replication due to a slip-replication mechanism [54]. This model assumes that mtDNA is replicated by the strand-asynchronous mechanism and that the light strand misaligns so that the 3′ repeat of the light strand anneals to the 5′ end of the heavy strand, generating a single strand loop, this loop would be susceptible to a single strand break and degradation [54, 55]. An alternative hypothesis was later proposed, which suggests mtDNA deletions are formed during mtDNA repair of double strand breaks, by the annealing of homologous repeats created by exonuclease activity at double strand breaks [53, 55].

To further investigate replication-dependent mechanisms for deletion formation, a mouse model with an inducible mitochondrially targeted restriction endonuclease [56] led to the suggestion that mtDNA deletions are formed during mtDNA repair of double-strand breaks. In these mice double-strand breaks were induced in adult neurons, resulting in the formation of deleted mtDNA molecules. Evidence suggests that double-strand breaks may be repaired either by non-homologous end joining or micro-homology-mediated end joining [55–58]. Both of these mechanisms only account for homology-dependent recombination, whereas there are reports of deletions without repeat sequences [17, 53]. More recent work looking at replication-dependent mechanisms has suggested that mtDNA deletions are instead formed by copy-choice recombination during active L-strand DNA synthesis [59]. This model is attractive since mtDNA deletions with direct repeats, imperfect repeats and no repeats have been detected and while this mechanism is enhanced by the presence of mtDNA repeats it can also occur without repeats. As such it is now thought that mtDNA deletions form either by copy choice recombination during mtDNA replication or by repair of double strand breaks via non-homologous end joining or micro-homology mediated end joining [60]. Furthermore, the mechanism for deletion formation may be dependent on the mechanism of mtDNA replication, which has been demonstrated to vary between tissues [61].

56.4.2 Sporadic mtDNA Deletions

In contrast to mtDNA point mutations, sporadically acquired mtDNA deletions are the most common sporadic, clonally expanded mtDNA mutation in post-mitotic cells.

The reason for this contrast remains an important unsolved mystery. High levels of clonally expanded mtDNA deletions have been reported in neuronal populations from the substantia nigra, hippocampus, striatum and spinal cord with both ageing and disease [12, 62–64], as well as aged skeletal muscle fibres [65]. These mtDNA deletions accumulate to high levels in both neurons and muscle fibres, leading to mitochondrial dysfunction. However, the mutation loads observed in different neuronal populations differ substantially, with the highest levels (over 50%) typically observed in the dopaminergic neurons of the substantia nigra [66, 67].

Extensive studies have demonstrated that clonally expanded mtDNA deletions are associated with mitochondrial OXPHOS dysfunction in both neurons and muscle fibres [9, 65] (Fig. 56.2). Furthermore, in the brain these have been found to be associated with neurodegeneration [62], and there is evidence that they are associated with muscle fibre atrophy [65]. Despite increasing knowledge about the consequences of clonally expanded mtDNA deletions, it is not yet fully understood how these deleted mtDNA species come to predominate in cells.

While much is known about mitochondrial genetics, the process by which a single mtDNA mutation accumulates still eludes us. Based on data from estimates of mtDNA turnover rate and mtDNA copy number from rat muscle fibres, modelling of random genetic drift by Elson et al. [18] predicts 4% of post-mitotic cells should become COX-deficient over an 80 year period in healthy individuals. However, that prediction does not include a range of uncertainty or confidence interval.

Data from human substantia nigra shows that respiratory chain deficiency is higher with 40% of neurons found to be COX-deficient at 80 years [68, 69], and this is likely to be an underestimate since approximately 5% of neurons are lost per decade [70]. Levels of COX-deficient cells in a single section of aged muscle are much lower with percentage of COX-deficient fibres typically less than 5% in people aged 75–88 years [71]. However when the length of muscle fibres in the quadriceps vastus lateralis is considered, the true percentage may be higher, approximately 6% at 49 years and approximately 31% by the age of 92 years [65]. Again, there is considerable unstated uncertainty about these predictions.

However, as well as assuming random genetics, the model of Elson et al. [18] also assumes that fibres are well mixed (i.e. it ignores diffusion of mtDNA along the considerable length of muscle fibres). If this model of random genetic drift underestimates the percentage of respiratory chain deficient cells, it may be that there are selective pressures at work that are preferentially allowing the accumulation of these deleted mtDNA species.

An alternative to purely random drift is that deleted mtDNA molecules have a replicative advantage [19]. Many studies *in vitro* have generated supporting evidence for this theory, finding that deleted mtDNA molecules repopulate cells faster than wild-type mtDNA under relaxed copy number control [72, 73]. However, following artificial depletion of the mtDNA, the cell may replicate its mtDNA at a faster rate in order to return to the required mtDNA copy number, under such conditions the time taken to replicate a single molecule could be much more rate limiting and therefore important, than it would be in post-mitotic cells. For example, work in human muscle has demonstrated no relationship between the size of mtDNA deletions and respiratory chain deficient segments, suggesting that

there is no replicative advantage [9]. However, we do not know when each of the mutations formed but must assume that if deletions form randomly with a mix of small and large deletions this should not impact results. Finally, work in *C. elegans* examining the behaviour of two differently sized mtDNA deletions, also found no evidence for a replicative advantage [74].

Another alternative hypothesis that has been proposed is a feedback mechanism whereby low levels of protein products from the deleted genes, triggers mtDNA replication [21, 22]. While this hypothesis is attractive, the authors suggest a subset of 'feedback genes'. However, examination of the spectrum of breakpoints reported on 'mitobreak' [75] demonstrates that no one gene is always deleted and indeed deletions occur that do not remove any of the suggested genes. Therefore, if such a mechanism exists, it is likely that the number of genes contributing to the feedback mechanism is either larger or all inclusive.

More recently, we have proposed a perinuclear niche hypothesis for the clonal expansion of deletions in skeletal muscle. This stems from the observation that the smallest regions of respiratory chain deficiency are both subsarcolemmal and perinuclear. The hypothesis suggests that the close proximity of the mtDNA deletion and dysfunctional mitochondria to the nucleus provides a driver of clonal expansion through retrograde stress signalling triggering a local increase in mtDNA replication. Such stress signalling may include a reduction in ATP/ADP or NAD+/NADH ratios, upregulation of the integrated mitochondrial stress response [76] or (as suggested in the original investigation) upregulation of mitochondrial biogenesis via the unfolded protein response [23]. Furthermore, if mtDNA replication is higher in the perinuclear region of muscle fibres as previously reported in HeLa cells [77], this may also lead to a higher frequency of replication errors and deletion formation in the perinuclear mitochondria. The perinuclear hypothesis would likely favour sporadic mutations arising in close proximity to the nucleus, and therefore it is possible that it would also favour mtDNA point mutations. However, point mutations are less commonly investigated in muscle and further work would need to be completed to investigate this.

This hypothesis only suggests a selective pressure after the mtDNA deletion has reached sufficient levels locally to cause respiratory chain deficiency, and it has yet to be determined whether such an advantage is sufficient to explain the high levels of respiratory chain deficient cells observed. However, it is also possible that such focal deficiency could form without an induction of replication if mtDNA replication is naturally higher in the perinuclear region, as previously suggested [77]. In muscle, the mitochondria are either packed around the edge of the fibre adjacent to the myonuclei or between the myofibrils [78] and as such transport is minimal with mitochondrial fission and fusion providing the main means for distribution of mtDNA and proteins throughout the cell [79–81]. These attributes of mitochondrial organization and dynamics, as well as muscle fibre structure are integral to the perinuclear hypothesis. Therefore, while we cannot yet rule out the existence of a similar mechanism in neurons, it is possible that an alternate mechanism may be present in neurons, despite perinuclear replication, given the more dynamic nature of the mitochondria in this cell type.

In comparison with muscle, the mitochondria in neurons must be transported from the site of biogenesis to areas of high energy demand (e.g. the synapse and nodes of Ranvier) and subsequently to the site of degradation. The majority of mitochondria are found to be stationary at the sites of ATP requirement, with around 10–20% of mitochondria being actively transported [82]. This small proportion of moving mitochondria and the sites at which mitochondrial biogenesis and degradation occur are likely to be important factors when we are considering how mtDNA mutations clonally expand in neurons. Previously, it has been demonstrated that when mitochondria lose their membrane potential they are transported back to the cell body for degradation [83, 84], this is further supported by a lack of mitophagy observed in dendritic arbours and axonal projections in mitoQC mice, with the majority of mitochondrial degradation observed in the soma [85]. Furthermore, if the majority of mtDNA replication occurs in close proximity to the nucleus as previously reported in cultured cells [77], it is likely that the site of clonal expansion is in the cell body with mtDNA mutations being distributed along the neuron by mitochondrial transport, fission and fusion.

In muscle the presence of perinuclear foci of mitochondrial dysfunction suggests that the nuclei play an important role in the accumulation of mtDNA deletions and mitochondrial dysfunction. Therefore, the perinuclear niche hypothesis shows promise for explaining how these mtDNA deletions accumulate. However, it will be necessary to understand the relative contributions of mtDNA replication and mito-nuclear signalling to this process and to systematically compare this to random genetic drift using *in silico* modelling (discussed in more detail below). The structure of neurons would favour a situation where the majority of mitochondrial replication would occur in the perinuclear area and thus would also support a similar perinuclear niche hypothesis for the clonal expansion of mtDNA deletions to that proposed for muscle. However, this is less definitively described in neurons than muscle and also needs to take into consideration the vastly different movement and dynamics of mitochondria within these two cell types. This may explain why perinuclear focal deficiency has not to date been reported in neurons; however, it is still possible that such focal deficiency occurs and is simply less frequent or more challenging to find.

56.4.3 Single, Large-Scale mtDNA Deletions

Similar to mtDNA point mutations, there is a disparity in what happens for inherited and sporadically acquired mtDNA deletions. Inherited mtDNA deletions such as those in patients with Pearson's syndrome are also lost from the blood [86]. However, it does appear in post-mitotic tissues that inherited mtDNA deletions loads are maintained throughout life or clonally expand. High levels of mtDNA deletions have been detected in individual muscle fibres and are associated with respiratory chain deficiency [87]. High levels of inherited mtDNA deletions have also been detected in neurons [88].

56.5 Important Challenges and Unanswered Questions

Despite interest in understanding clonal expansion during development and ageing, and in disease, there are several important challenges that have hindered progress in understanding this important mechanism. Furthermore, there are many unanswered questions which are inherently important for understanding clonal expansion.

56.5.1 How Does Clonal Expansion Occur during Development?

As stated above we know that inherited mtDNA variants are segregated unevenly between oocytes and that in a foetus tissue homogenates have a similar mtDNA mutation load [8]. However, we know little about the formation of somatic mutations *in utero*, or about the single-cell mutation loads in fetal tissues. The question of clonal expansion during development *in utero* presents many unknown parameters for which it is all but impossible for us to gather biological data independently, making it clear that there is an important role for mathematical modelling of our uncertainty about this process. We can build hypotheses about how inherited mutations expand from the initial condition of the progenitor oocyte and how new mutations arise during development and compare the predicted consequences (e.g. mutation load distributions in fetal samples) with observations. However, it is important to be explicit about our uncertainty about model parameters such as initial mutation load, replication rate and copy number control, many of which are difficult or impossible to measure directly, without assuming a model of how expansion happens.

56.5.2 Why Do Mitotic Cells and Post-Mitotic Cells Differ?

As discussed above, somatic mtDNA point mutations tend to accumulate in mitotic cells, while somatic deletions predominate in post-mitotic cells. The key to why this difference in the appearance of mtDNA mutations exists is likely to lie in the differences between the two types of cell. In mitotic tissues, mtDNA undergoes both strict (frequent) and relaxed (infrequent) replication, whereas in post-mitotic tissues, only relaxed replication occurs. As such this means that mtDNA is more frequently replicated in mitotic cells than post-mitotic cells. In addition, in replicating cells mitochondria are segregated to different daughter cell populations resulting in an asymmetric or symmetric distribution of mutant mtDNA between daughter cells, allowing for a bottleneck effect impacting clonal expansion [38].

Cells in mitotic tissues are regularly turned over and undergo apoptotic cell death. In comparison, in multinucleated skeletal muscle fibres it is thought that apoptosis occurs for single nuclei within a muscle fibre leading to muscle fibre atrophy [89], although this hypothesis is contentious [90]. Satellite cells may also fuse with a muscle fibre allowing regeneration of the fibre [91]. Both regeneration

and apoptotic processes are effectively sub-cellular, and as such, a whole cell (and indeed the whole population of mitochondria in a muscle fibre) is not removed on a periodic basis. In neurons where cell death occurs during neurodegeneration, there is limited means to replace cells that are lost. Some neuronal populations in particular show a steady decline overtime [70], it is not understood why these are lost but it may be these are the ones with the highest mutation load.

56.5.3 How Do Inherited and Acquired Mutations Differ?

There are clear differences between the changes in sporadic mtDNA mutations and inherited mtDNA mutations over time. Inherited mtDNA mutations are lost in rapidly dividing cells such as the blood, intestinal epithelium, buccal mucosa and urine [49–52, 92]. In post-mitotic cells not much is known about what happens to inherited mtDNA point mutations overtime, but it is commonly believed that single, large-scale mtDNA deletions clonally expand from birth in skeletal muscle fibres [9] and neurons, with higher mtDNA deletion loads in post-mitotic tissues than in mitotic tissues of the same patient [93, 94]. In comparison both somatic mtDNA point mutations and mtDNA deletions clonally expand over time. It has been hypothesized that, in neurons this accumulation of somatic mutations, may be due to a difference in how cells respond to a mutation that is acquired in comparison to one that has been inherited [95]. Such a hypothesis is intriguing and warrants investigation in other tissues.

56.5.4 How Do We Investigate a Process That Takes Place Over a Lifetime?

One of the greatest problems is that the accumulation of mutated mtDNA to pathological levels usually occurs over decades in humans. However, the most direct observations we can make are cross-sectional observations in patient tissue. Furthermore, clonal expansion is dynamic and is heterogeneous, occurring independently in individual cells. In order to capture this heterogeneity, observation of many single cells is required. However, even if it were possible to revisit the same cell repeatedly *in vivo*, it is not possible to track all of the individual mtDNA molecules within a cell over time. Direct, longitudinal observation of clonal expansion is simply not possible.

One helpful approach has been to examine spatial patterns within tissues (e.g. subcellular location of respiratory chain deficiency). From such studies we can deduce presence and location of mutations in single cells [9, 23], and make deductions or predictions as to how these arose. It is difficult to know what happened prior to the sample being collected and it is important that we incorporate that uncertainty into our predictions.

Given the practical constraints on making longitudinal observations throughout human lifespans, in order to understand clonal expansion, we need to work with a model system. Importantly, the goal of any model (mathematical, statistical or

biological) is that it should be a simpler, more tractable version of the real system, capturing its main characteristics. Models do not need to replicate the target system exactly in order to be useful, provided the differences are considered when interpreting the results. It is important that we carefully choose models appropriate for the question at hand.

56.6 Modelling Clonal Expansion

56.6.1 Biological Models

Examining mtDNA population dynamics in mitotic cell culture has a number of advantages, including the use of patient-specific human cells, which are an experimentally tractable model of clonal expansion during development. For example, with mitotic cell culture it is possible to observe point mutations arising, reaching loads as high as 25% and declining in continuously dividing cell culture in as little as 21 weeks [96]. However, there are substantial disadvantages to such culture based models, including: time scale (although, in principle, cells can be cultured almost indefinitely); the possibility that culture conditions may alter the selection of mtDNA mutants versus wild-type; we are likely only to be able to observe expansion of point mutations in continuously dividing culture; and many aspects of cell biology (mitochondrial dynamics, mtDNA replication rate etc.) we observe in one cell type may not be representative of others *in vivo* in particular post-mitotic cells (e.g. skeletal muscle fibres, neurons). One alternative for this last point is the growing work using patient derived induced pluripotent stem cells (iPSCs) [97], which can be differentiated into any cell type and also used to generate 2D or 3D co-cultures that are more representative of tissues. Such cultures are technically difficult to establish and maintain, however, and still suffer from many of the disadvantages listed above.

Animal models have the advantage of providing physiologically relevant conditions and cell types. For practical reasons, mouse models are a popular choice, however many do not accumulate the same levels of respiratory chain deficiency as humans. Differences between mouse and human tissue probably arise because of their much shorter lifespan but could also be due to significant difference in cell types (e.g. fibre type composition in muscle) and possibly mtDNA population size. This is important to consider when interpreting findings from animal models since, for example, Elson et al. [18] demonstrated a slower increase of mtDNA mutation load when the cellular mtDNA population is larger. Another advantage of animal models is the ability to manipulate process or use techniques to label cellular components and thus test hypotheses. However, animal models still present similar issues to human tissue samples since sampling tissue from mice still requires cross-sectional observations at single time points.

In addition to mouse models, *Caenorhabditis elegans* has been used in several studies which looked to investigate the clonal expansion of deleted mtDNA molecules [74, 98] and mtDNA point mutations [99]. *Caenorhabditis elegans*, similar

to mice, has a shorter lifespan than humans and as such does not have sufficient time for sporadic mtDNA mutations to clonally expand in wild-type animals [100]. However it provides a cost-effective means to investigate some of the possible selective pressures that could impact clonal expansion from low mutation loads in physiologically relevant tissues, such as the activation of retrograde signalling responses, and changes in mitochondrial biogenesis or mitophagy [74, 98].

56.6.2 Mathematical Models

Work on mathematical models of clonal expansion has continued since Chinnery et al. first modelled relaxed replication demonstrating that heteroplasmy can shift quickly over a short period of time [17]. This work was developed further by Elson et al. to explain between-cell heterogeneity in mtDNA mutation load dynamics [18]. Kowald & Kirkwood add the process of transcription to the same underlying model [21]. Stamp et al. incorporate the effect of asymmetric division of mitotic stem cells along with random drift [38]. Johnston & Jones consider the effect of various copy number control schemes on theoretical mutation load distributions between cells [101].

As stated before, random genetic drift presents an important, simple null hypothesis to which, new models can be compared. Indeed, while it does seem to explain clonal expansion of mtDNA point mutations in colonic crypts, it does not seem to fully explain clonal expansion of mtDNA deletions in post-mitotic cells. However, it is possible and indeed likely that this is due to the specific assumptions embedded in the modelling by Elson et al. [18] about fixed mtDNA copy number, copy number control, mtDNA mutation rate, mtDNA half-life and mixing of mtDNA populations within the cell, all of which we still know very little about. Furthermore, these parameters are likely to vary between different tissues and cell types, and so inferences based solely on information from other cell types and tissues should be avoided. In order to assess the predictions from the random genetic drift hypothesis, we need to take intrinsic stochasticity as well as uncertainty about parameter values and processes into consideration, ideally through formal statistical inference, using methods that can handle parameter uncertainty and stochastic models. Henderson et al. demonstrate the computational and analytical difficulties behind inference for a model of mtDNA population dynamics [102]. Indeed, Bayesian inference for stochastic simulation models remains an active area of research in applied statistics [103–105], but there are several tools currently available to help with exact Bayesian inference for deterministic models [106–108].

Building a mathematical model helps us to be explicit about our mechanistic hypothesis about how a system works. Simulating from that model helps us make predictions about its consequences. We can then test the hypothesis by comparing predictions with experimental observations.

We expect that in order to make progress, particularly on the difficult problem of understanding the expansion of mutations in post-mitotic tissue (e.g. muscle fibres and neurons), we will need to incorporate our considerable uncertainty

about the process of clonal expansion into our analysis of simulation model output. To make robust, statistical comparisons between predictions and observations, we need to include uncertainty about parameters into simulations from a model, propagating that uncertainty forward to uncertainty about predicted outputs. However, direct experimental observations of parameters relevant to clonal expansion (particularly the rate of mtDNA replication) is not currently possible. Assuming a dynamic model and fitting its output to cross-sectional, single-cell observations of copy number distributions and mutation load distributions over time is an important approach to learn about these processes in human cells.

Models are useful for bridging the gap between what can be observed and the process of interest. In this case we can make cross-sectional observations of mtDNA mutation load and copy number in hundreds of heterogeneous single cells, sometimes at multiple time points. We can use mathematical models to simulate mtDNA replication, including selection processes and potentially cell loss, to predict what observed mutation load distributions would look like under different hypotheses. For example, Elson et al. [18] created a model based on the random drift hypothesis and demonstrated that, for a plausible set of parameters (mtDNA copy number, mtDNA replication rate, mtDNA mutation rate), over a human lifespan, the proportion of post-mitotic cells where mutations arose de novo and expanded clonally by neutral drift during relaxed replication increased significantly with age.

Writing a model to describe clonal expansion forces us to be explicit about the mtDNA population we have in mind. For example, the model simulations carried out by Elson et al. [18] are of random genetic drift during relaxed replication, in well-mixed cells, with a fixed rate of mutation, a fixed replication rate and a fixed copy number. In reality, all of these parameters are likely to change with tissue or cell type and might even change in time and space. If we are to compare model predictions with spatial data (e.g. the spatial distribution of mutation loads along massive muscle fibres) then we need to update the model to include those spatial effects. Work by Elson et al. [18] and subsequent models (e.g. model of mtDNA population dynamics in mitotic epithelial crypts by Stamp et al. [38]) assume perfect control of mtDNA copy number. If copy number is not tightly controlled, then it is likely that mtDNA population dynamics will include nonlinear bottleneck effects.

The point here is that, even under the label ‘random genetic drift hypothesis’, there is quite a wide range of different ways to represent the mechanisms underlying this hypothesis. It is important to explore the full range of plausible mechanisms (formal model selection) as well as to explore the full range of plausible parameter values (formal parameter inference). In some cases, it is possible to frame the choice between different mechanisms or even different hypotheses as parameter inference. For example, including selective advantage into a model of random genetic drift, it could be possible to infer statistically that the parameter representing selective advantage is not significantly different from zero, thereby rejecting that hypothesis.

Experimental data derived from patient tissue are difficult to gather and are very valuable. By carrying out parameter inference for a specific model and validating its predictions by comparing with data [102], we make the best use we can of patient tissue; for example, we can use this approach to estimate tissue-specific mutation rates, mtDNA copy number, replication rates and the strength of any selective advantage, together with our uncertainty about them, even for individual patients. Although it is difficult work, requiring experimental design specifically targeted at a particular model and challenging computation, the payback is that we can infer estimates for parameters that are difficult to access as well as make and assess quantitative predictions of mutation load distributions (and their uncertainty) which we can use to assess the validity of models and their underlying hypotheses. Learning about long-term dynamic processes driving clonal expansion throughout human lifespans is extremely difficult. However, the availability of rich, single-celled datasets makes this approach more realistic and promising than ever before.

56.7 Conclusion and Future Perspectives

The ongoing revolution in technology allows single-cell observations of mtDNA populations and allows us to discriminate more precisely between hypotheses about clonal expansion. Single-cell analysis provides us with the opportunity to quantify the distribution of cellular outcomes within an individual patient. To further our understanding of clonal expansion, we should take advantage of these rich data by comparing directly with distributed model predictions. We should look to re-visit older hypotheses such as random drift, as well as newer ideas such as perinuclear niche hypothesis by building and assessing mathematical models. We would like to alert our computational biology colleagues that the impossibility of direct observation of clonal expansion makes this a field where modelling work will make a real and important contribution. While learning about clonal expansion, particularly in post-mitotic cells, the modelling, statistical and computational challenges will be difficult but rewarding. By understanding this puzzling phenomenon that holds important pathological relevance to a range of diseases, we can then start to look for ways to slow or indeed halt this process. Such a therapeutic target would have extensive applications; however, it will also be important to understand key differences between diseases, tissues and mutations if we are to hold any hope of developing treatments.

Ethics

Muscle biopsies from quadriceps were obtained via needle biopsy under local anaesthesia. Ethical approval was granted by the Newcastle and North Tyneside local research ethics committees (reference 2002/205), and prior informed consent was obtained from each participant. Formalin-fixed paraffin-embedded (FFPE) human midbrain tissue sections were obtained from the Newcastle Brain

Tissue Resource (NBTR, https://nbtr.ncl.ac.uk/), with approval from the Local Research Ethics Committee and adherence to the Medical Research Council's (MRC) Guidelines on the use of human tissue in medical research. Colon tissue was collected during surgery with prior informed consent and was approved by the Joint Ethics Committee of Newcastle and North Tyneside Health Authority (2001/188) and the National Research Ethics Committee London-Stanmore (11/LO/1613).

Disclosures and Conflict of Interest

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Chapter 57

Hallmarks of Cancer—the New Testament

Sasi S. Senga, MSc, and Richard P. Grose, PhD

Centre for Tumour Biology, Barts Cancer Institute, Queen Mary University of London, London, UK

r.p.grose@qmul.ac.uk

Keywords: 2′3′-cyclic GMP-AMP (cGAMP), adenocarcinoma, astrocyte, *Bacteroides, Bifidobacterium,* breast to brain metastasis (B2BM), cancer hallmarks, cancer stem cells (CSCs), chemokines, *Clostridium, c-MYC,* death receptor 4 (DR4), dedifferentiation, de-differentiation, Enterotoxigenic *Bacteroides fragilis* (ETBF), epigenetics, epigenetics, *Escherichia coli, Fusobacterium nucleatum* (*F. nucleatum*), induced pluripotent stem cells (iPSCs), into granulocytes, *Kruppel-like factor 4 (KLF4),* lung adenocarcinomas, melanoma, microbiome, multipotent adipose-derived stem cells (ASCs), natural killer (NK), neoplastic cells, neuronal signalling, nuclear factor-κB (NF-κB) signalling, *N*-Methyl-d-aspartate receptor (NMDAR), *octamer-binding transcription factor 3/4* (*Oct-3/4*), *sex-determining region Y-box 2* (*Sox2*), T cell immunoglobulin and ITIM domain (TIGIT), transdifferentiation, transposable element (TE), triple-negative breast cancer (TNBC), tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), tumourigenesis, Yamanaka factors, β-catenin

57.1 A Historical Perspective on Cancer

Ancient Egyptians believed cancer to be a curse, as seen from evidence as old as 3000 BC from the Edwin Smith Papyrus which described breast cancer [1] and Ebers Papyrus dated 1500 BC which described skin, uterus and other types of tumours [2]. Hippocrates proposed the idea of an excess of black bile to be the reason for cancer, the idea was further developed by Greek physician Galen, revered physician to the Emperor Marcus Aurelius, who suggested black bile caused incurable types of cancer while yellow bile caused curable variants of cancer [3]. This was refuted in the sixteenth century by the renowned anatomist

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Andreas Vesalius, who disproved the existence of black bile [4]. In the sixteenth century, Paracelsus identified the first correlation between cancer and the environment, showing deposits of arsenic salts and sulfur in the blood of mine workers were associated with cancer. This laid the foundation for later work by others, namely Percival Pott (chimney sweeps), John Hill (snuff) and Ludwig Rehn (aniline dyes) [5]. In 1914, Theodor Boveri was the first to hypothesize that abnormal segregation of chromosomes to daughter cells can lead to tumour development in '*Zur Frage der Entstehung Maligner Tumoren*' [6].

Fast forward to 2000, Douglas Hanahan and Robert Weinberg compiled the key concepts surrounding cancer into the hallmarks of cancer, discussing the various mechanisms that underpin tumour development (Fig. 57.1).

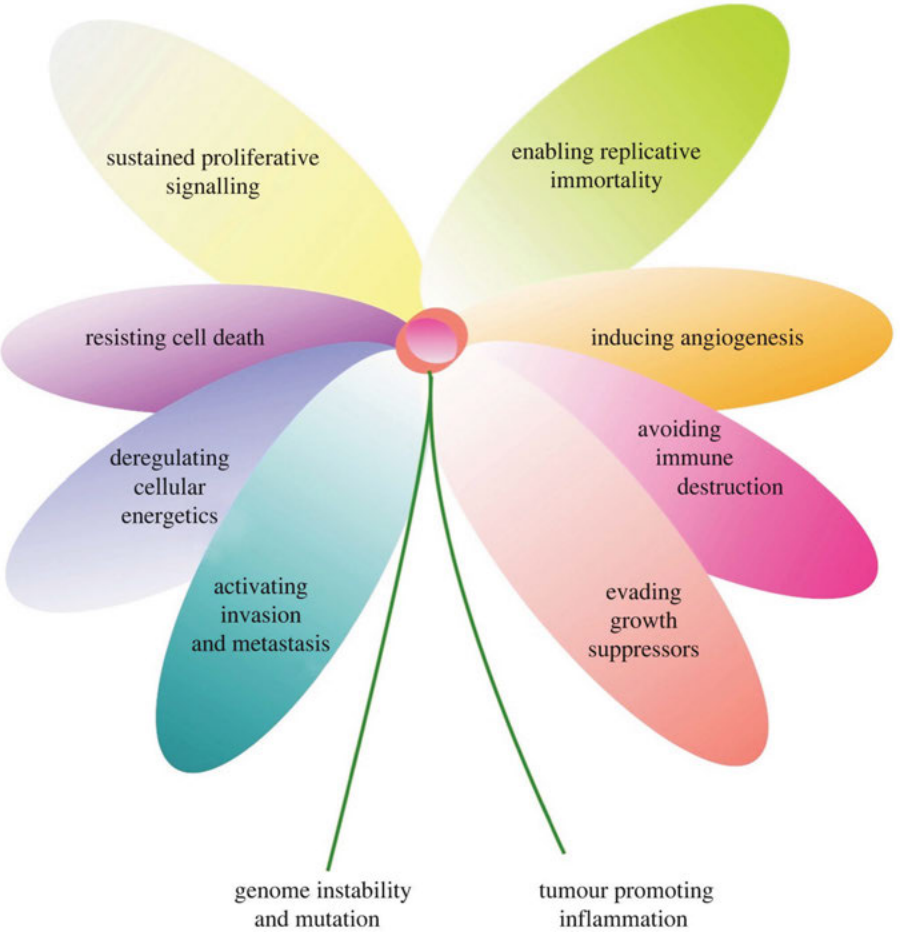

Figure 57.1 Hallmark flower.

57.2 Hallmarks of Cancer

The challenges presented by multiple roadblocks, which are in place to prevent excessive cell proliferation and the development of tumours, leads to the daunting complexity of cancer. Tumour cells do not invent new mechanisms, but rather manipulate existing molecular and cellular pathways to circumvent protective mechanisms which are in place to prevent the formation of a tumour.

These conceptually distinct capabilities of tumour cells have a powerful resonance in the field of cancer therapeutics. Despite our knowledge of specific mutations in tumour cells generated through global sequencing efforts, such as the International Cancer Genome Consortium, the reductionist view would be to just focus on the cancer cell. However, we are actually dealing with a complex heterotypic tumour microenvironment where the tumour cells are only the foundation of cancer as a disease but not its complete manifestation.

We propose four novel hallmarks of cancer, justify their importance in tumourigenesis and argue the need to incorporate them in the mainstream hallmark conceptualization (Fig. 57.2).

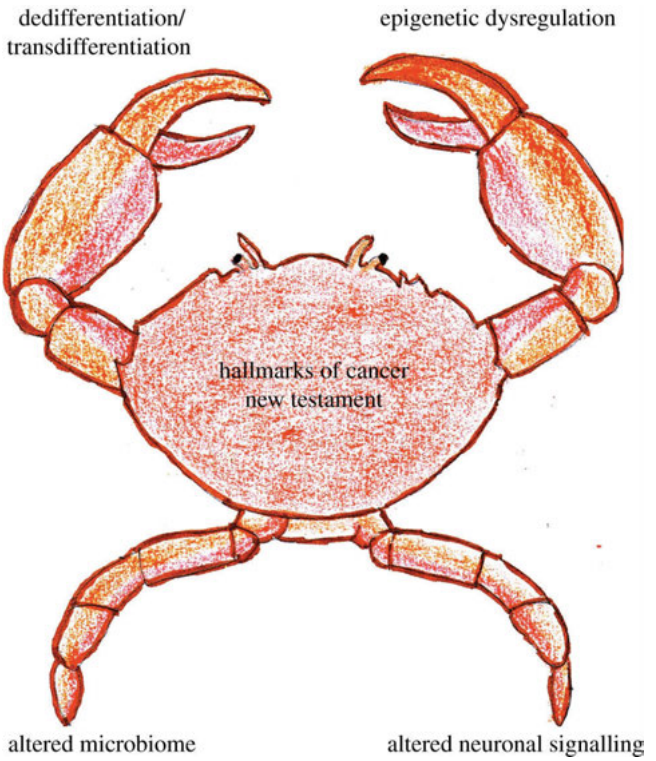

Figure 57.2 Novel hallmarks of cancer.

57.3 New Hallmark 1: Dedifferentiation and Transdifferentiation

In 1957, Conrad Waddington proposed the unidirectional developmental model, wherein pluripotent stem cells at the top of the hill progressively lose their pluripotency as they follow developmental pathways and end up among different valleys in a terminally differentiated state [7] (Fig. 57.3a). However, the concept of tumour cell plasticity goes against the Waddington landscape, where dedifferentiation allows non-cancer stem cells to acquire stem cell-like features.

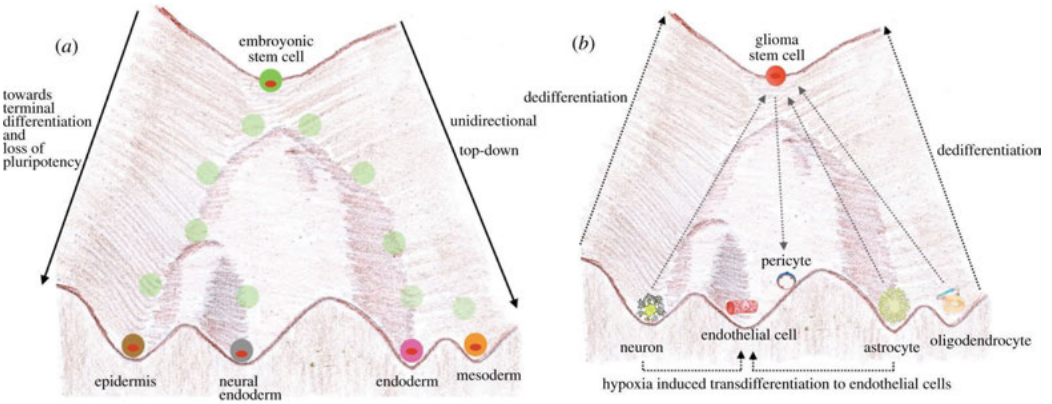

Figure 57.3 Tumour defiance of Waddington landscape. (a) Waddington landscape depicting the unidirectional nature of differentiation, adapted based on the concept of [8]. (b) The forgotten hallmark Dedifferentiation: Dedifferentiation from terminally differentiated neuron, astrocyte and oligodendrocyte, as well as transdifferentiation of neuron and astrocyte to endothelial cells.

In 1962, Sir John Gurdon challenged the unidirectional dogma of development with his ground-breaking study which showed the formation of a fully functional tadpole clone even when the nucleus of a frog zygote was replaced with a nucleus harvested from a terminally differentiated tadpole intestinal cell [9]. This proved his hypothesis that the genome of a mature specialized cell has all the information required to develop into the different cell types of an organism. However, Gurdon's experiment involved physical removal and transfer of cell nuclei and as such the question remained whether such a hypothesis could be replicated in intact cells. Forty years later, this question was answered with a proof of concept study which defied the Waddington landscape in intact cells. Introducing just four genes, the Yamanaka factors: *c-MYC*, *Kruppel-like factor 4* (*KLF4*), *Sex-determining region Y-box 2* (*Sox2*) and *Octamer-binding transcription factor 3/4* (*Oct-3/4*); Takashi and Yamanaka were able to develop what they termed induced pluripotent stem cells (iPSCs), which had the ability to differentiate into any of the cell lineages, endodermal, ectodermal and

mesodermal [10]. This forms the basis of the hypothesis that tumour cells, which are champion survivors, will hijack any mechanism in order to survive; as such, dedifferentiation is a lucrative hallmark for them to achieve immortality.

Cancer stem cells (CSCs) are a unique subpopulation which possesses the cardinal property of self-renewal. This population can underpin tumour heterogeneity and resistance to cancer therapeutics, leading to relapse. The dedifferentiation of non-CSCs to CSC gives a survival advantage to cancers. Turning the clock back in time to a stem cell progenitor state is not a mere manifestation of the existing hallmark but a pivotal hallmark in itself and it further confers the ability to switch lineages, as lineage plasticity enables resistance against therapeutics. Let us consider some key examples that reiterate dedifferentiation as an integral hallmark of cancer.

57.3.1 Evidence of Dedifferentiation in Glioblastoma

The interconvertible nature of cancer stem cells and non-cancer stem cells can be seen within glioblastoma multiforme, a highly lethal sub-type of brain cancer. In 2002, a study reported that even mature astrocytes and neurons can be the cell of origin in certain brain tumours. EGFR activation and dual inactivation of $p16^{INK4a}$ and $p19^{ARF}$ cause astrocytes to undergo dedifferentiation to a multipotent progenitor state dictating the emergence of the high-grade phenotype of gliomas [11]. The extent of dedifferentiation of astrocytes is radical enough to give rise to pluripotent cells which have the ability to differentiate into glia as well as neurons, as evidenced by expression of neuronal marker TUJ1 among such tumours which arise from dedifferentiated astrocytes [11]. Indeed, the majority of mature differentiated cells in the central nervous system, given the right permissive microenvironment, can undergo dedifferentiation to a progenitor state, generating a neural stem cell that can perpetuate tumour progression as well as tumour heterogeneity and resistance to treatment [12] (Fig. 57.3b). Tumour plasticity allows for vascular mimicry via the transdifferentiation of glioblastoma cells into vascular endothelial cells [13] and even pericytes, which can support the maintenance of tumour vessel function [14] (Fig. 57.3b).

57.3.2 Evidence of Dedifferentiation in Intestinal Tumours

Tumour initiating cells formed via dedifferentiation have also been reported in intestinal tumours. Enhanced nuclear factor-κB (NF-κB) signalling leads to activation of β-catenin/TCF transcription via stabilization of β-catenin, inducing dedifferentiation of non-stem intestinal epithelial cells to intestinal epithelial cells with tumour initiating stem-like properties [15]. If Wnt activity plays a role in dedifferentiation of non-stem intestinal epithelial cells to tumour initiating cells, then a further investigation into whether this activity is mediated by the tumour microenvironment is warranted. In colon cancer, myofibroblasts in the tumour niche orchestrate high Wnt activity via β-catenin localization through hepatocyte

growth factor secretion, which facilitates the reprogramming of the colon cancer cells to a stem cell-like progenitor state [16].

57.3.3 The Pliability of Cell Fate in Pancreatic Cancer via Dedifferentiation

There is a dynamic equilibrium between stem-like state and non-stem differentiated state. An activating mutation of the small GTPase KRAS is identified in about 90% of pancreatic tumours [17]. In a proof of concept study in pancreatic ductal adenocarcinoma, KRAS and its downstream target MYC were shown to rapidly reprogram differentiated mature cells to a stem cell-like state, poised to become malignant. Generation of metastatic pancreatic tumour cells with self-renewing capability is particularly shown to be controlled via MYC, which functions as a built-in amplifier [18].

Another study has shown that the major mechanism of initiation of pancreatic ductal adenocarcinoma lies in the synergism between the transcription factor SOX9 and activated KRAS, leading to the dedifferentiation of pancreatic acinar cells through a duct-like phenotype and the subsequent formation of pancreatic intraepithelial neoplasia [19]. Such genetic mutation induced dedifferentiation explains the differences in the kinetics of dedifferentiation of cells, at different states of differentiation in a tumour, which reiterates the need to target both cancer stem cells, and non-stem cancer cells to prevent re-initiation of tumourigenesis following therapy.

57.3.4 Therapy Resistance via Lineage Plasticity through Dedifferentiation

Despite achieving remission in metastatic melanoma with adoptive cell transfer therapies, there is frequent relapse. Relapse may be due to the secretion of the proinflammatory cytokine tumour necrosis factor (TNF)-α by T cells and macrophages in the tumour microenvironment, which results in reversible dedifferentiation of melanoma cells and thereby a loss of melanocytic antigens [20]. If dedifferentiation can aid in evasion from T cell immunotherapy, it raises the possibility of dedifferentiation as an enabling hallmark for immune evasion.

Dedifferentiation is also linked to resistance to targeted therapies in melanoma, for example, resistance to BRAF inhibition is conferred by the downregulation of microphthalmia-associated transcription factor (MITF), which plays a key role in melanocyte differentiation, and the upregulation of the receptor tyrosine kinase AXL, platelet-derived growth factor receptor and EGFR [21]. Dedifferentiation also provides cues for a potential susceptibility target, for example, the ability to induce ferroptosis, a form of iron-dependent necrotic cell death, in dedifferentiated melanoma cells [22]. As such, it can provide an option to induce a form of synthetic lethality by the combination of ferroptosis inducing drugs along with targeted therapies or immunotherapy in melanoma

patients. Dedifferentiation-based changes have been found among melanoma patients even within the first week of targeted therapy treatment [23], suggesting a combination regimen with ferroptosis inducing drugs could possibly be initiated up-front to prevent escape via dedifferentiation.

Dedifferentiation has also been implicated in therapeutic resistance among prostate and breast cancers. A gain and loss of function study identified that upregulation of the reprogramming transcription factor Sox2 can confer reversible lineage plasticity by switching prostate cancer cells to a neuroendocrine phenotype, in the context of concomitant loss of tumour suppressors p53 and Rb [24]. Earlier studies showed that multipotent adipose-derived stem cells (ASCs), used in soft tissue reconstruction following mastectomy, undergo a phenotypic alteration via myofibroblastic differentiation, leading to contraction and enhanced stiffness, ultimately promoting tumourigenesis [25]. The impact of physical stresses on the tumour microenvironment leading to tumour progression has also been shown to involve triggering dedifferentiation. When a tumour grows, it causes compression of the surrounding tissue. Physical stress caused by mammary adenocarcinoma via compression of surrounding adipocytes triggers Wnt/β-catenin signalling and their subsequent dedifferentiation to myofibroblasts, which then interact with breast cancer cells leading to enhanced tumour proliferation [26].

57.3.5 Reflecting Upon the Yamanaka Factors' Relationship to Oncogenesis

A definitive proof of the importance of re-programming in cancer ontogeny is that each of the four Yamanaka factors capable of playing a role in dedifferentiation has an ascertained role in oncogenesis among multiple cancers. Oct4 is a biomarker for seminomas [27] and has also been attributed to the maintenance of the undifferentiated cell population with proliferative capacity by blockage of progenitor cell differentiation [28]. Sox2 is a key driver towards a stem cell fate among Ewing's sarcoma, breast and brain tumours [29, 30]. Aberrant MYC expression has been strongly linked to several cancers [31] and KLF4 has been linked to colorectal cancer [32]. Though challenging targets themselves, Yamanaka factors may provide insight for the development of more targeted therapies.

The loss of *APC* maintains a progenitor state, following which oncogenic mutations such as KRAS can be acquired, driving tumourigenesis [33]. So, does dedifferentiation act as an enabling hallmark to grant time to acquire additional mutations to progress in the path towards tumour development? Or does dedifferentiation allow for lineage plasticity for tumour cells to alter their cell fate to a lineage more resistant to therapeutics?

In the case of acute promyelocytic leukaemia (APML), translocation results in promyelocytic leukaemia protein (PML) and retinoic acid receptor α (RARα) fusion. The expression of the *PML-RARα* fusion gene blocks the terminal differentiation of granulocytes, resulting in the maintenance of neoplastic cells in the promyelocytic

progenitor stage, but all-trans retinoic acid has been successful in overcoming the differentiation block by inducing differentiation of neoplastic cells into granulocytes [34]. The abrogation of terminal differentiation, as seen in APML, in order to maintain a progenitor-like state, supports the hypothesis of dedifferentiation as a logical hallmark. Even if cancer cells proceed to, or develop from, a state of terminal differentiation, they can revert back to their progenitor state and maintain their stemness via dedifferentiation. The Waddington landscape has been defied by cancer, providing tumour cells the plasticity to choose their fate by pushing the ball uphill against the landscape, to maintain cancer stem cells and underpin the basis of cancer as a lethal disease.

57.3.6 Therapeutic Interventions Based on the Hallmark of Dedifferentiation

An important aspect of hallmarks of cancer conceptualisation is to aid in advancing therapeutic strategies, so an understanding of the nuances of the hallmark of dedifferentiation is important. There are three therapeutic modalities that can be targeted towards the tumour cell plasticity conferred by dedifferentiation:

1. Blocking dedifferentiation via combination therapies. Target the differentiated cell lineage along with drugs that block dedifferentiation in order to prevent early resistance to therapeutics as a result of lineage plasticity conferred by dedifferentiation.
2. Target dedifferentiation with differentiation therapy towards a permanently differentiated state. Initially attempted in the context of teratoma [35], but a proof of concept study for this approach was treating APML with all-trans retinoic acid therapy [34]. Other studies also reported a differentiation therapeutic approach aimed at the conversion of dedifferentiated tumour cells into epithelial cells that are more sensitive to chemotherapy [36, 37].
3. Go with the flow and use the tumour plasticity to target the dedifferentiated cancer stem cells with transcription factors or small molecules to differentiate them into harmless cell lineages which lack tumourigenic potency. This final therapeutic approach necessitates an in-depth understanding of the hallmark of dedifferentiation. Recent work proved the efficacy of this approach by switching malignant breast cancer cells into harmless post-mitotic adipocytes. Combination of PPARγ agonist Rosiglitazone, an anti-diabetic drug, with a MEK inhibitor was used to force the breast tumour cells towards adipogenesis, resulting in harmless post-mitotic functional adipocytes [38] (Fig. 57.4).

These studies argue strongly that the dedifferentiation of tumour cells along a developmental pathway towards a progenitor or stem cell-like state among various cancers is a forgotten hallmark, a discrete acquired capability of cancer and certainly warrants further investigation for a better understanding of this novel trait of cancer cells. Hanahan and Weinberg's Hallmarks of Cancer had generic nature as one of the features of every hallmark, as something that is prevalent

in the majority of cancers despite the heterogenetic nature of the disease. Dedifferentiation certainly qualifies as a generic hallmark distinct from the other hallmarks of cancer. The reported interplay between transcription regulators Sox2 and Sox9 as an epigenetic switch between high proliferation and high invasiveness [39] leads to our next hallmark of cancer that influences tumourigenesis—epigenetic dysregulation (Fig. 57.5).

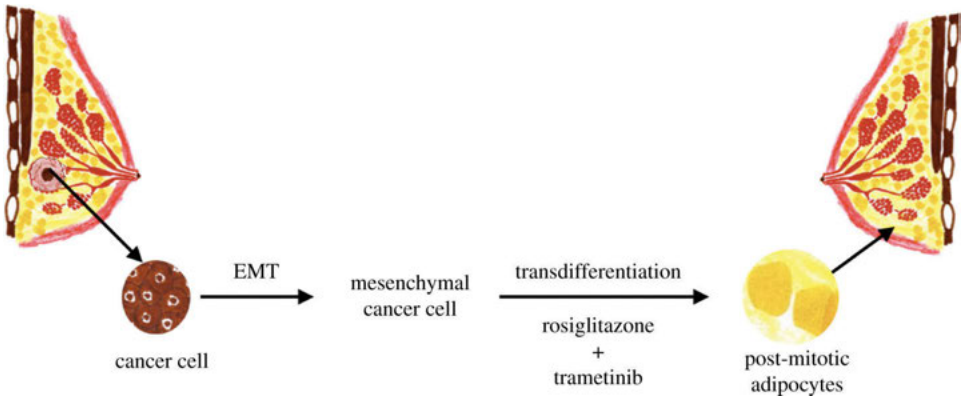

Figure 57.4 Transdifferentiation approach to therapy. Following combination of PPARγ agonist rosiglitazone (an anti-diabetic drug) + MEK inhibitor—cancer cells are converted to functional adipocytes, adapted based on [38].

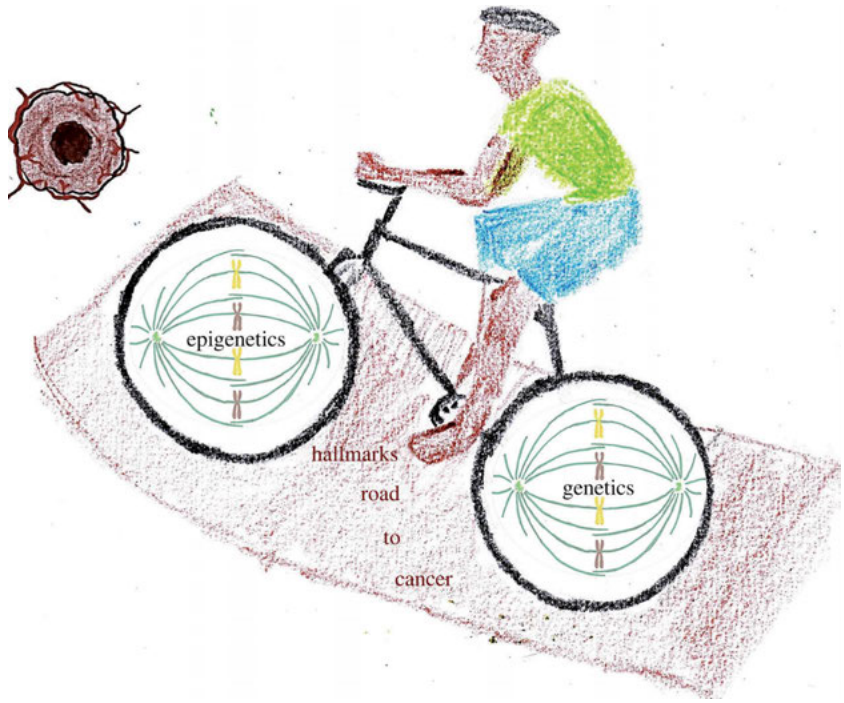

Figure 57.5 The road better not taken: intertwined nature of epigenetic instability and genetic instability in riding over the road of hallmarks towards tumourigenesis.

57.4 New Hallmark 2: Epigenetic Dysregulation

Dedifferentiation to a progenitor state is a rate-limiting step in melanoma formation but it is underpinned by epigenetic machinery [40]. Although Yamanaka factors provide the possibility of reprogramming differentiated somatic cells to a pluripotent state, the blockage of histone H3 lysine 9 (H3K9) methylation has been shown to enhance this reprogramming capability [41, 42]. Similarly, in the context of DNA methylation, another key epigenetic alteration, the promotion of DNA demethylation via stimulation of TET (ten-eleven-translocation) enzymes using vitamin C enhances reprogramming to a pluripotent state [43]. Epigenetics can also regulate the process of winding back the clock to a pluripotent state on the basis of chromatin state and the expression levels of chromatin-modifying enzymes [44], providing a conceptual link with our first hallmark of dedifferentiation.

57.4.1 What Is Epigenetics?

Among several phenomenal works, Theodor Boveri laid the foundation of the role of epigenetics in cancer through his observation of abnormal chromatin structures in tumour cells, described over 90 years ago [45]. The term 'epigenetics' was first coined by Conrad Waddington, defining it as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' [8]. Vogelstein and Feinberg, in an attempt to dissect the mechanism underlying the higher frequency of mutations among tumours, compared normal tissue with tumour tissue and revealed the loss of DNA methylation in a substantial proportion of tumour tissues, positing that hypomethylation of CpG islands could lead to oncogene activation in cancer [46] and revealing the prevalence of global hypomethylation among tumour genomes.

Holliday refined the definition of epigenetics as heritable changes in the gene expression without alteration in the DNA sequence, that is altering the phenotype without altering the genotype [47]. With epigenetics playing a fundamental role in the development and progression of various cancers via modification of gene expression, such as hypermethylation of tumour suppressor genes in retinoblastoma [48] and epigenetic silencing of microRNAs [49], it is a pivotal hallmark of cancer.

57.4.2 Epigenetic Fingerprints as the 'Midas Touch' Driving Tumourigenesis—the Hallmark of Hallmarks

As discussed in the features of a hallmark, epigenetic dysregulation is an active functional capability, it is a unique feature among cancer cells and there are epigenetic fingerprints on tumour cells which reflect its chronic nature. Epigenetic dysregulation viewed as a bystander would be a relegation of its active role in tumourigenesis, as several studies have pointed out its role in tumour initiation. In 2006, Feinberg proposed the epigenetic progenitor model of tumourigenesis,

wherein epigenetic dysregulations of the progenitor cell population give rise to tumours [50] (Fig. 57.6).

Figure 57.6 Epigenetic crossroads: multitude of studies supporting the epigenetic progenitor model.

57.4.3 Sustained Proliferative Signalling

Many tumours display a gain of function mutation of isocitrate dehydrogenase (IDH) [51, 52], leading to the generation of the oncometabolite 2-hydroxyglutarate, which disrupts the function of hydroxylases such as TET — a key catalyst in the process of DNA demethylation [53, 54]. The result is a hypermethylated phenotype as seen with the CpG island methylator phenotype (G-CIMP) in IDH mutant glioma [55]. This alters the binding affinity of the DNA binding protein

CTCF (CCCTC-binding factor) which is very sensitive to methylation states [56]. CTCF has a critical function as an insulator, setting the boundaries which limit the interactions between an enhancer and a gene in the context of topologically associated domains (TADs) [56]. This insulation is lost as a result of the reduced binding of CTCF, facilitating aberrant interactions between promiscuous enhancers and genes as a result of the altered chromosomal topology caused by epigenetic dysregulation [57]. In this context, *Platelet-derived growth factor receptor A* (*PDGFRA),* a predominant oncogene among gliomas [58] is activated as a consequence of the epigenetic dysregulation-led decrease in CTCF insulation, with a potent promiscuous enhancer driving constitutive *PDGFRA* expression, driving sustained proliferation in gliomas [57]. The loss of CTCF insulation may even be preserved in subsequent cell divisions, compromising the genomic topology otherwise maintained by this insulation, leading to further oncogene activation not limited to just *PDGFRA* [59].

This mechanism is not limited to gliomas, as CTCF sites which are adjacent to oncogenes have been reported as mutational hotspots and are frequently mutated in multiple tumours, such as endometrial [60], colorectal (CRCs), oesophageal and liver cancer [59].

57.4.4 Evading Growth Suppressors

Cyclin-dependent kinase inhibitor 2A (CDKN2A) encodes a potent tumour suppressor $p16^{INK4a}$, that binds to cyclin-dependent kinase 4/6 (CDK4/6), which leads to an allosteric conformational change inhibiting the cyclin D–CDK4/6 complex formation. As a result of the lack of this complex, retinoblastoma protein (Rb) is maintained in a hypophosphorylated state, promoting the formation of Rb/E2F repressive complex. This leads to the suppression of growth, as a result of cell cycle arrest in G1 [61]. Epigenetic silencing of tumour suppressors such as $p16^{INK4a}$ via promoter hypermethylation mediates evasion of growth suppression, as evident from multiple studies on epigenetic alterations enumerated below (Table 57.1).

Table 57.1 Epigenetic instability mediating evasion of growth suppressors

Type of cancer	Frequency of promoter hypermethylation	Reference
Pancreatic adenocarcinoma	24.6%	[62]
Oesophageal squamous cell carcinoma	81.7%	[41, 42]
Melanoma	25.9%	[63]
Burkitt's lymphoma	72.5%	[64]

Similarly, the hyperactivity of enhancer of Zeste homologue 2 (EZH2), a catalytic subunit of polycomb repressive complex 2 (PRC2) involved in the trimethylation of histone H3 lysine 27 to form H3K27me3, is implicated in the

evasion of growth suppression via *CDKN2A* repression [65–67] reiterating the role of epigenetic dysregulation in facilitating the hallmarks [62].

57.4.5 Invasion and Metastasis

An integral component of the hallmark of invasion and metastasis is a reversible epithelial–mesenchymal transition (EMT), orchestrated by the interaction between epigenetic modulators of chromatin configuration and EMT inducing transcription factors. Expression of E-cadherin, a key coordinator of epithelial phenotype, is lost during EMT. Epigenetic repression of *CDH1,* which encodes E-cadherin, is mediated by the recruitment of EMT inducing transcription factor Snail to the *CDH1* promoter, leading to a repressive mark H3K27me3 [68]. Further to this, Snail can associate with Mi-2-nucleosome remodelling and deacetylase (NuRD) repressive complex, which can repress *CDH1* activity via deacetylation of *CDH1* promoter [69].

57.4.6 Replicative Immortality

Alternative telomere lengthening (ALT) is a telomerase-independent homologous recombination-based pathway which cancer cells use to overcome the Hayflick limit to maintain telomere length [70]. An interplay between epigenetics and genetic mutations leads to perturbations of histone variant H3.3 and its specific chaperone proteins α-thalassemia X-linked mental retardation protein (ATRX) and death domain associated protein (Daxx) which impairs the incorporation of the histone variant H3.3 at telomeres, disrupting their heterochromatic state and facilitating ALT [71].

57.4.7 Inducing Angiogenesis

Epigenetics plays a key role in angiogenesis. Histone deacetylases have been shown to downregulate the expression of von Hippel–Lindau (VHL) and p53, but promote an increase in hypoxia-inducible factor-1α and vascular endothelial growth factor (VEGF), thereby stimulating angiogenesis by the suppression of hypoxia-responsive tumour suppressor genes [72, 73]. Choriocarcinoma, a highly vascular tumour derived from trophoblasts, displays epigenetic silencing of *FLT1* via promoter hypermethylation. Normal placental trophoblasts express abundant levels of an anti-angiogenic factor, Soluble Fms-like tyrosine kinase-1 (sFLT1) from the *FLT1* locus. Epigenetic silencing of *FLT1* blocks expression of this negative regulator, thereby facilitating angiogenesis in choriocarcinoma [74].

57.4.8 Resisting Cell Death

Glioblastoma multiforme is a very aggressive cancer with a dismal prognosis. Nevertheless, a promising therapeutic strategy is to induce tumour cell death via tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-based therapy

that binds to human death receptor 4 (DR4). However, epigenetic silencing via promoter methylation of *DR4* attenuates TRAIL/DR4-mediated apoptosis [75]. Further evidence of epigenetics mediating resistance to cell death is resistance to anthracycline therapy in acute myeloid leukaemia (AML), due to impaired DNA damage response from defective nucleosome remodelling, as a result of mutation of epigenetic regulator DNA methyltransferase 3A [76]. CXCL14, a chemokine which can influence apoptosis, is shown to be a frequent candidate for epigenetic silencing among lung tumours. Tumour specific methylation of the CXC-subfamily of chemokines was observed in 75% of lung adenocarcinomas [77].

57.4.9 Immune Evasion

Epigenetics is fundamental to the normal functioning of immune cells. Antigen presentation through MHC class I is pivotal for CD8+ T cells activity. The class I transactivator *NLRC5* is a transcriptional regulator of the MHC class I genes, but the promoter region of *NLRC5* is frequently methylated among cancers, resulting in the reduction of MHC class I gene expression [78].

57.4.10 Deregulating Cellular Energetics

In order to adapt to a hostile microenvironment and to satisfy their high metabolic needs, cancer cells can use glycolysis, instead of oxidative phosphorylation to metabolize glucose, even in aerobic conditions. The central activators implicated in the glycolytic phenotype are the PI3 K/AKT/mTOR pathway along with MYC and HIF-1 signalling [79].

Tumour suppressors that repress this pathway, namely *PTEN* [80], *VHL* [81, 82], *LKB1* [83] and prolyl hydroxylases [84] are epigenetically silenced via promoter hypermethylation, contributing to deregulation of cellular energetics.

57.4.11 Genomic Instability and Mutation

Faithful genome replication and the maintenance of genomic integrity are underpinned by epigenetic mechanisms. Transposable elements (TE) are highly repetitive sequences of DNA in the human genome, and have their own regulatory sequence, allowing for independent expression and ability to alter the expression of neighbouring genes. Since TE activity has a high propensity to disrupt genomic integrity, these are usually silenced epigenetically, but this regulation is lost in cancer [85].

57.4.12 Tumour Promoting Inflammation

DNA demethylation triggers transcription of inflammation-related genes, including *chemokine receptor 4* (*CXCR4*) and *serum amyloid A* (*SAA*) in advanced clear cell renal cell carcinoma (ccRCC), contributing to tumour promoting cancer cell-intrinsic inflammation via epigenetic remodelling [86].

The above studies highlight an epigenetic foundation for each of the established hallmarks of cancer provides compelling evidence of the indispensable nature of epigenetic dysregulation as a pivotal enabling hallmark of cancer. However, just as cancer involves more than just tumour cells, so our bodies are more than simply an assemblage of human cells. This sobering thought leads us to our third hallmark, the microbiome.

57.5 New Hallmark 3: Altered Microbiome

The concept of the human body being a vessel for other microorganisms is well established—the microbial metagenome in our body outnumbers our genome by at least 100-fold [87]. Microorganisms first appeared around 3.25 billion years ago [88] and over the 1.25 billion years of co-existence with multicellular eukaryotes [89] (Fig. 57.7), the interaction with microbes has shaped evolution, as illustrated by the microbial control of host homeostasis [90]. It has been estimated that nearly half of the metabolites in plasma are of microbiotal origin [91], but the human microbiome plays a duplicitous role. *Helicobacter pylori* is nearly ubiquitous among humans, colonizing about 50% of the world population, having co-evolved with humans in an association spanning over 50 000 years [92]. *H. pylori* colonization has been shown to decrease the risk of gastroesophageal reflux disease and its subsequent sequela, oesophageal carcinoma [93]. It may also confer protection against asthma [94], demyelinating diseases such as multiple sclerosis [95], tuberculosis [96] and inflammatory bowel disease [97]. *H. pylori* also has been shown to modulate energy homeostasis via cooperation with gut microbiota, impacting on circulating metabolic gut hormones [98].

In contrast with these beneficial roles, as a component of the gut microbiome it is also linked to 90% of gastric cancers [99]. The carcinogenicity of *H. pylori* is associated with the expression of *vacuolating cytotoxin gene A* (*vacA*) and *cytotoxin-associated gene A* (*CagA*) [100, 101]. *CagA* positive *H. pylori* promotes genetic instability via perturbation of the mitotic spindle checkpoint, causing chromosomal instability [102] and epigenetic instability. Increased levels of DNA methyltransferases (DNMTs) [103] lead to hypermethylation of *MLH1,* a key DNA mismatch repair gene [104], and have been suggested to mediate a mutator phenotype in a hit and run fashion, promoting tumourigenesis [101]. Given these data, it is worthwhile to postulate whether *Escherichia coli* has a role in cancer, being among the first bacteria to colonize the gastrointestinal tract of neonates [105] and advocated to promote gut health in multiple off the shelf probiotics [106].

Commensal *E. coli*, within few days following birth, establish a favourable anaerobic environment in the gut facilitating colonization of other species including *Bifidobacterium*, *Clostridium* and *Bacteroides* [107]. Certain strains of *E. coli* harbour a gene cluster hybrid non-ribosomal peptide synthetase-polyketide synthase (*pks*) island which produces genotoxic colibactin [108]. Colibactin has been described as a bacterial ‘warhead’, forming bulky unstable DNA adducts

via alkylation [109]. Among epithelial cell lines, *pks*$^{+}$ *E. coli* has been shown to induce double-stranded DNA breaks [110] and interstrand cross-links [111].

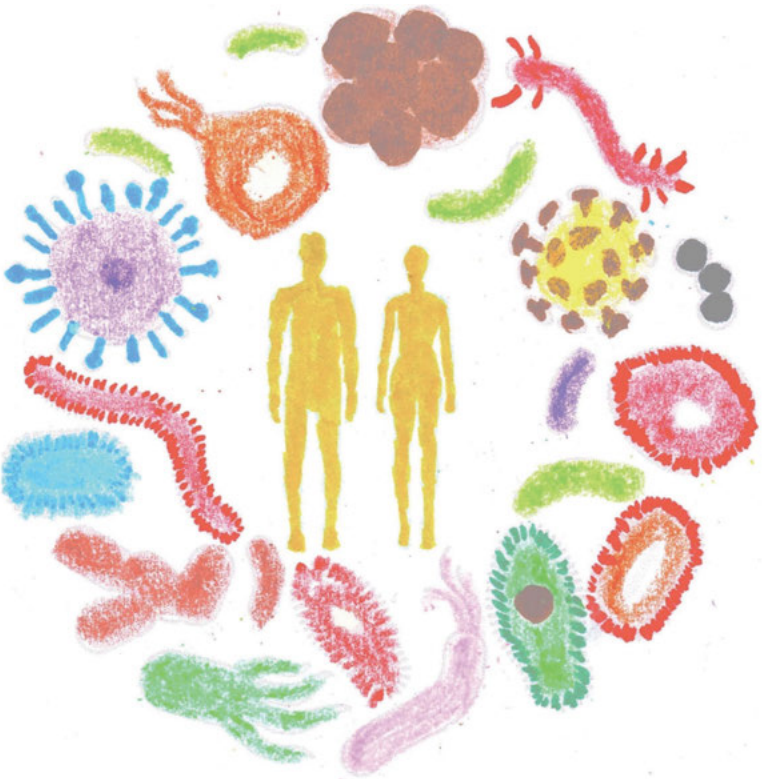

Figure 57.7 Coexistence.

Whole-genome sequencing studies have described mutational signatures from colorectal crypts of healthy individuals where, in a subset of crypts, an unknown mutagenic agent caused co-occurrence of single-base substitution-A (SBS-A) and insertion/deletion A (ID-A). The two motifs were described to be from mutagenic insult which occurs in early childhood [112]. The cause of these two motifs surfaced while investigating the long-term effect of colibactin using single-cell-derived organoids, with *pks*-mutational signature strongly matching the two motifs SBS-A and ID-A [113]. Since *pks*$^{+}$ *E. coli* is the mutagenic agent responsible, and the study was performed on organoids that cannot precisely mimic inflammation or the immune environment, the inference is that colibactin can directly initiate tumourigenesis via mutations [113]. Interestingly, the impact of these data reaches beyond the gut, suggesting a similar role in head and neck as well as urogenital cancers [113]. So, perhaps one should reconsider probiotics containing genotoxic *E.coli* and consider screening for *pks*$^{+}$ *E. coli* in the context of colorectal cancer prevention.

Arguing for a microbiotal impact merely on the hallmark of genomic instability as being sufficient for its contribution to tumourigenesis is to grossly

underestimate the role of the microbiome. Paget's seed (cancer cells) and soil (tumour microenvironment) hypothesis [114] is highly relevant to the role of the microbiome in tumourigenesis. The microbiome can exploit the inflammatory milieu to a pro- or anti-tumour state, cultivating the soil which is apt for sowing the seeds of tumourigenesis. This can be clearly substantiated by findings from a study using the first identified oncovirus [115], where Rous sarcoma virus does not induce tumours in sterile embryos despite expression of the *v-Src* oncogene [116].

In 1990, Fearon & Vogelstein [117] proposed the Vogelgram model of multi-step colon cancer pathogenesis. A key reason for the success of colon screening in CRC prevention is due to the long latency period from tumour initiation to overt clinically detectable CRC. Here, we consider this long latency in the context of the proposed hallmark of microbiome dysbiosis.

57.5.1 Microbiome Tug-of-War Hypothesis

The tug-of-war between microbiome species may underlie the long latency in CRC. Enterotoxigenic *Bacteroides fragilis* (ETBF) promotes the colonization of *pks*$^{+}$ *E. coli*, together with leading to genetic and epigenetic instability. Following this, colonization by pro-tumourigenic *Fusobacterium nucleatum* further promotes tumourigenesis by aiding in the development of an immunosuppressive microenvironment and seeding metastasis, whereas anti-tumourigenic bacteria act to prevent malignancy. The long latency period, which may ultimately lead to subsequent accumulation of genetic/epigenetic mutations and overt malignancy, depends on the balance between pro/anti-tumourigenic microbes (Fig. 57.8).

ETBF secretes a 20 kDa zinc-dependent metalloprotease toxin, *B. fragilis* toxin (BFT). BFT degrades E-cadherin, leading to increased intestinal epithelial cell proliferation and permeability of the intestinal barrier [118]. BFT further leads to activation of β-catenin signalling and induces STAT3 (signal transducer and activator of transcription 3) activation [119] and the T helper 17 (TH17) immune response [120]. ETBF modulates the colonic niche to select for bacteria with a colonization advantage, inducing upregulation of antimicrobial peptide lipocalin 2 [121] that causes sequestration of bacterial siderophores. Siderophores are iron-binding complexes that are pivotal for bacteria to thrive in iron limiting environments, hence bacteria which are resilient to lipocalin 2, such as *E. coli*, begin to thrive along with ETBF [122]. Therefore, the first hit in our hypothesis of CRC tumourigenesis is orchestrated by ETBF followed by the co-colonization of ETBF along with *pks*$^{+}$*E. coli*, following which *Fusobacterium nucleatum* comes into play.

An anaerobic Gram-negative bacterium, *Fusobacterium nucleatum* is typically resident in the oropharynx, participating in dental biofilm formation [123]. Its virulence factor FadA adhesin binds to the extracellular domain of E-cadherin and promotes tumourigenesis via β-catenin/Wnt signalling [124]. *F. nucleatum* is also immunosuppressive, causing inhibition of T cell responses while allowing for the expansion of tumour promoting myeloid-derived immune cells [125].

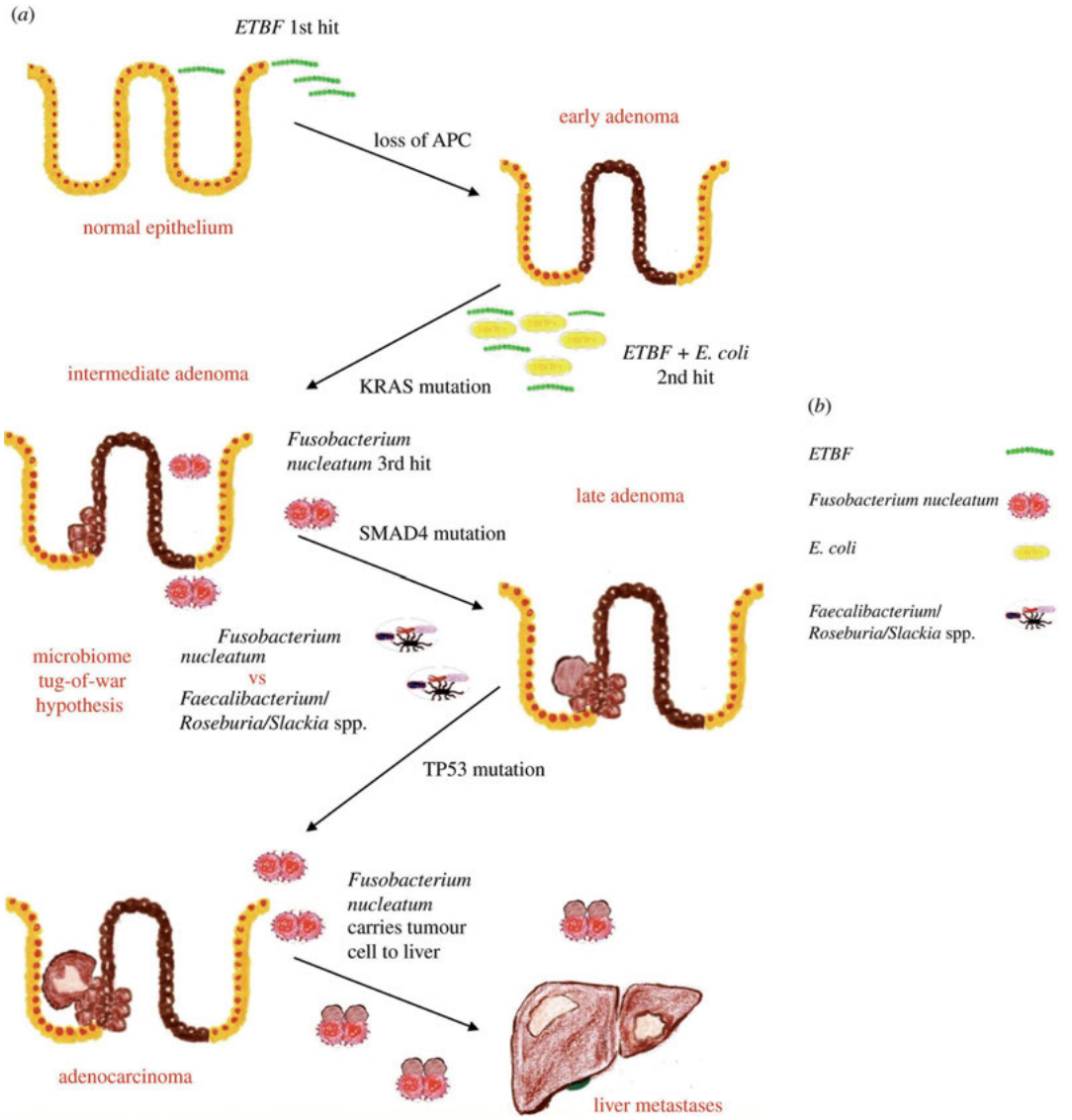

Figure 57.8 (a,b) Microbiome tug-of-war hypothesis for CRC latency: improvisation of the Vogelgram [117], with an explanation of the role of bacteria in multi-step CRC progression.

Fap2 protein of *F. nucleatum* binds directly to the inhibitory receptor—T cell immunoglobulin and ITIM domain (TIGIT), and inhibits natural killer (NK) cell activity, leading to immune evasion [126]. In support of this hypothesis, *F. nucleatum* did not initiate tumour formation *in vivo* [127], but sustained the pro-tumourigeneic momentum in the latter part of multi-step CRC tumourigenesis and facilitated metastasis [128]. Intriguingly, a study based on biopsies from CRC patients and mouse xenografts revealed that *F. nucleatum* can accompany primary colorectal adenocarcinoma cells to distant metastatic sites, being maintained

among patient-derived xenografts of CRC even through multiple passages. Moreover, treatment with metronidazole, an antibiotic to reduce *F. nucleatum* load, resulted in reduced tumour growth [128], suggesting that tumour cells are rewarded for carrying *F. nucleatum* by its modulation of the microenvironment at the distant metastatic site in favour of tumour growth. Meanwhile, there is a subset of anti-carcinogenic bacteria including *Faecalibacterium*, *Roseburia* and *Slackia* spp. which generate catabolites such as short-chain fatty acids (SCFAs), for example, butyrate [129] and the antioxidant equol [130]. Butyrate downregulates proinflammatory gene expression and suppresses tumour growth via inhibition of histone deacetylases [131]. The outcome of the tug-of-war depends on epigenetic factors such as diet which will give the final upper hand aiding in colonization by either pro- or anti-carcinogenic bacteria.

The majority of pancreatic ductal adenocarcinoma (PDAC) patients have a dismal prognosis, but a small subset of patients survive longer than 5 years [132]. Intriguingly, long-term survivors have higher tumour microbial diversity with distinct tumour microbial signatures compared to short-term survivors [133]. The tumour microbial diversity was shown to exert an immune-activating effect via improved immune cell infiltration to the tumour milieu. Furthermore, colonization of pancreatic tumours by gut microbiota was identified, with 25% of PDAC microbial composition matching that of the gut. Preclinical data from the same study showed that faecal microbial transplant (FMT), from patients who were long-term survivors, into tumour-bearing mice led to immunoactivation in the murine tumour microenvironment and a significant reduction in tumour growth, reiterating the role of tumour microbiome in disease progression and outcome, as well as the potential of FMT in treating PDAC [133].

57.5.2 The Microbiome Is More Than Bacteria

We need to consider more than simply bacteria and viruses. Fungal infiltration from the gut to the pancreas was shown to occur via the sphincter of Oddi (Fig. 57.9a), which serves as a direct link between the pancreatic duct and the gut. Taxonomic diversity analysis identified the dominance of the genus *Malassezia* in PDAC tissues compared to that of the gut, in mouse models. Comparison of sequencing data of PDAC patient faecal samples to that of paired tumour tissue corroborated these findings. Antifungal ablation with amphotericin B mitigated pancreatic dysplasia in mouse models and was shown to work synergistically with gemcitabine in reducing tumour burden [135]. Through repopulation experiments, *Malassezia globosa* was identified as being responsible for PDAC disease progression, via fungal-mediated activation of the mannose-binding lectin (MBL)–C3 cascade (Fig. 57.9b). MBL is a protein of the innate immune system which serves as an opsonin. Upon binding to the sugar motifs on the fungal wall, it triggers the complement cascade, in particular C3, a pivotal component downstream of MBL [135]. Based on the inference from the study, we can speculate

that diagnostic assay using the taxonomic composition of stool samples may be appropriate for early detection of PDAC, and that antifungal therapy may be efficacious.

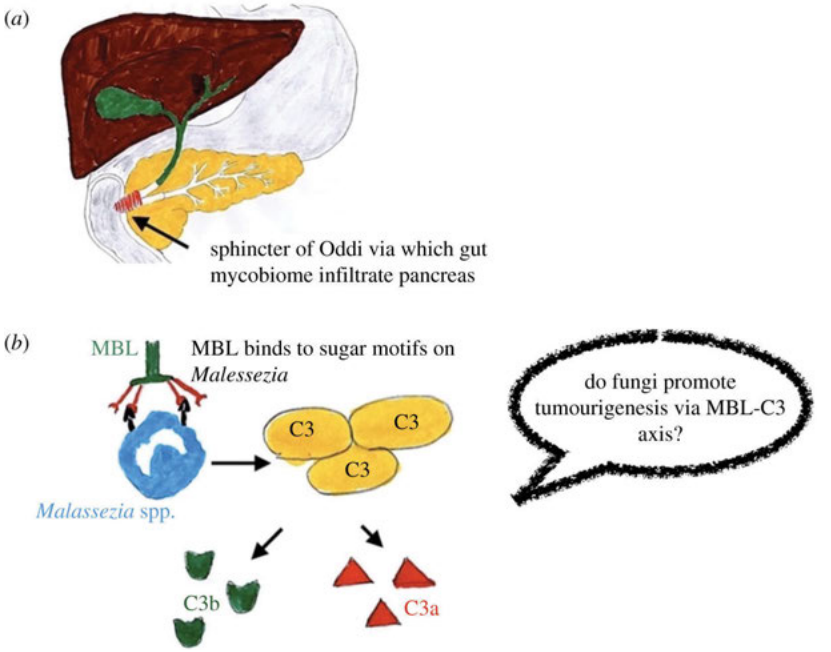

Figure 57.9 Tumour promoting inflammation triggered by mycobiome. (*a*) Sphincter of Oddi. (*b*) The mannose-binding lectin (MBL) pathway of complement activation, adapted based on [134].

More than 85% of human papillomaviruses (HPV) are cleared spontaneously [136], so why can the remaining 15% mediate progression to cervical neoplasia? The answer lies with the vaginal microbiome, dysbiosis of which plays an important role even in HPV-related cervical cancers [137]. *Lactobacillus* species are dominant in the vaginal niche and are characteristic of vaginal health [138]. They maintain the vaginal microenvironment in an acidic state (pH < 4.5) via the production of lactic acid [138] and protect against invading pathogens such as herpes simplex virus [139], human immunodeficiency virus [140], *Neisseria gonorrhoeae* [141] and even *E. coli* [142]. The depletion of *Lactobacillus* species has been linked to an increased risk of acquisition of HPV infection and its reduced clearance [137], and reduction in *Lactobacillus* dominance and increased vaginal microbiome diversity correlated strongly with cervical neoplasia severity [137, 143].

The microbiome also plays an important role in deciding the outcome of both conventional chemotherapies and immunotherapeutic interventions. It can alter the bioavailability of drugs [144], and DNA damage induced by platinum-based regimens is severely attenuated in the absence of commensal microbiota [145]. Oral administration of *Bifidobacterium* in mice controlled melanoma growth on

a par with checkpoint blockade using programmed cell death ligand 1 (PD-L1) specific antibody and co-administration resulted in near eradication of tumour growth [146]. Furthermore, the efficacy of blocking CTLA-4, a major negative regulator of T cell activation, depends on *Bacteroides* species and tumours in axenic or antibiotic-treated mice do not respond to CTLA-4 blockade [147]. The microbiome also has a role in immunosurveillance, as seen with the hygiene hypothesis which links an increase in the incidence of some cancers to decrease in exposure to certain microbes [148, 149].

In conclusion, the microbiome exerts both beneficial and nefarious effects over the human body. We argue that it has a role in each of the triumvirate of immunoediting [150], namely elimination, equilibrium and escape during tumourigenesis and as such is a pivotal enabling hallmark of cancer. Antibiotic mediated alteration of gut microbiota has been shown to alter the cerebral tumour microenvironment, thus affecting glioma progression [151], which bring us to our final enabling hallmark of cancer—nerves/neuronal signalling.

57.6 New Hallmark 4: Altered Neuronal Signalling

Vesalius, in his book *De corporis humani fabrica libri septem*, described the tandem nature of blood vessels and nerves [4, 198] (Fig. 57.10), innervation and blood supply being indispensable for growth and survival. Since angiogenesis has an established role, it is enticing to delve further into the role of nerves in cancer, a topic that is often overlooked. Perhaps the reason might be the difficulty involved in observing nerves during routine histology of tumour specimens, but nerves are one of the most significant aspects of tumour progression. Metastasis involving the central nervous system/peripheral nervous system results in manifold increased morbidity/mortality.

In 1840, surgeons attempted to transect the trigeminal nerve, which runs along the face, and the accompanying blood vessels, in order to cure tumour of the lips. It provided symptomatic control but failed to cure the patients and eventually mandated complete resection of the tumour [152]. However, with recent advances in the understanding of the function of the nervous system, its role in tumour initiation and progression can be better elucidated, to derive therapeutic benefits. The density of nerve fibres in tumour tissue correlates with the aggressiveness of the disease among multiple cancers, including breast [153], lung [154], colorectal [155] and prostate cancers [156]. Based on these observations, one might advocate neuronal transection to control tumour progression. However, in a PDAC mouse model, sub-diaphragmatic vagotomy, targeting the vagus nerve—a mixed nerve with both sensory and parasympathetic components, resulted in enhanced tumour growth and reduction in survival [157]. Contrastingly, transection of the same nerve in gastric cancer models resulted in suppression of tumourigenesis [158]. Instead of the radical approach of transection, an alternative approach is to use chemical denervation, as performed in specific targeting of the sensory nerves in ductal carcinoma, and the use of capsaicin

inhibited pancreatic ductal adenocarcinoma (PDAC) progression [159]. Another approach is via injection of botulinum toxin A (Botox), a neurotoxin, into the gastric wall. This inhibited progression to overt adenocarcinoma among preneoplastic models and inhibited disease progression in advanced gastric cancer models [158].

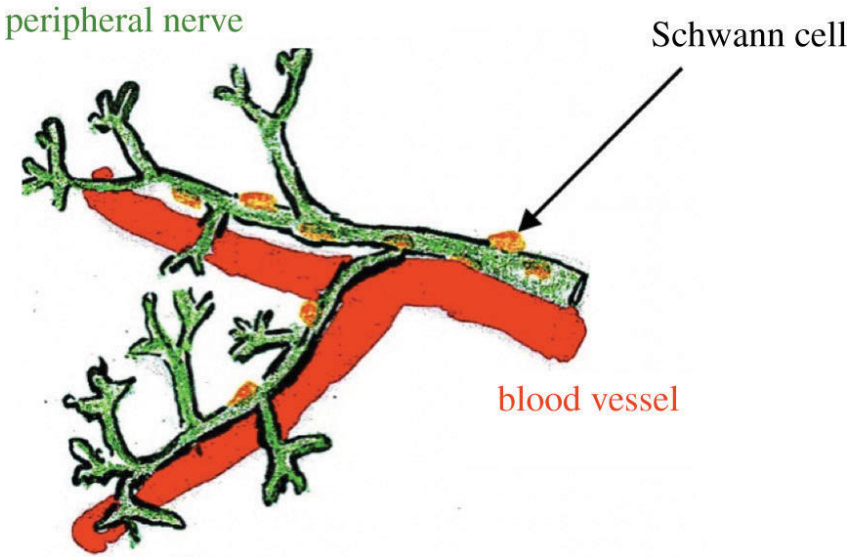

Figure 57.10 Blood vessels and nerves in tandem.

57.6.1 β-Blockers for Inhibiting Tumour Progression

Sympathetic nerves are implicated in blood vessel patterning during early development [160]. Sympathetic nerves release noradrenaline, the circulating levels of which increase during chronic stress [161]. β-adrenergic receptors mediate most of the effects of noradrenaline. Chronic stress has long been attributed as a risk factor for cancer [162]. Reproduction of the effect of chronic stress in transgenic mouse models of breast cancer via long-term administration of isoprenaline, a non-selective β-adrenergic agonist, resulted in increased lymph node metastasis, while inhibition of adrenergic signalling with propranolol, a non-selective β-blocker, resulted in inhibition of metastasis to the lymph node [163]. Reiterating the role of β-adrenergic receptors, a similar effect was also observed in pancreatic cancer models of chronic stress, with a reduction of tumour volume following administration of propranolol [164].

The pro-tumourigenic effects exerted by sympathetic nerves in response to stress are mediated by β-adrenergic receptors. This was demonstrated elegantly in work showing that an adrenergic nerve-derived signal-mediated activation of an angiogenic switch in a transgenic mouse model of prostate cancer [165].

Sympathetic nerves in prostate tumours release noradrenaline which, via the β2-adrenergic receptor on endothelial cells, triggers an angiogenic switch by inducing a change in endothelial cell metabolism from oxidative phosphorylation

towards aerobic glycolysis, driving angiogenesis and fuelling tumour progression. Blockade of β-adrenergic receptor signalling reverts endothelial cell metabolism from aerobic glycolysis towards oxidative phosphorylation through cytochrome C oxidase assembly factor 6 (Coa6) activity, thereby inhibiting angiogenesis and curtailing tumour progression [165].

57.6.2 Perineural Invasion in Pancreatic Ductal Adenocarcinoma

Perineural invasion is linked to worse prognosis in PDAC [166], with PDAC cells recruiting nerves via nerve growth factor (NGF) [167]. In murine PDAC models, chronic stress-dependent sympathetic nerve signalling triggers tumour growth via a feedforward loop, wherein adrenergic signalling stimulates NGF, which promotes further innervation of tumour cells via axogenesis, resulting in increased noradrenaline accumulation in the tumour microenvironment, inducing β2-adrenergic receptor-dependent PDAC progression [168] (Fig. 57.11).

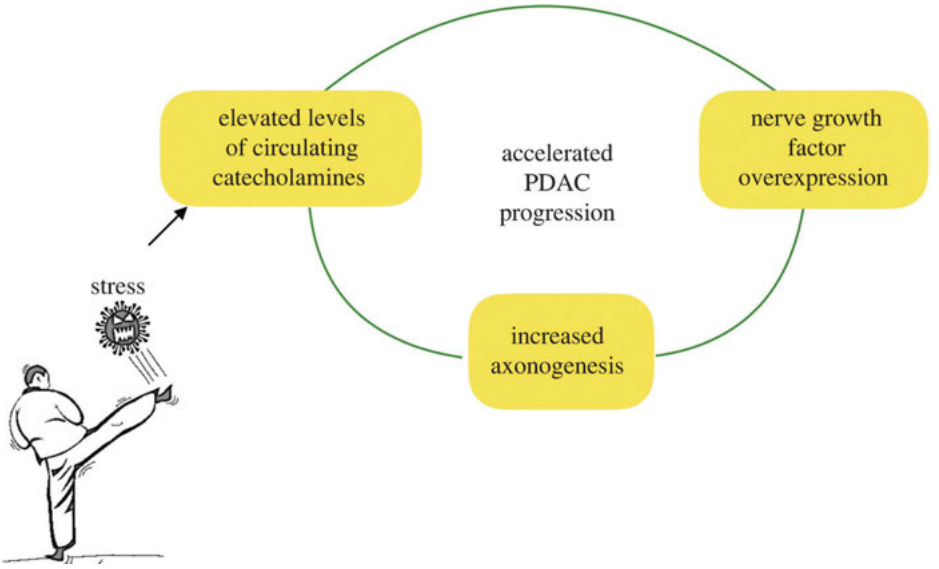

Figure 57.11 Chronic stress-dependent sympathetic nerve signalling triggering tumour growth via a feedforward loop.

Blockade of the β2-adrenergic receptor, or the NGF receptor tropomyosin-receptor kinase A (TRKA), disrupts this feedforward loop and inhibits tumour progression [167, 168]. Clinical studies have reported improved survival among PDAC patients with the use of β-blocker [169]. This provides a window of opportunity to treat patients with pancreatic intraepithelial neoplasias (PanINs) with a non-selective β-blocker regimen to potentially prevent progression to overt PDAC, although the challenge of early detection remains. This might be facilitated using a diagnostic assay based on the taxonomic composition of stool samples, as discussed earlier.

While β-adrenergic signalling is pro-tumourigenic in the aforementioned solid cancers, there is the caveat of an opposite effect adding to the complexity of targeting the hallmark of nerves/neuronal signalling. Noradrenaline-mediated sympathetic nerve signalling has been linked to the maintenance of the steady-state condition of haematopoietic stem cells (HSCs) in the bone marrow niche in a circadian manner [170]. Attrition of the β-adrenergic signalling leads to an increased propensity of myeloproliferative neoplasms [171, 172], therefore the implementation of β-blocker targeting sympathetic signalling in malignancy is context-dependent.

57.6.3 Putting the Cart Before the Horse: Whether Nerves Migrate Towards Tumours or the Tumour Cells Migrate towards Nerves?

Schwann cells, the glial cells responsible for myelinating peripheral nerves, are key to neural homeostasis, participating in Wallerian degeneration, neural repair and regeneration [173]. In an *ex vivo* model using rat sciatic nerve, Schwann cells displayed a high affinity towards pancreatic and colon tumour cells, but not normal cells, migrating towards tumour cells, thereby outlining a pathway for tumour driven neurogenesis [174]. Nerve growth factor (NGF), and its receptors TRKA and p75NTR are critical regulators of gland innervation and neurite outgrowth. They are also implicated in neural tracking [175], the ability of tumour cells to migrate along axons.

Pro-NGF, the precursor of NGF, serves as a reservoir for NGF [176]. Immunohistochemical studies in prostate cancer suggested that pro-NGF production by tumour cells might drive axonogenesis [177]. Thus, there is an element of reciprocal interaction between nerves and tumour cells driving tumourigenesis; Schwann cells migrate towards tumour cells while prostate tumour cells in turn recruit nerves via pro-NGF.

57.6.4 Synaptic Interaction between Brain Tumour Cells and Neurons

Clues to the interaction between tumour cells and neurons come from the study of synapses between neurons and oligodendrocyte precursor cells, demonstrating a neuron to non-neuron synapse [178], as well as the finding that glutamate secretion confers a growth advantage to glioma cells [179]. These preliminary studies were bolstered by the identification of functional synapses between neurons and glioma cells, with transcriptomic analysis further confirming that the glioma cells express GluA2, a subunit of the ionotropic glutamate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR). Treatment with an AMPAR antagonist inhibited glioma progression, suggesting glioma cells can co-opt glutamatergic signalling to facilitate invasion and tumour progression [180, 181] (Fig. 57.12). Based on both of these studies, one can speculate whether anti-epileptic drugs that act presynaptically, such as levetiracetam [182], might inhibit glioma progression. An alternative approach could be a non-competitive AMPAR antagonist such as perampanel, which has good

penetration to the brain, for use in glioma treatment [183]. Key to note is that the AMPARs mentioned in both the studies [180, 181] are calcium permeable, which means the target of the drug candidate must be calcium permeable AMPAR.

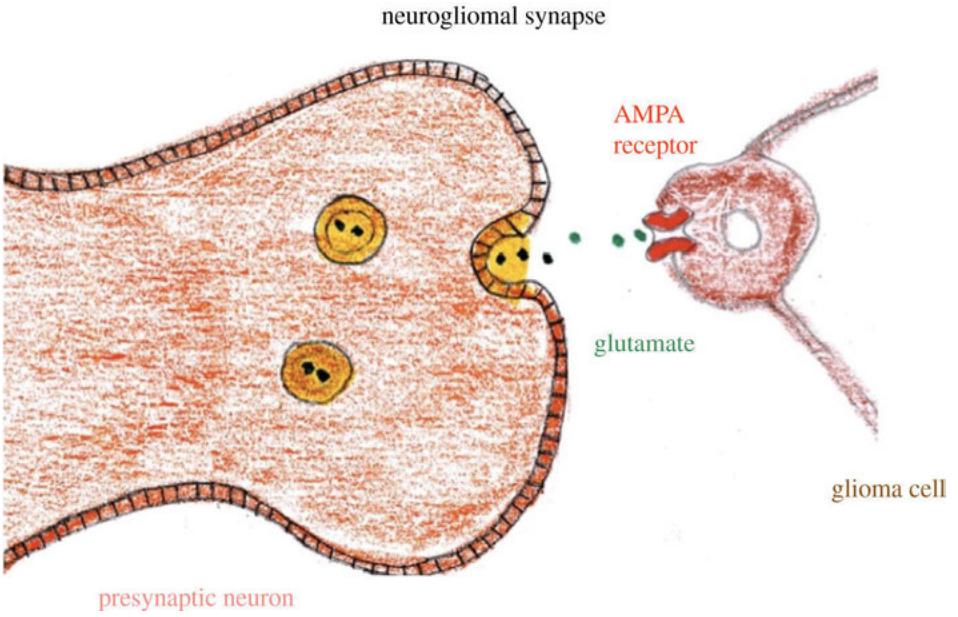

Figure 57.12 Synaptic interaction between presynaptic neuron and glioma via AMPA receptor.

Metastasis to the brain presents a checkmate scenario to clinicians but a breakthrough finding, deciphering the interaction between neurons and metastatic cells [184], can now pave way for new therapeutic approaches.

57.6.5 Parasitic Tripartite Synapse

Triple-negative breast cancer (TNBC) carries a poor prognosis as it lacks the expression of targetable hormone receptors and human epidermal growth factor receptor 2, coupled with a propensity to metastasize to the brain [185]. *N*-Methyl-*d*-aspartate receptor (NMDAR), a type of glutamate receptor, plays a key role in the synaptic plasticity of the central nervous system, but has also been implicated in ovarian and pancreatic tumour progression [186]. Transcriptomic data identified higher expression of NMDAR among basal sub-types of breast cancers such as (TNBC), in particular the NMDAR GluN2B subunit, which contains phosphorylation sites critical for NMDAR signalling. An autocrine source of glutamate-mediated NMDAR signalling in the breast to brain metastasis (B2BM) microenvironment was excluded, with B2BM cells found to express neuroligin-2 [184], the expression of which by non-neuronal cells has been shown to induce presynaptic differentiation and trigger *de novo* formation of pseudo-synapses [187, 188].

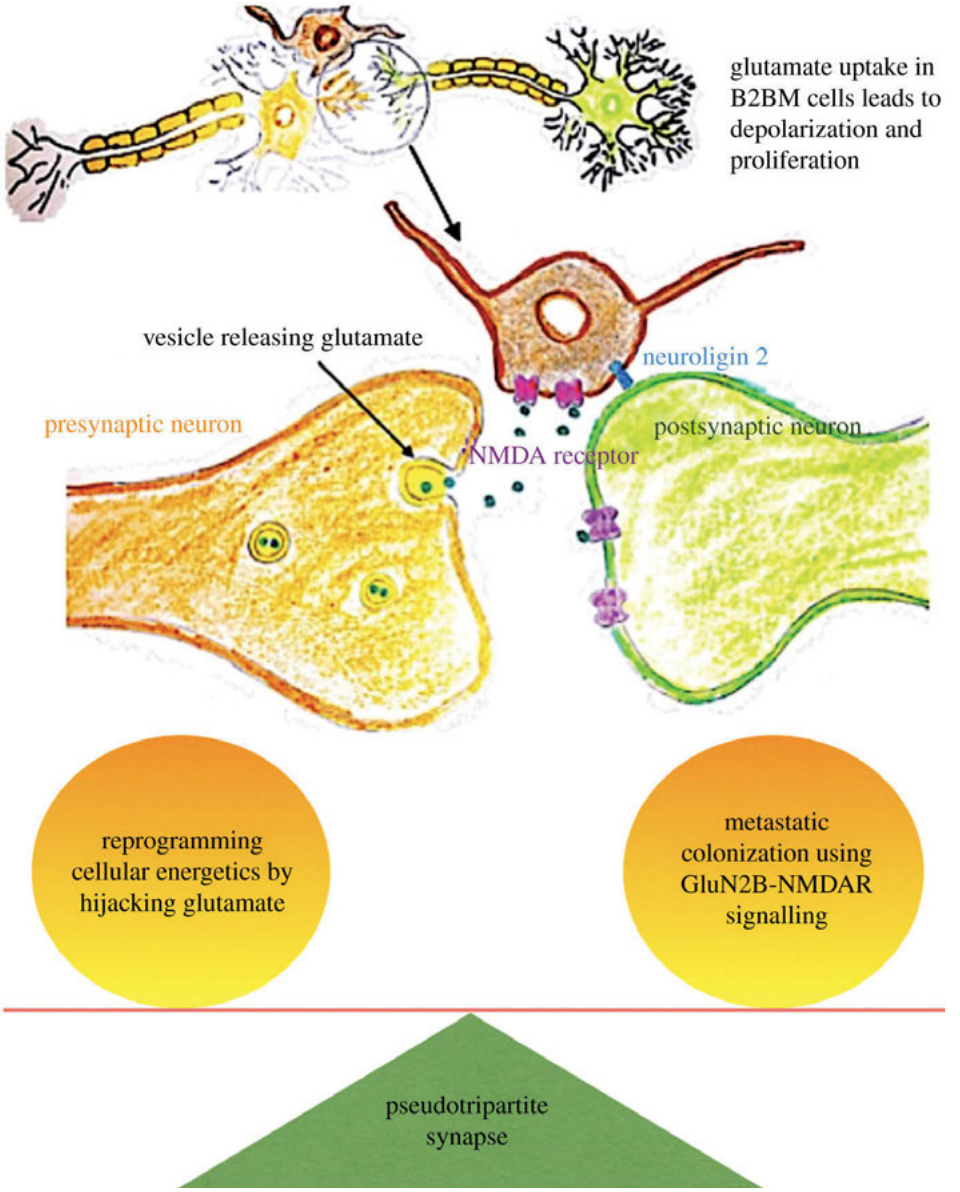

Figure 57.13 Parasitic tripartite synapse: B2BM colonization of brain using glutamate from the fake tripartite synapse, adapted based on [184].

Microscopic analysis of mouse B2BM models revealed a pseudo-tripartite synapse phenomenon. Finger-like projections emanated from the B2BM cells towards excitatory synapses, forming a fake tripartite synapse [184]. In normal neurophysiology, glutamate released from presynaptic neurons is endocytosed by postsynaptic neurons that express the glutamate receptor NMDAR, as well as by astrocytes which are located adjacent to the synaptic cleft [189]. This tripartite phenomenon is mimicked by the B2BM cells, which take the position

of the astrocyte next to the synaptic cleft and use the glutamate from the presynaptic neuron to promote further metastasis and colonization in the brain (Fig. 57.13).

Modulation of GluN2B expression demonstrated that NMDAR signalling was not necessary for the initial seeding of breast tumour cells to the brain but, rather, was critical for the proliferation of B2BM cells [184]. Thus, B2BM cells effectively tune the neural niche to their advantage without disrupting the existing synaptic infrastructure. Tumour cell–astrocyte gap junctions can also be co-opted to promote brain metastasis via 2′3′-cyclic GMP-AMP (cGAMP)-mediated signalling. This can potentially be disrupted by the gap junction modulator meclofenamate, which has oral bioavailability and can pass through the blood–brain barrier [190]. Similarly, based on the inference that B2BM cells co-opt NMDAR signalling for metastatic progression in the brain [184], one could exploit the parasitic tripartite synapse for therapeutic and diagnostics purposes. One approach might be to repurpose memantine, an NMDAR antagonist used for treating Alzheimer's disease, to curtail the progression of B2BM in TNBC patients. Furthermore, radiolabelled glutamine can potentially be used for imaging triple-negative breast cancer brain metastasis [191].

57.6.6 Nerves and the Tumour Microenvironment

Nerves can also play a role in immune evasion during tumourigenesis by orchestrating an immune-suppressive tumour microenvironment. β2-adrenergic receptor signalling by adrenergic nerves can inhibit lymphocyte egress, effectively reducing the recruitment of antigen primed T cells [192]. Manipulation of autonomic nerves using a novel viral-vector based neuro-engineering technique revealed accelerated breast cancer progression with sympathetic nerve stimulation in tumours, while local sympathetic denervation curtailed tumour growth and reduced the expression of immune checkpoint molecules, such as programmed death-1 (PD-1) and PD-L1, as well as FOXP3, that mediates immunosuppression [193]. Such a strategy of the localized intervention targeting neural input, using genetic neuro-engineering techniques, may hold promise to stimulate the immune system while offsetting the deleterious side-effects of the systemic use of checkpoint inhibitors.

Nerves and neuronal signalling are an indispensable part of tumourigenesis, playing an active role in modulating the tumour microenvironment. They are involved in the recruitment of blood vessels to the tumour, control constriction/relaxation of blood vessels, alter the expression of immune checkpoint molecules and provide cues for proliferation to tumour cells, yet the nervous system has been largely disregarded in cancer therapeutics. Nerves and neuronal signalling are an enabling hallmark of cancer that provides tumours with a means of interacting with its microenvironment to facilitate metastatic progression. Future treatment regimens must work around the neural circuit to offer better control over tumour progression.

57.7 Conclusion

The understanding of cancer from a curse, to that of a heterogeneous group of diseases that lack the fundamental ability to respond to principal signals regulating proliferation, differentiation, and cell death is a phenomenal leap of understanding. From multiple resections without anaesthesia in ancient times, to targeted cancer therapeutics is certainly a remarkable feat of achievement. The Hallmarks of Cancer [194] marked the Millenium era for cancer researchers, laying the framework for honing our understanding of cancer as a disease. We present four novel hallmarks, the traits of which are the language which cancer cells use to interact with the microenvironment to facilitate proliferation and survival. We consider two additional core hallmarks: dedifferentiation/transdifferentiation and epigenetic dysregulation, alongside two enabling hallmarks: altered microbiome and altered neuronal signalling.

Seminal studies, we have discussed, overturned the unidirectional landscape of differentiation [9, 10], yet the hallmark of dedifferentiation has long been ignored in the field of cancer therapeutics. The lineage plasticity conferred by the proposed hallmark of dedifferentiation, hijacked by tumour cells, can also be used for targeting tumour cells at their most vulnerable state to potentially transdifferentiate them to lineages which lack metastatic potential.

Two of the hallmarks proposed to confer a vantage point for therapeutic manipulation due to their reversible nature: epigenetic dysregulation and the microbiome. Epigenetic dysregulation provides numerous opportunities to intervene in cancer progression and development. For example, dietary factors can influence serum methionine levels, which in turn can affect histone methylation [195]. Microbiome dysbiosis can be manipulated by enhancing our ability to identify anti-carcinogenic (friend) and pro-carcinogenic (foe) among the microbiome. Microbiome composition must be integrated and used as a tool to enhance the outcome of therapeutics.

Finally, the hallmark of altered neuronal signalling consists of multiple clues to halt metastasis. The two factors which cancer cells use to design their micro-environment to their advantage are microbiome and nerves. Tumour cells use nerves to establish blood vessels and garner proliferative cues. Cancer can be associated with excruciating pain, a key being that cancer cells recruit numerous nerves, a trait that can be intercepted for pain management. Two modalities for managing the hallmark of altered neuronal signalling are either to include resection of nerves in surgical protocols for tumour management (significantly more challenging than resecting lymph nodes), or to target the nerve growth factor/localized intervention of neuronal signalling within the tumour microenvironment. Future studies may look into possibilities of targeting artemin which has an established role in the migration of sympathetic precursors [196, 197].

Considering cancer as the conductor of a malign symphony and the hallmarks as the musicians, we need to tune our hearing to appreciate every key nuance of the piece. By identifying new performers, we can adapt our interventions, re-educating the orchestra and re-establishing the rhythm of life.

Abbreviations

ALT: alternative telomere lengthening
AML: acute myeloid leukaemia
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
APML: acute promyelocytic leukaemia
ASCs: adipose-derived stem cells
ATRX: α-thalassemia X-linked mental retardation protein
B2BM: breast to brain metastasis
BFT: *B. fragilis* toxin
Botox: botulinum toxin A
CagA: *cytotoxin-associated gene A*
ccRCC: clear cell renal cell carcinoma
CDK4/6: cyclin-dependent kinase 4/6
CDKN2A: cyclin-dependent kinase inhibitor 2A
cGAMP: cyclic GMP-AMP
Coa6: C oxidase assembly factor 6
CRCs: colorectal
CSCs: cancer stem cells
CXCR4: *chemokine receptor 4*
Daxx: domain associated protein
DNMTs: DNA methyltransferases
DR4: death receptor 4
EMT: epithelial–mesenchymal transition
ETBF: enterotoxigenic *Bacteroides fragilis*
EZH2: enhancer of Zeste homologue 2
FMT: faecal microbial transplant
G-CIMP: CpG island methylator phenotype
HPV: human papillomaviruses
HSCs: haematopoietic stem cells
ID-A: insertion/deletion A
IDH: isocitrate dehydrogenase
iPSCs: induced pluripotent stem cells
MBL: mannose-binding lectin
MITF: microphthalmia-associated transcription factor
NGF: nerve growth factor
NK: inhibits natural killer
NMDAR: *N*-methyl-d-aspartate receptor
NuRD: Mi-2-nucleosome remodelling and deacetylase
PanINs: pancreatic intraepithelial neoplasias
PDAC: pancreatic ductal adenocarcinoma
PDGFRA: platelet-derived growth factor receptor A
pks: polyketide synthase
PML: promyelocytic leukaemia protein

PRC2: polycomb repressive complex 2
RARα: retinoic acid receptor α
Rb: retinoblastoma protein
SAA: serum amyloid A
SBS-A: single-base substitution-A
SCFAs: short-chain fatty acids
sFLT1: soluble Fms-like tyrosine kinase-1
TADs: topologically associated domains
TE: transposable elements
TIGIT: T cell immunoglobulin and ITIM domain
TNBC: triple-negative breast cancer
TNF: tumour necrosis factor
TRAIL: tumour necrosis factor-related apoptosis-inducing ligand
TRKA: tropomyosin-receptor kinase A
vacA: vacuolating cytotoxin gene A
VEGF: vascular endothelial growth factor
VHL: von Hippel–Lindau

Disclosures and Conflict of Interest

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Chapter 58

Adipose Tissue in Health and Disease

Innocence Harvey, PhD,[a] Anik Boudreau,[a] and Jacqueline M. Stephens, PhD[a,b]

[a]*Adipocyte Biology Laboratory, Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA*
[b]*Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA*

jsteph1@lsu.edu

Keywords: adipocyte-specific secretory factor (ADSF), adiponectin, adipose tissue (AT), apoptosis-associated speck-like protein containing a CARD (ASC), brown adipose tissue (BAT), caspase-1, caveolin-1 (Cav-1), Cushing's syndrome, forkhead box O1 (FOXO1), hyperinsulinaemia, insulin receptor (IR), insulin resistance, insulin-like growth factor 1 receptor (IGF-1R), interleukin 6 (IL6), leptin, metabolic disease, metabolic syndrome, metabolically activated (MMe), metabolically oxidized (Mox), microRNAs (miRNAs), monocyte chemoattractant protein 1 (MCP1), neuroregulin 4 (NRG4), Nod-like receptor (NLR), NRG4 receptor (ErbB), peroxisome proliferator-activated receptor gamma (PPARγ), portal theory, resistin, tumour necrosis factor alpha (TNFα), type 2 diabetes, vascular endothelial growth factor (VEGF), white AT (WAT)

Adipose, or fat, tissue (AT) was once considered an inert tissue that primarily existed to store lipids, and was not historically recognized as an important organ in the regulation and maintenance of health. With the rise of obesity and more rigorous research, AT is now recognized as a highly complex metabolic organ involved in a host of important physiological functions, including glucose homeostasis and a multitude of endocrine capabilities. AT dysfunction has been implicated in several disease states, most notably obesity, metabolic syndrome and type 2 diabetes. The study of AT has provided useful insight in developing strategies to combat these highly prevalent metabolic diseases. This review highlights the major functions of adipose tissue and the consequences that can occur when disruption of these functions leads to systemic metabolic dysfunction.

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58.1 Introduction

Adipose tissue (AT) is now fully recognized as a metabolically active organ. Historically, AT was thought to provide fuel or insulation to organs, and to serve as a connective tissue. Studies in the last two decades have demonstrated that AT plays a critical role in systemic metabolic health. While AT is indeed the primary site for energy storage in the form of lipid, it is also a major endocrine organ, producing and secreting adipose-tissue-specific hormones known as adipokines. In addition to hormones, fat tissue secretes various forms of genetic material, lipid and proteins that all contribute to its substantial endocrine activity. AT also responds to a variety of circulating metabolites and hormones, including lipids, growth hormone, cortisol, insulin, catecholamines and many others. Moreover, AT is known to be a major metabolic organ, along with the liver and skeletal muscle, critical to maintaining proper glucose homeostasis [1]. Disruption in any one of the three primary functions of adipocytes (lipid storage, endocrine function and responsiveness to insulin) can have major impacts on overall metabolic health. Excess adiposity, or obesity, is a major risk factor in several disease states including type 2 diabetes, cardiovascular disease, hepatic steatosis and at least 13 types of cancers [2–5]. Although research in adipocyte biology and physiology has advanced dramatically in recent years, our understanding of the complex processes governing the role of AT in health and disease is still emerging. This review highlights our knowledge of AT pathologies and how they contribute to metabolic diseases, as well as gaps in our understanding of AT biology that require further study.

58.2 Adipose Tissue Expansion and Development

58.2.1 Cellular Growth and Development

AT is the primary organ for the storage of lipids. Excessive lipid accumulation results in obesity, also known as excessive adiposity or AT expansion, which is driven by adipocyte hyperplasia and/or hypertrophy. Hyperplasia refers to the formation of new adipocytes from preadipocytes through adipogenesis, a highly complex and tightly regulated process involving many hormones and transcription factors [6], most notably peroxisome proliferator-activated receptor gamma (PPARγ), which is absolutely required for adipocyte differentiation and is considered the master regulator of adipogenesis [7]. Although adipogenesis *in vitro* is well understood, the control of this developmental pathway *in vivo*, in the presence of other tissues and a plethora of circulating factors, is less understood, due in part to the limited methodologies available to study adipogenesis *in vivo*. However, recently developed model systems featuring fluorescent labelling of adipocytes have allowed for a more rigorous *in vivo* assessment of adipogenesis [8–10]. For example, Tang and colleagues were able to detect newly formed adipocytes with the use of the AdipoTrak mouse model and demonstrated that

PPARγ agonist treatment enhances adipogenesis *in vivo*, supporting previously established *in vitro* studies [8]. Utilization of fluorescence-activated cell sorting and liquid chromatography–tandem mass spectrometry methods has led to the identification of several unique types of adipocyte progenitor cells in adipose tissue, and provided insight into adipocyte origin, development and heterogeneity in both mice and humans [11–13]. Furthermore, other recent technologies such as single-cell RNA sequencing have advanced our comprehension of the genes and processes governing adipocyte commitment of precursors, progenitors and adipocyte stem cells [14, 15]. The study of adipogenesis *in vivo* is still an emerging field and we have much left to learn. A recent review provides a detailed overview of the complex nature of adipocytes, as well as other cells within adipose tissue, and highlights the advantages of using of single-cell RNA sequencing in the study of adipocyte biology and development [16]. Further utilization of these new methods will enhance our understanding of overall AT expansion.

It is well established that inhibiting adipogenesis in mice can lead to metabolic dysfunction. For example, loss of PPARγ inhibits adipocyte hyperplasia and total AT accumulation, while promoting adipocyte hypertrophy, insulin resistance and other markers of metabolic dysfunction [17]. PPARγ agonists not only promote adipocyte differentiation [18], but also improve overall glucose homeostasis and metabolic health [19–21]. Deuterium labelling has allowed for further study of adipogenesis in humans *in vivo.* In line with the studies above, there is evidence that PPARγ agonists promote femoral adipocyte differentiation and improve insulin sensitivity in humans [22]. New adipocytes resulting from PPARγ-driven adipogenesis facilitate increased lipid storage in AT and are associated with reduced circulating lipids, enhanced glucose disposal and increased fat oxidation in diabetic patients [8, 23]. Notably, this formation of new adipocytes is associated with reduced ectopic lipid storage and a decrease in other markers of metabolic syndrome in patients with fatty liver disease [24, 25]. Although PPARγ agonists are highly effective insulin sensitizers for type 2 diabetes treatment, their clinical use has been drastically reduced in recent years due to considerable side effects, including weight gain, fluid retention, congestive heart failure and bone fractures [26–29]. Conversely, there is recent data to suggest that enhanced adipocyte turnover negatively impacts metabolic health [30]. However, as the authors indicated, these data are correlative and it is still unclear whether adipocyte death was a driver of the increased adipogenesis. Clearly, more research needs to be performed in this area.

In contrast to hyperplasia, hypertrophy is the enlargement of individual adipocytes by lipid accumulation. Hypertrophy can occur through uptake of dietary lipids from the circulation, or through the fatty acid synthesis pathway in adipocytes, known as *de novo* lipogenesis (reviewed in [31]). Many rodent studies have suggested that larger adipocytes are a characteristic of metabolic dysfunction [32–34]. This notion is supported by clinical studies reporting that increased adipocyte size is associated with insulin resistance, hepatic steatosis and other markers of metabolic dysfunction [27, 28, 35]. Similarly, adipocyte

volume was higher in patients who did not show improvements in insulin resistance following bariatric surgery [36]. Adipocyte hypertrophy has also been associated with insulin resistance and inflammation in healthy patients who are genetically predisposed to type 2 diabetes [37]. While it is generally accepted that impaired adipogenesis and excessive adipocyte hypertrophy are drivers of insulin resistance in obese states, data from several mouse models indicate that the relationship between fat cell size and metabolic dysfunction is not straightforward, and that changes in metabolic parameters can occur in the absence of altered adipocyte size, and vice versa. For example, ablation of *Siah2*, a ubiquitin ligase, results in obesity and enlarged adipocytes, but preserved insulin sensitivity [38]. Conversely, adipocyte-specific mTORc1 depletion in mice leads to smaller adipocytes accompanied by systemic insulin resistance [39]. Similarly, mice with ectopic expression of nuclear SREBP-1c in adipocytes have overt metabolic dysfunction and lipodystrophy, despite having notably smaller adipocytes when compared to controls [40]. Mice lacking collagen VI have large adipocytes due to uninhibited expansion, but have substantially improved whole-body energy and glucose homeostasis [41]. Also, a mouse model with ectopic expression of endotrophin, a proinflammatory adipokine, in adipocytes displayed increased AT inflammation and fibrosis, as well as systemic metabolic dysfunction, while adipocyte size was unchanged [42]. These data and many other examples make it clear that fat cell size is not an absolute indicator of systemic metabolic health. Overall, a balanced combination of adipocyte hypertrophy and hyperplasia is required for appropriate AT expansion and maintenance of metabolic health.

58.2.2 Extracellular Development

Angiogenesis and vascularization are also important contributors to AT development, as they are required not only for oxygenation, but also for endocrine functions and nutrient transport to and from AT. Insufficient vascularization during AT expansion promotes hypoxia, which may trigger further complications including inflammation, fibrosis and apoptosis [43], contributing to adipose tissue dysfunction (Fig. 58.1). While many proteins participate in AT remodelling during expansion, vascular endothelial growth factor (VEGF) is considered the primary player in this process [44]. Several rodent studies demonstrate that reduced AT vascularity in obesity leads to systemic metabolic dysfunction. For example, mice with adipocyte-specific deletion of VEGF that are exposed to high-fat feeding have reduced vascularity, increased inflammation and significantly reduced glucose handling abilities despite a reduction in fat mass [45]. Notably, adipocyte overexpression of VEGF reverses these outcomes [45]. Several review articles have highlighted the implications of impaired VEGF signalling in obesity-induced metabolic disease in humans [46, 47]. The anti-angiogenic transcription factor forkhead box O1 (FOXO1) and the angiogenic adipokine neuroregulin 4 (NRG4) are also known to contribute to vascular regulation. FOXO1 levels are elevated in obesity, and mice with reduced endothelial expression of FOXO1

had improved vascular remodelling in AT and enhanced glucose tolerance [48]. However, the FOXO1-deficient mice also had reduced body and fat mass, confounding the interpretation of these findings. NRG4 has recently been recognized as a pro-angiogenic adipokine [49]. Constitutive expression of NRG4 in adipocytes leads to improved glucose tolerance, increased adipose blood vessel formation and reduced hypoxia in AT of obese mice when compared to control mice of the same body weight [49]. Moreover, pharmacological inhibition of angiogenesis or blockade of the NRG4 receptor (ErbB) in the transgenic mice prevented the enhancement of angiogenesis and the favorable metabolic effects noted above [49]. These data suggest that NRG4-induced angiogenesis is a positive regulator of metabolic health in AT. Taken together, these data underscore the notion that AT development as a whole is crucial to systemic health.

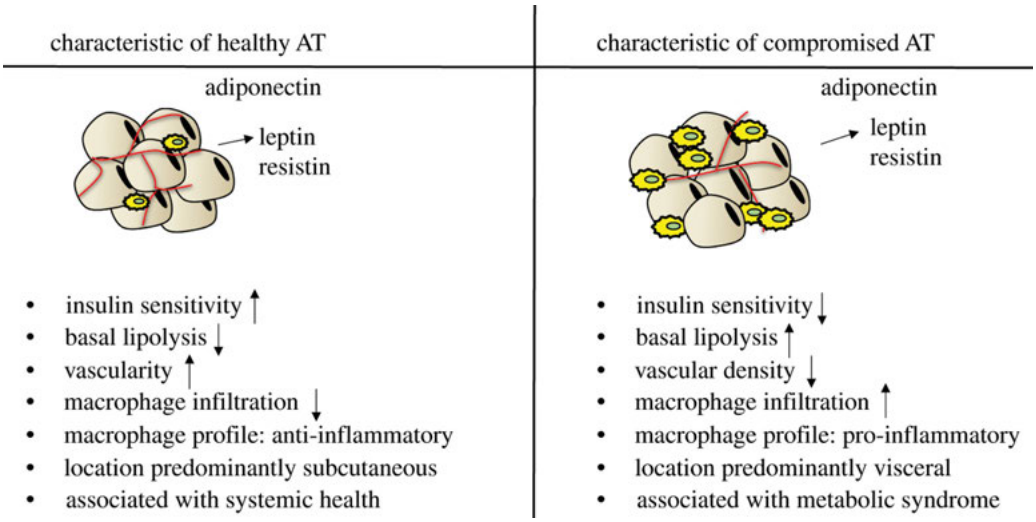

Figure 58.1 General classifications of metabolically healthy and unhealthy white adipose tissue (AT). Metabolically healthy AT is generally stored subcutaneously, is highly vascularized with low levels of macrophages and has appropriate adiponectin secretion. Healthy adipocytes also have less production and secretion of leptin and resistin. Healthy adipocytes are insulin sensitive with low basal lipolysis that is associated with overall systemic metabolic health. In contrast, metabolically compromised AT is primarily stored in the visceral cavity, has reduced vascularity with increased infiltration or presence of proinflammatory macrophages, and enhanced secretion of leptin and resistin. Typically, unhealthy adipocytes are insulin resistant and have increased basal lipolysis. The increased fatty acids from lipolysis contribute to systemic metabolic dysfunction.

58.3 Inflammation

Inflammation is a normal and necessary acute physiological response to a variety of stimuli, such as injury, but is often chronically elevated in several disease states including obesity and metabolic dysfunction. AT inflammation has been extensively studied over the past decade, and obesity is known to be associated

with chronic low-grade inflammation and metabolic disease [50–52]. Numerous proinflammatory molecules in AT are involved in obesity-related metabolic disease, including tumour necrosis factor alpha (TNFα), interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1) and various adipokines (reviewed in [53], and discussed below in 'Endocrine functions within adipose tissue'). A high-profile report of obesity-induced inflammation was a study in the early 1990s showing elevated TNFα expression in the AT of genetically obese mice [54]. TNFα is known to induce insulin resistance in adipocytes through several mechanisms, including downregulating the expression of both the insulin receptor and the insulin-sensitive glucose transporter [55, 56], as well as impeding insulin signalling events, antagonizing PPARγ action and inducing expression of proinflammatory genes (reviewed in [57]). Conditions such as hyperinsulinaemia and excess circulating lipids result in the recruitment of macrophages and other immune cells to the AT (Fig. 58.1), where they act as primary drivers of inflammation through the production of various paracrine factors, including inflammatory cytokines such as TNFα and IL-6 [43, 58–62]. In obesity, the chronic overproduction of these inflammatory mediators can cause impaired adipocyte insulin signalling, further inflammation and a continued deterioration of AT function [43, 60, 63]. Traditionally, macrophages have been classified as either M1 or M2. The M1 type is associated with a proinflammatory environment and enhanced secretion of proinflammatory cytokines from macrophages and adipocytes; whereas the M2 type is considered immunosuppressive and typically plays a more protective or restorative role following inflammatory insults [64]. However, in the last few years, studies have identified several subtypes within the M1/M2 classifications, as well as additional classifications including the newly defined obesity-associated, metabolically activated (MMe) and metabolically oxidized (Mox) macrophages [62, 65]. Moreover, new techniques such as single-cell RNA sequencing allows for highly sophisticated analysis of the cellular composition within adipose tissue and has revealed that immune cells represent a substantial percentage of adipose tissue cells and encompass an even greater variety than previously thought [15, 66]. The understanding of the contributions of adipose tissue macrophages to adipocyte function and metabolic regulation continues to be an active and expanding area of research.

Inflammasomes, which also contribute to AT inflammation, are also known to influence glucose homeostasis. These multiprotein complexes promote the maturation and secretion of inflammatory cytokines and mediate inflammatory responses to a variety of stress signals, including microbial infection, as well as endogenous mediators such as free fatty acids or extracellular ATP (reviewed in [67]). The most thoroughly characterized is the NLRP3 inflammasome, which is associated with AT inflammation and systemic insulin resistance [67–71]. Its components include a Nod-like receptor (NLR), caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein. Whole-body knockouts of inflammasome components have alleviated metabolic disturbances from diet-induced obesity [72, 73]; however, these global knockouts

also result in reduced body weight and fat mass, complicating the overall interpretation of the metabolic data. More recently, macrophage-specific knockouts have been developed and have yielded similar results [74]. There is evidence to suggest these data are translatable to humans as monocyte-derived macrophages from newly diagnosed type 2 diabetics expressed higher levels of NLRP3, ASC and proinflammatory cytokines, including several interleukins and TNFα, when compared to non-diabetic control macrophages [75]. Moreover, the release of proinflammatory cytokines was significantly elevated in the culture media as well as in the serum of diabetic patients at baseline and following stimulation from fatty acids, when compared to non-diabetic controls [75]. These findings are consistent with previous studies documenting macrophage infiltration into AT as a feature of obesity-associated metabolic dysfunction in mice [59]. While our understanding of AT macrophages and the involvement of other AT immune cells in metabolic health and disease continues to evolve, it is clear that proinflammatory conditions are implicated in the pathology of obesity and associated metabolic disease states. However, in a field that is rapidly changing, it is worth noting that some degree of inflammatory signalling appears to necessary for normal AT function. Two proinflammatory cytokines, TNFα and oncostatin M, are known to be required for proper AT expansion and maintenance of insulin sensitivity in mice [76–79]. Moreover, recent findings suggest that inhibition of a high-fat diet induced inflammation specifically in adipocytes interferes with proper glucose handling [80]. Although AT inflammation clearly has detrimental effects in obesity, it also has adaptive and homeostatic roles in AT expansion and function and its impact on systemic metabolic regulation.

58.4 Location of Lipid Storage

On the whole, excess adiposity poses an increased risk of developing metabolic syndrome [81] and type 2 diabetes [82, 83]. However, there are individuals who have increased adiposity in the absence of metabolic dysfunction, and are considered metabolically healthy obese [84]. Clearly, factors beyond simple adiposity are involved in regulating systemic metabolic homeostasis (Fig. 58.1). It is recognized that AT is highly heterogeneous, and that its many functions are impacted by parameters such as its constituent adipocyte types and anatomical locations. First, it is important to understand that there are several different types of adipocytes and AT, each with unique metabolic profiles. These types include white, brown and beige (or 'brite') fat. White AT (WAT) is the most abundant, and is the main focus of this review. Its metabolic characteristics are largely dependent on its anatomical location, as discussed below. Brown AT (BAT) is characterized by its ability to generate heat by uncoupling fuel oxidation from ATP generation, a process of metabolic inefficiency that has been speculated to be favorable to weight loss. Beige/brite AT is a newer designation and typically refers to white AT that has acquired some characteristics of brown fat. There is controversy regarding beige fat. While a large body of literature

suggests that beige fat is metabolically beneficial [85, 86], it has also been considered a stress response to a large variety of conditions [87, 88]. The functional differences among the AT varieties are not only dependent on location or energy production and utilization, but also on differences in gene expression, lipid droplet size, innervation and mitochondrial density. BAT and beige AT biology and function have been extensively reviewed [89]. Adding to the complexity of AT biology, recent studies reveal significant heterogeneity *within* the individual AT depots that likely impacts overall function and metabolic health [15, 90, 91]. Characteristics of AT heterogeneity are an emerging area of investigation; therefore, our understanding is still in its infancy.

The anatomical location of AT also influences systemic metabolism and overall health. Fat depots found in humans are not metabolically or anatomically identical to those found in rodents [92]. This information should be considered when interpreting AT studies. In general, WAT depots are broadly categorized as subcutaneous (located under the skin) or visceral (surrounding internal organs), and these distinctions are widely used and accepted in the study of AT. Typically, subcutaneous AT is considered metabolically healthy, especially when located around the gluteal-femoral region [93]; whereas visceral AT is associated with inflammation and increased metabolic disease risk. Specifically, human clinical studies have reported strong positive correlations between visceral fat and metabolic syndrome components including HOMA-IR and triglycerides, as well as hepatic steatosis, fibrosis and inflammation [94–96]. Moreover, visceral fat is positively associated with cardiovascular disease [95] and inflammatory markers [94, 97]. Patients with severe obesity that had an omentectomy (where less than 1% of total fat was removed) in addition to gastric banding had significantly improved glucose handling 2 years after surgery when compared to gastric banding control patients, without significant differences in weight [98]. However, it is important to note that while not statistically significant, the omentectomy group lost more weight and had significantly lower BMI, potentially confounding these data. Metabolic improvement from omental removal has also been observed in lean dogs where omentectomy resulted in enhanced glucose uptake when compared to sham-operated dogs without significant alterations in visceral or total adiposity [99]. Similarly, metabolic improvements are observed when visceral fat is removed from mice, but not from the removal of other AT depots [100]. Conversely, subcutaneous AT is generally positively associated with metabolic health. Findings across several studies indicate that subcutaneous AT is negatively correlated with circulating triglycerides, insulin and glucose in humans [93, 101]. This is supported by evidence in obese rodents showing that transplantation of subcutaneous depots from the same mouse or a donor mouse into the visceral cavity improves metabolic profiles without altering total fat mass; however, this was not true of visceral depot transplants [102, 103]. Collectively, these data suggest that the location of AT may be a stronger predictor of metabolic health than total fat mass is, and that large amounts of AT in the visceral cavity are detrimental.

One major hypothesis as to why visceral fat has such detrimental effects on metabolic health is its proximity to the portal vein, such that anything released from visceral depots, including fatty acids and inflammatory molecules, have direct access to the liver. This idea is referred to as 'the portal theory' (see review [104]). In contrast, subcutaneous fat depots drain into the vena cava and enter the systemic circulation. In support of this theory, one study showed that donor epididymal AT depot transplants into the mesenteric cavity of recipient mice (i.e. portal drainage) resulted in significant glucose intolerance, increased IL-6 expression and macrophage infiltration [105]. On the contrary, when a depot of equal size from the same donor was transplanted to the visceral side of the peritoneum of another littermate (i.e. caval drainage) modest improvements in glucose tolerance were observed. Interestingly, ablation of IL-6 prevented glucose disturbances and reduced other inflammatory markers in the portal-drained group, indicating that inflammatory mediators released from the visceral AT may play a significant role in the associated pathology. Another study in obese rats showed that mesenteric visceral fat, which drains into the portal vein and most closely resembles human visceral AT, played a greater role in insulin resistance when compared to perirenal and epididymal fat depots (typically considered to be visceral depots in rodents, although they do not drain portally) [106]. These data further support AT location as a major driver of metabolic symptoms associated with AT dysfunction.

Though the data described in support of the portal theory are sound and rigorous, there is evidence that glucose disposal rate is negatively associated with the amount of subcutaneous fat, suggesting that subcutaneous depots can also contribute to poor glucose metabolism [107, 108]. Intrinsic depot-specific properties, such as inflammatory profiles and lipolytic rates are difficult to separate from the potential impacts of anatomical position (such as portal versus caval drainage), and more research in this area is warranted to determine the relative contributions of these factors to metabolic health. Also, the characteristics of AT that are primarily responsible for improvements in metabolic health are still largely unknown. Hopefully, future research and methods will identify more specific drivers of metabolic health and disease. Nonetheless, we can conclude that both the metabolic profile and the location of fat tissue can substantially contribute to metabolic disease.

Finally, in instances where there is insufficient AT mass or elevated AT lipolysis (discussed in further detail below), lipids can be stored ectopically across several tissues resulting in metabolic dysfunction. This phenomenon is commonly observed in lipoatrophy, a condition in which there is very little AT present, thereby preventing proper lipid storage. As a consequence, individuals with lipoatrophy have elevated circulating lipids, as well as ectopic fat storage in the liver and muscle [109, 110], conditions that are commonly associated with impaired glucose homeostasis [111, 112]. Indeed, ectopic lipid accumulation in cardiac and skeletal muscle can result in tissue-specific and systemic insulin resistance [113–117]. As a whole, these findings underscore the importance of

proper lipid storage within the AT and away from internal organs or skeletal muscles.

58.5 Adipose Tissue Lipolysis and Insulin Resistance

Insulin resistance and the progression to type 2 diabetes are among the most common metabolic syndrome co-morbidities associated with obesity [83, 118]. AT contributions to systemic insulin resistance have been previously addressed and are discussed throughout this review, but insulin resistance within the AT should also be considered when evaluating the role of AT in metabolic diseases. Although skeletal muscle is responsible for the majority of insulin-stimulated glucose uptake [119], proper insulin signalling in AT is also important for systemic regulation of blood glucose as revealed by a variety of different mouse models. For example, adipocyte-specific ablation of GLUT4, the primary glucose transporter responsible for insulin-stimulated glucose uptake in AT and muscle, impairs insulin signalling in liver and muscle, and induces systemic insulin resistance and glucose intolerance in mice [120]. Likewise, adipocyte-specific insulin receptor knockouts have similar basal glucose uptake, but significantly reduced insulin-stimulated glucose uptake in adipocytes when compared to controls [121]. Notably, these mice have improvements in systemic glucose tolerance and this discrepancy may be a result of an upregulation in other signalling pathways to combat the loss of adipocyte insulin signalling from congenital gene ablation. For instance, one study investigated the effects of insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) on outcomes of metabolic disease in chow-fed mice by generating adipocyte-specific inducible knockout (KO) models of one (IRKO or IGF-1RKO) or both of these receptors (double KO; DKO [122]). Despite all KO groups having similar or reduced fat mass when compared to controls (depot dependent), the IRKO and DKO mice displayed systemic insulin resistance and hepatic steatosis when compared to the control and IGF-1RKO groups, with the combined deletion of these receptors resulting in the greatest disturbances in glucose handling. These findings suggest that a compensatory mechanism may be activated in other insulin responsive tissues, potentially including non-insulin dependent signalling pathways, to combat systemic glucose intolerance when there are defects in insulin receptor signalling in the AT from birth. Nevertheless, these data indicate that proper insulin signalling within the AT is key for systemic health.

One way in which AT insulin sensitivity impacts systemic health is through regulation of AT lipolysis (the breakdown of triglycerides into free fatty acids and glycerol). Lipolysis is induced by adrenergic stimulation to mobilize energy stores in conditions such as fasting, exercise and stress. In the fed state, insulin inhibits lipolysis and promotes lipid storage. Disruption of insulin signalling in AT, therefore, can result in elevated basal lipolysis (reviewed in [123]). The chronic low-grade inflammation associated with obesity also contributes to excessive release of lipids by adipocytes, as the inflammatory cytokine TNFα can also induce

lipolysis in a manner independent of insulin signalling (reviewed in [124] and [57]). Indeed, obesity and insulin resistance are known to be associated with high basal lipolysis rates (Fig. 58.1). The resulting increase in circulating fatty acid levels promotes further metabolic dysfunction through ectopic lipid accumulation, particularly in liver and muscle [123]. The vicious cycle of insulin resistance and elevated basal lipolysis in adipocytes is represented in Fig. 58.2. Type 2 diabetes and hepatic lipid accumulation are often observed in conditions associated with elevated basal lipolysis, including Cushing's syndrome [125, 126], as well as in conditions of lipoatrophy where there is an excess of circulating lipids [111]. Although AT may not be directly responsible for the majority of whole-body glucose uptake, it is clear that impaired glucose uptake and lipid storage in AT affect other insulin-responsive organs and thus modulate overall systemic health.

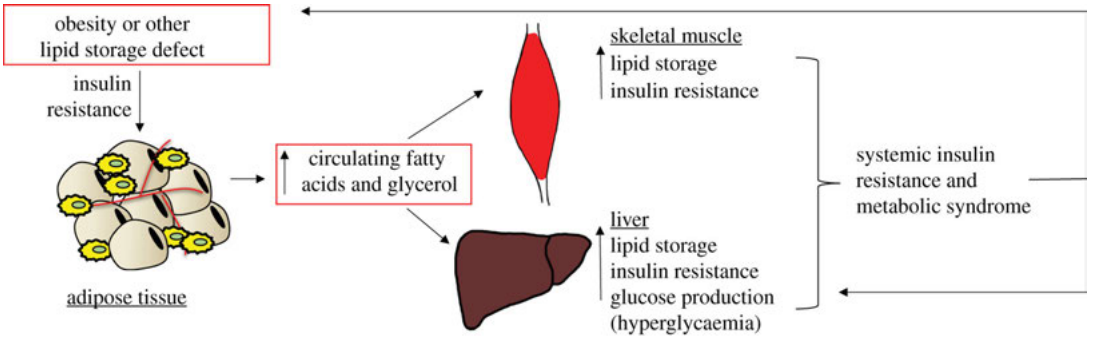

Figure 58.2 Contribution of adipose tissue dysfunction in the perpetuation of metabolic disease. Disturbances in lipid storage, such as in obesity or lipodystrophy, will interfere with proper adipocyte function and can contribute to insulin resistance. Insulin resistance within the adipose tissue will disrupt normal adipocyte signalling and metabolism resulting in elevated lipolysis. Chronically elevated circulating lipids can lead to ectopic lipid storage and insulin resistance in other tissues, including skeletal muscle and liver. Insulin resistance in the liver in particular is problematic as insulin signalling tightly regulates hepatic glucose production. All of these events can have significant consequences on metabolic health, ultimately resulting in a vicious cycle that perpetuates systemic metabolic disorder.

58.6 Endocrine Functions within Adipose Tissue

In addition to being highly insulin-responsive, AT also secretes several molecules involved in glucose regulation and metabolic health (Fig. 58.1). These molecules, collectively known as adipokines, can be anti- or proinflammatory. Adipokines can act as endocrine regulators, released into the circulation and affecting several other tissues and organs, but can also regulate local signalling in a paracrine or autocrine manner. Several adipokines have now been discovered (reviewed in [127–129]), but we will focus here on the three adipokines that are produced in mouse adipocytes: leptin, resistin and adiponectin. In 1994, leptin was the first adipocyte-derived endocrine hormone to be discovered.

Leptin is released from adipocytes proportionally to AT mass and is acutely regulated by fasting [130–132]. In a normal physiological setting, high levels of leptin signal to the brain to cease food intake and, therefore, is known as an anorexigenic hormone [133–136]. An absence of leptin signalling due to genetic mutations in leptin or the leptin receptor leads to severe obesity from hyperphagia in both mice and humans [137], and restoration of signalling will reverse these effects [138, 139]. Leptin's anorectic effects, and its ability to rescue obesity in deficient states initially fuelled enthusiasm that leptin would effectively combat obesity. However, leptin is positively correlated with adiposity in humans [140, 141], and leptin resistance is a common occurrence in obese states [141, 142]. Therefore, leptin treatment in individuals with obesity who exhibit adequate or even elevated leptin levels has not been as beneficial as once hoped. On the contrary, recent findings from two mouse models of reduced leptin expression exposed to high-fat diet suggest that lower levels of leptin during the progression of obesity are protective against weight gain as well as the associated metabolic dysfunction [143]. In fact, a recent review features several studies that support the notion that a reduction in leptin signalling in the context of obesity is associated with weight loss and metabolic improvements [144]. Moreover, leptin has been recognized as a proinflammatory adipokine, so not only is it not beneficial in enhancing weight loss in the general population, it can actually be detrimental to metabolic health when chronically elevated [145]. Collectively these data suggest that low, but sufficient leptin may be beneficial for maintaining metabolic health. Leptin has also been shown to regulate endogenous cortisol production, thereby indirectly modifying glucose homeostasis [146]. These data highlight the importance of leptin production and signalling in the regulation of food intake and body weight.

Resistin is a proinflammatory adipokine, so named for its ability to promote insulin resistance. It was discovered in 2001 in an effort to identify genes suppressed by the PPARγ agonist and antidiabetic drug, rosiglitazone [147]. Interestingly, this endocrine hormone was also identified by another laboratory as an inhibitor of adipogenesis, and named 'adipocyte-specific secretory factor (ADSF)'. Not surprisingly, given its relationship with insulin resistance, resistin is elevated in obesity in mouse and man [147, 148]. Loss of resistin through gene ablation or inactivation improves glucose metabolism in obese mouse models [149, 150]. Evidence indicates that resistin enhances the protein levels and activity of SOCS3, which is required for resistin's ability to reduce insulin signalling in adipocytes [151]. Though most of the data surrounding resistin are negative in terms of how it affects metabolic health, there is also evidence that resistin is important in the regulation of fasting blood glucose [152]. Therefore, it is likely that resistin is necessary for glycemic control, further illustrating the importance of adipocyte endocrine function in sustaining metabolic health. It bears mention that while resistin is primarily secreted from adipocytes in rodents, macrophages are the predominant source of resistin in humans. Nevertheless, resistin's function remains the same across species [153, 154].

Adiponectin, an endocrine hormone released by adipocytes, is known to have anti-inflammatory effects and can enhance insulin sensitivity in several tissues, most notably the skeletal muscle and liver. Adiponectin acts via two G protein-coupled receptors called Adipor1 and Adipor2 and highly expressed in muscle, liver and heart [155, 156]. In contrast to leptin, adiponectin circulating levels are lower in obesity and type 2 diabetes [157]. Adiponectin exerts its anti-diabetic effects mainly through suppression of hepatic glucose production [158–160], but also enhances glucose uptake in skeletal muscle *in vitro* [161, 162]. Administration of adiponectin significantly lowers blood glucose in diabetic mice without affecting insulin levels [158], and has not been shown to induce hypoglycemia, an added benefit in the treatment of diabetes. Adiponectin can also act in an autocrine manner, as underscored by the fact that it was first discovered in an effort to identify genes involved in adipogenesis [163]. It has now been shown that adiponectin can increase insulin-independent and insulin-stimulated glucose uptake within primary rat adipocytes [164] and regulate lipid accumulation and glucose uptake within the adipocyte [165]. Adiponectin signalling is also important in cardiac muscle, as low hormone levels are associated with coronary artery disease [166]. Furthermore, adiponectin is reported to enhance multiple signalling events including antioxidant, vasodilation and anti-inflammatory activities thought to promote cardiomyocyte health [167, 168]. However, it should be noted that there is some controversy regarding adiponectin and cardiac health, as high adiponectin levels have been linked to cardiac dysfunction [169]. Although still not widely recognized, AT is a *bona fide* endocrine organ, releasing hormones and participating in interorgan communication to regulate glucose homeostasis and systemic health.

58.7 Emerging Approaches to Combat Adipose Tissue-Derived Metabolic Dysfunction

The studies described in this review highlight the substantial complexities associated with AT in health and disease. As described, alterations in any adipocyte function can be detrimental to overall health. However, as our knowledge of adipocyte biology has expanded, a variety of interventions have emerged as potentially viable therapeutic strategies to ameliorate these metabolic disturbances. Listed below are a few strategies that have recently been investigated to combat adipocyte-mediated contributions to systemic metabolic disease states.

58.7.1 Exercise

Exercise is known to be extremely beneficial for health, and it has been shown to improve glucose homeostasis [170, 171]; however, AT-specific effects of exercise have not been studied until recently. There is now evidence that exercise may drive improvements in inflammatory profiles and insulin signalling in AT.

Specifically, in a rat model of HFD-induced obesity, aerobic-interval exercise training significantly improved macrophage and inflammatory profiles, as well as capillary density in AT when compared with controls [172]. Moreover, transplantation of WAT from exercise-trained mice into sedentary mice significantly improved systemic glucose tolerance and insulin sensitivity in chow-fed and HFD-fed animals when compared to sham controls or transplantation of sedentary tissue from donor mice given the same diet [53]. Lastly, exercise-trained mice displayed significant elevations in the expression of genes involved in browning in their WAT [173], potentially enhancing energy expenditure and improving overall metabolism. These data suggest that exercise has direct effects on adipocytes that could mitigate the AT dysfunction associated with systemic metabolic perturbations.

58.7.2 microRNAs

microRNAs (miRNAs) are small non-coding RNAs that generally function as inhibitors of genes by binding to their target mRNA transcripts, thereby preventing gene translation and protein expression. These molecules were discovered in 1990, but AT has only recently been identified as a major source of circulating miRNAs [174]. The importance of miRNA expression and activity within the AT is a fairly new topic and still being explored; however, recent evidence indicates that they are crucial for maintenance of adipocyte function. An adipocyte-specific gene knockout of dicer (the enzyme involved in processing miRNAs) results in significant reductions in all WAT depots and severe insulin resistance [175]. Interestingly, with evidence that circulating miRNAs are altered in individuals with obesity and type 2 diabetes, miRNAs are now being considered as potential biomarkers of metabolic health in humans, and are being investigated as potential therapeutics in the treatment of metabolic disease (reviewed here [176]), clearly illustrating miRNAs as promising targets in the regulation of metabolic syndrome.

58.7.3 Exosomes

Exosomes are a particular type of extracellular vesicles that can transport a wide range of materials, including proteins, lipids, metabolites and different species of RNA. In recent years, exosomes have been identified as mediators of disease pathology and as potential therapeutics (reviewed in [177]). Adipose-derived exosomes are currently the subject of intense study, as they are now known to have a critical role in interorgan communication, and to modulate whole-body metabolism (reviewed in [178]). Recent evidence suggests that AT exosomes are significant transporters of circulating miRNAs [174]. A study in diet-induced obese mice showed that intraperitoneal injections of exosomes from isolated adipose-derived stem cells originating from the epididymal WAT of lean mice promoted a shift in macrophage polarization from M1 to M2 and resulted in

significant reductions in markers of inflammation within the circulation [179]. Additionally, the administration of exosomes resulted in significant improvements in glucose tolerance, as well as significant reductions in hepatic lipid accumulation. Exosomes are also implicated in paracrine signalling within the AT, providing transport from different cell types and allowing for intra-organ communication. One group reported the surprising finding that caveolin-1 (Cav-1) protein was detected in adipocytes where the *Cav1* gene had been successfully deleted [180]. It was determined that Cav-1 was being transported via exosome from nearby endothelial cells and taken up by the adipocytes. Furthermore, data from this paper also suggest that exosome production in response to stimuli such as the fasting/feeding transition is blunted in obesity. These studies support the use of exosomes as a treatment for metabolic disease. In fact, exosomes are currently being investigated for their ability to package and deliver microRNAs as therapeutics [176].

58.8 Concluding Remarks

Novel methodologies and technical advances continue to drive the elucidation of complex mechanisms involved in the contributions of AT to health and disease. We have summarized the principal features of AT function and dysfunction in Fig. 58.1. In addition to the many unresolved questions we have discussed in this review, it should be noted that mechanistic data from animal models are largely derived from studies on male rodents, and that sex differences in metabolism and AT function are known to exist in rodents as well as humans [181, 182]. Therefore, special emphasis should be placed on the study of sex differences in the context of AT in health and disease in the future. In conclusion, while much remains to be learned about how AT contributes to metabolic disease, there is no question that AT is central to systemic health and that disruption of any of its functions can have substantial impacts.

Disclosures and Conflict of Interest

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Chapter 59

Viral Pathogenesis of SARS-CoV-2 Infection and Male Reproductive Health

Shubhadeep Roychoudhury, PhD,[a] Anandan Das, MSc,[a] Niraj Kumar Jha, PhD,[b] Kavindra Kumar Kesari, PhD,[c] Shatabhisha Roychoudhury, PhD,[d,e] Saurabh Kumar Jha, PhD,[b] Raghavender Kosgi, MBBS, MS, MCh,[f] Arun Paul Choudhury, MBBS, MD, PhD,[g] Norbert Lukac, PhD,[h] Nithar Ranjan Madhu, PhD,[i] Dhruv Kumar, PhD,[j] and Petr Slama, PhD[k]

[a]*Department of Life Science and Bioinformatics, Assam University, Silchar, India*
[b]*Department of Biotechnology, School of Engineering and Technology, Sharda University, Greater Noida, UP, India*
[c]*Department of Applied Physics, School of Science, Aalto University, Espoo, Finland*
[d]*Department of Microbiology, R. G. Kar Medical College and Hospital, Kolkata, India*
[e]*Health Centre, Assam University, Silchar, India*
[f]*Department of Urology and Andrology, AIG Hospitals, Gachibowli, Hyderabad, India*
[g]*Department of Obstetrics and Gynecology, Silchar Medical College and Hospital, Silchar, India*
[h]*Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic*
[i]*Department of Zoology, Acharya Prafulla Chandra College, New Barrackpore, West Bengal, India*
[j]*Amity Institute of Molecular Medicine and Stem Cell Research, Amity University, Noida, India*
[k]*Department of Animal Morphology, Physiology and Genetics, Faculty of AgriSciences, Mendel University in Brno, Brno, Czech Republic*

shubhadeep1@gmail.com

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protein-1alpha (MIP-1α), Middle East Respiratory Syndrome Coronavirus (MERS-CoV), monocyte-chemoattractant protein (MCP), monocyte-chemoattractant protein-1 (MCP1), natural killer (NK), N-terminal domain (NTD), oxidative stress (OS), platelet-derived growth factor (PDGF), programmed cell death protein-1 (PD1), reactive oxygen species (ROS), secondary costimulatory immune checkpoint molecule (OX40), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), severe acute respiratory syndrome-related coronavirus (SARS-CoV), spermatozoa, superoxide radical (O_2•), T-cell immunoglobulin domain and mucin domain-3 (TIM3), testicular damage, transmembrane protease/serine subfamily member 2 (TMPRSS2), tumour necrosis factor (TNF), tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF)

59.1 Introduction

In early December 2019, several pneumonia cases of unknown aetiology were reported in Wuhan, China. Genome sequencing studies confirmed these to be the result of a novel viral infection named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19) [1]. As of 22 December 2020, the viral outbreak has spread globally across as many as 222 countries, thereby infecting more than 76 million people and causing over 1.6 million deaths [2]. SARS-CoV-2 mainly enters the cell by binding to angiotensin-converting enzyme 2 (ACE2), a receptor found predominantly on the surface of epithelial cells in the lungs [3]. This is believed to be the main reason behind the vulnerability of the respiratory system to SARS-CoV-2 infection. However, ACE2 is also expressed in various other tissues of the body, and as a result, there is a high probability of SARS-CoV-2 infection of other organ systems, including the digestive, urogenital, circulatory, central nervous and reproductive systems [4].

Due to the high expression of the ACE2 receptor in testicular tissue in both somatic and germ cells, such as seminiferous duct cells, Leydig cells, Sertoli cells and spermatogonia, there is increasing concern about the possible impact of SARS-CoV-2 infection on male fertility [5, 6]. Moreover, ACE2-mediated SARS-CoV-2 invasion may lead to viral infection, which may also cause damage to testicular tissues [7]. This indicates that the testis is a potential target of SARS-CoV-2 invasion and that damage to testicular cells may severely hamper the process of spermatogenesis. A recent study reported significant impairment of sperm quality in a COVID-19 patient [8]. Moreover, young men, if infected, may be at a greater risk of testicular damage due to higher expression of the ACE2 receptor in comparison to patients more than 60 years of age, who show comparatively lower levels of expression and are hence less prone to such testicular damage [9]. Single-cell RNA sequencing data of adult human testes indicated a higher positive rate of ACE2 in infertile men. The authors further suggested that such men with reproductive disorders may be susceptible to

SARS-CoV-2 infection through a pathway activated by ACE2 [9]. However, infection in testicular organs does not necessarily mean direct damage to sperm cells. In a recent study, a semen sample of only 15.8% of COVID-19 patients under surveillance was found to be positive for SARS-CoV-2 particles, even in recovering patients [10]. By contrast, *in situ* hybridization studies could not confirm the presence of any viral genetic material in testicular tissues, and the damage was attributed to the infiltration of inflammatory molecules in the testicular tissue during the immunological response of the virus [11]. Recently, another group of researchers have also reported the absence of SARS-CoV-2 in the semen and testis of men in the acute infection and recovery phases [12]. This review discusses the origin of SARS-CoV-2 and its mechanism of invasion along with potential infection of the reproductive system of the affected male.

59.2 SARS-CoV-2: History, Origin and Transmission

Coronavirus was first observed during the mid-1930s [13], and the earliest human infection of coronavirus was documented in 1960 as a cold [14]. Much later, in 2002, a new species of coronavirus, originating from bats and transmitted to humans through palm civet cats as intermediate hosts, occurred and was named SARS-CoV (Fig. 59.1). In 2012, another coronavirus of bat origin, namely, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged, with camel as an intermediate host [19]. Very recently, SARS-CoV-2 has caused the largest pandemic in recent human history and the first documented coronavirus pandemic of such a large magnitude [20].

Coronaviruses are divided into four genera, comprising α-, β-, γ- and δ-coronaviruses, of which only α- and β-coronaviruses are capable of infecting animals. SARS-CoV-2 is a β-coronavirus belonging to the family Coronaviridiae and the subfamily Orthocoronavirinae. It is an enveloped and non-segmented positive-sense, single-stranded RNA virus [21]. Human coronaviruses generally have zoonotic origins, and the genome sequence of SARS-CoV-2 shares 96.2% identity with the bat coronavirus RaTG13. This suggests that SARS-CoV-2 originated in bats and was transmitted to humans through unknown intermediate hosts (Fig. 59.1) in the Wuhan seafood market in China in December 2019 [22, 23]. Metagenomic sequencing has revealed that pangolins might have acted as intermediate hosts between bats and humans because of the similarity of the pangolin coronavirus to SARS-CoV-2 [24, 25]. However, no intermediate host sample could be obtained in an initial cluster of infection from the Wuhan seafood market, and the earliest symptomatic patients did not have any exposure to the wet market of Wuhan [26]. This left the matter of intermediate host of the virus unresolved, warranting more confirmatory evidence to settle the argument [20].

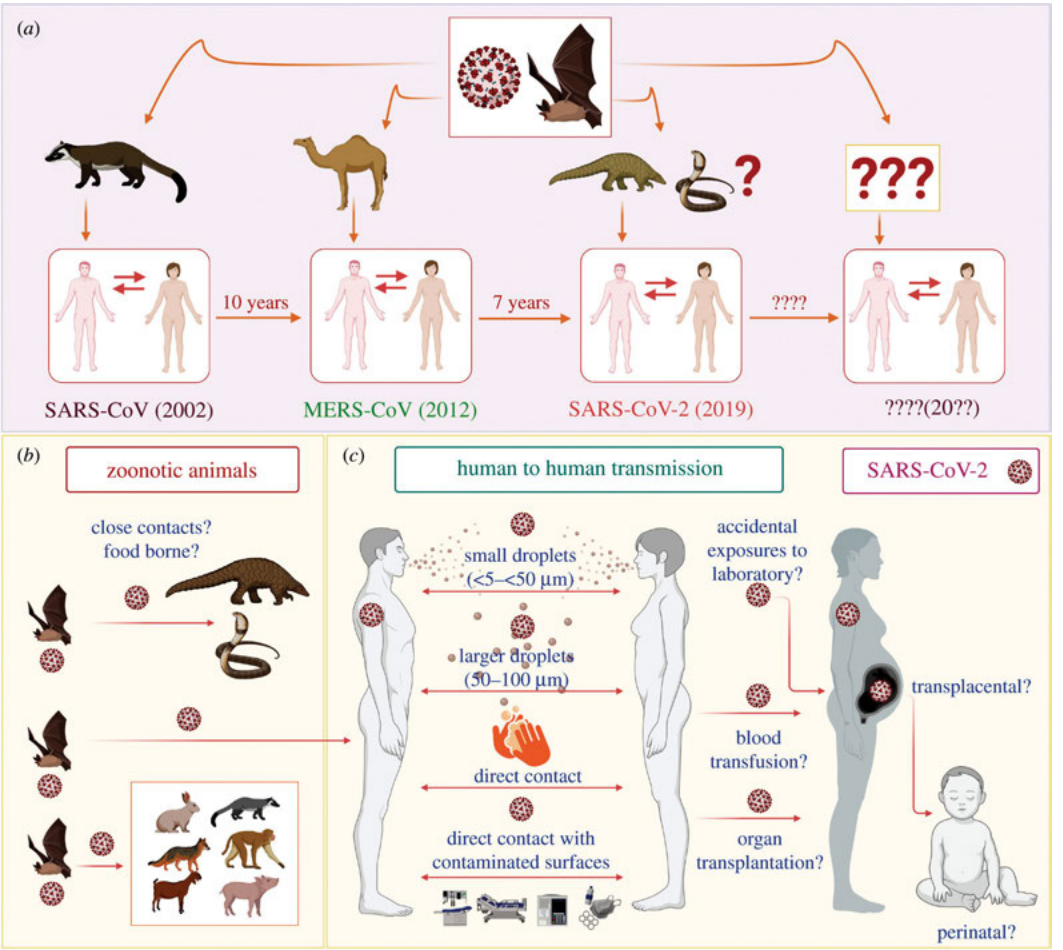

Figure 59.1 Origin of coronavirus and potential routes of transmission of SARS-CoV-2. (*a*) The origin of coronavirus. Like SARS and MERS, coronavirus is an emerging virus that has crossed the species barrier from wild animals to humans. The origin of SARS-CoV-2 is also suspected to be from an intermediate animal host, and the likelihood of crossing the species barrier for a fourth time cannot be ruled out. The current COVID-19 outbreak caused by SARS-CoV-2 has already been predicted and will also be contained sooner or later, similar to earlier outbreaks [15]. However, the real issue is how we plan to counter the next zoonotic CoV pandemic that is likely to occur in the next 5 to 10 years, if not sooner. (*b*,*c*) The potential routes of transmission of SARS-CoV-2. SARS-CoV-2 is alleged to have zoonotic (animal-to-human) origin with further human-to-human transmission [16], and the likelihood of food-borne transmission should be ruled out pending further investigation [17]. In addition, it can potentially be transmitted through direct contact, as in other respiratory viruses, such as by shaking contaminated hands or exposure to contaminated surfaces (fomite transmission). Nevertheless, other possible routes of SARS-CoV-2 transmission, such as accidental exposure to the laboratory, blood transfusion, organ transplantation [18], and transplacental and perinatal routes, need to be adduced more concretely. *Abbreviations*: SARS-CoV, severe acute respiratory syndrome-related coronavirus; MERS-CoV, Middle East respiratory syndrome-related coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

59.3 Possible Mechanism of SARS-CoV-2 Invasion into Host Cells and Immune Pattern of Infection

According to existing clinical data, COVID-19 is not limited to respiratory ailments, but it may also give rise to complications such as acute renal injury and renal necrosis in some patients [27, 28]. During the earlier coronavirus outbreak in 2002–2003, SARS-CoV-infected men presented with orchitis as a major complication, which led to reproductive dysfunctions in such men [11]. SARS-CoV and SARS-CoV-2 are similar in that both viruses invade host cells through the ACE2 surface receptor present in the host cell, and it is worth mentioning that ACE2 exists not only in respiratory tissues but also in reproductive tissues, including spermatozoa, seminiferous tubules, Leydig cells and Sertoli cells [29]. This evidence fuelled the possibility of SARS-CoV-2 infection in the male reproductive tract and potential damage to male fertility [30].The expression of ACE2 was also reported in the proximal regions of the heart, kidney, lung, ileum and bladder [31]. Inside the lung, epithelial cells have a higher expression of ACE2. The binding of SARS-CoV-2 with the ACE2 receptor (ACE2-R) allows its entry into cells and completes its replication [6]. This may, in turn, activate direct viral invasion and cause tubular epithelial and podocyte damage, resulting in acute cardiac and lung injury. This is because of the potential SARS-CoV-2-mediated downregulation of ACE2 expression, which may further contribute to an increase in angiotensin 2 (Ang-II)-induced lung injury [6].

In coronaviruses, the entry process is mediated by surface-located spike (S) glycoproteins, which are embedded in the viral envelope [32]. The S protein of SARS-CoV-2 resembles the typical characteristics of the coronavirus S protein, which is divided into two subunits, S1 and S2, responsible for receptor recognition and membrane fusion, respectively. The S1 subunit can be further subdivided into an N-terminal domain (NTD) and a C-terminal domain (CTD). Immunostaining and flow cytometry assays identified the S1 CTD as the key region in SARS-CoV-2 that interacts with the ACE2 receptor. SARS-CoV also uses the S1 CTD as the receptor-binding ligand, and the overall mode of binding is similar to that of the SARS-CoV-2 receptor-binding domain. However, the SARS-CoV-2 CTD has higher atomic interactions with the receptor than the SARS-CoV CTD, which indicates that the SARS-CoV-2 CTD has a higher affinity for the ACE2 receptor [33]. This evidence is important to establish the fact that SARS-CoV-2 is much more infectious than SARS-CoV.

Recent studies have indicated that a particular transmembrane serine protease, designated transmembrane protease/serine subfamily member 2 (TMPRSS2), has a major role in viral entry. ACE2 and TMPRSS2 interact in cellular exocytic pathways and at cell surfaces, resulting in the cleavage of the ACE2 receptor. Proteolysis of the ACE2 receptor by deglycosylation enhances the capability of coronaviruses to enter the cell [34]. SARS-CoV-2 uses the serine protease TMPRSS2

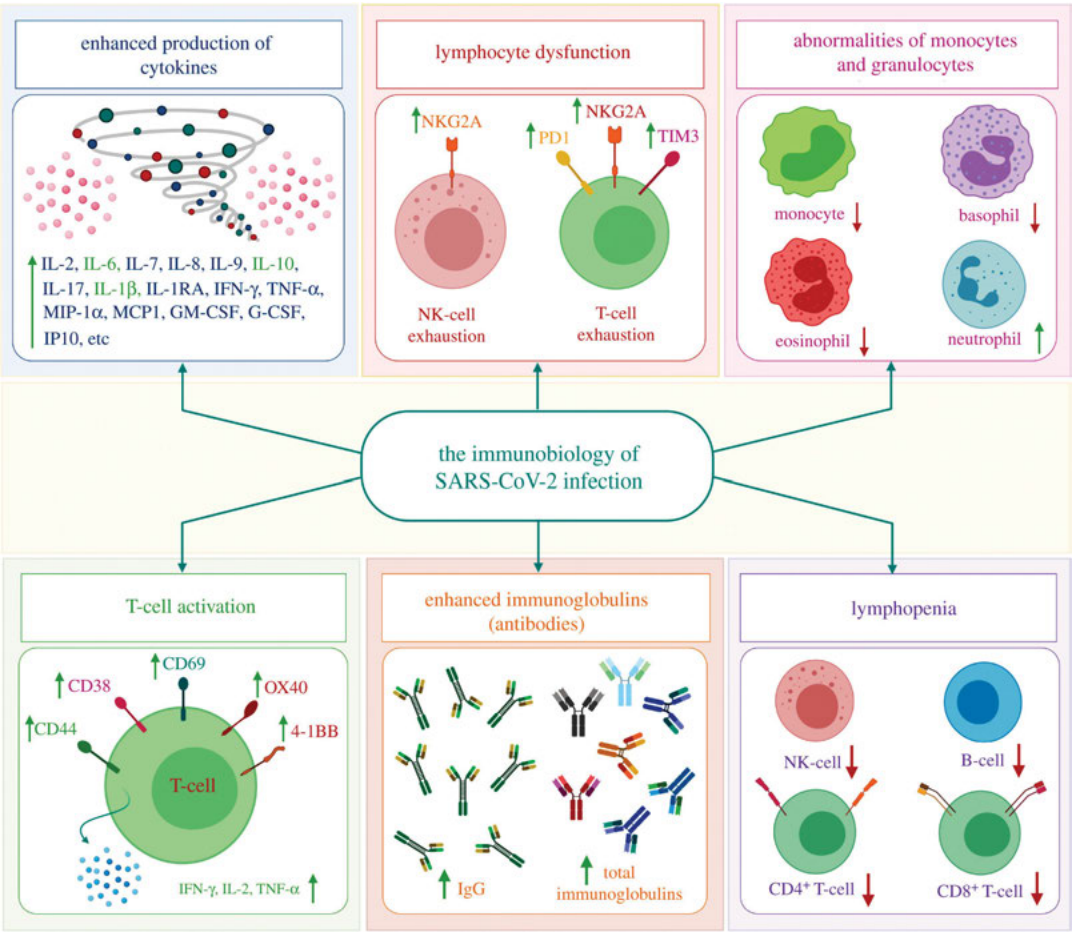

Figure 59.2 The immunopathology of SARS-CoV-2 infection. SARS-CoV-2 uses the ACE2 receptor to gain entry into the cell (airway epithelial cells), leading to an increase in pro-inflammatory cytokines and the development of cytokine storms, which lead to infection and augment COVID-19 severity. In addition, SARS-CoV-2 infection includes abnormalities of granulocytes and monocytes, lymphopenia, lymphocyte activation and dysfunction, enhanced production of cytokines and increased antibodies [38]. *Abbreviations*: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; IL, interleukin; TNF-α, tumour necrosis factor alpha; IFN-γ, interferon gamma; MIP-1*α*, macrophage inflammatory protein-1alpha; MCP1, monocyte chemoattractant protein-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IP10, interferon gamma-induced protein 10; NKG2A, killer cell lectin-like receptor subfamily C member 1; PD1, programmed cell death protein 1; TIM3, T-cell immunoglobulin and mucin domain-3; CD, cluster of differentiation; OX40, secondary costimulatory immune checkpoint molecule; 4–1BB, a member of the tumour necrosis factor receptor superfamily T-cell costimulatory receptor; NK, natural killer.

for S protein priming in cells, which significantly increases the cell susceptibility of the virus [35]. ACE2 is highly expressed in spermatogonia, Leydig cells and Sertoli cells, whereas its expression is low in spermatocytes, spermatids and

other somatic cells. In a recent study, SARS-CoV-2 RNA was measured in throat and semen samples of infected men. The organ distribution of ACE2 mRNA and protein in human tissue in The Human Protein Atlas Portal revealed relatively high levels of ACE2 protein and RNA expression in the testis [36]. However, the expression of TMPRSS2 is concentrated in spermatogonia and spermatids, with relatively low levels of expression in other cell types. Thus, SARS-CoV-2 may pose a real threat to male fertility due to the expression of both ACE2 and TMPRSS2 in testicular cells [37].

The immune patterns of SARS-CoV-2 infection include abnormalities of granulocytes and monocytes, lymphopenia, lymphocyte activation and dysfunction, enhanced production of cytokines and increased antibodies (Fig. 59.2). Lymphopenia is a key feature of COVID-19 patients, especially in severe cases. CD44, CD69 and CD38 are highly expressed on $CD4^+$ and $CD8^+$ T cells of patients, and virus-specific T cells from severe cases exhibit a central memory phenotype with high levels of IL-2, TNF-α and IFN-γ. However, lymphocytes show an exhaustion phenotype with killer cell lectin-like receptor subfamily C member 1 (NKG2A), programmed cell death protein-1 (PD1), and T-cell immunoglobulin domain and mucin domain-3 (TIM3) upregulation (Fig. 59.2). The percentages of eosinophils, basophils, and monocytes are reduced in severe patients, while neutrophil levels are significantly elevated. Increased cytokine production, especially of IL-6, IL-1β and IL-10, is another key characteristic of SARS-CoV-2 infection and severe COVID-19. IgG levels are also increased, and there is a higher titre of total antibodies [38].

59.4 Effect on the Male Reproductive System

Several important findings have already been reported regarding the pathology of COVID-19 on the respiratory system as well as other organ systems. Data on the histopathological changes in various other organ systems due to SARS-CoV-2 infection are also emerging [39]. Figure 59.3 summarizes the potential impact of SARS-CoV-2 infection on the reproductive system in males. As mentioned earlier, the presence of the ACE2 receptor on germ cells, Leydig cells and Sertoli cells recently indicated the testis as a potential target of SARS-CoV-2 infection [29]. The serine protease receptor TMPRSS2 is also present in male reproductive tissues and plays a crucial role in mediating the entry of SARS-CoV-2 [42]. By contrast, another recent study reported a normal testicular appearance in COVID-19 patients [43].

Pathological studies conducted on deceased COVID-19 patients aged 42–87 years confirmed that Sertoli cells are mostly affected, showing swelling, vacuolation, cytoplasmic rarefaction and detachment from the tubular basement membrane. A wide range of changes can be observed in the seminiferous tubules along with a reduction in their number. One major change has been a loss and sloughing of the intratubular cell mass into the lumen. The extent of severity varies from patient to patient, with mild tubular injury in the majority of cases.

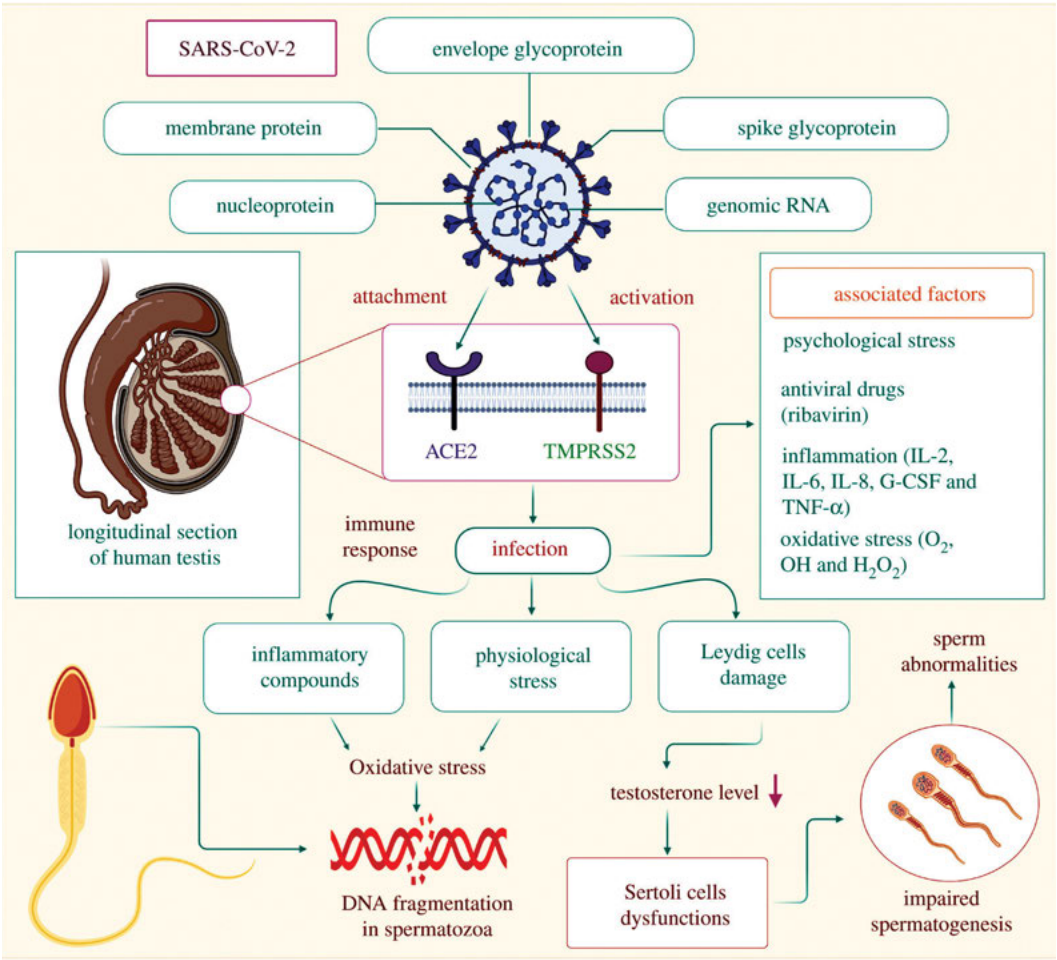

Figure 59.3 Possible mechanism of SARS-CoV-2 invasion in the reproductive system of infected men and the potential health impacts associated. SARS-CoV-2 gains entry into the reproductive system through the ACE2 and TMPRSS2 receptors present on testicular tissues. The immune response triggered by viral entry produces various inflammatory substances, such as cytokines, which induce OS in testicular cells, which in turn damages the DNA of developing spermatozoa. Various psychological stresses due to SARS-CoV-2 infection may also lead to the production of ROS. SARS-CoV-2 also causes damage to Leydig cells, lowering the production of testosterone, which may ultimately hamper the proper functioning of Sertoli cells. Impaired functioning of Sertoli cells may further disrupt the process of spermatogenesis. However, recent studies have reported low testosterone levels in SARS-CoV-2-infected men with other comorbidities [40, 41]. This suggests that normal testosterone may reveal antiviral immune responses to combat SARS-CoV-2 infection in men. *Abbreviations*: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease/serine subfamily member 2; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; TNF-α, tumour necrosis factor alpha; O_2•, superoxide radical; OH•, hydroxyl radical; H_2O_2, hydrogen peroxide.

The number of Leydig cells in the interstitium is also significantly reduced in SARS-CoV-2-infected patients compared to healthy individuals. Various degrees of alterations in spermatogenesis can be observed with considerable consistency in patients of different age groups [5, 44]. An immunohistochemical study validated the presence of oedema and mild inflammatory infiltrates composed of CD3-positive T-lymphocytes in the interstitium [44]. It is worth mentioning that viral particles can hardly be traced in the testicular tissues of infected patients. Thus, it may be hypothesized that viral membrane proteins might play a role in injury to seminiferous tubules and Leydig cells. There is also a possibility of hyperthermia due to fever, secondary infection, hypoxia and steroids being the key mediators of testicular damage in SARS-CoV-2 patients [44].

Viral membrane proteins of SARS-CoV-2 may reach the testicular interstitium via blood, and Leydig cells may be one of the first target sites of infection. In the case of extreme viraemia, the virus itself may infiltrate testicular tissues. Invasion by either the virus or its proteins in Leydig cells is followed by alterations in steroidogenic pathways, which may cause Leydig cell dysfunction and a decrease in the ratio of serum testosterone and luteinizing hormone (LH) [45]. SARS-CoV-2-mediated induction of cytokines and chemokines in non-reproductive tissues may be transported to Leydig cells and Sertoli cells. It is likely to be followed by the recruitment of peripheral immune cells, including macrophages and virus-specific T-cells, which may cause inflammation and orchitis [46]. Segmental vascularization of the testis may also account for an orchitis-like syndrome [47]. However, orchitis is not a common symptom reported in COVID-19 patients due to the immunosuppressive properties of Sertoli cells and testicular macrophages, which may play a critical role in suppressing inflammation and limiting virus-associated testicular damage to some extent [48]. Sertoli cells elicit immune privilege to germinal cells with the help of the blood-testis barrier, which includes tight junctions between adjacent Sertoli cells [49]. Apart from working as a barrier between maturing germ cells and immune cells, Sertoli cells can also modulate the immune response by expressing several immunoregulatory factors [50]. However, this immunosuppression is believed to be compromised when there is severe infiltration of viral particles in testicular tissues, as in extreme cases of SARS-CoV-2-associated inflammation. The transient adverse effects on blood-testis barrier integrity may subsequently hamper the normal process of spermatogenesis [48]. This is in contrast to SARS-CoV infection, wherein the elicited immune response is stronger. High fever associated with SARS-CoV infection might add up to the cause of developing orchitis followed by testicular dysfunction. In SARS-CoV-infected testes, leucocyte infiltration has been shown to damage the blood–testis barrier along with the subsequent loss of immune-protective properties [11]. Furthermore, the development of pro-inflammatory cytokines and IgG antibodies in the germinal epithelium, basement membrane, interstitium, vascular endothelium and degenerated germ cells may predispose SARS-CoV-infected men to autoimmune orchitis [11].

Some recent studies have reported the presence of SARS-CoV-2 particles in human semen. Although their route of entry into semen is not fully understood,

it may be hypothesized that the virus may reach semen via the impaired blood-testis barrier in the presence of systemic local inflammation. In some patients, viral infiltration into the semen may be manifested by increased viraemia [10]. However, there are contradictory findings, as some other studies failed to detect any viral protein or DNA in the semen of infected COVID-19 patients [8, 51]. Hence, it may be suggested that there is a possibility of transmission of the virus from infected men to their female partners during sexual intercourse, although more evidence is necessary to arrive at a definitive conclusion.

Furthermore, SARS-CoV-2 infection has recently been implicated in the disruption of the normal process of autophagy in the testis [30]. Autophagy is believed to aid in the process of degradation of damaged organelles and needless metabolites from the cell apart from elimination of intracellular pathogenic microorganisms, which is of paramount importance. Autophagy is especially important in the reproductive system of men, as it ensures the smooth conduction of spermatogenesis by helping in the formation of specific components and by preventing the unnecessary accumulation of cytoskeleton in sperm cells [52]. Recent studies have found increased expression of autophagy receptor SQSTM1/p62 in SARS-CoV-2-infected cells, thereby suggesting a decrease in autophagic flux [53]. Apart from the virus itself, viral proteins may either induce or inhibit the autophagy pathway directly to achieve viral survival [30]. This suggests that SARS-CoV-2 may limit the level of autophagy, eventually impairing reproductive function in males. The potential health impacts of SARS-CoV-2 infection on the male reproductive system are summarized in Table 59.1.

Table 59.1 Pathophysiology of SARS-CoV-2 infection on the male reproductive system (the specific receptors present in various tissues as well as the immunological response in the form of cytokines elicited by the virus are also highlighted)

	Features	Refs.
Reproductive tissues showing receptor expression	(i) ACE2: seminiferous tubule, Leydig cells, Sertoli cells, spermatozoa	[42]
	(ii) TMPRSS2: epididymis, prostate gland, seminal vesicles	[52]
Immunological response	Increase in IL-1β, IL1RA, IL-7, IL-8, IL-9, IL-10, basic FGF, GCSF, GMCSF, IFN-γ, IP10, MCP1, MIP1α, MIP1β, PDGF, TNF-α and VEGF	[54]
Effect on reproductive system	***Testis*:** • testicular epithelium damage • seminiferous tubules damage • Leydig cells and Sertoli cells dysfunction • inflammation due to infiltration of pro-inflammatory cytokines • hamper in spermatogenesis leading to decreases in sperm count • increased ROS production leads to OS, which affects semen parameters, such as sperm function and motility; lipid peroxidation; and DNA damage	[42]

Abbreviations: ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease/serine subfamily member 2; IL, interleukin; FGF, fibroblast growth factor; GCSF, granulocyte colony-stimulating factor; GMCSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IP, inflammatory protein; MCP, monocyte-chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; OS, oxidative stress.

59.5 SARS-CoV-2 and Male Fertility

These are still early days for understanding the effect of SARS-CoV-2 on male fertility due to the lack of sufficient short- and long-term studies. However, emerging evidence indicates the possibility of testicular damage due to SARS-CoV-2 infection, which in turn may compromise the fertility potential of such men. Both the reproductive and general well-being of patients infected by SARS-CoV-2 may be at risk, as large proportions of vulnerable men are of reproductive age [55]. Proper hormonal balance is an important prerequisite to male fertility potential as well as outcome. Improper endocrine functioning may compromise reproductive health. SARS-CoV-2 is known to induce inflammatory responses that may disrupt the activity of the hypothalamic–pituitary–testicular (HPT) axis, leading to reduced LH, follicle stimulating hormone (FSH) and testosterone levels [42, 56]. However, there are contradictions to this theory, as lower serum testosterone levels, higher LH levels and lower testosterone to LH ratio in COVID-19 patients have recently been reported in comparison to healthy men [45]. This exposes the missing links in the association of SARS-CoV-2 infection with modulation of sex hormones, which needs prompt attention to clarify our understanding of SARS-CoV-2 infection and fertility in males.

Oxidative stress (OS) is widely regarded as an important aetiology of male infertility [57–60]. OS is induced when the balance between oxidants and reductants (antioxidants) is disrupted due to increased production of reactive oxygen species (ROS) or reduced generation of the latter. Elevated levels of ROS can affect sperm structural and functional integrity, including motility, morphology, count and viability [61]. High OS is also a threat to sperm DNA integrity, as high ROS concentrations have been linked with DNA fragmentation and chromatin packing. Moreover, the capacity to repair sperm DNA damage is also severely compromised during excessive viraemia, which is attributed to the disruption of the nucleoprotein-mediated defence system that the spermatozoa originally had [62]. This may in turn decrease fertilization rates, reduce implantation, impair embryonic development and increase the rate of pregnancy loss [63, 64]. Increased production of ROS is further manifested by the disruption of sperm membrane integration and induction of apoptosis in spermatozoa [65, 66]. SARS-CoV-2 can activate oxidant-sensitive pathways via inflammatory responses, thereby inducing OS [42]. As already discussed, this virus has the potential to cause orchitis, which can also trigger disruption of oxidative balance in the testis. According to a study conducted on adult male Sprague–Dawley rats, OS impairs sperm quality even after one complete cycle of spermatogenesis, which is suggestive of its long-term consequences on fertility. It has also been found that the epididymis is largely affected by OS, in contrast to the testis, and it is in the epididymis, where spermatozoa are rendered more vulnerable to oxidative damage [67]. This may be because the developing spermatozoa are somewhat protected in the testes due to the nutritive effects of Sertoli cells and antioxidant protection through superoxide dismutase [68, 69]. These observations have been supported by previous studies that concluded that spermatozoa collected

from the epididymis of OS-induced rats after 24 h of treatment still had increased DNA oxidation and reduced motility, indicating their long-term effects [70]. From these observations, it may be hypothesized that SARS-CoV-2-induced OS may elicit long-term deleterious effects on male fertility, particularly on developing spermatozoa, but only concrete evidence can settle this argument.

Various side effects conferred by some of the drugs used for the treatment of COVID-19, including ribavirin and glucocorticoids, may serve as a potential aetiology of male infertility [71]. Ribavirin is a broad-spectrum antiviral drug, and as reported in animal experiments, ingestion of this drug resulted in a decrease in testosterone concentration and impairment of spermatogenesis [72]. This drug has also been found to cause sperm abnormalities in rats [73]. As confirmed by clinical studies, ribavirin can cause sperm DNA fragmentation, and when combined with interferon treatment, this antiviral drug can hamper male fertility by decreasing sperm count [74–76]. Glucocorticoids are steroidal drugs that are only used for a short time in COVID-19 patients with progressive deterioration of oxygenation indicators and excessive activation of inflammatory reactions in the body. Small doses of glucocorticoids administered over a short period of time do not have any negative impact on the male reproductive tract, but overdose may expand the interstitial space of the spermatogenic epithelium, followed by destruction of cell connections and the blood-testis barrier, thus making the testicular tissue vulnerable to harmful substances [71].

The rapid global emergence of COVID-19 has created a situation of socio-economic crisis and psychological distress among people across many parts of the world. Modern-day humans are not used to current restriction protocols, and social distancing and isolation regimes often lead to feelings of frustration, stress, anxiety and even depression [77–79]. This forms an important consideration from the perspective of male infertility, as the relationship between stress and infertility has been a topic of serious debate over the years [80]. A prevalence study of psychological symptoms of infertility concluded that 25–60% of infertile individuals report psychiatric symptoms and that their levels of anxiety and depression are significantly higher than those of fertile men [81]. SARS-CoV-2-infected men should be provided with psychological consultation in time to avoid irrational fear and excessive stress, as these may indirectly affect their reproductive health and well-being [77]. Poor fertility potential during psychological stress may be linked with manifestations of lower sperm quality and sexual dysfunctions, which ultimately interfere with the probability of a couple conceiving. Stress and anxiety have been able to influence semen parameters such as lower sperm count and concentration, lower semen volume and higher sperm DNA fragmentation [82, 83]. Poor fertility performance in men with psychological disorders is also due to less sexual activity, hypoactive sexual desire and erectile dysfunction [84]. This evidence suggests that SARS-CoV-2-mediated psychological stress may also play an important role in male infertility.

Viral infection might be associated with androgen secretion, and hence an appropriate treatment regimen should consider the androgen levels of the patients [9]. Management strategies such as cryopreservation and assisted

reproductive technology (ART) may also be considered vital approaches in tackling specific clinical conditions of male infertility. To employ these strategies for COVID-19 patients, extra precautionary measures should be undertaken during the handling of semen to reduce the chances of viral transmission [85]. Some of the measures for the elimination of the risk of cross-contamination and transmission through cryobanking services include testing both partners for SARS-CoV-2 before initiating treatment, use of closed-carrier cryodevices and sanitary cryostorage protocols [86]. Some embryologists have advocated placing all new cryopreserved specimens into a quarantine tank until patients are determined to have negative viral test results at some future time, especially when dealing with donor semen. Furthermore, all gametes and embryos should go through extensive washing to dilute out potential viral contamination to reduce the possibility of contamination with SARS-CoV-2 [86, 87]. The use of high-security straws may also minimize the risks associated with cryopreserving sperm during the pandemic. During the pandemic, a thorough evaluation (especially in the setting of a multidisciplinary team) and molecular confirmation of the absence of SARS-CoV-2 in seminal fluid from asymptomatic cancer patients may assist in ensuring the safety of sperm cryopreservation [88].

59.6 Gender-Based Susceptibility

Epidemiological studies conducted across different parts of the world have reported higher COVID-19 morbidity and mortality rates in men than in women [89–91]. A recent meta-analysis of 3 111 714 reported global cases also confirmed three times higher demand of intensive treatment unit in male patients as compared to that of female [92]. The vulnerability of men to this disease may be explained by analysing the genetic, immunologic and behavioural differences in both males and females [93].

A positive correlation between ACE2 expression and SARS-CoV-2 infection is already well established. Moreover, there are studies quantifying the expression of ACE2 receptors in human cells based on sex. Single-cell RNA sequencing revealed that males have higher expression of ACE2 in the lungs than females [94]. Another report reported higher circulating levels of ACE2 in healthy and diabetic men as well as in renal disease patients in comparison to women [95]. TMPRSS2 is another protein necessary for SARS-CoV-2 invasion, and its expression has been found to be several-fold higher in prostate epithelium than in other tissues, leaving SARS-CoV-2-infected men more vulnerable to the disease [96].

Immunological studies concluded that sex-based differences contribute to variations in susceptibility to infectious disease and response to vaccines [97]. Experiments conducted in animal models suggested that male mice were more susceptible to SARS-CoV than female mice of similar age, and the enhanced susceptibility was attributed to the accumulation of inflammatory monocytes, macrophages and neutrophils resulting in vascular leakage and alveolar oedema. By contrast, the decreased vulnerability of female mice was probably due to the

protective effect of oestrogen receptor signalling [98]. Human studies have also indicated stronger humoural and immune responses against viral infection in females than in males, which holds true in the case of SARS-CoV-2 infection as well [99].

In fact, gender-based differences in behaviour and lifestyle have been considered responsible for the sex-based variation in the pattern of vulnerability to SARS-CoV-2 infection and COVID-19 [84]. Higher smoking and consumption of alcohol among men compared to women may be considered an important factor behind this hypothesis [90]. Recent studies have also reported that women have a more responsible attitude towards the COVID-19 pandemic, which affects their level of compliance with the guidelines issued by the governments and in undertaking preventive measures such as frequent hand washing, using masks and maintaining social distancing protocols, resulting in lower chances of SARS-CoV-2 infection [100].

59.7 Conclusion

Preliminary findings so far suggest the possibility of both direct and indirect infection of SARS-CoV-2 in the reproductive system of males and possible impact on general health and well-being potentially leading to infertility. Evidence indicates a possible long-term effect of the pathogenicity of SARS-CoV-2 infection on testicular tissue, which may further impact reproductive performance. Moreover, the possibility of sexual transmission of SARS-CoV-2 cannot be ruled out.

59.8 Future Perspectives

The presence of SARS-CoV-2 nuclei has been confirmed in the testicular tissue of infected men using RT-qPCR technique, which is indicative of the direct viral invasion on the male reproductive system [101]. However, the evidence is not yet considered to be conclusive enough to definitely determine as to whether there are asymptomatic patients who need to avoid sexual intercourse with their female partners in order to prevent possible viral transmission [102]. SARS-CoV-2-infected men should be provided with psychological consultation in time to avoid irrational fear and excessive stress, as these may indirectly affect their reproductive health and well-being [71]. The effects of SARS-CoV-2 on the reproductive system of such men may also be elicited by viral infection-mediated immunomodulation and progressive inflammation [103]. Further research is also needed to develop specific treatment strategies for men with an impaired male reproductive system resulting from SARS-CoV-2 infection. In this regard, several therapeutic methods have been developed recently for the treatment of COVID-19 patients, such as mesenchymal stem cells [104], miRNA-based therapy (responsible for changing ACE-2 expression) [105] and hormone therapy [106]. Therefore, treatment regimens should also consider the androgen

levels of men, as SARS-CoV-2 infection is believed to be associated with androgen secretion [9]. Management strategies such as cryopreservation and ART may be considered vital approaches in tackling specific clinical conditions of male infertility. To employ these strategies for COVID-19 patients, extra precautionary measures should be undertaken during the handling of semen to reduce the chances of viral transmission [85]. Accordingly, clinical trials should be conducted on SARS-CoV-2-infected male subjects of reproductive age, along with longitudinal studies in paediatric patients to understand the long-term effects of SARS-CoV-2 infection on testicular functions and spermatogenesis in such men [85]. In summary, existing evidence on the impairment of the reproductive system in men who have suffered and/or are suffering from COVID-19 is still preliminary in nature, and further research can only reveal the exact mechanisms and impacts of SARS-CoV-2 infection clearly together with specific short- and long-term approaches for the management of these men.

Abbreviations

ACE2: angiotensin-converting enzyme 2
ACE2-R: ACE2 receptor
Ang-II: angiotensin 2
ART: assisted reproductive technology
CD: cluster of differentiation
COVID-19: Coronavirus disease 2019
CTD: C-terminal domain
FGF: fibroblast growth factor
FSH: follicle stimulating hormone
GCSF: granulocyte colony-stimulating factor
G-CSF: granulocyte colony-stimulating factor
GMCSF: granulocyte-macrophage colony-stimulating factor
H_2O_2: hydrogen peroxide
HPT: hypothalamic–pituitary–testicular
IFN: interferon
IFN-γ: interferon gamma
IL: interleukin
IP: inflammatory protein
IP10: interferon gamma-induced protein 10
LH: luteinizing hormone
MCP: monocyte-chemoattractant
MCP1: monocyte chemoattractant protein-1
MERS-CoV: Middle East Respiratory Syndrome Coronavirus
MIP: macrophage inflammatory protein
MIP-1α: macrophage inflammatory protein-1alpha
NK: natural killer
NKG2A: killer cell lectin-like receptor subfamily C member 1

NTD: N-terminal domain
O_2•: superoxide radical
OH•: hydroxyl radical
OS: oxidative stress
OX40: secondary costimulatory immune checkpoint molecule
PD1: programmed cell death protein-1
PDGF: platelet-derived growth factor
ROS: reactive oxygen species
SARS-CoV: severe acute respiratory syndrome-related coronavirus
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2
TIM3: T-cell immunoglobulin domain and mucin domain-3
TMPRSS2: transmembrane protease/serine subfamily member 2
TNF: tumour necrosis factor
TNF-α: tumour necrosis factor alpha
VEGF: vascular endothelial growth factor

Disclosures and Conflict of Interest

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Chapter 60

Elevated CO_2 Modulates Airway Contractility

Masahiko Shigemura, PhD, and Jacob I. Sznajder, MD

Division of Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

j-sznajder@northwestern.edu

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60.1 Introduction

Cells and tissues sense and respond to changes in the concentration of gaseous molecules through specific signalling pathways. Oxygen- and nitric oxide-activated cellular signalling pathways have been extensively studied [1–3], but much less is known about the mechanisms by which non-excitable cells sense and respond to changes in carbon dioxide (CO_2) concentrations [3–5]. CO_2 is a primary product of oxidative metabolism and its physiological levels in mammals are significantly higher than atmospheric levels (approx. 5% versus approx. 0.04%, https://scripps.ucsd.edu/programs/keelingcurve/) [4, 6], suggesting that CO_2 is inextricably linked to physiological conditions. In humans, the elevation of CO_2 levels in tissues and the bloodstream (hypercapnia) is a consequence of inadequate alveolar gas exchange in patients with lung diseases such as the acute respiratory distress syndrome (ARDS) [7–9], chronic obstructive pulmonary disease (COPD) [10–12]

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and others [13–15]. In clinical situations, hypercapnia has been initially proposed to be benign or even protective in the lung since hypercapnia and its associated acidosis have been shown to attenuate systemic cytokine response in mechanically ventilated patients with acute lung injury and ARDS [7, 8, 16]. However, it is becoming increasingly evident that elevated CO_2 conditions have deleterious pathophysiological effects on various organs, including the lung [9, 17–19], skeletal muscles [20–22] as well as innate immunity system [18, 23–27]. In the lung, recent studies suggest that high concentrations of CO_2 activate specific gene expression [19, 28, 29] and signal transduction pathways with adverse consequences on alveolar epithelial function (alveolar fluid clearance) [17, 30–35] and epithelial cell repair [36–39].

Hypercapnia is also reported to modulate the tone of lung airways which is a dynamic equilibrium between various excitatory and inhibitory mechanisms. The effects of hypercapnia on the airways and airway smooth muscle are controversial, as there are reports attesting to it causing increased airway contractility [19, 40–49] or airway relaxation [50–61]. Here, we review recent advances in our understanding of how elevated CO_2 conditions modulate the airway tone, focusing on the effects of hypercapnia and respiratory acidosis.

60.2 Hypercapnia-Induced Bronchoconstriction

Evidence suggesting that changes in the level of CO_2 in the blood influence the airway tone was first reported by Einthoven in 1892 [40]. He described that inhalation of high concentrations of carbonic acid (CO_2-rich mixtures) caused bronchoconstriction in dogs, which was confirmed in various models of normoxic hypercapnia-exposed dogs [41–45] and cats [46, 47]. Airway tone is regulated by interaction of the sympathetic and parasympathetic nerves [46, 62] and the stimulation of vagal efferent nerves can increase the tone, resulting in bronchoconstriction [46, 62–64]. As the hypercapnia-induced bronchoconstriction was abolished by blocking the vagus nerve, it was understood to be dependent on the integrity of vagal conduction [40–44, 46, 47]. In healthy humans, there have been few reports describing that the inhalation of high CO_2 concentrations decreases specific airway or pulmonary conductance which is the mathematical inverse of airway resistance [48, 49]. The increases in airway resistance during high CO_2 exposure were interpreted as extrathoracic airway narrowing [48] such as larynx narrowing [49], because the hypercapnic effect was not blocked by atropine or β_1/β_2 adrenergic receptor agonists. However, the direct studies of laryngeal resistance during high CO_2 exposure indicated no change in anaesthetized animals [65] and healthy human subjects [66]. Furthermore, several reports of bronchoconstriction in the hypercapnia-exposed animals [42, 44, 46] showed that the blockage of the vagus nerve did not entirely abolish the bronchoconstrictor response to the high CO_2 exposure. These reports suggest that other mechanisms can contribute to the airway response to hypercapnia. Recently, we have reported that CO_2 operates as a signalling molecule that increases contraction of mouse

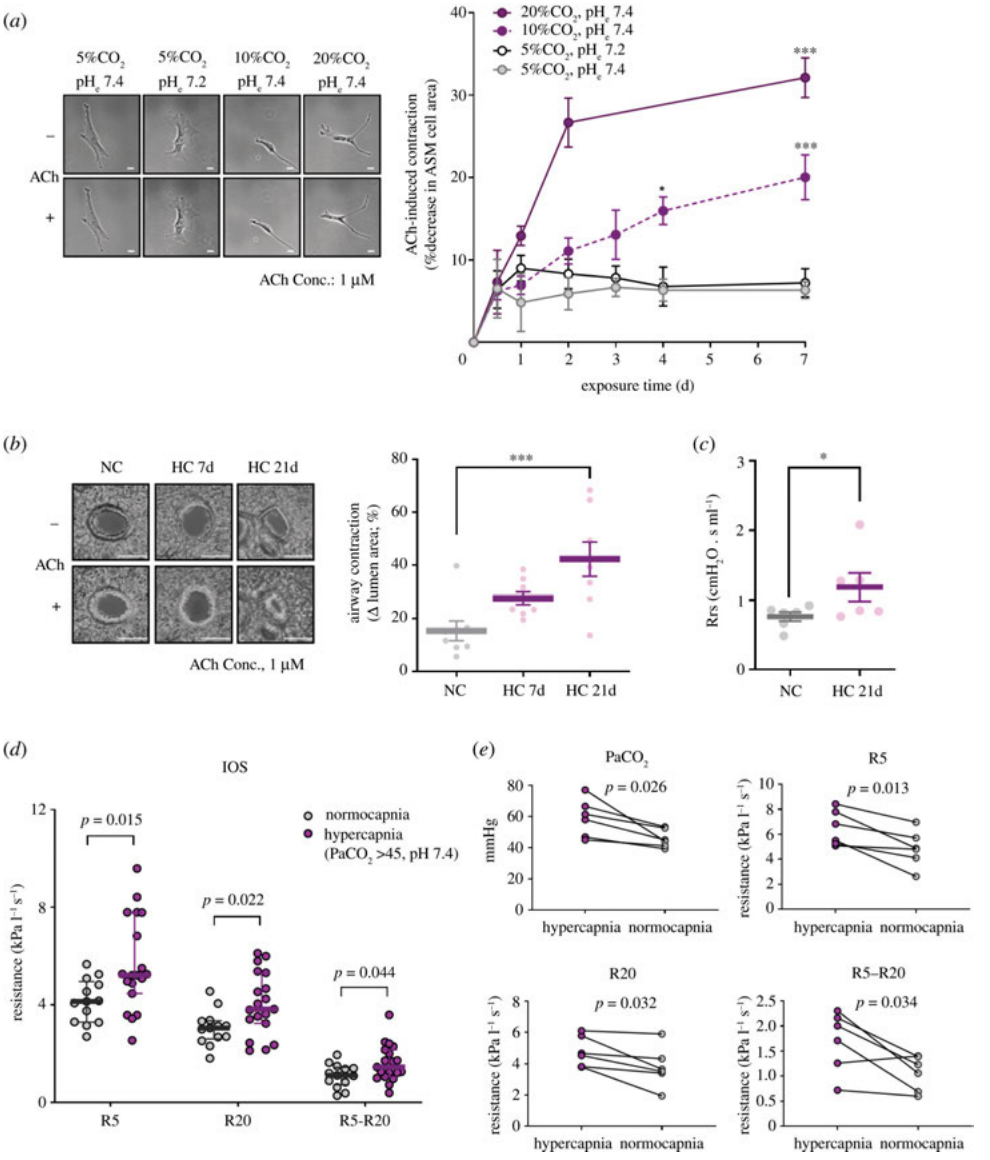

Figure 60.1 Normoxic hypercapnia increases airway smooth muscle contractility. (a) Acetylcholine (ACh)-induced cell contraction in mouse airway smooth muscle cells exposed to different conditions. Left, representative images from 7-day exposure conditions (scale bar, 50 µm). Right, time-course quantification of ACh-induced cell contraction. (b,c) C57BL/6 J wild-type mice were exposed to 21% O_2 and 10% CO_2 (HC) or maintained in room air (NC) for up to 21 days. Representative images (top; scale bar, 100 µm) and quantification (bottom) of ACh-induced airway contraction in precision-cut lung slices (b). Total resistance of the respiratory system (Rrs) at baseline on a FlexiVent instrument (c). (d) Comparison of respiratory resistance measured by impulse oscillometry between normocapnic and hypercapnic patients with chronic stable COPD. Values of R5, R20 and R5–R20 indicate total, proximal and peripheral respiratory resistance, respectively. (e) Changes of respiratory resistance in hypercapnic patients. All data are expressed as means ± s.e.m. (a–c) or median with interquartile range (d,e). *$p < 0.05$, ***$p < 0.001$. Adapted from [19]. Copyright © 2018 American Association for the Advancement of Science.

and human airway smooth muscle cells [19]. We found that high concentrations of CO_2, independently of hypoxia and extracellular pH, increased acetylcholine (ACh)-induced cell contraction, which is both time- and dose-dependent in cultured cells (Fig. 60.1a). In a murine model, the exposure to normoxic hypercapnia, particularly chronic hypercapnia, increased ACh-induced airway contraction in precision lung cut slices (Fig. 60.1b) as well as airway resistance (Fig. 60.1c). Furthermore, we found that, in a small cohort of patients with chronic COPD, patients with hypercapnia had higher airway resistance (Fig. 60.1d), which improved after correction of hypercapnia (Fig. 60.1e). Our study also provided novel insights into the molecular mechanisms by which hypercapnia promotes airway smooth muscle cell contractility via calcium–calpain signalling. The signalling was mediated by caspase-7, which by cleaving the transcription factor myocyte-specific enhancer factor 2D (MEF2D), leads to downregulation of the microRNA-133a (miR-133a) and consequent upregulation of Ras homologue family member (Rho) A and myosin light-chain (MLC) phosphorylation (Fig. 60.2). Our data suggest that the elevation of CO_2 levels activates specific signal transduction pathways in airway smooth muscle cells, which results in deleterious changes in the airway tone, leading to bronchoconstriction. Taken together, these reports suggest that hypercapnia can contribute to airway constriction by activating vagus nerve and high CO_2-responsive signal transduction pathways. In lung disease conditions, hypercapnia may worsen airway constriction and limit ventilation to poorly functioning lung units setting up a feedback loop that could culminate in respiratory failure.

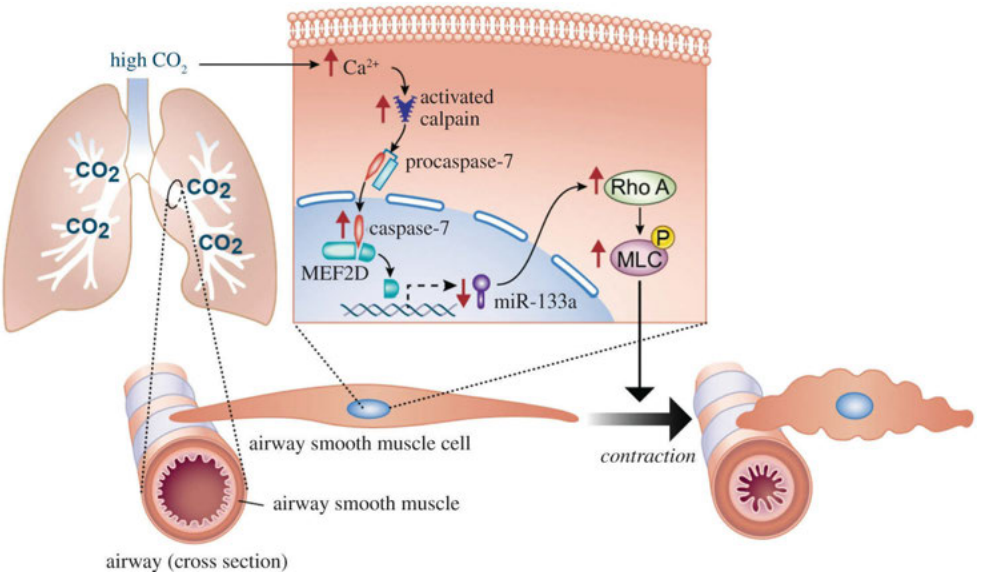

Figure 60.2 Schematic of calcium–calpain signalling in airway smooth muscle cell contraction during hypercapnia. Hypercapnia promotes airway smooth muscle contractility through an increase in intracellular calcium (Ca^{2+}) and consequent activation of calpain which cleaves caspase-7. Cleaved caspase-7, in turn, cleaves the transcription factor myocyte enhancer factor 2D (MEF2D) that reduces miR-133a expression, thereby increasing Ras homologue family member A (RhoA) abundance and MLC phosphorylation. Reproduced from [19]. Copyright © 2018 American Association for the Advancement of Science.

60.3 Respiratory Acidosis-Related Bronchodilation

There have been studies reporting that hypercapnia shows airway relaxation [50–61]. We have reported that airway smooth muscle relaxation occurs during acute hypercapnia-associated acidosis, but it was transient and modest [19]. We reason that hypercapnia may acutely contribute to bronchodilatation when the tone of airways is previously increased by various constrictor stimuli such as drugs [52–54], hypoventilation [55, 67–69] or when the reduction of ventilation in one lung following the occlusion of its pulmonary artery leads to bronchoconstriction in response to local airway ischaemia [70] and hypocapnia [50–52, 71]. The inhalation of high CO_2 concentrations reduces constriction of airways as well as the tension developed by isolated bronchial rings caused by drugs such as 5-hydroxytryptamine [52–54]. It also reverses the airway constriction associated with pulmonary artery occlusion in ventilated animal models [50, 52]. In humans, the administration of high CO_2 can relax the constriction of airways in the patient with unilateral pulmonary artery occlusion [51] and young asthmatic adults with hyperventilation (hypocapnia) [55] or exercise-induced bronchoconstriction [55, 56]. These *in vivo* and *in vitro* effects of hypercapnia were not stimulated by the nerve reflexes and were understood to be a result of changes in extracellular/intracellular pH level, possibly elevated CO_2-related acidosis (respiratory acidosis) in airway smooth muscle cells. Many of the cellular responses to CO_2 elevation are thought to be a consequence of acidosis because of the rapid conversion of CO_2 in solution into H_2CO_3 and subsequently HCO_3^- and H^+ [5, 72]. Several *in vitro* reports show that respiratory or normocapnic (metabolic) acidosis produced a reversible reduction in active tension of bronchial rings [53, 54, 57, 58]. Extracellular pH can alter airway smooth muscle tone by changing the levels of pH and intracellular calcium (Ca^{2+}) [58, 59, 73]. Intracellular acidification has been reported to decrease intracellular Ca^{2+} levels through voltage-dependent Ca^{2+} channels in the potassium-induced contractile model, thereby inhibiting airway smooth muscle cell contraction [60]. On the other hand, an *in vitro* study reported that high concentrations of CO_2, independently of extracellular pH, enhanced airway smooth muscle relaxation via the epithelium-dependent mechanism induced by substance P in the model of methacholine-precontracted bronchial smooth muscle [61]. Collectively, elevated CO_2 conditions, specifically showing acute respiratory acidosis, appear to have a potent relaxing effect on contracted airways.

60.4 Effect of Hypocapnia on Airway Contractility

Low levels of CO_2 (hypocapnia) have been also reported to increase airway constriction in humans with pulmonary artery occlusion [51, 71], hyperventilation [67, 68] and exercise-induced asthma attacks [55, 56] and other models *in vivo* [50, 52, 70, 74] and *in vitro* [59, 61, 75, 76]. The bronchoconstrictor effect of hypocapnia is largely attributed to local mechanisms on the bronchial smooth muscle since it was not abolished by vagotomy or atropine in intact animals

[50, 70] and asthmatic patients [55, 56]. Several reports suggest that the hypocapnic response involves additional contribution of cholinergic reflexes in the airways [67, 68]. The cellular mechanisms involved in local airway response to hypocapnia are likely dependent on intracellular alkalosis elicited by hypocapnia on airway smooth muscle cells. *In vitro* studies suggest that intracellular alkalosis can increase airway smooth muscle contractility [77] by increasing intracellular Ca^{2+} levels through voltage-dependent calcium channels in airway smooth muscle [60, 73, 76].

60.5 Conclusion

A proposed model for the effects of CO_2 levels on the airway tone, airway smooth muscle contractility or relaxation, is presented in Fig. 60.3. Lung airway cells appear to sense and respond to changes in CO_2 levels via specific mechanisms of the vagus reflexes, molecular CO_2 and pH effects. Thus, the effect of elevated

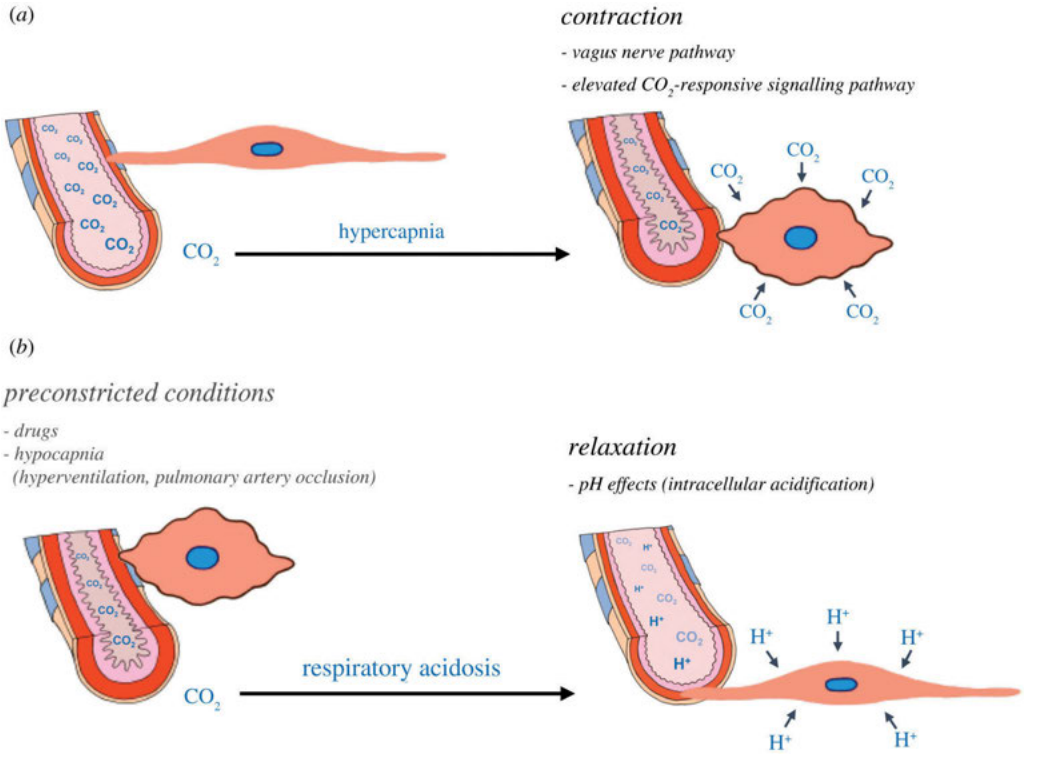

Figure 60.3 A proposed model for the modulation of CO_2 in airway tone. Lung airway cells sense and respond to changes in CO_2 levels, which modulates the tone of airways, airway contraction or relaxation, via specific mechanisms of the vagus reflexes, molecular CO_2 and pH effects. (*a*) Hypercapnia. Acute and chronic hypercapnia promote airway contractility via either vagus reflexes or molecular CO_2 effects. (*b*) Respiratory acidosis. Elevated CO_2 conditions particularly showing acute respiratory acidosis can have a potent relaxing effect on contracted airways via pH effects.

CO_2 levels to lung diseases is somewhat controversial. Hypercapnia is associated with worse outcomes in patients with obstructive lung diseases such as asthma [13], obesity hypoventilation syndrome [14] and COPD [10–12]. Furthermore, the recently reported strategy of mechanical ventilation aimed at reducing the partial pressure of CO_2 in arterial blood can provide beneficial effects including improvement of airway resistance, health-related quality of life and mortality for patients with COPD and hypercapnia [11, 12, 19]. Understanding the elevated CO_2 effects on airway contractility is of significant clinical interest for those patients and could help with the design of innovative therapeutic approaches.

Disclosures and Conflict of Interest

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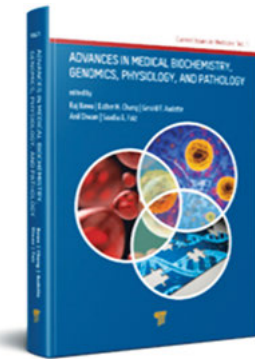

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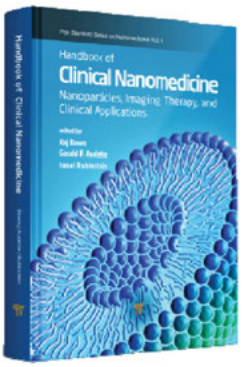

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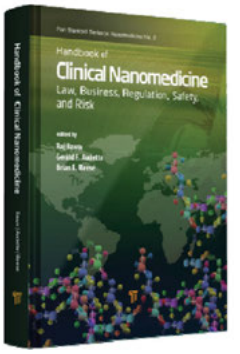

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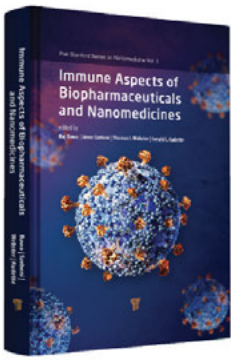

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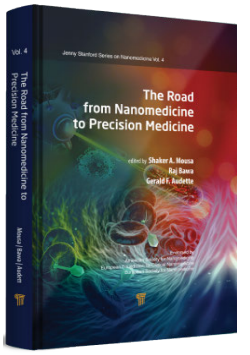
The Road from Nanomedicine to Precision Medicine

Shaker A. Mousa, PhD, MBA, Raj Bawa, PhD, MD, and Gerald F. Audette, PhD (Editors)



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The enormous advances in nanomedicine and precision medicine in the past two decades has necessitated a growing need for an authoritative and comprehensive reference source that can be relied upon by biomedical researchers, clinicians, pharmaceutical scientists, regulators, and lawyers alike. This stand-alone, full-color book provides a broad survey of various interconnected topics, all accomplished in a user-friendly format. Each chapter contains key words, tables, and figures in color, future projections, and an extensive list of references. It is intended to be a standalone reference volume that broadly surveys and highlights innovative technologies and advances pertaining to nanomedicine and precision medicine. In addition, it also addresses often-neglected yet key issues such as translational medicine, intellectual property law, FDA regulatory issues, nanomedicine nomenclature, and artificial nanomachines—all accomplished in a user-friendly, broad yet interconnected format. The book is essential reading for the novice and expert alike in diverse fields such as medicine, law, genomics, pharmaceutical sciences, biomedical sciences, ethics, and regulatory science. The book's multidisciplinary approach will attract a global audience. It will serve as a valuable reference resource for the industry, academia, and government.

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The pace and sophistication of advances in medicine in the past two decades have been truly breathtaking. This has necessitated a growing need for comprehensive references that highlight current issues in specific sectors of medicine. Keeping this in mind, each volume in the *Current Issues in Medicine* series is a stand-alone text that provides a broad survey of various critical topics in a focused area of medicine—all accomplished in a user-friendly yet interconnected format. However, unlike other series on medicine or medical texts, this series focuses on current trends, perspectives, and issues in medicine that are central to healthcare delivery in the 21st century. Medical practitioners today continue to improve upon techniques and technologies to provide procedures for patients that are safer, faster, less invasive, and more accurate—a direct consequence of advances in technological breakthroughs from a variety of medical and engineering fields. In order to render modern patient care, it is imperative that surgeons and medical practitioners stay current with these latest advances in their respective specialties. Given this backdrop, the specific topics covered in this volume and the expertise of the contributing authors accurately reflect the rapidly evolving areas within surgical and medical specialties. While recognizing how expansive and multifaceted medicine is, *Advances in Surgical and Medical Specialties* addresses crucial recent advances in surgical and medical specialties, integrating the knowledge and experience of experts from academia and practicing surgeons. The multidisciplinary approach reflected here makes this volume a valuable reference resource for medical practitioners, medical students, nurses, fellows, residents, undergraduate and graduate students, educators, venture capitalists, policymakers, and biomedical researchers. A wide audience will benefit from having this volume on their bookshelf: health care systems, the pharmaceutical industry, academia, and government.

About the Series Editor

Raj Bawa, PhD, MD, is president of Bawa Biotech LLC (founded 2002), a biotech/pharma consultancy and patent law firm based in Ashburn, Virginia. Trained as a microbiologist and biochemist, he is an inventor, author, entrepreneur, professor, and registered patent agent licensed to practice before the US Patent & Trademark Office. He is currently scientific advisor to Teva Pharmaceutical Industries, Israel; visiting research scholar at Pharmaceutical Research Institute of Albany Pharmacy, Albany, New York; and full professor (adjunct) at NOVA in Annandale, Virginia. He is VP/chief IP officer at Guanine, Inc., in Rensselaer, New York, a company focused on rapid, accurate detection of infective pathogens. Dr. Bawa has served as a principal investigator of various NCI research grants, and most recently as a principal investigator of a CDC grant to develop an assay for carbapenemase resistant bacteria. Previously, he was an adjunct professor at Rensselaer Polytechnic Institute, Troy, New York, from 1998 to 2018. He held various positions at the US Patent Office, including primary examiner from 1996 to 2002. He earned a BSc (Honors School) in microbiology, MS in cancer biology, PhD in biophysics/biochemistry, and MD. Currently, he is a life member of Sigma Xi, cochair of the nanotech and precision medicine committees of the American Bar Association and founding director of the American Society for Nanomedicine (established 2008). He has authored over 100 publications, edited 10 texts, and serves on the editorial boards of numerous peer-reviewed journals, including serving as an associate editor of *Nanomedicine* (Elsevier).

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