Systems Thinking in Medicine and New Drug Discovery

Volume One

Robert E. Smith

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Systems Thinking in Medicine and New Drug Discovery:

Volume One

By

Robert E. Smith

Cambridge Scholars Publishing

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By Robert E. Smith

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This book is dedicated to my grandchildren. I hope they can work with others in their generation to end civilization's addiction to fossil fuels and reverse the damage that my generation has done to the environment.

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CONTENTS

Preface xi

Chapter One 1
Overview of Systems Thinking
1.1 Introduction 1
1.2 Reductionist Thinking and DNA 7
1.3 Systems Thinking 15
1.3.1 Definition and the importance of change 15
1.3.2 Misconceptions that are clarified by systems thinking 16
1.3.3 Characteristics of systems thinking 21
1.3.4 The roots of systems thinking 42
1.3.5 Autopoiesis and Gaia 43
1.3.6 Systems view of autopoiesis and stem cells 50
1.3.7 The importance of networks in life 53
1.3.8 Genetics and systems thinking 66
1.3.9 Nonlinearity and rhythms in living systems 81
1.3.10 The –omics, from genomics to personal –omics 104
1.3.11 A multidimensional, non-linear view of diseases 109
1.3.12 Drugs that bind to multiple targets 110
1.3.13 Modularity of biological networks 111
1.3.14 Going beyond genomics and looking for hidden connections 112
1.3.15 Tools for understanding metabolism better 113
1.3.16 Evidence based medicine and the Critical Path Initiative 115
1.3.17 Cancer Genome Characterization Initiative, CGCI 120
1.3.18 Systems-based thinking in education, in contrast with reductionist thinking in advertisements and propaganda 122
1.4 More systems thinking is needed 141
1.5 Reductionist thinking is still useful 147

Chapter Two 167
The Deep Ecology of the Human Biology
2.1 The human ecosystem, holobionts, hologenome and viruses 167
2.2 The widespread existence of microbes in the human body 170
2.3 Gut microbiome and the enteric nervous system (ENS) 172
2.3.1 Composition of the gut microbiome 172

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2.3.2 Interactions and communication between the ENS and the rest of the body 175
2.3.3 Communication between the gut microbiome, ENS, brain and immune system 180
2.3.4 The gut microbiota and ENS comprise an entero-endocrine organs 184
2.4 The role of the gut microbiome in preventing diseases..... 187
2.5 The role of gut microbiome dysbiosis in causing diseases 191
2.5.1 The importance of biodiversity 191
2.5.2 The gut microbiome and inflammation 193
2.5.3 The benefits of vegan and vegetarian diets 193
2.5.4 The gut microbiome and cardiovascular diseases 196
2.5.5 The role of the gut microbiome in stress, anxiety and neurodegenerative diseases 197
2.5.6 The importance of healthy microbiota in obtaining an effective response to Vaccines..... 201
2.5.7 The importance of oral health and preventing periodontal disease..... 203
2.6 Factors affecting the composition of the microbiome..... 206
2.7 Viruses are important, too..... 215
2.8 Eukarya in the human microbiome 219
2.8.1 Introduction to the microbial eukaryotic cells in the human microbiome..... 219
2.8.2 Fungi 220
2.8.3 Protozoa and intestinal worms (helminths) 223

Chapter Three 241
Systems Thinking in New Drug Development
3.1 How new drugs are developed..... 241
3.1.1 Select a disease..... 245
3.1.2 Select a therapeutic target or phenotype..... 250
3.1.3 Find a lead compound 284
3.1.4 Improve the lead compound..... 336
3.1.5 Drug delivery..... 339
3.1.6 Manufacturing and cGMP 347
3.1.7 Clinical trials 356
3.1.8 Many drugs that were developed and approved had a single therapeutic target 358
3.1.9 Combinations of drugs are also used and are being developed..... 360

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

3.1.10 Some targets are now known to have formerly unexpected biological roles 365
3.1.11 Some drugs may be useful in treating other diseases 366
3.1.12 The Reemergence of Phenotypic Screening 369
3.1.13 Other new approaches to new drug discovery 373
3.2 The difference between how prescription drugs, dietary supplements and 'super-foods' are developed and used 380
3.2.1 'Superfoods' 387
3.2.2 Dietary supplements: from amphetamine to fish oil to vitamin D and folic acid 393
3.2.3 A complex, nonlinear dynamic systems perspective on Ayurveda and Ayurvedic research 415
3.2.4 Discovery and development of innovative drug from traditional medicine by integrated chinmedomics strategies in the post-genomic era 417
3.2.5 A complex systems science perspective for whole systems of complementary and alternative medicine research 417
3.2.6 A Pilot Feasibility Study of whole-systems Ayurvedic medicine and yoga therapy for weight loss 418
3.2.7 Prescription drugs and weight loss supplements for weight loss 419
3.2.8 Prescription drugs: strict protocols and quality management systems, take much longer to reach the market 422
3.3 Immunotherapy – using one's own body to cure one's self 424
3.4 Synthetic biology and CRISPR technology in new drug development 437
3.5 Rapid prototyping by 3D and 4D printing 447
3.5.1 Introduction 447
3.5.2 Biodegradable scaffolds 448
3.5.3 Other applications of 3D and 4D printing 448
3.5.4 Other personalized medical devises 451

Appendix 494
Primer on Physical and Analytical Chemistry
A1.1 Introduction 494
A1.2 Ultraviolet-Visible (UV-Vis) Spectrophotometry and Fluorescence Spectroscopy 506
A1.3 HPLC and UPLC 511
A1.4 Gas Chromatography, GC 515
A1.5 Mass Spectrometry, GC-MS and LC-MS 518
A1.6 Quantitative Analysis 539

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

A1.7 IR and NMR 540
A1.8 Magnetic Resonance Imaging, MRI 555

Abbreviations and Acronyms Used 558

Index 576

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PREFACE

Medicinal chemistry is undergoing an important paradigm shift (or way of thinking) from reductionist to systems thinking. This is based on a similar paradigm shift in medicine and the pharmaceutical industry. Network science is an integral part of this. It has led to the emergence of network medicine. It not only aims to develop safe and effective new prescription drugs for patients who become sick, but also to recommend diets, lifestyles and even some dietary supplements that can prevent diseases. Instead of focusing on just one aspect of health, network medicine uses systems thinking to predict peoples' susceptibilities to diseases and find ways to prevent them. In addition, patients who have specific cancer-causing oncogenes are being identified before clinical trials begin. That way, only the patients who are likely to benefit from the anticancer therapy will be recruited into clinical trials. This will increase the success rates of these trials and help to reduce the cost of new drug development and healthcare.

Network medicine also emphasizes precise, personalized treatments, in which patients and their caregivers can actively participate. It recognizes the important human need for patients and caregivers to be involved in preventing and curing their diseases. This is done with the types of extensive teamwork, open collaborations and continuous feedback which are hallmarks of Total Quality Management (TQM) and Total Quality Leadership (TQL) that are used in modern businesses. This leads to new insights. In business, it means that every employee is important because they all make crucial contributions to the organization. It also means listening to the voices of the customers, so their demands can be met. Similarly, TQM and TQL in systems medicine means listening to the voices of the patients and their caregivers, so their health needs can be met. It also means that the needs of every part of the human body are recognized by other parts of the body that interact synergistically and communicate with each other constantly. In addition, there is a deep ecology in the human body in which even the lowliest viruses and Bacteria make crucial contributions to the health of their human hosts and serve as essential parts of the neuroendocrine immune system. So, the human body operates under the principles of total quality, in which every component interacts, communicates and undergoes nonlinear feedback and feed

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forward mechanisms. However, the human body is not a machine that is involved in manufacturing. Machines don't make themselves. Humans and other living creatures make themselves in the process called autopoiesis (self-making). So, TQM in the human body means total quality making, not total quality manufacturing, like it does in some industries.

The main goals of this two-volume set are to inform people with different backgrounds about the new ways that we are looking at human life and medicine, help healthcare professionals do their jobs better, provide background information and references for patients and their caregivers, as well as clarify some serious misconceptions that have emerged. For example, some people believe that the FDA and other governments' regulatory agencies are in a conspiracy with pharmaceutical companies and physicians to keep people sick, so they can maximize profits and continue to sell patients prescription drugs that don't cure any diseases. This thinking can lead some people to avoid seeing physicians, who prescribe 'chemicals'. Some people believe that all dietary supplements are always safe and effective – especially if they are labeled 'natural'. Many misconceptions like these can be exposed by using systems thinking.

The first volume provides an overview of systems thinking. The first chapter compares and contrasts reductionist and systems thinking in biochemistry and medicine. Some basic principles of systems thinking and network science are introduced. The new ways that diseases are prevented, treated and cured are described. This includes changing how new drugs and dietary supplements are being developed. Network science is crucial in this change, as is using one's own body and natural resources to prevent, treat and cure diseases. At the same time, a huge, interactive network supports healthcare. It starts with the smallest viruses and Bacteria. It includes patients, caregivers, nurses, physicians, hospitals, research institutes, universities, industry and many government agencies. It is based on a holistic view. Doctors no longer engage in a man-to-man struggle to cure the disease. Instead, they work with large interdisciplinary teams to focus on treating the whole patient and not just the disease. Moreover, pharmaceutical companies have realized that the former, secretive approach to developing new drugs was inadequate. Now, they collaborate with each other and with academia and even individual patients working at home. Part of this paradigm shift is the realization that there is a deep ecology.

So, the second chapter in the first volume also includes a description of how the human body is an ecosystem, consisting of not just human cells, but also viruses, Bacteria, Archaea, and Eukarya. At the same time, a type

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of deep ecology has emerged in science and medicine. That is, biology and medicine are just as important as math, physics, chemistry, engineering and industry.

The third chapter in the first volume describes how systems thinking is used in every stage of new drug development. In this new paradigm, some of the best work is done by people who are educated in more than one field, so they can do interdisciplinary research using systems thinking. They are using 3D printing to produce plastic models based on CT and/or MRI images of patients, so they will know what to expect when they start surgical procedures. Such models are also quite useful in teaching surgical techniques to new residents. Interdisciplinary teams are also using 3D and 4D printing to make devices that are specific for each patient and can alter their shape as young patients grow and recover. In addition, interdisciplinary scientists are using 3D printing to make personalized organs on a chip to test the safety and efficacy of new drugs. This is eliminating the need for animal toxicity testing. Moreover, 3D printing may be able to provide personalized food and feed the world's growing population.

However, it's also important to look for hidden connections. For example, one substance used in 3D printing (UVR-6105) became popular decades ago because it's resistant to photobleaching by UV light. Also, it is less genotoxic than the substance it replaced (bisphenol A diglycidyl ether, or BADGE). However, UVR-6105 can undergo hydrolysis that can be catalyzed by either esterase enzymes or the acid in gastric fluid. The hydrolysis products are produced in an autopoietic system that resembles death more than life. The hydrolysis products are more bioavailable and more carcinogenic than BADGE. Instead, a silicone-based material is resistant to hydrolysis and has very low toxicity. It might be better for regenerative medicine and for making devices that will go into patients' bodies.

CRISPR technology is another important advance. It may be able to produce new cures for diseases as well as make new organisms. Sterile mosquitoes are being made that may eliminate malaria and the Zika virus. Genetically modified Bacteria and algae may be able to remediate pollution and make biofuels. However, there are many potential pitfalls. Just as many fear the uncontrolled dissemination of genetically modified foods into the environment, some fear the release of genetically modified Bacteria and plant seeds. Also, CRISPR could potentially make designer babies and people with super-human powers.

In the meantime, many people think that only natural remedies should be used and chemicals (prescription drugs) should be avoided. As a result, dietary supplements are widely consumed. Some are quite dangerous and

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potentially deadly. So, a quick, easy way to see if your turmeric is adulterated with carcinogenic chromium (Cr^{6+}, hexavalent chrome) is presented. In addition, the different ways that prescription drugs and dietary supplements are developed, brought to market and sold are discussed.

Systems thinking is an interdisciplinary process requires scientists to learn other scientific disciplines. So, there is an Appendix in volume 1 that describes some basic chemistry to biologists. There is another Appendix in volume 2 that explains some basic principles of neurology, immunology and endocrinology to chemists and non-specialists.

Volume 2 starts by describing predictive, preventive, personalized and participatory P4 medicine. This includes the use of systems thinking in primary care medicine and an explanation of how our understanding of causality has changed. It also tells what P4 medicine does and how all the stakeholders are collaborating and using evidence based medicine. This includes the Advancing Regulatory Science Initiative and listening to the voices of the patients, in the spirit of TQM. There is also a description of how metabolomics shows what is happening within a patient's body and provides fundamental insight into the causes of diseases. Targeted radiation therapy is also described, along with precision systems medicine that targets cancer stem cells. The second chapter discusses the important role of inflammation. This includes dispelling some previous misconceptions on how dietary antioxidants work to prevent diseases, including cancer. Since nutrition and lifestyle are important in preventing diseases and maintaining good health, they are discussed in chapter 3. This includes an explanation of how the superfood cult is misleading many people – especially women. In fact, the only true superfood is mother's breast milk. The second volume finishes by describing the harmful effects of a toxic environment, with recommendations about trying to reverse the effects of global climate change through systems thinking. Then, there is an Appendix that describes the basic principles of the neuroendocrine immune system.

It's also important to be able to speak each other's languages. So, several examples are given of the same word, abbreviation or acronym being used in very different ways in different fields of science. Also, since many people who don't speak or write English as a native language can easily become confused by the way that numbers are written in English. The English language uses commas (like 10,000) where other languages use periods (like 10.000). So, in this book, the number ten thousand (and all larger numbers) are written using a space (like 10 000) instead of a

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comma or period, except when directly quoting sources that were written in English.

This book also provides information that people can use in their work and their lives. For example, if a healthcare professional is ever challenged by someone who believes that he or she is part of a conspiracy to keep dietary supplements out of the hands of people who need them, he or she only needs to talk about folic acid. It's a dietary supplement that physicians and governmental regulatory agencies help people find and take to prevent birth defects and many types of cancer. It can be found in most breads and cereals, as well as vitamins for pregnant women. It tells how mother's breast milk is the only true superfood. Also, many databases, government websites and other internet sources are provided, along with over 1600 references to the scientific literature. There is also a list of hundreds of toxic substances that have been found in dietary supplements and information (*m/z* values that can be seen in a mass spectrometer) that chemists can use to detect them. Moreover, there have been cases where seemingly harmless supplements like vitamin D can be formulated wrong by manufacturers. So, educating physicians and patients on the adverse effects of high doses may be the most important way to prevent unnecessary or excess supplementation. Moreover, the FDA and other governments' regulatory agencies are not in a conspiracy to keep people sick. It is just the opposite. They and many other organizations and individuals are collaborating to make modern medicine predictive, preventive, personalized and participatory.

This work should not be taken as having an impact on the FDA or any other governmental regulatory agency.

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CHAPTER ONE

OVERVIEW OF SYSTEMS THINKING

1.1 Introduction

Systems thinking has become an important paradigm in many areas of science. Fritjof Capra described how it is important in modern physics and biology [1, 2]. He and Pier Luigi Luisi further showed how it is important in mathematics, biology and medicine [3]. This includes a deep ecology, in which humans are viewed as just one of many equally important parts of the global ecosystem [2]. However, medicinal chemistry has been viewed as being conservative, since it only recently started emphasizing the need for systems thinking [4-6]. So, one of the primary goals of this book is to describe how the tools and methods of systems thinking are being used to improve medical science and accelerate new drug development. This contrasts with the ways that medicine and new drug development were done previously using reductionist thinking. That is, many prescription drugs were developed based on their abilities to either activate or inhibit a single therapeutic target, such as an enzyme or an ion pump [4]. This was based on reductionist thinking that preached that one should look for the single root cause of a disease. More recently, many new drug candidates and medical devices are being developed based on their ability to modify the activities of many therapeutic targets [4-6]. This is based on systems thinking, which recognizes that complex problems (like diseases) have many causes. Not all of them are obvious upon initial inspection. So, researchers and pharmaceutical companies are looking for hidden connections, which is another important feature of systems thinking [7]. This includes looking for ways to affect protein-protein and protein-DNA interactions and the development of allo-network drugs that don't bind to an enzyme's active site [6]. This contrasts with reductionist thinking, in which a drug should bind to the active site of an enzyme or the ligand-binding site on a protein receptor or ion pump. This is how medicinal chemistry was once taught.

However, there is a growing consensus that systems thinking is improving healthcare and new drug development dramatically [8]. In some

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ways, this is a return to the way drugs were developed before the 1970s – before many therapeutic targets were known. Not until the 1980s was the paradigm shifted from observations of physiology to the studies on the molecular level. In the late 20th and now in the 21st Century, a Renaissance in systems thinking is occurring and it is being used to develop new, improved prescription drugs and medical devices. This was due, in part, to new advances in robust high-throughput platforms that produce much data, the construction and free public access to curated databases, and the emergence of interdisciplinary teams that conducted research that enabled us to understand the data better. This has led to a new code of behavior, in which data are shared, with an emphasis on quality rather the quantity of data. It also led to the development of predictive toxicology. Still, datasets are growing exponentially in size. The data describing the three billion nucleotides in the human genome grew to about one petabyte (2^{50} bytes, 1024 terabytes or about a million gigabytes) of data by 2008 and one Exabyte (billion times billion bytes) by 2012. This increase has inspired the creation of new computational tools that help us understand better the complexities of human health and disease. In this context, complexity has been defined as follows: "in a complex system the whole is more than the sum of its parts". There are several properties of complex systems. They have many heterogeneous interacting parts. They operate on many different scales. There are many possible states, as well as relatively complicated laws governing their transitions. Complex systems have emergent properties and are sensitive to initial conditions. There are path-dependent dynamics and a networked hierarchical connectivity. Autonomous agents interact. There is self-organization, as well as collective shifts and non-equilibrium dynamics. Complex systems can adapt to changing environments. Finally, there are co-evolving subsystems, ill-defined boundaries and multilevel dynamics [8].

So, extreme reductionist thinking is seldom useful any more when developing new drugs [8]. However, the other extreme that tries to take into account all the possible variables and their interactions is not feasible. Still, complex systems can be understood well enough to make systems thinking very valuable. The structure of complex living systems is modular and has many redundant or expendable parts. So, it is important to identify the most important segments of the system. Moreover, the state space of complex living systems has a relatively low number of major attractors. So, there is some simplicity in complex systems. As a result, many of the emergent properties of complex living systems can tolerate some error in the data that are used to predict them or in the biochemical factors that determine them [8].

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Systems thinking has led to a better understanding of diseases [8]. They are now understood to be pertubations in the complex networks in the human body. In addition, network analysis is becoming crucial in new drug development. Networks are usually visualized by graphs containing points (nodes) that are connected by line segments (edges) that may or may not have a direction (making them a vector). However, the nodes in complex, cellular systems are not points, but rather biopolymers (DNA, RNA, proteins) that have their own network structures. Moreover, a good understanding of network dynamics is crucial for understanding the complexities of diseases and the actions of drugs. So, edges can have a direction and a sign (activation or inhibition). They can also have conditionality, since some edges are active only if one of their nodes have another edge [8].

An excellent review article listed several tools that are available for visualizing networks as well as human disease-related networks [8]. It also provided data on networks, and made network-based predictions of disease-related genes that can serve as biomarkers. It described different methods that are available for comparing networks. There is also a list of chemical compound similarity networks, a list of resources for studying protein-protein interaction networks and signaling network resources, as well as lists of metabolic network resources and resources related to drug design. Finally, there is a list of system-level hallmarks of drug quality and trends of network-related drug design that are designed to help achieve the hallmarks [8].

It is also important to understand network theory when developing new drugs using systems thinking [8]. Networks exist throughout living systems [5]. The human brain contains a network of neurons that are connected by axons and supported by glial cells. Cells are networks of ions and molecules, connected by biochemical reactions. Some ions and molecules (such as H^+, adenosine triphosphate or ATP and H_2O) are highly connected nodes in the biochemical network. On the other hand, some compounds (like 1,3-bisphosphoglycerate) are intermediates in just one metabolic pathway and are not hubs. At the same time, people, animals and plants cannot exist without networks of symbiotic relationships. The average adult human has about 10^{15} cells, only 10% of which are human. Almost all the rest are Bacteria. They help us digest our food while properly treating and excreting wastes. Moreover, societies are networks of people, connected by family, friends and work. Food webs and ecosystems consist of networks of organisms [5].

Properly organized networks are essential for life and help make us human [5]. We are complex, self-regenerating, autopoietic organisms,

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whose bodies are ecosystems living in larger ecosystems. Life is sustained by a network of production processes, in which the function of almost every component is to participate in the production or transformation of itself and the other components in the network [2, 3, 9]. So, network theory and systems thinking have emerged as guiding principles in biology and medicine [9].

Information storage and processing in cells, along with the execution of cellular programs exist in distinct levels of organization. Our genes may contain essential information for life, but they are not sufficient. RNA, proteins, enzymes, hormones and proper organization are also needed. Genes interact with histones and protein transcription factors, as well as with RNA and with other genes. The activation and repression of the expression of genes depend on the environment. There is structural coupling [5].

There has also been a change in how 3D printing (rapid prototyping) is used. That is, rapid prototyping and reductionist thinking were once used to make models of proteins with a single ligand or inhibitor bound to it [10]. This was used to demonstrate the lock and key model of ligand binding in the Smithsonian Institute of American History's exhibit "Chemistry in American Life". Plastic models of proteins and DNA were used in the Brookhaven National Laboratory to show their three-dimensional crystalline structures, based on X-ray diffraction. It ignored the fact that proteins actually have many different structures when they are dissolved in an aqueous solution, such as the cytoplasm of cells. It also assumed that all proteins are the same in everybody. Reductionist thinking preceded personalized medicine. Rapid prototyping has now improved so that it can be used with systems thinking to make personalized devices such as a bioresorbable tracheal splint for an infant who was critically ill [11]. It is now called three- or four-dimensional (3D or 4D) printing. A social, medical and scientific network has emerged from systems thinking. It enables predictive, preventive, personalized and participatory (P4) medicine. The days when reductionist thinking taught that diseases can only be cured by a man-to-man struggle between the doctor and the disease are gone forever. We now treat the person and not just his or her disease. We also realize that we all need some personalized treatments, even though some preventive treatments (like vaccines) are useful to almost everybody. All people (including homozygotic twins) are unique. Medical science is becoming much better at predicting every individual's susceptibility to different diseases by analyzing his or her genetics, epigenetics and metabolism. In addition, proper diet, physical activity and positive thinking, as well as avoiding unhealthy habits can help prevent

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diseases. When illnesses do emerge, patients and their caregivers can participate with healthcare professionals to come up with the best treatments and cures. All of this is compatible with the very natural process of total quality management (TQM), which has been adopted throughout modern industry and which has always been a hallmark of good health and wellness. It is an essential part of systems thinking. Moreover, systems thinking is needed for proper TQM. Note that the term total quality leadership (TQL) is often used synonymously with TQM.

Systems thinking has been integrated into TQM for many years [12]. It is essential for survival in an increasingly competitive environment that requires efficient use of modern technologies, such as 3D and 4D printing. The health and productivity of an organization requires using a holistic approach. Traditional operating methods must be continuously evaluated and replaced when they become dysfunctional or unnecessary, just like ancestors of cetaceans (whales and dolphins) did when they no longer needed legs as they evolved into creatures that spent their entire lives in water [12].

One of the most important tools of TQM in industry and living systems is system dynamics [11]. The process helps associates work together while leveraging their knowledge and skills by applying the tools of system dynamics. The acronym LEARN sprang from a similar term used in by educators when teaching in classrooms. LEARN is used with the PDSA (Plan, Do, Study and Analyze) quality model. The steps in LEARN are locate, employ, assess, reflect and nominate. First, locate a core interest for systemic evaluation. This step identifies key variables and uses both positive and negative feedback to establish a proper balance. Second, employ system dynamics tools for leveraging and increasing understanding. Connections between variables are identified to help recognize causal loops. Third, assess the adequacy of the current systems and repeat step two as often as necessary. Fourth, reflect on the process and the results and make important suggestions. Fifth, nominate or propose solutions and enter the PDSA cycle. In this step, a hybrid of many solutions may be produced [11]. So, like TQM in living systems, this is a cyclic process.

Even though it has not been recognized by most people, the same principles of TQM are used by our bodies to maintain good health and to prevent diseases. Just as businesses listen to the voices of customers and communicate with them, cells within our bodies communicate with each other. Many medicinal chemists now realize that the human body is an ecosystem with a deep ecology. Human eukaryotic cells are just one component of the ecosystem. This is similar to the concept in TQM in

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which all associates are important to the organization. In modern biology, we are changing the way we think about what it is to be human. That is, the viruses, Bacteria, Archaea and other microorganisms in us help make us healthy humans. As in TQM, there is an extensive communication network among different microbes and between microbes and human (eukaryotic) cells. However, TQM in industry is often involved in manufacturing tools and machines. They don't make themselves. They are made by people. In contrast, living systems are not machines. We make ourselves in a process called autopoeisis (self-making). We make ourselves and we team with other people and organizations made by humans to improve all aspects of our healthcare. So, in biology, TQM means Total Quality Making, not Total Quality Manufacturing.

The principles of TQM are used by an extensive networking system in many governments. For example, the FDA's Center of Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER) are developing a more systematic approach for incorporating the "Patient's Voice" into drug development. The goal is to build on the series of public workshops that occurred under the current patient-focused drug development program. This is being used to produce guidances to assess the burdens of diseases and the treatments that are most important to patients, as well as impact measures, clinical outcome assessments, and endpoints to inform drug development and regulatory decisions.

In addition, physicians are distinguishing between care that concentrates on the patient's disease and care that looks at the whole patient [13]. Healthcare that is centered on the patient's disease usually focuses on individual visits. It can be oriented towards a specific episode while managing diseases that emerge. It is primarily concerned with the progression of a patient's disease, while thinking of the many systems and organs in the human body as being distinct. It defines co-morbidity as the number of chronic diseases occurring simultaneously. In contrast, healthcare that is focused on the individual looks for changing interrelationships over time. It also views episodes in illness as being part of experiences with health that occur throughout the course on one's life. It considers diseases to be interrelated as well affecting the evolution of one's health problems. Moreover, organs and systems in the body are also thought to be interrelated, while morbidity is a combination of interacting illnesses. This is called multi-morbidity. One of the major limitations of primary care in the USA is the underestimation of the importance of long-term relationships with patients that is independent of the care that is given for each individual episode of an illness. Instead, it is important to

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consider the patient's (or individual's) viewpoint of the relative importance of each illness and symptom. It is also important to pay attention to an individual's illnesses in the context of multi-morbidity. Finally, good primary care and an individual patient's health are not just the sum of the care that is given for each illness. It is the care of the whole person that is important [13].

1.2 Reductionist Thinking and DNA

To understand systems thinking better, it is important to compare it to its opposite - reductionist thinking. By doing so, we can see some of its strengths and weaknesses. It is an important part of a good childhood education. It is needed to learn how to add, subtract, multiply, divide and do quantitative analyses [4]. In our first science classes, we were taught to do controlled scientific studies. Only one independent variable should be changed at a time and only one dependent variable should be measured. We learned that the universe is made up of basic building blocks (protons, neutrons, electrons, atoms and molecules). It was thought that the best way to understand something was to break it down into its smallest components. Once the smallest building blocks of matter were understood completely, this could be combined with a thorough knowledge of math and physics to make predictions. All we needed to know was the position, mass and momentum of an object to be able to predict the future position and momentum of that object when it is affected by a force. For example, if you kick a ball with a force of 20 Newtons on a smooth, frictionless surface, you can calculate exactly how far it will go. If you kick it again, it will go the same distance. If there is no friction, no strong wind or any other interference from the environment, the calculations will be accurate. That is, reductionist thinking teaches that we are separate from the environment. Every individual proton, neutron, electron, atom and molecule is separate, and interactions are always additive. The whole is said to be equal to the sum of its parts. To determine the properties of a group of atoms, molecules, cells, tissues or organisms, all that is needed is to just add up their individual properties [4].

Sir Isaac Newton used reductionist thinking to describe the law of gravity and many aspects of classical mechanics [4]. These laws worked rather well in helping people understand many physical phenomena. This led 18th and 19th century societies to believe that the universe was predictable. Even now, many scientists think that it is possible to predict all the properties of matter and energy, if we could just learn more about the so-called fundamental particles and apply the 'hard' sciences of math

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and physics [4]. Living organisms, including people, are made of matter and were thought to be like machines [2]. Metabolic pathways were studied independently and were described as a linear sequence of reactions such as in glycolysis, or a single cycle, such as the tricarboxylic acid cycle. In business, hard management skills such as technical knowledge and individual sales figures were of paramount importance. They can be taught in traditional business schools. They can also be quantified and shown on colorful graphs. Soft skills such as being able to communicate effectively and become genuinely interested and involved with one's employees were not so important.

Reductionist thinking accurately described many of the mechanics of the human heart. The vascular system of the body was thought of as pipes in plumbing in a house. If they became clogged with too much cholesterol, it could cause a heart attack. Also, specific functions were assigned to every organ in the body. For example, the alimentary tract and human gut were responsible for digestion and the production of solid waste. Bacteria were thought to be separate from the human body, most of which contained no Bacteria or viruses. Nobody had any idea that the human gut was an essential part of the nervous, immune and endocrine systems. The renin-angiotensin system (RAS) was thought to be responsible only for controlling water and electrolyte homeostasis as well as blood pressure. The brain was for thinking, even though a myth began that claimed that we only use 10% of our brain when we think. People who had psychosomatic illnesses were simply considered to lack proper self-discipline. Normal diseases were dealt with by a single physician in a kind of man-to-man struggle. Nurses were there simply to obey doctor's orders and were discouraged from ever questioning a doctor's decisions. Files on a patient's health history were kept in large manila folders. There was often important information in them, but it was very hard to find. The system worked like a poorly controlled machine or inanimate object that often broke down.

One of the properties of inanimate objects is a linear response. Toxicologists looked for a linear response when estimating the excess relative risk of mortality from solid cancer due to exposure to ionizing radiation. Despite the large amount of scatter in the data at each dose, the results were fit to a straight line that did not pass through the origin. That is, when the straight line was extrapolated to a very low dose (near zero), there was still some mortality. This led to the conclusion that there is no safe dose of radiation. Fortunately, it is widely accepted that the low dose of ionizing radiation that patients receive during diagnostic imaging by X-rays, computed tomography and other methods is safe and does save lives.

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Similarly, reductionist thinking can lead some to think that linear dose-response curves also apply to nutrition. That is, if a nutrient or biochemical is healthy at the relatively low dose that exists in a food or beverage (like green tea), then higher doses must be better. This has led to the widespread sale and consumption of dietary supplements, such as vitamins, green tea extract and epigallocatechin-3-gallate or EGCG (the most abundant antioxidant in green tea). It also led to the idea that dietary antioxidants exert their health benefits by reacting directly with dangerous free radicals (also known as reactive oxygen species or ROS). So, foods like kale (*Brassica oleraceae*) and açaí (*Euterpe edulis*) that have very high antioxidant activities *in vitro* were called superfoods. For several years, the U.S. Department of Agriculture listed the *in vitro* antioxidant activities of many foods, beverages and spices.

Reductionist thinking also teaches that you gain weight if the number of Calories that you consume exceeds the number of Calories that you burn up through metabolism and exercise. All Calories are thought to be the same – regardless of their source. It doesn't matter if they come from carbohydrates, fats or proteins. Also, endurance exercises were thought to be the best way to burn off Calories.

However, reductionist thinking does have its uses. It is important in current Good Manufacturing Practices (cGMP) and Good Laboratory Practices (GLP). That is, one of the first steps in new drug development is to synthesize the drug in accordance with cGMP and measure its toxicity and pharmacokinetics in accordance with GLP. When the U.S. National Toxicology Program determines the toxicity of industrial chemicals a toxicokinetic study is done under GLP. In both cGMP and GLP, a method is needed to quantify the new drug or potentially toxic chemical. The first step in validating the method is to analyze several different concentrations of the chemical that one wants to quantify. Analytical methods such as high performance liquid chromatography (HPLC) are used to separate the compounds being analyzed (the analytes) from other chemicals in the sample. Either ultraviolet (UV) absorption or mass spectrometry (MS) can be used to detect each separate analyte. Ideally, the detector response is linear. The results are used to construct what is called a calibration curve – even though there is no curvature, as shown in Figure 1.

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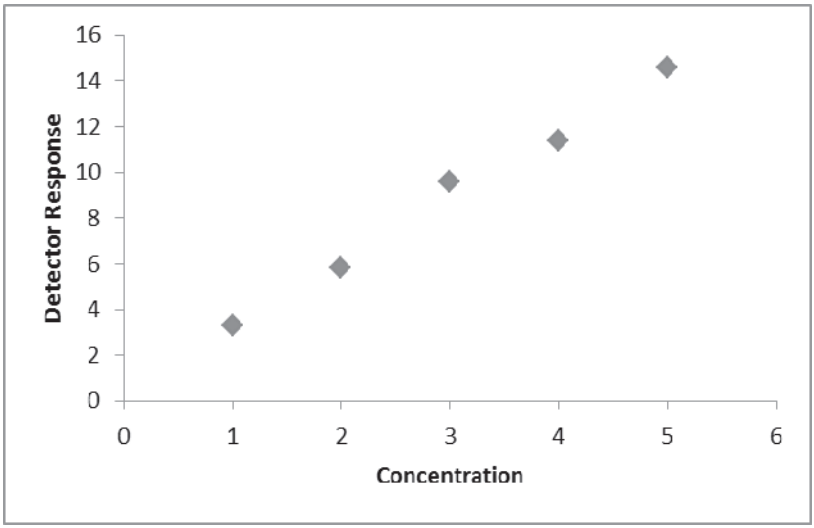

Figure 1. Simulated Calibration Curve for cGMP or GLP**.**

Next, the data are fit to a straight line using linear regression analysis, as shown in Figure 2.

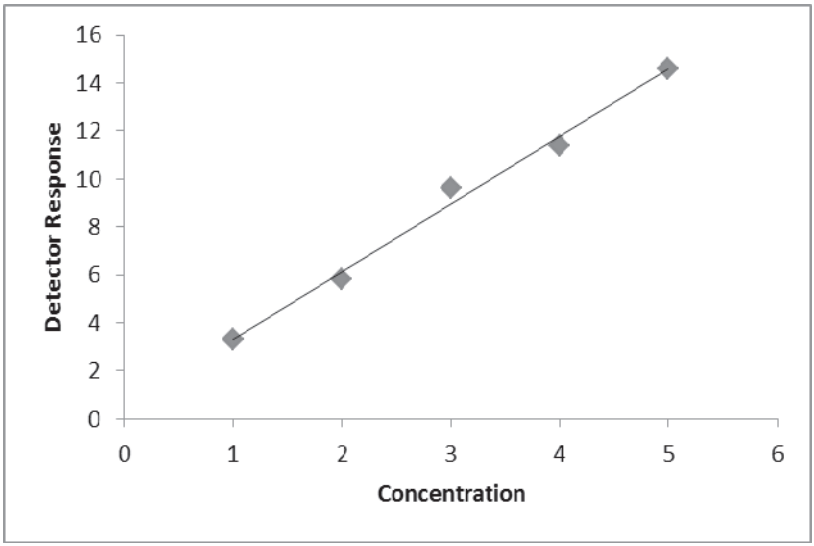

Figure 2. Simulated calibration curve fit to a straight line by linear regression analysis.

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This illustrates an important point in reductionist thinking. It tends to separate different scientific disciplines from each other. Almost all areas of science have their own special vocabulary that often contradicts the vocabulary in other areas of science. This tends to reduce science into distinct disciplines that have trouble communicating with each other. For example, if one considers math to be the only true science, then the nomenclature of the analytical chemist is wrong. There is no curvature in the calibration 'curve' and the detector response is nonlinear. Still, the terminology is widely accepted and understood by analytical and medicinal chemists. Moreover, the use of 'linear' calibration 'curves' is required by the laws of the USA and many other countries when developing new drugs or testing potentially toxic substances.

The different uses of the same word and concepts in physics and biology can also be problematic. For example, the theory of evolution is the foundation of all of biology. No biology makes sense without evolution. Microbiologists and virologists can even show that Bacteria and viruses do evolve in real time. So, many biologists say that the theory of evolution states that all life on Earth evolved from the same single cell. A physicist may ask the biologist to define the word cell with mathematical equations, just like the terms force, mass, velocity, momentum and acceleration have exact mathematical definitions. The biologist may say that we are still learning more about cells. For example, some very large viruses that were once thought to be cells are now classified as viruses. The physicist would probably still want a mathematical definition of a cell. Without a set of mathematical equations, there is a significant gap in the definition of a cell. That is, how can a biologist say that we all evolved from something that we don't know much about and can't even define in terms that a mathematician or physicist would accept? So, widely differing vocabularies and definitions have historically made scientists specialize and reduced science into many distinct disciplines.

Reductionist thinking also implies that there is a hierarchy [4]. Mathematicians might feel that theirs is the only true science. Physicists might accept this as they use mathematics to prove many principles in physics. However, biologists might see a problem. I remember one of the first meetings of mathematicians and biologists in 1994. A mathematician gave a talk that only half of the audience understood. A biologist asked, "Does your work have any practical applications"? The speaker proudly answered no and about half the audience applauded. Many of them were skeptical of almost anything a biologist might claim. Then, a biologist talked about two different species of deer that looked almost identical, but one had twice as many protein-coding genes than the other. A

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mathematician asked if the ratio was exactly two and the biologist answered no. The mathematician then said that the answer indicated that the difference in the number of genes meant nothing. She was right.

There can be good reasons for such skepticism. For example, the complete title of Charles Darwin's book describing evolution was "On the Origin of Species by Means of Natural Selection. Preservation of Favoured Races in the Struggle for Life" [14]. At that time, slavery and subjugation of 'inferior races' was rampant. The misuse of the term race helped lead to the Holocaust in World War II. Fortunately, the scientific process requires all hypotheses to be tested experimentally and be modified as new facts emerge. So, we now realize that there is only one race – the human race. However, new technologies may make it possible to produce designer babies and people with super-human powers.

Reductionist thinking led scientists into thinking that there is a hierarchy in biochemistry. It was thought that all the information needed to produce an organism was in its DNA. Supposedly, there was a one-way flow of information, producing a cause and effect. That is, genes make RNA, which makes proteins, which make cells, which make organs, which make tissues. Because of this hierarchy, RNA and proteins were not thought to influence the production of DNA. There was even an idea that our genes were selfish [15]. In the first 12 chapters of his book, The Selfish Gene, Richard Dawkins suggested that we are like machines, created to spread our selfish genes. We exist and perform acts of self-sacrifice so our genes can survive. We can be thought of as vessels that contain our genes. Much of human behavior was supposedly under the control of our selfish, human genes. Professor Dawkins wrote that "genes swarm in huge colonies, safe inside gigantic lumbering robots sealed off from the rest of the outside world … They are in you and me; they created us, body and mind; and their preservation is the ultimate rationale for our existence" [15]. This book helped explain why some of our human genes might make us do things that are altruistic. This includes helping society at the expense of the individual, since the genes survive and are reproduced. However, in chapter 13, Professor Dawkins went on to write, "We, alone on earth, can rebel against the tyranny of the selfish replicators" [15]. In that chapter, he introduced many principals of evolutionary psychology. That is, we give our children not only our human genes, but also a society, complete with education, technology and civilization; all which help us survive and reproduce, regardless of our genetic differences. So, Professor Dawkins' idea of selfish genes was a metaphor, meant to stimulate debate and thinking and not a mathematical law [15].

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Before the human genome was solved, it was thought that humans must have at least 100 000 genes that code for proteins because other simpler organisms that were already analyzed had as many as 20 000 genes. DNA was thought to be the book of life or the blueprint of life. Since humans were the most complex organisms, our book of life must be more complex and longer than that of other species. Bacterial and viral DNA was ignored. Even though it was known that Bacteria helped us digest our food, they were thought of as being more like digestive enzymes. Moreover, they were not considered to be human. They usually caused diseases and even death. There was even a time when nursing students were taught that when a doctor could not determine why a patient was sick, it must be due to a viral infection.

Diseases were thought to be caused by something becoming defective in the living machine. To cure the disease, all that was needed was to identify the single root cause of the problem and fix it by giving a medicine with a single active ingredient. So, many prescription drugs approved by the United States Food and Drug Agency (FDA) and other countries' medical regulatory agencies were thought to have a single molecular target. Doctors were also taught to act like machines. They were taught to avoid any emotional bond with the patient, just as a car mechanic should avoid becoming emotional when repairing a car [4]. Moreover, nurses were told that they were subordinate to doctors and should never question their authority, much like union workers before the advent of TQM.

In reductionist thinking, the whole is equal to the sum of its parts [2-4]. In this type of thinking, mathematics is always quantitative and is the foundation of all science [4]. Physics is thought to be the basis of all of chemistry and chemical principles can potentially describe all of biology. It may be possible eventually (with enough research) to describe all of biology, chemistry and physics with mathematical equations. If we can just learn all the mathematical and physical properties of all the chemicals in the human body, we will know everything about human biology and medicine. Reductionist thinking teaches that it is theoretically possible to find the smallest fundamental particles and fundamental laws of physics. This idea has been expressed as, "Nothing exists but atoms and space. Everything else is opinion" [16]. Even though chemists and physicists know that there are smaller particles than the atom, reductionist thinkers feel that there must be a small set of fundamental particles, and all of them should be governed by the same fundamental laws. Still, scientists are quite interested in learning more about subatomic particles, regardless of whether they mostly use systems thinking or reductionist thinking. So,

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they noticed with interest when the search for smaller particles in the super collider in Geneva, Switzerland resulted in success in 2012. Scientists found the fundamental Higgs boson, which is believed to give all other particles their mass. Reductionist thinking and the search for the Higgs boson were explained more thoroughly by the Nobel Prize winner, Leon Lederman, in his book, The God Particle (not because he thought that the particles would tell us anything about God, but because the book editor didn't like his originally proposed title) [16].

Reductionist thinking is often very effective in selling products and is used during political debates. A clever salesman or politician knows that one of the most effective ways of lying is to over-simplify and tell just part of the truth. They can use simple expressions that evoke strong emotions. One of the best examples is when products are advertised as being "all natural", as if everything that is made by nature is good and things made by people are bad. Slavery was once called "the natural order". Other popular examples of over-simplifications include "this product contains no toxic chemicals", "it has been scientifically formulated and clinically tested" and "it has been clinically proven to be safe and effective". Two of the most misused terms are prove and proof, as in "We will prove that the defendant is guilty beyond a shadow of doubt" or "Where is the proof to support your claim?" Scientists would use the term 'evidence' instead of proof. In contrast, systems thinking teaches that science can't prove anything [3]. The main role of science is to learn how to ask the right questions. If done properly, almost every answer can generate ten or more questions and open new avenues of exploration.

Reductionist thinking can also produce arbitrary rules that are not supported by scientific evidence. For example, "All the living things on earth died - birds, domestic animals, wild animals, small animals that scurry along the ground, and all the people" [17]. Reductionist thinkers may also tend to label something as strong or weak. When asked to identify their greatest strength and greatest weakness, they will give two different answers. For example, they might say that their greatest strength is their extensive knowledge and their greatest weakness is not being able to put up with ignorant people. They may not realize that certainty is an enemy of wisdom. A specialist might be very certain about all that their discipline teaches but be quite ignorant of other disciplines. This can lead to serious mistakes and miscommunication when people from each discipline only know their own specific vocabulary. For example, the fact that we can be infected with pathogenic viruses and Bacteria could be one the greatest weaknesses of the human body, while many other Bacteria and

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viruses are essential for life. Moreover, some viruses and Bacteria can be used to cure diseases. This comes from systems thinking.

In reductionist thinking, viruses and Bacteria are outside the human body and are often causes of diseases [4]. Also, one must find the root cause of a disease and fix it. Physicians were involved in man-to-man combat with the disease that a patient had. Also, DNA was thought to be the blueprint of life. All genes were considered to be pieces of DNA that coded for mRNA, which coded for proteins, the 'machinery of life'. All the instructions for making a new human, or for keeping a living human healthy, were said to be in the DNA, which was thought to be the blueprint of life. If a gene was defective or became mutated, it would code for an mRNA molecule that coded for a defective protein. So, it should be possible to give the patient the proper gene and the disease could be cured. Furthermore, when studying pharmacology or toxicology one should test only one compound at a time [4].

So, many drugs were developed with a single protein as the therapeutic target. Information was thought to flow from DNA to mRNA to proteins. Drugs acted by either binding to the active site of an enzyme or to the ligand binding site in a protein receptor. There was even a 'one gene one protein hypothesis' in which each gene was thought to code for a single protein [4]. This limited the ability of medicinal chemists to develop new and more effective drugs. It also led to a misunderstanding of how our bodies can make millions of different antibodies that target millions of different antigens. This is done by clonal selection. That is, there is a pre-existing group of B-lymphocytes (white blood cells made in the bone marrow) that make their own unique antibodies by splicing gene segments that code for the variable (V), diversity (D) and joining (J) regions of the antibodies. When an antigen enters the body, it binds to the particular B-lymphocytes that have the cognate antibodies that recognize the antigen. The B-lymphocytes that have the cognate antibodies are selected and cloned. That is, many exact copies of them are made. So, by mixing and matching (or recombining) the V, D and J regions, millions of different antibodies can be made. Since the D region is optional, this is called V(D)J recombination.

1.3 Systems Thinking

1.3.1 Definition and the importance of change

Barry Richmond has been credited with coining the term "system thinking" [18]. He wrote the following, "As interdependency increases, we

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must learn to learn in a new way. It's not good enough simply to get smarter and smarter about our particular 'piece of the rock.' We must have a common language and framework for sharing our specialized knowledge, expertise and experience with 'local experts' from other parts of the web. We need a systems Esperanto. Only then will we be equipped to act responsibly. In short, interdependency demands Systems Thinking. Without it, the evolutionary trajectory that we've been following since we emerged from the primordial soup will become increasingly less viable" [18, 19]. Systems thinking is a system of thinking about systems [18]. However, most definitions of systems thinking use reductionist terms that describe the elements of systems thinking. For example, it has been defined as, "the art and science of making reliable inferences about behavior by developing an increasingly deep understanding of underlying structure" [19]. On the other hand, systems thinking was recently defined using the terminology of systems thinking. That is, it is "a set of synergistic analytic skills used to improve the capability of identifying and understanding systems, predicting their behaviors, and devising modifications to them in order to produce desired effects. These skills work together as a system" [19]. This is an important change in how we think and try to solve problems.

Change is constant in the universe [2]. It does not mean that it is always good or bad. However, our way of thinking (paradigm) can affect how we address problems and solve them. System thinkers realize that some of today's problems can come from yesterday's solutions and today's solutions can come from yesterday's problems. On a molecular scale, this means that tumor suppressors like the p53 protein protect us from cancer when we are young, but can cause cancer if and when they become mutated later in life. On a larger scale, it means that some of the deadly viruses that have caused epidemics (like polio) can be modified by genetic engineering to cure cancer and maybe even bring back formerly extinct species, like the wooly mammoth.

1.3.2 Misconceptions that are clarified by systems thinking

In reductionist thinking, when a seemingly complex problem occurs (like a disease), it could be solved once the single root cause was identified. This is especially popular in politics, in which the root cause is assigned to the opposition party. The goal is to fix the blame – not the problem. This was evident in the attempts of one party to fix the healthcare system by blaming the former President and head of the opposing party. It succeeded temporarily by winning the election, but failed to solve the

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problems in healthcare. No single political party or one simple change (such as repealing an existing law) can change the fact that the much of the American population is aging, inactive, obese and suffering from diseases that are very expensive to treat and almost impossible to cure. However, it does not help to assign blame or try to shame people who are overweight. That would only make the problem worse and would ignore important scientific and medical facts. First, nobody is fat. Fat is a type of nutrient and not a type of person. Second, a mere 10% decrease in body weight can have very significant health benefits and lower the need for expensive healthcare – even though the person may not meet society's standards of beauty. Third, even though fat shaming may seem to be successful at first, it may not be in the long run. If fat shaming leads to anorexia and bulimia it will cause much more human suffering and be expensive to try to treat. Fourth, fat shaming can lead to clinical depression in the victim as well as the victim's family and friends. This is also expensive to treat and causes much human suffering. So, systems thinking is better. We should realize that we are all in this together. Happiness and compassion trump hatred and insensitivity.

So, systems thinking is changing the way we think of diseases [8]. That is, we try to fix the problem – not the blame. When reductionist thinking was used, diseases were recognized and defined by the symptoms that emerge in its later stages – once the pathological impact became apparent. So, therapeutic strategies almost always focused on the pathological phenotypes in the advanced stages. This led to the development of many life-saving drugs. However, this approach often neglected the earlier causes of a disease and can obscure important differences between various groups of people who have different genotypes or other properties that existed before symptoms become apparent. So, diseases are being recognized by systems thinkers "as an emergent property of a pathobiological system, i.e. a property, which cannot be predicted by studying only the parts of the system, but emerges from the complex interrelationships" [8].

In systems thinking, we realize that complex problems have many interacting causes and effects. At the same time, today's solutions can come from yesterday's problems. The cigarettes, sweetened soda pop and red meat that were freely given to servicemen and women during World War II are now known to cause serious health problems. In the 1940s and 1950s, nurses were taught that if a doctor can't figure out what is causing a disease it must be caused by a virus. When the HIV pandemic emerged, there were no cures for any viral infections. In the 21st Century, viruses are being genetically modified and used as oncolytic viruses to treat and even

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cure many types of cancer. We also realize that much of what makes us human are pieces of ancient viral DNA that have been incorporated into our human eukaryotic DNA.

System thinkers also realize that a person's body weight depends on much more than just the difference between the number of Calories consumed and the number of Calories burned off due to metabolism and exercise. Not all Calories are the same and not all carbohydrates are the same. Complex carbohydrates are much healthier than simple sugars, including the 'natural' sugar, fructose. Proteins and fats make most people feel satiated (full) faster than carbohydrates. Saturated fats in red meat are unhealthy, while polyunsaturated fats and omega-3 fats in olives, açaí and many types of seafood are healthy. Also, high intensity interval training is healthier than endurance exercises [20]. It is especially important to exercise one's abdominal muscles. This converts white adipocytes (fat cells) to brown adipocytes that secrete hormones like leptin that go to the brain and increase satiety [5].

Systems thinkers also realize that it may be impossible to predict the properties and behavior of living system based on the mathematical and physical properties of the individual components [4]. That is, entirely new properties can emerge at higher levels of organization. The whole is greater than the sum of its parts. Individual ions, compounds, cells and tissues can interact to produce effects that can't be seen with the individual components. DNA is not the blueprint of life. The properties of DNA and the messages it conveys to a cell depend on the internal and external environments and needs of the organism. Proteins, RNA and even ions like H^+, Ca^{2+} and Mg^{2+} affect the production of DNA. Although almost every type of human cell (except blood platelets and mature red blood cells) has the same sequence of bases in their DNA, the code is read differently by different cells in different tissues and different environments. Moreover, accessibility to the code can be affected by epigenetics that affect the availability of genes to transcription into messenger RNA (mRNA) and by post-translational changes of the proteins coded for by the mRNA. Moreover, most genes code for different types of RNA and not proteins.

At the same time, we are coupled with the environment through metabolism. We change our structure in response to environmental conditions, and we change our internal and external environments in response to our needs. For example, when our lymph nodes are exposed to potentially harmful Bacteria from the extracellular environment, some of the genes in immature white blood cells are turned on. The genes code for proteins that interact with other proteins and DNA to turn some other

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genes off and some on. They do this by binding to promoters that promote the transcription of other genes. This can change the internal environment of the cell, causing it to differentiate into a specialized cell or secrete ROS that can kill the invading Bacteria, thus changing the extracellular environment. It should be noted that some biologists don't like the term ROS because it misuses the word species. That is, obviously reactive oxygen species (ROS) like hydrogen peroxide and the superoxide anion will not mate and produce fertile offspring.

Another piece of evidence that supports systems thinking is that the genetic code is not fixed in one place. That is, some pieces of DNA are mobile. They can move around on the same or different chromosomes. These mobile genetic elements are active in the brain and other parts of the body. They help make us human. So, the internal and external environments of the cells change all the time. Living systems are dynamic. Moreover, the environment (like sunlight) that is external to our bodies can change our internal environment, which is subject to a circadian rhythm. Hormones, neurotransmitters and even the activity of the gut microbiome are different during the day and night. In metabolism, we consume molecules from the environment and these molecules change us. So, our structures are coupled with the environment and the environment is coupled to our structures [4].

This is also true at the cellular level [4]. Genetically identical cells often have very different cell fates and behaviors, due to their stochastic nature. That is, cellular metabolism, cell fate and behavior depend on both predictable and random variables. The RNA and/or proteins that can be coded for by a gene may appear to be predictable under a controlled experiment on a single cell at a single moment in time. However, the interactions between promoters and inhibitors of gene transcription depend in part on the random movement of proteins and/or RNA. Once the proteins and/or RNA reach their DNA targets, the effects may be predictable, but getting to the target involves some random motion. Cells in the human body take advantage of their inherent stochastic variability to respond to continuous changes in their microenvironment, so they can survive at the expense of the rest of the identical (clonal) cell population [4]. We are not under the strict control of our DNA or genes.

In contrast to reductionist thought, we now know that there is not a strict hierarchy of DNA causing mRNA to be made, which causes proteins to be made [4]. There are mobile genetic elements, epigenetics and post-translational modifications that affect one's phenotype and health. There are also some pieces of DNA are transcribed into long, precursor-mRNAs (pre-mRNAs) that contains insertion elements (introns) and exons. The

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introns are excised (cut out), while exons are spliced together to make mRNA. The excision of introns is catalyzed by a spliceosome, which is made up of five small nuclear RNAs (snRNAs) and over 150 proteins. Exons can be mixed and matched in different ways. Also, pre-mRNA can be modified to make different mRNAs that are translated into different proteins. There are also other types of RNA (such as long non-coding RNA, lncRNA, and micro RNA, miRNA) that can affect the ability of a gene to be transcribed or an mRNA to be translated into a protein. Proteins that bind to RNA can affect DNA transcription. So, many types of RNA exist. They play central roles in gene expression and the generation of phenotypic complexity [4].

Moreover, some types of ions (especially phosphate), methyl groups, small molecules, RNA and proteins are needed to make DNA function properly, turn genes on (or off), or activate regulatory proteins [4]. The epigenetic control mechanisms that lie above the genes also affect DNA transcription. That is, some nucleotides can be modified by adding methyl groups and by later removing them. Moreover, histone proteins and ribonucleoproteins are integral parts of chromosomes that can be modified to affect transcription [4]. As a result, "Heredity does not reside solely in the DNA of the genome; it resides also in an epigenetic cellular state" [21].

That is, systems thinking rejects the idea that heredity is based solely on our DNA [21]. Reductionist thinking once led many to believe that we are merely vehicles for our genes and inheritance is an individual concept that is not related to the environment. Our growth and development from the initial zygote were thought to be due to running a genetic program (similar to a computer program). Inheritance of traits was thought to be innate, not acquired and not affected directly by the environment. The individual is under-valued, even though we are all part of the environment. Reductionist thinking believed that random mutations produce new characteristics. Those that provide reproductive advantages are selected [21]. This led some to think that there was a separation between the body and the mind [1, 2, 21]. It is almost as if biology and genetics were studied without living humans (or other organisms) [21]. In contrast, in systems thinking, we not only exist and are important, but we continuously evolve in harmony with other organisms in our ecosystems and environment. Moreover, it recognizes the importance of horizontal gene transfer (HGT). HGT occurs between and among viruses as well as Bacteria, Archaea and Eukarya. Systems thinking also recognizes that the reductionist concept of an arms race between viruses, Bacteria and Eukarya is anthropocentric and has a faulty political ideology. The metaphor of an arms race implies that

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nature follows political doctrines. Instead, systems thinking leads us to understand that nature follows "a pattern of continuous association and integration" [21]. So, our traits are based on much more than our DNA. They also depend on epigenetic changes to the DNA, as well as the RNA that is transcribed from it and the proteins that are translated from the mRNA.

That is, after proteins are made they can be modified by the addition of ions, functional groups and molecules, such as phosphate, acetyl groups, sugars, and lipids [4]. These are not coded for by DNA. So, there is no single code or blueprint of life. Many genes have very different functions as a newly fertilized egg develops into a fetus, then a baby, and even further in development. Also, the same gene can do very different things in different cells and at different times in the cell cycle. So, the properties of genes depend on their environment and the needs of the cells, tissues and organism. Finally, there are epigenetic changes that can be caused by environmental pollutants and dietary compounds, such as endocrine disruptors. These changes can even be passed down to future generations [4]. However, these changes have not always been obvious.

1.3.3 Characteristics of systems thinking

1.3.3.1 Holistic approach and synergy

In systems thinking, a holistic approach is used to look for interactions and hidden connections between objects in a whole unit [2, 3, 7, 22]. For example, new drugs are being developed that bind to a protein in one part of the cell that then interacts with other proteins and/or DNA in other cells, causing the therapeutic response. One example is the prescription drug vorinostat that binds to the enzyme histone deacetylase. This affects the interactions between DNA and transcription factors on cancer cells, making them recognizable by natural killer cells in the patient [4]. This is a type of immunostimulation based on nonlinear processes. Moreover, the whole is more than the sum of its parts, which can interact in a nonlinear way to produce unexpected effects [2, 3]. Some interactions are synergistic and others are inhibitory [4]. Sometimes the product of an enzyme-catalyzed reaction can stimulate the production of the original susbstrate, causing even more product to be formed. This is positive feedback. For example, some cytokines (interferons, interleukins and growth factors) that are secreted by cells in the immune system circulate in picomolar (10^{-12} M) concentrations during normal, healthy conditions. However, the concentrations can increase up to 1000-fold during trauma or infection.

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Cytokines are multifunctional proteins that can act on the cells that produce them (autocrine mode). That can also act on nearby cells in the paracrine mode. At other times, the product of a metabolic pathway can inhibit previous reactions in the pathway. This decreases the formation of the product in negative feedback. Also, many complex illnesses (like AIDS, many types of cancer and cardiovascular diseases) are treated and sometimes cured with a combination of drugs that work synergistically [23]. However, many of the methods used to try to quantify such synergy are based on the results of pharmacokinetic studies that determine the effects of increasing doses on the results of the treatment. These methods require many resources and they are usually unable to interpret the results and explain the mechanisms of synergy. Instead, methods for systematic synergy modeling have emerged. This includes network methods for studying protein-protein interactions (PPIs), pathway dynamic simulations, synergy network motif recognitions and integrative drug feature calculations, as well as analyses of the genome, metabolome and various other types of -omics. The goal is to provide a comprehensive and deep insight into the mechanisms of drug synergy. This is opening new ways of rational drug design using combinations of drugs [23].

Drug synergy is possible because whole new properties can emerge when atoms, ions and molecules are organized to form organelles, cells, tissues, organisms, communities and ecosystems. Dynamic systems do emerge.

1.3.3.2 Feedback and criticality in dynamic systems

So, systems thinkers realize that living systems are dynamic. They study systems dynamics, which is the identification and modeling of feedback relationships and rhythmic changes that characterize a particular system. They work with all areas of science and most of society to link the structure of complex systems to their behavior over time. So, system dynamics modeling can help physicians and medicinal chemists assess the impact of new drugs in both the short and long term. Moreover, the construction of a system dynamics model enables all stakeholders to provide important input and receive feedback [23].

Feedback has been defined as “a situation in which two (or more) dynamical systems are connected together such that each system influences the other and their dynamics are thus strongly coupled” [24]. Feedback is used in biology and signal transduction to regulate gene expression and cellular metabolism. It’s also used to control muscular, hormonal, immunological and cardiovascular functions as well as affect

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locomotion, vision, proprioception, attention and consciousness. Each of these examples use feedback to determine what works, how it works, and what can be done to affect it. Positive feedback is used to create switch-like behavior through autoregulation of genes as well as the proteins and RNA that they encode. It is also used to create oscillations such as those present in the cell cycle, circadian rhythms and in the brain as waves [24]. Dynamic systems are also complex. They usually have many interacting elements, each exhibiting nonlinear dynamics [25]. Many of the interactions are indirect and not obvious upon initial examination. There are many hidden connections [7].

Dynamic systems also exhibit criticality [25, 26]. That is, they can exist in critical states far from equilibrium. A small change can cause a much larger change in the system. Many features of the central nervous system (CNS) are scale free. The absence of a specific scale makes them scale free. It means that their properties remain similar at any scale. Scale-free networks are poised at criticality. This is a common property of self-similar processes or objects. Operating at a critical state enables a system to have a maximal dynamic range as well as optimal information storage and transmission capacity. Mathematically, scale-free networks follow a power law. That is, the probability that a node will have k links, N, having a particular number of connections, k, follows the equation: $N(k) \sim k^{-\gamma}$. That is, the probability that a node will have k links is directly proportional to the number of connections raised exponentially to the power, γ. The exponents (γ) for long-range temporal correlations (LRTCs) can predict behavior dynamics. LRTCs are important in human cognition and can contribute to brain disorders when they are dysfunctional. Critical brain dynamics can be modulated using closed-loop neurofeedback stimulation. Automatic LRTC modulation may become useful in studying the functional roles of brain criticality in healthy subjects and for developing novel therapies for brain disorders associated with abnormal LRTCs [25].

Critical transitions are also important in many chronic diseases [27]. Human biology and health often exhibit nonlinear dynamics. Changes in the system can lead to tipping points that change it from one stable state to another. This change is called a critical transition or a regime shift. These types of transitions are familiar in chemistry and physics. Examples include phase transitions (gas to liquid to solid and back) and exothermic reactions. Similarly, critical transitions are also being described in biology and biochemistry. Living systems can have sudden changes in their dynamical states, as described by mathematical catastrophic bifurcation theory. That is, at critical junctures, a system can reach a crossroad or bifurcation point, that can lead it into one of two very different directions.

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Unfortunately, it also means that changes in the global climate can occur fast and lead to unforeseen consequences. It also means that a person can go from being quite healthy to severely ill very fast. For example, diseases from microbiome dysregulation to depression often respond in a nonlinear manner to perturbations. Sudden transitions have been seen in pulmonary diseases, depression, type 1 and 2 diabetes, inflammation, the start and termination of epileptic seizures and cancer, as well as cardiovascular and neurodegenerative diseases. Alternative stable states with high resilience can also occur in people after repeated prolonged exposure to antibiotics. Detailed understandings of critical transitions that happen when diseases begin and subsequently progress are helping with healthcare. For example, it is important to identify early warning signs so a disease can be prevented or stopped in its earliest stages [27].

Almost all natural phenomena (from superconductivity to gravity and from economics to life) result from the underlying collective dynamics of their individual components [25]. Neuroscience, cognitive, emotional and motor behaviors will be understood ultimately as the emergent properties of an underlying set of neurons, glia and their connections. However, neurons are nonlinear elements. This can make understanding them rather difficult. Even though the problem can be described in terms that are familiar to biologists, the solution can also be written in terms that are very familiar to physicists [25].

For example, many physicists are familiar with the ferromagnetic-paramagnetic phase transition [26]. When an iron magnet is heated, its magnetization decreases until it reaches zero above a critical temperature, Tc. At higher temperatures, the individual spin orientations change continuously in small groups. As a result, the mean magnetization, which expresses the collective behavior, is zero. At temperatures below Tc, the system will be very ordered and exhibit large domains of equally oriented spins. In this condition or state, there is negligible variability over time. In between these two states, at the Tc, the system has very different properties in both space and time. At temperatures close to Tc, large correlated structures (up to the size of the system) emerge, even though the interactions are with their nearest-neighbors. Also, the largest fluctuations in the magnetization are observed at Tc. At this temperature, the system is highly susceptible to change. A single spin perturbation has a small but finite chance of starting an avalanche or tipping point that reshapes the entire system's state. This is unthinkable in a non-critical state. When these dynamical properties are properly translated to neural terms, there are striking analogies to brain dynamics. Neuromodulators that alter brain states act globally and affect non-specific targets. They can be thought of

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as control parameters, as is temperature in the ferromagnetic-paramagnetic phase transition. It has been suggested that the complexity of brain dynamics is due to underlying critical processes. There are many metastable states at a critical point near possible transitions. As a result, the brain can access a large repertoire of behaviors in a flexible way. This hypothesis proposes that the most fundamental properties of the brain are only possible because it stays close to this critical instability [26]. It is an emergent property of the brain.

1.3.3.3 Emergent properties and scale-free networks

So, the term emerge is essential in systems thinking and has become extremely popular in the 21st century. Scientists and non-scientists frequently say that something has emerged or is emergent. However, the term emergent has been used for much longer in medicine. It implies an unexpected emergency. Emergent conditions are those that appear unexpectedly in patients – often with dire consequences. When this happens, they may have to go to the Emergency Room of a hospital. Still, it is important to note that emergent properties in systems thinking can be good and healthy. That is, life itself is an emergent property that arises when inanimate ions and molecules are organized into cells and non-random networks.

The properties of a network depend on the type of network and its topology, or structure [5, 28]. Networks are made up of nodes (like cells) that are connected or linked with each other. Living systems and the internet are scale free networks in which the properties of the processes they undergo are similar at any scale. Also, they tend to grow, while new nodes are added and new links are formed between nodes. Nodes with the most links are called hubs. Scale-free networks are resilient to random attacks. For example, if a toxin or pathogen were to randomly knock out, or inhibit a reaction that is not a hub in the metabolic network of the cell, it would have little effect. In contrast, if a toxin were to inhibit a reaction catalyzed by a hub protein (like p53) it would have huge effects. So, scale-free networks are dominated by a few well-connected nodes, called hubs. Most nodes in a scale-free network have a few connections, but a small number of nodes can have an almost unlimited number of connections. Such networks have common architectures and properties. They obey fundamental laws that apply equally to cells, computers, language and society. Their organizing principles are important in drug discovery, the treatment of diseases, halting the spread of epidemics, and defending the Internet from hackers [29].

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In clinical trials, another aspect of systems thinking emerges - the placebo effect. Some patients get better, even if given a placebo, which contains no drug, and is often just a sugar pill. It is important to stress that the patients receiving the placebo are told that they can expect a positive response. They simply need to be told by some friendly, respectable or authoritarian person that they look better, or that they are getting better. That is, for the placebo effect to work, the patient must believe that it will be beneficial.

The opposite of the placebo effect also occurs [4]. This nocebo effect is when patients get worse, just because they are told that they are susceptible to a disease. This was seen in the Framingham Heart Study. Women who believed they were prone to heart disease were four times as likely to die from heart disease as women with similar risk factors who didn't believe they were prone to heart disease. Unfortunately, this idea can be taken too far. Patients won't necessarily get better simply by having a positive attitude. Some patients will get better, even though they have a negative attitude. For example, it was once thought that cancer was a disease against one's self, caused by a negative attitude and ulcers were caused by worrying too much. We now know that this is not true. Attitude is not linked to cancer survival. On the other hand, a positive attitude may improve the quality of peoples' lives and help them stay healthy, whether they have cancer or not. Still, bad (healthy) things happen to good people and good things (such as health and longevity) can happen to people (like Sir Winston Churchill) who had very unhealthy lifestyles. Cancers are not caused by low self-esteem, even though being diagnosed with cancer does frequently lower one's self esteem. Moreover, we now know that most ulcers are not caused simply by a bad attitude or by worrying, but by a type of Bacteria, *Helicobacteria pylori*. We don't cure ulcers with laughter or psychotherapy, but with the antibiotic amoxicillin. Still, people can make themselves sick by worrying too much or by self-induced stress, which can encourage the growth of *H. pylori*. Worrying and a feeling of hopelessness can be very bad for one's health. Moreover, almost all diseases have a mental aspect and can be thought of as being at least partially psychosomatic [3]. This can't be predicted from the basic physical and chemical properties of the atoms, ions and molecules that are part of a human ecosystem. Instead, the placebo and nocebo effects are emergent properties that occur when molecules are organized into human and bacterial cells that interact with each other to make up the ecosystem that is a person's human body [4]. Still, happiness and a positive attitude can help prevent diseases and even slow the aging process, while depression can be deadly.

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1.3.3.4 Interdisciplinary and iterative processes

Systems thinking is an inherently interdisciplinary and iterative process. For example, a combination of computer science and biology had led to a joint approach to studying the algorithmic properties of biological systems (molecular, cellular, organismal and ecological). A new view has emerged. Biological processes are inherently algorithms that nature uses to solve computational problems. At the same time, IBM's supercomputer (Watson) is learning medicine and much modern surgery is computer controlled.

The interdisciplinary process requires systems thinkers to become almost multilingual and to know the first rule of effective communication, 'Know your audience'. Just as gift and Gift (poison) mean very different things in English and German, so does linear in math and medicinal chemistry. When analyzing blood for the concentration of a developmental drug, chemists use a 'linear' calibration 'curve'. In math we learn that two points define a line and there is no curvature in a line. There is another example. A physician may read an article in a food chemistry journal that reports the fatty acid content of a vegetable oil. It is important that he or she knows that food chemists use the terms 'acid' and 'fatty acid' very differently that other scientists. So, physicians should (and almost always do) use the word fat instead of fatty acid when talking about the importance of avoiding *trans* fats and saturated fats with their patients. If they think that fish oil is acidic, they might not take it to treat dysplipidemia (high cholesterol) or to prevent many different diseases. Similarly, the labels on foods and beverages must indicate the total fat content and not use the word acid. That way, people who suffer from acid reflux can eat olive oil and take fish oil, which are rich in unsaturated fat, not fatty acids. Still, food chemists are going to continue to say that many foods contain fatty acids – even though they are simply a dietary source of fatty acids, produced by the digestion of triacylglycerides.

1.3.3.5 Avoid dogma in systems thinking

Finally, system thinkers realize that there are very few rules that apply to everything and there is seldom an absolute truth in chemistry or biology. For example, biologists who use systems thinking also realize that not all living creatures will die, despite popular belief [16]. Some organisms may be hundreds of millions of years old and could live for billions of additional years. This concept may even change how we identify 'self', 'mother' and 'offspring'. Bacteria, including those in our

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human body, frequently reproduce by dividing to make identical copies of themselves. When their host dies, they have a feast. They can multiply and spread into the environment to find a new host. There is no way that we can determine how long an individual bacterial cell has been alive. When a bacterium divides in two, which one is the parent and which is the offspring? Similarly, there is a very small eukaryotic organism called the immortal jellyfish (*Turritopsis nutricola*) [30]. They can revert to the sexually immature (polyp) stage after becoming sexually mature. They can make many copies of themselves – as immature polyps. So, if one anthropomorphizes this, every polyp is its own mother, daughter and sister. They can all reverse their life cycle and avoid death. They can still be killed by predators, but their population is increasing. They are originally from the Caribbean Sea, but have spread throughout the world's oceans as ships take up ballast water and discharge it in ports around the world [30]. The point is that systems thinkers try to keep an open mind. They are continuously re-thinking what it is to be human. At least some parts of us (our commensal Bacteria) are reused by new humans and other organisms. More important, not just our eukaryotic cells with 23 pairs of chromosomes make us human, but so also do other cells, as well as viruses and viral DNA.

1.3.3.6 Some systems are more widespread and multifunctional than previously thought

We are also discovering that some systems are more widespread, interconnected and multifunctional than previously thought. The nervous system is no longer thought to be separate from the endocrine and immune systems. All three can be studied using systems thinking. So, we talk about the neuroendocrine immune system. Some of the biochemicals that are produced by our immature immune systems help regulate the growth and development of our nervous systems. There are other biochemicals that are used in both the immune and nervous systems to communicate with each other. Both systems make decisions. The brain helps us recognize and defend ourselves against external threats, while the immune system recognizes and defends us against internal threats caused by invading organisms, pathogens and even cancer. Still, the brain's connection with the endocrine system is part of classical endocrinology [5]. The hypothalamus connects the nervous and endocrine systems through the pituitary gland, or hypophysis. It helps control body temperature, hunger, parenting and attachment behavior, thirst, fatigue, sleep, circadian rhythms and other activities of the autonomic nervous system. When it is dark, the

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pineal gland secretes *N*-acetyl-5-methoxytryptamine, which is better known as melatonin. It helps regulate the sleep-wake cycle. It causes drowsiness and lowers the body temperature. The anterior lobe of the pituitary gland secretes growth hormone, beta-endorphin, luteinizing hormone, follicle stimulating hormone, melanocyte stimulating hormone, adrenocorticotropic hormone, thyroid stimulating hormone and prolactin. The posterior lobe stores vasopressin, also known as antidiuretic hormone and oxytocin. The brain is often called the largest sex organ in the body. It is just as important as the testes, ovarian follicle and corpus luteum in the reproductive system. However, other parts of the body are now known to be part of the neuroendocrine immune system. Naturally, this includes the thyroid and adrenal glands, as well as the sex organs [5].

However, it also includes organs that are not part of the classical endocrine system [5]. This includes the kidneys, which secrete renin, erythropoietin (EPO), calcitrol and thrombopoietin. They are a pair of organs about 10-13 cm long that are located at the rear of the abdominal cavity. The term renal refers to the kidneys, so renal damage and metabolism are synonymous with kidney damage and metabolism. The kidneys regulate pH, electrolytes and blood pressure. They are also part of the endocrine system. They release the hormones renin, EPO, calcitriol and thrombopoietin, while being regulated by antidiuretic hormone, aldosterone and parathyroid hormone. They receive about 20% of the cardiac output [10] and work with the heart, liver, vascular endothelium, and adrenal cortex as part of the renin-angiotensin-aldosterone system (RAAS). The kidneys can also play a major role in heart failure, which is now understood as a multi-organ syndrome in which several deleterious cellular pathways are activated. EPO is an essential glycoprotein hormone that controls the production of red blood cells. About 40% of its molecular weight is due to attached sugars (glycosylation). It promotes the survival of progenitors and precursors of red blood cells by inhibiting apoptosis. Also, EPO plays an important role in the brain's response to neuronal injury and in the process of healing wounds. The EPO receptor is also found in bone marrow as well as the peripheral and central nervous systems. Calcitrol (also called 1,25-dihydroxycholecalciferol or 1,25-dihydroxyvitamin D_3) increases the concentration of calcium (Ca^{2+}) in the blood by increasing its absorption from the gastrointestinal tract (GIT). Thrombopoietin is a glycoprotein protein hormone. It is also known as megakaryocyte growth and development factor. It is produced in the kidneys and liver and regulates the production of blood platelets by the bone marrow. It stimulates the production and differentiation of

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megakaryocytes, the cells in the bone marrow that fragment into many platelets [5].

The adrenal glands are on the top of the kidneys [5]. They make hormones in response to stress. This includes cortisol, adrenaline (epinephrine) and noradrenaline (norepinephrine). They also produce androgens in their innermost cortical layer. The adrenal glands affect kidney function by secreting aldosterone. They also produce androgens in their innermost cortical layer. The adrenal glands affect kidney function by secreting aldosterone. Adipose tissue produces adipokines. The stomach secretes gastrin, ghrelin, neuropeptide Y, somatostatin, histamine and endothelin. Ghrelin increases food intake and fat mass, while activating the reward network in the mesolimbic system of the brain. Neuropeptide Y (NPY), like ghrelin, stimulates the appetite. It is a neurotransmitter in the brain and autonomic nervous system. It is a vasoconstrictor and makes adipose cells grow in fatty tissue. It is also produced in the hypothalamus and other parts of the brain. It increases food intake and the storage of energy as fat (triglycerides). It also helps to reduce anxiety and stress, reduces the perception of pain, affects the circadian rhythm, reduces voluntary alcohol consumption, lowers blood pressure and controls epileptic seizures. NPY is also important in eating disorders and obesity, in which there is an increase in the concentrations of glucocorticoids in blood plasma. This causes an increase in not just glucose, but also NPY, through activation of type II glucocorticoid receptors and by blocking the negative feedback of corticotropin-releasing factor (CRF) on the synthesis and release of NPY. In the meantime, insulin resistance causes the NPYergic activity to be abolished. Insulin resistance is a resistance to the beneficial effects of insulin that leads to increasing concentrations of glucose in the blood. It should be noted that a diet high in fat and sugar stimulates the release of NPY, causing abdominal fat to increase. Also, elevated concentrations of NPY may be associated with resilience against and recovery from posttraumatic stress disorder [5].

The heart and skeletal muscles are also endocrine organs [5]. They have cardiomyocytes and myocytes that secrete hormones. This includes atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), the C-type natriuretic peptide, adrenomedullin, proadrenomedullin N-terminal peptide and endothelin-1. They help maintain the volume of the extracellular fluid and proper blood pressure. ANF suppresses the sympathetic outflow of the heart. Both ANF and BNP reduce vascular smooth muscle tone and peripheral vascular resistance while decreasing one's desire for salt and water, thus leading to a reduction in the volume of extracellular fluid. ANF also inhibits the secretion of vasopressin from the pituitary gland, thus

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decreasing water reabsorption in the kidneys. Myocytes secrete myokines. They are secreted when muscles contract and then affect other tissues. Also, exercise causes an increase in cytokine levels, which can affect the immune system. Interleukin-6 (IL-6) is a major myokine that is released when skeletal muscles contract. Myokines like IL-6 not only help the neuroendocrine immune system, but also mediate changes in metabolism that occur during exercise. Skeletal muscles also secrete members of the tumor necrosis factor-β (TNF-β) superfamily of transforming growth factors that induce signaling molecules that help regulate cell growth, regeneration, differentiation, transformation, and death. Skeletal muscles also produce a glycoprotein called follistatin, which is not in the TNF-β superfamily. Follistatin inhibits the synthesis and secretion of the FSH from the pituitary gland. It also inhibits the activities of activin and myostatin in the activin-myostatin-follistatin (AMF) system. Skeletal muscles produce another protein called irisin that also inhibits activin and myostatin. Irisin is made by hydrolyzing the extracellular portion of fibronectin type III domain–containing protein (FNDC). Irisin is secreted into the blood. Bone morphogenic protein (BMP) is also produced by skeletal muscles. It is in the TGF-β family of proteins that are involved in skeletogenesis and differentiation as well as embryonic development. C1q/TNF-related proteins (CTRPs) are also secreted by skeletal muscles. One of them, CTRP15, is also known as myonectin. Its concentration increases after exercise, but like BMP, its physiological role is not well known. Finally, skeletal muscles also produce and secrete brain-derived neurotrophic factor (BDNF), which helps regenerate myocytes and induce myogenesis [5]. It also produces osteocalcin, sclerostin, fibroblast growth factor 23 and lipocalin 2. Lipocalcin 2 is released to prevent bacterial infections and it functions in the brain to help control appetite.

Even though the skeletal system was once thought to be just connective tissue that also produced blood cells and helped regulate Ca^{2+} homeostasis, it is now known to be part of the neuroendocrine immune system [5]. Bones undergo continual autopoiesis. Osteoclasts remove bone, while osteoblasts re-make bones. Also, osteocytes help regulate the processes of bone remodeling and mineralization. That is, the bone marrow produces hematopoietic stem cells (HSCs) that can differentiate into myeloid cells (monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes/red blood cells, megakaryocytes/platelets and dendritic cells) and lymphoid cells (T-cells, B-cells and natural killer cells/NK cells). At the same time, bones interact with adipose tissue and muscles to regulate bone density and body fat. Bone marrow also produces adipocytes. Obesity can lead to osteoporosis in part by reducing muscle

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mass. On the other hand, exercise increases muscle and bone mass, while decreasing fat mass. Adipose tissue secretes several pro-inflammatory cytokines such as IL-6 and TNFα that affect osteoclasts. Bones produce osteocalcin and osteopontin that help to control body weight and glucose homeostatis. So, there is feedback between the skeleton and other endocrine organs. Also, myostatin affects fat and bones. Adipokines affect the liver and skeletal muscle and affect insulin sensitivity. Adipose tissue also produces aromatase enzymes which catalyze reactions that are in the biosynthetic pathway for estrogen. That is important because estrogens have important roles in bone homeostasis. In post-menopausal women, circulating estrogen levels decrease, which leads to an increase in the number of adipocytes and decrease in osteoblasts. Ligands that bind to another nuclear hormone receptor, PPARγ, induce adipogenesis in the bone marrow, as well as inhibit osteogenesis. Parts of the Wnt signaling system can inhibit adipogenesis while promoting osteogenesis. Members of the epidermal growth factor family, such as Pref-1, or pre-adipocyte factor-1, can inhibit both adipogenesis and osteogenesis [5].

Adipose tissue contains not only white and brown adipocytes, but also cells known as either beige fat or brite fat (from brown and white) [5]. These beige fat cells emerge when white visceral adipose cells (VATs) transdifferentiate. VAT cells differentiate further into heat-producing brown adipose tissue (BAT) that has anti-obesity and anti-diabetic effects. These interactions may be due to the fact that adipocytes, osteoblasts and myoblasts originate from a common progenitor, pluripotent mesenchymal stem cells, or MSCs. There is an inverse relationship between MSC differentiation into either adipocytes or osteoblasts. This is mediated by cross-talk between signaling pathways that are activated by PPARγ, steroid receptors and other cytokines. Also, environmental factors and lifestyles affect adipogenesis in the bone marrow despite osteoblastogenesis. This means that bone marrow MSCs can undergo several types of differentiation. They can dedifferentiate and transdifferentiate in response to changes in the microenvironment. Finally, myostatin is an important link between fat, bone and muscle homeostasis. It is an important regulator of MSC proliferation and differentiation. It is expressed in the early steps in healing bone fractures. It affects the proliferation and differentiation of osteoprogenitor cells, as well as muscle mass and bone strength. MSCs can modify and influence almost all the cells of the innate and adaptive immune systems. MSCs derived from the bone marrow are also important in organ repair and may be used eventually to produce new organs for people who now need to wait for an organ transplant [5].

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So, it has been proposed that there is a bone-adipose axis [5]. The number of mature osteoblasts and adipocytes in the bone marrow depends partly on the way that MSCs differentiate. So, factors that increase bone marrow adipogenesis inhibit osteoblastogenesis, resulting in decreased osteoblast numbers, diminished bone formation and, potentially, inadequate bone mass and osteoporosis. In addition, mature osteoblasts and adipocytes secrete factors (including PPAR-γ2, Wnt, IGF-1, GH, FGF-2, estrogen, the glycoprotein 130 signaling cytokines, vitamin D and glucocorticoids) which can change the cell fate of MSCs and function of each other. That is, cytokines in the IL-6 family exert their effects through transmembrane receptor complexes comprised of ligand-specific receptors and the shared transmembrane receptor glycoprotein 130, or GP130. These cytokines are pro-osteogenic and anti-adipogenic. PPAR-γ2 regulates adipocyte differentiation and lipid storage in mature adipocytes. Wnts are glycoproteins that regulate many processes that occur during development. Wnt signaling plays an important role in bone formation. GH and IGF-1 correlate with bone marrow adiposity. IGF-1 is produced not only in the liver, but also in osteoblasts, independent of control by GH (which controls IGF-1 biosynthesis in the liver). As the level of IGF-1 decreases in the elderly, it can cause bone loss and osteoporosis. Also, a loss of estrogen can increase the amount of adipose cells in the bone marrow. FGF-2 is found in marrow stromal cells, osteoblasts and osteocytes and is stored in the extracellular matrix of bone. It is pro-osteogenic and anti-adipogeneic. Vitamin D promotes osteoblast differentiation and inhibits bone marrow adipogenesis. Finally, mature osteoblasts and adipocytes express some of the same genes, but the proteins that they encode have different functions in these different cells [5].

Bone is also an energy-regulating endocrine organ that regulates mineral metabolism [5]. That is, osteoblasts secrete the hormone osteocalcin, which helps to regulate energy metabolism. It does this by regulating insulin secretion by pancreatic β-cells, testosterone secretion by testicular Leydig cells, fatty acid metabolism in the liver, and the sensitivity of muscle and fat to insulin. There is an osteocalcin-bone-pancreas-testis axis that helps to regulate energy metabolism and the production of sex hormones. Osteocalcin helps to regulate male fertility. It has glutamic acid residues that can be carboxylated. When it is under-carboxylated, it is biologically active and can stimulate pancreatic β-cells to secrete insulin, while promoting insulin sensitivity in other organs and stimulates the biosynthesis of testosterone in the testis [5].

Both osteoblasts and osteocytes secrete FGF23 [5]. It affects the kidneys, parathyroid gland, choroid plexus, and pituitary gland. FGF23

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helps to regulate the concentration of phosphate (HPO_4^{2-} and $H_2PO_4^-$) and 1,25-dihydroxyvitamin D_2 in the blood [5].

The bone marrow also produces early outgrowth cells (EOCs) that secrete water-soluble hormones that reduce progressive organ fibrosis in chronic kidney disease (CKD) and heart failure [5]. That is, fibrotic injury occurs in many diseases, including CKD. It also has adverse effects on the heart. The EOCs secrete factors that inhibit pro-inflammatory tumor growth factor β (TGF-β) and the production of collagen. This means that these factors may become useful in cell-free treatments of heart and kidney damage in CKD [5].

Finally, it has been shown that phytoestrogens, saponins and flavonoids can affect the osteogenic and adipogenic pathways of differentiation [5]. The shift towards osteogenesis occurs by inhibiting PPAR-γ and C/EBPα transcription factors. So, phytoestrogens, saponins and flavonoids may help to prevent or minimize bone loss and other metabolic diseases [5].

New properties and functions of the renin-angiotensin system or RAS (not to be confused with the oncogene *RAS* or oncoprotein Ras) have also been discovered [31]. RAS controls blood pressure as well as thirst, salt retention, and fluid homeostasis. For decades, the main effector for the RAS was known to be angiotensin II (AngII). AngII is made from the precursor protein angiotensinogen by the reactions catalyzed by renin, angiotensin converting enzyme (ACE), chymases, and various carboxy- and amino-peptidases. AngII exerts its pressor effects on the cardiovascular and renal systems by binding to AngII type 1 and AngII type 2 receptors (AT1R and AT2R). These receptors are mutually antagonistic, enabling them to control blood pressure, water, and electrolyte homeostasis. The RAS is currently targeted by prescription drugs that control high blood pressure. The main therapeutic targets are ACE and AT1R. So, there are ACE inhibitors (ACEIs) and AT1R blockers (ARBs). However, much more is now known about the RAS. It is an example of how today's problems can come from yesterday's solutions. That is, the RAS may play an important role in autoimmune diseases, cancer, and neurodegenerative disorders. Many, if not all, components of the RAS can be produced by resident or infiltrating cells, such as mast cells, as a consequence of infection or injury. Also, there is an alternative pathway for angiotensin in which a newly described enzyme, ACE2, liberates angiotensin 1-7 (Ang1-7). It acts on the Mas receptor, which was originally identified in 1986 through the Mas protooncogene that codes for proteins that help regulate cell growth and differentiation. More recently, the ACE2/Ang1-7/Mas pathway was found to have nothing to do with controlling blood pressure. We also know now that RAS affects

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inflammation, can modulate the immune system and help in tissue development and repair. We also know that the systems that respond to inflammation and wounds use white blood cells and cytokines for repair and remodeling. However, categorizing white blood cells as being simply immune cells is reductionist. It keeps us from realizing that their function changes over time and depends on the environment. Moreover, an inflammatory response is not always just an immune response. That is, inflammation can suppress adaptive immune responses. So, RAS it has a direct role in healing. The discovery of the ACE2 enzyme and Ang1-7 has increased commercial interest in the role of the RAS in wound healing and is becoming a hot topic [31].

The lymphoid immune system is also more widespread than previously thought. All the tissues in the human body benefit from two types of vessels [32]. Blood vessels deliver oxygen (O_2) and nutrients, while lymphatic vessels remove waste products from tissues. Immune cells also use these blood vessels to access tissues and lymphatic vessels as their exit routes. Moreover, many biochemicals that were first identified as being part of the immune system are now known to be crucial in the development of the CNS and its function. So, the immune and nervous systems are closely linked and communicate extensively with each other [32]. The immune system communicates with the nervous system by using cytokines, while the nervous system communicates with the immune system by innervating it [33].

Even though the brain was once thought to enjoy an immune privilege, we now know that it contains microglia in the parenchyma (functional parts of the brain) and an extensive immune repertoire in the meninges and choroid plexus [33]. So, the CNS is affected by immune activity from embryonic development through old age. Microglia in the brain help prune and maintain neuronal circuits both early in life and in adulthood - during learning. We also know now that the microglia and other cells of the nervous system are affected by signaling from the peripheral immune system. So, neuronal activity is influenced by the immune system and is even dependent on it [33].

For many decades, it was known that the adaptive immune system had lymphoid cells that produced important cytokines [34]. It is now known that there are also innate lymphoid cells (ILCs) that produce their own cytokines, such as T-helper cells, or T_H [34, 35]. ILCs not only orchestrate the development of the immune response in the fetus, but coordinate the adaptive immune response later in life. There are three types of ILCs: ILC1, ILC2 and ILC3. They don't express cell-surface markers that are associated with other immune cell lineages. Moreover, they don't express

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a T cell receptor and don't respond to antigens [34]. After cells are infected with viruses or Bacteria, ILC1s express interferon-γ (IFN-γ). This induces the activation of macrophages and the production of oxygen radicals. For example, natural killer cells are cytotoxic ILC1s. Some ILCs (ILC2) produce interleukins that induce vasodilation, the production of extracellular matrix and mucus and the activation of M2 macrophages (also called 'alternatively activated macrophages'). Finally, extracellular microbes such as Bacteria and fungi activate ILC3s to produce IL-17, IL-22 and lymphotoxins. They lead to the production of anti-microbial peptides (AMPs) by epithelial cells and the generation and recruitment of neutrophils. So, ILC1s, ILC2s and ILC3s are the innate counterparts of the T_H1, T_H2 and T_H17 subsets of helper T cells [35].

At the same time, we now have a better understanding of the lymphatic system. It was once thought to be like a sewer that cleared fluids, proteins and debris from the interstitial space, but was not present in the brain [36]. It was known to transport dietary fat and absorb drugs. However, we now know that the lymphatic system is important in fluid homeostasis, lipid metabolism, cancer, transplant rejection, infection, inflammation, metabolic diseases and in controlling the immune system. In cancer, lymph nodes drain solid tumors and are the place where metastasis begins. Moreover, there are extensive links between lymphatic function, lipid deposition, obesity and atherosclerosis. So, some researchers are investigating the possibility of targeting the lymphatic system with new drugs. This includes using nanotechnology to mimic endogenous biopolymers and lipid conjugates that can use endogenous lipid transport processes [36].

We all know that the skull and cerebrospinal fluid (CSF) protect our brains from blunt force [36]. At the same time, it was thought that the blood-brain barrier (BBB) was a like a fortress that restricted the entry of pathogens into the brain and limited its inflammation. It was also thought that the size of the response within the CNS was less robust than that in other tissues. So, when antigens entered the brain, it was thought that they elicited a relatively weak inflammatory response. This concept was termed "immune privilege". It implied that healthy human brains are well-protected from the potentially adverse consequences of immune actions. One of the corollaries to this theory of the immune privileged brain was there was no functional lymphatic system in the brain, even though it is present in almost all other organs. The lymphatic system was known to transport interstitial fluid and waste products out of tissues and into the blood, where they are targeted for removal. At the site of infections, this

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transport system delivers antigens and professional antigen presenting cells to lymph nodes for education of the adaptive immune response [36].

However, a paravascular transport pathway was discovered in the brain that serves as a lymphatic system that clears interstitial fluids and is called the 'glymphatic system' to emphasize the importance of glial cells in it [36, 37]. That is, recirculation and clearance of interstitial solutes use an aquaporin-4 water channel. Thus, the lymphatic vessels that are associated with the sinuses are connected to the glymphatic system in one unified network. Moreover, the efflux pathways used to clear solutes from the CNS make them important in several diseases, including protein-based pathologies and immune controlled demyelination. For example, clearance of β-amyloid in Alzheimer's disease as well as α-synuclein in Parkinson's disease, Lewy body disease, multisystem atrophy, and diseases caused by prions can emerge if the functional competence of these efflux pathways is compromised. If they become dysfunction, it could lead to the suppression of not only solute clearance, but also fluid efflux itself. This can lead to problems such as intracranial hypertension and glaucoma [37].

The glymphatic system is activated during sleep, so that neurotoxic waste products that accumulate while awake (like amyloid-β) can be removed [36, 37]. More recently, conventional lymphatic vessels have been found in the meninges (membranes that enclose the brain and spinal cord) [36, 38]. The presence of glymphatic and lymphatic systems in the brain has important implications for immune control within the brain and the accumulation of neurotoxic plaques formed by amyloid-β [38]. Moreover, unexpected accumulation of prescription drugs and neurotoxins may occur during sleep deprivation [38].

The immune system is now known to be more widespread than previously thought [39]. That is, the CNS was thought to be able to tolerate an influx of antigens without generating an inflammatory response. Supposedly, the BBB isolated and protected the CNS from potentially harmful actions caused by the immune system. It was thought that many immune-based diseases of the CNS happened when immune cells breached the BBB. We now know that lymphocytes circulate through the healthy brain and immune responses can occur in the parenchyma without lasting consequences. Moreover, there is extensive cross-talk between the brain and the periphery. The glymphatic system removes waste and enables an extensive exchange between cerebral spinal fluid (CSF) and interstitial fluid (ISF). The CSF enters the brain parenchyma and spinal cord via aquaporin 4 water channels on astrocytes that surround the vasculature in the brain. As CSF enters, it forces the ISF towards the perivenous space where it collects and drains toward the cervical lymph

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nodes. The ISF carries extracellular proteins and solutes with it. At the same time, the lymphatic vasculature lines the dural sinuses and meningeal arteries [39, 40]. These functional lymphatic vessels express all of the molecular markers of lymphatic endothelial cells [38]. They carry both fluid and immune cells from the CSF, and are connected to the deep cervical lymph nodes. The unique location of these vessels may have impeded their discovery previously. The discovery of the CNS lymphatic system is causing a reassessment of many basic assumptions in neuroimmunology and is providing important information about the etiology (causes) of neuroinflammatory and neurodegenerative diseases [40].

The CSF−ISF exchange moves the fluid through the parenchyma [37]. It flushes extracellular debris from the parenchyma. Also, pathogenic antigens that may be present in the CNS at the time of an infection can be removed. This process accelerates during resting sleep, so it may be one important reason why sleep exists. So, “a good night’s rest may literally clear the mind” [37].

In addition, the field of neurology has been linked to virology and immunology to produce the fields of neurovirology and neuroimmunology [37]. There are many interactions between viruses and neurons, as well as immune cells and neurons. As a result, the brain is no longer considered to be like a satellite in the body that is isolated from the effects of the host immune response. Instead, it is actively monitored by, and accessible to blood-borne lymphocytes and their signaling molecules. Moreover, even though some viral infections are often fatal, some viruses like the cytomegalovirus (CMV) can establish long-term quiescent infections that may be reactivated later in life and may cause glioblastoma multiforme. This broadens our knowledge about the pathogenic potential of neurotropic viruses, and how the host response can limit or eliminate these threats [37].

Moreover, immune dysregulation is linked with neuroinflammation, which is the way the CNS responds to disturbed homeostasis [41]. Smoldering neuroinflammation is an important causative factor in neurodegenerative diseases [5, 41]. Responses to neuroinflammation can be healthy during normal brain development [41]. Microglia enter the CNS very early in embryonic development and help form healthy brains. Other reactive components of the CNS include infiltrating myeloid cells, astrocytes, oligodendrocytes, cytokines and cytokine signaling pathways. Also, B-cells, myeloid cells and T cells mediate CNS disorders, paraneoplastic neurological diseases and multiple sclerosis [41].

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The interactions between the CNS and peripheral nervous system with the immune system have been reviewed [32, 42]. The immune system not only defends us against pathogens, but it also responds to danger signals released from damaged tissues, while playing important roles in tissue homeostasis and maintenance [32]. T cells in the meninges affect brain function, including cognitive function. Many, if not all neurodegenerative diseases are associated with the immune system [32]. Not only do immune cells support and shape the brain, but also the CNS affects immune responses [33]. Immune cells within the CNS (microglia) as well as the surrounding meninges and choroid plexus are important in maintaining proper brain function [43]. Proper CNS function depends on immune activity from embryonic development to old age. Microglial cells prune and maintain neuronal circuits, while signaling from the peripheral immune system affects microglia. The immune system communicates with the CNS through cytokines, while the nervous system communicates with the immune system by innervating lymphoid organs [43]. As we will see in Chapter 2, the gut microbiota also communicates and interacts with the immune and nervous systems.

The lymphatic vessels drain ISF and allow leukocytes to migrate from the CNS to the cervical lymph nodes that drain the fluids [39]. There is a continuous flow and exchange of proteins within the brain and the blood. Debris is taken out of the brain and deposited into the blood for removal. In addition, activated CD8+ T cells provide holistic protection from pathogens. If a person is infected with several antigens that are restricted to different tissues, the CD8+T cells that are activated in response to one pathogen might migrate to a tissue in which none of that antigen is present. We are only beginning to understand the various possible responses of hosts to multiple antigens, allergens, vaccines and viruses. So, the field of heterologous immunity has emerged. It studies the beneficial and detrimental effects of T cells that interact with different antigenic peptides from distinct pathogens. It has been shown that subacute viral infections of the CNS can occur. Such viruses may establish long-term quiescent infections that can be reactivated later in life [39]. For example, over 80% of the people who have glioblastoma multiforme have the human CMV in their blood and brain tumors [5, 44]. So, researchers are exploring the potential ability of adoptive T-cell therapies to control the reactivation of CMV as well as the progression of solid tumors that contain it [45]. Moreover, CMV is being evaluated as a vaccine vector for eliciting potent T-cell responses [5, 44, 46]. Note that a vector in biotechnology is not a line segment (in math and physics), but a vehicle that is used to transfer genetic material, such as DNA.

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Not only have new functions been identified for organs that we've known about for a long time, a completely new organ was identified by re-evaluating computed tomography (CT) images of the mesentery, as part of the Visible Human Project® (VHP) [47-49]. Data that were already present in the VHP were used to produce a digital model and reference atlas of the mesentery, which was previously thought to have a fragmented structure. This model showed the contiguity of the mesentery region [47]. Distinctive anatomical and functional features showed that the mesentery is an organ in and of itself [48]. It starts in the superior mesenteric root region and fans out to span the intestine from duodenum to rectum. However, the continuity only became apparent when the mesentery was exposed in a new way. When the peritoneum was divided, a plane that is formed by the mesentery and underlying fascia became apparent. When peeled away from the fascia, the mesentery became apparent as a discrete entity. When this process was repeated from the duodenum to the rectum, the entirety of the mesentery became obvious. It clarified the anatomy of the mesentery and produced an improved surgical nomenclature that has become applicable to all forms of resectional colorectal surgery. Moreover, previous research had been done under a variety of different classifications. Now that the mesentery is recognized as a unique, contiguous organ, seemingly disparate findings were brought together in a unified vision. This is helping to improve our understanding of colorectal cancer, inflammatory bowel disease, diverticular disease, cardiovascular disease, diabetes, obesity, and metabolic syndrome [48].

It is interesting to note that Leonardo Da Vinci recognized this long before CT images were available. He saw that the mesentery was associated with the small intestine and colon, was contiguous and converged centrally [48]. This is consistent with him being a systems thinker who was way ahead of his time [50].

The mesentery is part of the gut, or gastrointestinal tract (GIT), which is now recognized as not just an organ that digests food and generates solid waste. It is also a sensory organ that presents the largest and most vulnerable surface in the human body to the outside environment [51]. It must perform a balancing act. It must allow nutrients to enter the body, while excluding and/or detoxifying pathogens and environmental toxins. To do this, the GIT has a variety of systems to sense the environment as well as send and receive signals from the rest of the body. Sensory information is exchanged with the enteroendocrine hormonal signaling system, the nerves in the gut (enteric nervous system or ENS), the neuroimmune system and the local tissue defense systems. There are nutrient receptors on enteroendocrine cells. There are 12 major types of

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enteroendocrine cells that secrete over 20 hormones, including ghrelin and leptin (for appetite control), histamine and gastrin (to stimulate the secretion of gastric acid), serotonin (also known as 5-hydroxytryptamine or 5-HT) and glucagon-like insulinotropic peptide (GIP), which stimulates the release of insulin. When toxins or irritants enter the GIT, serotonin is released from enteroendocrine cells. It acts on vagal nerve endings in the wall of the gut. This activates the vomit center of the brain. There are also taste receptors that were first identified in the oral cavity and fatty acid receptors that release bile salts and lipases to help digest fatty acids and triacylglycerides. There are also receptors for amino acids, peptides and proteins as well as phytochemical receptors for which specific phytochemicals in herbs and spices are ligands. This includes cinnamaldehyde and cinnamic acid in cinnamon and allyl isothiocyanate (from wasabi and mustard). Some of the phytochemical receptors are in a family or group called transient receptor potential (TRP) ion channels. They are ligand-gated ion channels that produce an inward current when they are activated. They are also important in vision, taste, olfaction, hearing, touch, thermosensation and osmosensation. So, the sight, smell or even thought of food can stimulate and activate the digestive tract [51].

There are also extrinsic and intrinsic neural pathways that carry sensory information in the GIT [51]. There are primary afferent neurons that lead to active sensation when they are activated. There are also four extrinsic sensory pathways: the vagal, thoracolumbar, lumbosacral and viscerofugal pathways. They can be classified based on the arrangement and morphologies of their nerve endings. Some have intraganglionic laminar endings, while others have mucosal afferents, muscular–mucosal afferents, intramuscular arrays and vascular afferents. Vagal sensory nerve endings contain receptors for hormones like ghrelin and serotonin that are released from enteroendocrine cells. In addition, Bacteria in the gut release biochemicals that activate the vagus afferent nerves. The intestinofugal neurons exist only in the GIT. Their cell bodies are in the wall of the gut and have processes that extend to pre-vertebral ganglia, other digestive organs and the CNS. There are also large multi-polar intrinsic primary afferent neurons (IPANs) with unique electrophysiological properties that limit their firing rates. IPANs, along with interneurons and motor neurons respond directly to mechanical stimulation. There are also Bacteria in the gut that can metabolize some components of food that our eukaryotic cells can't digest. Bacteria are also important in the development and maturation of the gut and the host's immune system. They produce bacterial antigens that activate toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors. Once the TLRs and

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NODs are activated, antimicrobial defense molecules (ADMs) are produced that defend the commensal Bacteria and their host from pathogenic Bacteria [51].

In addition, there is a gut-brain axis with interactions between the gut microbiota, autonomic nervous system (ANS), ENS and CNS [52]. It connects the emotional and cognitive centers of the brain with functions in the GIT. There is bidirectional signaling between the gut microbiota and the brain. Bacteria in the gut have important roles in anxiety, depression, Parkinson's disease, Alzheimer's disease and autism spectrum disorders. Gut Bacteria are also important in maintaining the integrity of the cells lining the gut and preventing a loss of permeability and the release of pro-inflammatory lipopolysaccharides. So, functional gastrointestinal disorders (FGID) such as irritable bowel syndrome (IBS) have been linked to mood disorders and a disruption of the gut-brain axis. At the same time, Bacteria in the gut make melatonin, which is also released by the pineal gland at night. Melatonin is biosynthesized at levels up to 400 times that of the pineal gland (which reaches its maximum after eating). It is a pleiotropic and multitasking hormone. Melatonin plays important roles in the circadian rhythm, which has many important effects on the immune system. Moreover, melatonin is a powerful antioxidant that stimulates the production of other endogenous antioxidants. The decline in the production of melatonin with age is thought to contribute to immunosenescence and potential development of many forms of cancer. Melatonin is also used clinically to help treat bipolar disorder, Alzheimer's disease, Parkinson's disease, depression and fibromyalgia [52].

The importance of the gut microbiome in human health and disease will be discussed further in Chapter 2.

1.3.4 The roots of systems thinking

The roots of systems thinking are found in traditional medicine, in which humans were thought to be a part of nature, not separate from it and should not be in conflict with it [4]. Traditional medicine looked to nature to find medicines [4]. This is part of the foundation of holistic medicine. It is only natural that humans should use medicinal plants to treat diseases. Our ancestors have been doing it for a long time [53-55]. For example, oral tradition indicates that a Tanzanian medicine man was trying to find a cure for a dysentery-like illness about 100 years ago. He saw that a porcupine with the same symptoms was eating small amounts of the roots of a poisonous plant, called mulengele (*Vernonia amygdaline*) [53]. This led to it being used to treat the dysentery-like illness, along with similar

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diseases. Chimpanzees that were infected with intestinal nematodes were also seen chewing the pith of *V. amygdaline*. In contrast, chimps that were not infected did not eat it. To get to the pith, sick chimps had to use their intelligence and learning skills to remove the outer bark and leaves. So, chimps are similar to humans. We both learn what to eat by observing others. We also learn how to treat illnesses from others. Natives who were infected with the intestinal parasite *O. stephanostonum* learned from their elders and treated it by chewing the pith of *V. amygdaline*. Through effective communication and feedback, several African ethnic groups started using a concoction from *V. amygdaline* to treat malarial fever, schistosomiasis (sleeping sickness), amoebic dysentery and other gastrointestinal illnesses. There are other species of *Vernonia* that have been used by people in India and native America. Chimps and humans avoid eating the toxic leaves, but goats do eat them and often die from poisoning [53]. However, a central nervous system and higher cognitive skills are not needed. Even moths, ants and fruit flies self-medicate [54]. Janzen et al [55] may have been the first to document such behavior. So, self-medication has been defined as the anti-parasitic use of secondary plant chemicals or other non-nutritive substances [55]. It is older and more traditional than the human race.

This tradition is continuing. The U.S. FDA has approved a topical ointment (sinecatechins 15%, Verege ®) made from a mixture of natural ingredients extracted from green tea [56]. It is used to treat external genital and perianal warts caused by the human papillomavirus. The active ingredients are catechins that are extracted from green tea. They are immunomodulators that inhibit major viral functions [56]. Also, at least eight different herbal medicines are undergoing clinical trials using Good Clinical Practice (GCP) as defined by the FDA and other governments' regulatory agencies [57]. So, even the FDA is permitting complete plants and herbs to be tested for safety and efficacy and not just individual new molecular entities (NMEs), also known as new chemical entities (NCEs). This is based on systems thinking.

1.3.5 Autopoiesis and Gaia

Another aspect of systems thinking is the realization that we continuously break down and re-make the many proteins, subcellular organelles, cells and tissues in our bodies, unlike any machine [2-4]. This is called autopoiesis, or self-making. It is the organization of the living. Machines do not make themselves. They are made by humans (or chimpanzees). In contrast, living systems are self-organizing. For example,

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the turnover of blood cells in someone who weighs about 70 kg is close to 1 trillion cells per day, including 200 billion erythrocytes (red blood cells) and 70 billion neutrophilic leukocytes (white blood cells). This remarkable process of cell renewal is done by the small population of bone marrow cells called hematopoietic stem cells. Even our bones are being broken down and re-made. Osteoclasts break down bone tissue, while osteoblasts re-make it. Our memories, structures, shapes and forms stay almost the same, even though many of the proteins are being broken down and re-made. Somehow, some people can remember things for over 100 years, even though many of the molecules in their bodies don't last nearly as long. Most last just a few days or weeks. Brain cells are continuously interacting and changing their connections with other brain cells. Cognition, thinking and the mind are not defined by molecular events, but by interactions between cells, tissues and organs. The mind is not a thing that is located in the part of the machine called a brain, but it is process that is an integral part of life [2, 3].

It is widely recognized that humans (and other organisms) break down and re-make much of their body. This almost always refers to the human eukaryotic cells and tissues. However, the Bacteria in our bodies are also being broken down and re-made. If we start to think of our microbiome as being part of what makes us human, then we should remember that this includes Bacteria, which play many essential roles in keeping us alive and healthy.

The lifespan of a cell depends largely on how harsh the environment is to which it is exposed [58]. The cells that line the acidic surface of the stomach last only about five days. Inside the intestines, cells live an average of 15.9 years. Epidermal (skin) cells live only about two weeks. Skeletal cells live a little more than 10 years and muscle cells in the ribs last 15 years. However, neurons in the part of the brain called the visual cortex can last a lifetime. There are also stem cells throughout the body that can divide to produce another stem cell plus a differentiated cell. When a stem cell divides by meiosis, it produces haploid germ cells (spermatozoa and oocytes). When a stem cell divides by mitosis, it produces two diploid cells. They can be two stem cells (identical parent and daughter cells) or a stem cell plus a differentiated cell, or two differentiated cells. Stem cells can last a lifetime, but cancer stem cells can end a life. Stem cells provide the opportunity to regenerate cells that die, or to produce more specialized cells (such as white blood cells or cancer cells). So, when a stem cell divides, it can produce another stem cell and a differentiated cell, each with the same DNA, but with very different ways of 'reading' the genetic code to produce very different cells [58].

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There has been some controversy about whether or not all of our body is broken down and re-made [58]. Though not stated in that article [58], it is very doubtful that anyone (especially a particle physicist [1]) actually believes that one of the most widely connected hubs in the cellular network, the proton (H^+), is ever broken down into its smaller subnuclear particles in a cell. Another widely connected hub, H_2O, is not broken down into H_2 and O_2 in human cells. On a larger scale, there are other exceptions. Neurons in the cerebral cortex, basal nuclei and many other parts of the brain are not re-made if and when they die [59]. Similarly, photoreceptor cells, auditory cells in the organ of Corti in the cochlea (inner ear) and tooth enamel are not broken down [59]. Stem cells are another exception. Some of them last a lifetime. Embryonic stem cells are totipotent and can differentiate into any of the various types of cells in the body, while hematopoietic stem cells are pluripotent and can make any type of blood cell.

Moreover, the components of the cell membrane (lipids, carbohydrates and proteins) and internal organelles, along with intracellular proteins, are continuously being broken down and re-made, even if the cell lasts a lifetime. Even the DNA in the cell nucleus is copied during every cell division. Mistakes that are made are corrected by reactions catalyzed by DNA-repair enzymes. Cells that are supposed to last a lifetime no longer undergo mitosis, so they don't copy their DNA, but mutations can occur, and these are continuously fixed by DNA-repair enzymes that help prevent cancer. Also, the structure of the chromosomes which contain DNA is continuously being modified as genes are either activated or inactivated.

Unlike machines, this process of autopoiesis is controlled by the individual. It is a network of production processes, in which the function of each component is to participate in the production or transformation of itself and the other components in the network [2, 4, 5]. The theory of autopoiesis starts by recognizing that there is a boundary (cell membrane, skin, etc.) that separates the individual from the environment. The plasma membrane on the outside of human eukaryotic cells is essential in intercellular communication. It contains many proteins that act as receptors, which bind primary intercellular messengers and transduce them into secondary messengers that affect inter- and intracellular metabolism. That is, some membrane-bound proteins secrete hormones or neurotransmitters that affect other cells. As mentioned previously, the first rule in communication is "know your audience". So, plasma membranes have glycosylated proteins called epitopes. They are antigens that can bind to antibodies. The portion of the antibody that binds to the antigen is called the paratope or antigen-binding site. It is a small part or fraction of the

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variable region (Fv) of the antibody. Antigens on pathogenic organisms elicit an immune response when they bind to antibodies that are produced by our white blood cells, also known as leukocytes. On the other hand, leukocytes have human leukocyte antigens (HLA) that are coded for by the major histocompatibility complex (MHC) genes. Note that HLA and MHC are often used synonymously as names for these antigenic proteins. They are the antigens that define self. Class I HLAs are present on all human eukaryotic cells (except red blood cells, or erythrocytes), while class II HLAs are only on immunocompetent cells, such as B lymphocytes and macrophages. Class I HLAs (or MHCs) display fragments of intracellular proteins on their cell surface plasma membranes, so they will be identified as self and not be attacked by T-lymphocytes. Many of them have covalently bound sugars, so they are said to be glycosylated. Cancer cells often have different patterns of glycosylation. Some drug delivery systems contain parts that recognize these altered patterns of glycosylation, as will be discussed in Chapter 3. At the same time, class II HLAs present extracellular protein antigens to helper T cells, activating them and triggering an immune response. Antigens bind to antibodies, which are proteins produced by our immune cells. So, every cell that produces an HLA is making an image of itself that is recognized by antibodies in immune cells. Once an immune cell recognizes a self-antigen, chemokines bind to it and initiate programmed cell death (apoptosis) of the immune cell. If an immune cell recognizes non-self-antigens, the response depends on the source of the antigen. If it comes from food, the immune cells should undergo self-destruction. If it comes from a piece of a parasite, virus or bacterium, signaling molecules will cause the immune cells to grow, differentiate and make more antibodies to kill the invading organisms. At the same time, our immune system must be able to tell the difference between pathogenic Bacteria and the commensal, healthy Bacteria that we need in our digestive system to help us digest our food. So, our immune system (or immune network) communicates and makes decisions, just as our brain and CNS do. If knowledge and intelligence are defined as the ability to make correct decisions, then both the immune and nervous systems are part of the same whole body that makes us smart [2, 4, 5].

So, the plasma membranes form a boundary on the outermost part of human eukaryotic cells [2, 4, 5]. These membranes are an important part of our living, autopoeitic system. That boundary and most things inside it are being continually broken down, re-made and organized, all under the control of the individual. When a cell is supposed to die, it undergoes programmed cell death, also called apoptosis. It is regulated by proteins

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called apoptosis antigens (APO or Apo) and cysteine-aspartate proteases (caspases) that catalyze the proteolysis or breakdown of other proteins that are released by dead or dying cells [2, 4, 5].

Since almost all viruses require a cellular host to make them, they are not considered to be living organisms in the autopoietic theory of life. However, this has been questioned since it was found that some viruses are so large that they can be infected by other viruses. For example, there are viruses that can infect the giant *Acanthamoeba polyphaga mimivirus*. These infective viruses use the *A. polyphaga mimivirus* host to make them. Moreover, viruses are often classified with a genus and species. This is another example of how the natural human tendency to classify everything can lead to confusion, since only living organisms have a genus and species. In addition to being classified like cells (by genus and species), viruses can also be classified by the way that their mRNA is made [5]. It can be coded by the host's double strand DNA (dsDNA), single strand DNA (ssDNA), double strand RNA (dsRNA), (+)-sense single strand RNA (ssRNA), or (-)-sense ssRNA. Those with RNA can be reverse transcribed, while those made from DNA are transcribed by the host's DNA polymerase. Viruses are also classified into different types, subtypes and strains. There are three types of influenza virus. The most common hosts for Type A viruses are birds. However, Type A viruses can also infect humans and other animals. Type B viruses are almost always found only in humans. Although they have caused epidemics, they have not caused pandemics. Type C viruses can cause mild illnesses, but do not cause epidemics or pandemics [5]. So, whether we classify viruses as living organisms or not, they are an important part of the human ecosystem. Viruses that infect Bacteria (bacteriophages) can control their population. This ensures that a proper balance is maintained, so the rest of the body can survive and undergo autopoiesis.

In the theory of autopoiesis, life is considered to be a cyclic process that produces the components of a living system. The average human cell has about 10^5 macromolecules. Each of these is destroyed and re-made about 10^4 times in a cell's life time. The pancreas replaces most of its cells every 24 hours. The cells of our stomach lining are replaced every five days [2]. Skin and red blood cells live for a few days to a week. Teeth and bones are regenerated every 10-15 years. Even the proteins within long-living neurons are recycled every 90 days. Still, the form and organization of the cells and the organism stay the same and under the control of that organism or cell. This is quite different from a computer or a machine, whose components are designed by humans and made by humans.

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Autopoiesis occurs at all levels of life and in all ecosystems [60]. Moreover, ecosystems are embedded in other ecosystems. None of our best efforts to improve human health, lower healthcare costs and cure diseases will succeed if the delicate fiber that binds civilization breaks. If we don't cure our addiction to fossil fuels and limit Global Climate Change, civilization could collapse. One way to prevent this could be to use clustered regularly interspaced short palindromic repeats (CRISPRs) to make genetically modified organisms (GMOs) that can remediate pollution and produce biofuels. However, the uncontrolled spread of GMOs can be quite controversial [3]. Whether they are used or not and whether the human race survives, our living planet will survive. That is, the biosphere of Earth is can thought of as a living system, called Gaia, in which autopoiesis occurs [2, 60].

Autopoiesis also applies to ecosystems [60]. The role of every component of a food web is to transform other components in the web. Plants take up energy, carbon dioxide (CO_2), inorganic compounds and ions from the environment and produce organic and organometallic compounds, including DNA, RNA, proteins and other biochemicals. These compounds are passed through the ecosystem, serving as food to herbivores, which are food to carnivores. Animals eliminate waste, which provides fertilizer and nutrients for plants. The entire network is a hypercycle that is self-regulated through multiple feedback loops [61]. Individual components (organisms and their cellular components) continually die, decompose and are replaced by the network's own transformation processes. Living organisms, including Gaia, continuously make and change the structure of the environment, which feeds information back and changes the structure of the organisms, through constant, interacting cycles. This is called structural coupling [60].

The atmosphere is Gaia's boundary and it is far from equilibrium. This was first recognized by the atmospheric scientist, James Lovelock [62]. Lovelock used Gaia, the name given to the ancient Greek goddess of the Earth, to describe the biosphere of our planet. Lovelock realized that our planet's atmosphere contains oxygen (O_2) at a much higher concentration than methane (CH_4) or carbon dioxide (CO_2). In contrast, the atmosphere of Mars is mostly carbon dioxide, with very little oxygen, strongly suggesting that Mars has little or no life. This is because, given enough time, methane and oxygen will react to form carbon dioxide and water, so an equilibrium will be established. Non-living objects are usually at equilibrium, especially if they have been around for a few billion years. The atmosphere of Mars appears to be at equilibrium (although large amounts of frozen water, or ice, may have been found recently on the

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Martian south pole). In contrast, our planet's atmosphere is far from equilibrium. Earth, or Gaia, has gone through drastic changes. Four billion years ago, there was little or no O_2, but mostly methane and carbon dioxide. The sun was weaker and fewer cosmic rays reached our planet. The first life forms were obligate anaerobes. Had O_2 been present, it would have been a deadly poison. After a couple of billion years, aerobic organisms emerged, and they caused an increase in the amount and concentration of O_2 in the atmosphere. The sun became stronger and more cosmic rays reached our planet. Eventually, some of these oxygen molecules reacted, rose to the upper atmosphere and formed an ozone (O_3) layer, which blocks cosmic rays and much UV light. Our planet's temperature was stabilized. Moreover, everything beneath the atmospheric boundary is being continuously broken down and re-made. The atmosphere, hydrosphere, biosphere and even the geosphere are continually being broken down and re-made in a set of inter-connecting cycles. Lovelock's ideas were developed further by Lynn Margulis, who explained the role of Bacteria in maintaining our planet's autopoietic system [63]. Bacteria can be thought of as the catalysts that help maintain the atmosphere, water and soil in their present state, far from equilibrium, but stable. This is like homeostasis in a cell, organ or organism. Bacteria also continually create, transform and maintain the biosphere's metabolic processes. They are the catalysts of a hypercycle that links oxidation and reduction, turning O_2 into CO_2 and vice versa. Photosynthetic Bacteria produce O_2, nitrogen fixing bacteria convert N_2 into nitrate (NO_3^-), thermophilic Bacteria oxidize H_2S and CH_4, while other bacteria produce H_2S, CH_4 and other gases. Bacteria in soil help accelerate the weathering (or break down) of rocks, which combine with rain water and carbon dioxide to form carbonates. Plants and animals recycle enormous amounts of CO_2, but the amount of CO_2 remained stable, until the industrial revolution and the more recent acceleration of the production of CO_2 by burning more fossil fuels.

To help us understand Gaia better, think of it as being like an old tree [2, 60]. As the tree grows, there is only a thin layer of living cells around its perimeter, just beneath the bark. All the wood inside is dead. Similarly, Gaia is covered by a thin layer of living organisms that we call the biosphere. It reaches down to about seven miles below sea level to about five to six miles above sea level. Neither the atmosphere above it, nor the rocks, lava and molten iron in the troposphere below it, are alive. Just as the bark of a tree protects the thin layer of living cells beneath it, Gaia's atmosphere protects the biosphere with its ozone layer. Outer space and

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the Earth's molten interior make up of Gaia's external environment [2, 60].

Autopoiesis can also be used when discussing ecosystems. The function of every component of a food web is to transform other components in the web. Plants take up energy, CO_2, inorganic compounds and ions from the environment and produce organic and organometallic compounds, including macromolecules. These compounds are passed through the ecosystem, serving as food to herbivores, which serve as food to carnivores. The animals eliminate waste, which provides fertilizer and nutrients for plants. The entire network can be described as a hypercycle [61]. It is self-regulated through multiple feedback loops. Individual components (organisms and their cellular components) continually die, decompose and are replaced by the network's own process of transformation. Life makes and changes the environment, which feeds back and changes the organisms, through constant, interacting cycles.

1.3.6 Systems view of autopoiesis and stem cells

There is a dynamic systems view of stem cell biology [64]. That is, different cellular states are represented as points in multidimensional state space. The three axes in that space represent the abundance of genes, proteins and metabolites. Perturbations of the interactions between them can cause the state of a cell to shift from a stem cell to a differentiated cell. Stem cells and differentiated cells must be able to remain in the proper state when they are perturbed slightly by fluctuations caused by noise. At the same time, the cells must be able to differentiate when they are perturbed by additional appropriate signals. This balance is maintained during healthy childhood development and in healthy adults. This is done by fluctuating and oscillating the expression of genes [64].

Through clever research and systems thinking, Japanese scientists were able to convert mouse skin cells into embryonic stem cells (ES cells) [65]. They only had to insert four genes (Oct4, Sox2, Klf4, and c-Myc) into the adult cells, using retroviruses as transduction vectors. This produced induced pluripotent stem (iPS) cells, or iPSCs. This may prove to be an excellent way to mass produce one's own stem cells, which could be further stimulated to produce different organs for organ transplants to one's own self. The goal is to eliminate the current difficult problem of organ rejection when organs are donated by someone else [65]. So, it is noteworthy that a vascularized and functional human liver was made from liver buds produced from iPSCs [66]. So, it may become possible to make

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organs from a patient's own cells and transplant them back into the patient, without fear of them being rejected.

Until recently, it was not known whether iPSCs could ever be reprogrammed to make any human cell, like ES cells can [67]. However, Japanese scientists were able to make female germ cells in mice develop and produce fertile offspring. They intend to do the same thing with other mammals, including humans [66].

In many ways, stem cells can be thought of as masters of autopoiesis, since they control the making (poiesis) of the body [68]. Embryonic stem cells can make any of the 407 types of human eukaryotic cells. They are found in four to five-day old embryos and have the potential ability to differentiate into any type of human cell in the body. They are no longer present after about five days. However, embryo-like, pluripotent stem cells are also present in adults and circulate in the body. Also, adult gonads may contain a reservoir of embryo-like, pluripotent stem cells. On the other hand, many embryonic stem cells develop into organ-specific adult stem cells that can produce different cell types in those organs. These organ-specific stem cells are senescent. They stay unchanged for years, until they are needed, or until they are mutated and become cancer stem cells. For example, slightly differentiated neural stem cells differentiate further in the developing fetus, baby and child's nervous system to make the various types of cells in the brain and spinal cord. Throughout a person's lifetime, hematopoietic stem cells will differentiate into all the different types of blood cells [68]. Throughout this process, DNA is being copied and repaired. During the lifetime of the cell or the organism, ionizing radiation, free radicals and other factors can damage the DNA temporarily. Each cell contains DNA-repair enzymes that proofread DNA and make repairs when necessary.

Another potential application for stem cells is making new donor organs through induced pluripotent stem cells (iPSCs) [68]. Unlike embryonic stem cells, iPSCs are made *in vitro* by treating differentiated cells with appropriate stimuli. It is quite likely that they will transform drug discovery and development [69]. The aim is to use iPSCs instead of animal models to evaluate the toxicities and clinical efficacies of drugs. This would replace expensive and controversial pharmacokinetic and toxicokinetic studies that were done on animals – usually mice and rats. However, the results from animals seldom predict what will happen in humans. This is indicated by the fact that the attrition rate of drug candidates was about 96% and that 2644 people died during clinical trials for 475 new drugs between 2005 and 2012. Also, only about one in 10 000 new drug candidates make it through all four stages of clinical trials and

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enter the market as drugs that are approved by the FDA and other governments' regulatory agencies. Hopefully, patient-specific iPSCs can be prepared and used to prepare 'mini-livers', 'mini-brains' 'mini-kidneys' and 'mini-lungs' that will have all the properties of whole organs. They could be used to test the efficacies of new drugs and to look for toxicities, as well as to build human disease models on a chip. This would be much cheaper, faster and productive than animal models. It may also be possible to treat or even cure diseases by permanently reversing the causes of the disease condition in a patient's iPSCs and then re-injecting them back into the patient. Finally, an international effort is being made to produce a bank of chemically-induced iPSCs that can be used for drug discovery [69].

Not only iPSCs, but also a patient's own T-cells can be used for personalized therapy. One example of this is the therapy called CTL019, which the FDA named as the breakthrough therapy of the year in 2014 for treating acute lymphoblastic leukemia (ALL) [5]. First, a patient's T-cells are removed by aphereis. They are genetically modified so that the causes of ALL were reversed. That is, a chimeric antigen receptor was coupled to an anti-CD19 single chain F_v domain that contained intracellular T-cell signaling domains from the T-cell receptor. They were put back into the patient's blood, leading to a 90% remission rate for up to two years in the 30 children and adults who received it [5]. So, stem cell technology and personalized cellular therapy are transforming personalized medicine.

1.3.6.1 Different ways that cells die

There are several ways that the body and its internal components are continuously being broken down [70]. This includes apoptosis, oncosis, necropsis, necroptosis, autophagy, autophagic cell death, ferroptosis, cornification, pyroptosis and netosis. Millions of human eukaryotic cells die each day and are replaced by newly differentiated cells to maintain tissue homeostasis. The most prominent and widely studied form of cell death is apoptosis, which is often called programmed cell death [71]. However, this is not entirely correct. Cell death during physiological development can have non-apoptotic features. Apoptosis, autophagy and necroptosis are three different types of programmed cell death. When apoptosis happens, pseudopods are retracted, cellular volume decreases (pyknosis), chromatin condenses, the nucleus fragments and the plasma membrane first blebs, but then maintains its integrity until the final stages. Finally, it is engulfed by resident phagocytes [71]. The Nomenclature Committee on Cell Death (NCCD) proposed a unified set of criteria for defining cell death and its different types or morphologies [72]. Instead of

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programmed cell death, they prefer the term regulated cell death (RCD). This involves genes that encode the molecular network that causes the RCD. There is an initiation phase that sets into motion the signal transduction events that activate executioner mechanisms. The initiation phase is reversible, but the executioner phase is not. The execution of RCD involves a network of biochemical processes that truly causes the final death of the cell. According to the NCCD, programmed cell death is a subset of RCD. It occurs during development and adult tissue homeostasis. Programmed cell death was incorrectly thought to be immunologically silent. We now know that PCD does not always occur with apoptotic morphology and it can induce an immunological response. Apoptosis has been defined as a type of RCD that depends on caspases. This is different than accidental cell death, which is caused by severe physical, chemical or mechanical insults. It was once thought that necrosis was the same as accidental cell death. We now realize that RCD can have both apoptotic and necrotic features. They previously proposed three criteria for defining cell death: "(1) the permanent loss of the barrier function of the plasma membrane; (2) the breakdown of cells into discrete fragments, which are commonly referred to as apoptotic bodies; or (3) the engulfment of cells by professional phagocytes or other cells endowed with phagocytic activity" [72]. However, it was recently discovered that some engulfed cells can be released by phagosomes and retain their viability. So, the NCCD recommended in 2015 that dead cells are those that undergo irreversible permeation of their plasma membranes or have been completely fragmented [72].

Programmed cell death is important in the development of the nervous system [73]. Babies, infants and young children have an excess of neurons. They compete for factors that promote survival. Apoptosis is the major mechanism of cell death in the developing nervous system. There is much apoptosis in the early stages of CNS development, before the peripheral nervous system (PNS) develops. Apoptosis can eliminate neurons that have been specified or positioned wrong or have migrated to the wrong places. Programmed cell death also remodels the post-embyronic CNS and leads to metamorphosis during puberty [73].

1.3.7 The importance of networks in life

Networks permeate all living systems [5]. Brains are a network of nerve cells that are connected by axons and supported by glial cells. Cells are networks of molecules, connected by biochemical reactions. Some ions and molecules (such as H^+, ATP and H_2O) are highly connected hubs in

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the network. Moreover, people, animals and plants can't exist without symbiotic relationships. The average adult human contains about 10^{15} cells, only 10% of which are eukaryotic. The rest are Bacteria, which help us digest our food, properly treat and excrete our wastes, as well as being as an essential part of our immune and nervous systems. Societies are made up of networks of people, connected by family, friends and work. Food webs and ecosystems are networks of various organisms [5].

Within living networks, organisms have symbiotic or syntrophic relationships [5]. Syntrophy is when one organism lives off the products of another organism. An example of syntrophy is when two different strains or types of Bacteria require each other for growth. Symbiosis is the close association of two organisms, usually for a long time. Ectosymbiosis is when one species lives on the body or surface of the host. For example, the Egyptian Plover bird will enter the open jaws of a crocodile and eat parasites that feed on crocodiles. This relationship is good for the bird because not only is the crocodile's mouth a ready source of food, but it is a safe one because few predators would dare attack the bird when it is in the jaws of the crocodile. Many symbiotic relationships are beneficial to both organisms (mutualism), some to just one (commensalisms), while some are harmful. Neutralism is when neither organism is harmed. If one organism benefits and the other is damaged or dies, it is parasitism. Competition is when both organisms harm each other. Frequently, symbiosis is essential. Most plants must have fungi living on their roots to provide them nutrients, such as nitrogen (N_2). In hydrothermal vents and cold seeps on some parts the ocean floor, giant clams, mussels and tube worms contain symbiotic Bacteria. We are more than a collection of genes and other building blocks. Organization and networks are essential for life and they help to make us human. In cells, information storage and processing, along with the execution of cellular programs, exist in distinct levels of organization [5].

The genes that code for proteins may contain essential information for life, but they are not sufficient [5]. RNA, proteins, enzymes, hormones and proper organization are also required. Genes interact with many types of RNA, histones and other proteins, as well as with other genes. They are also affected by the external environment. For example, ants, bees, wasps have a queen, who makes one mating flight and stores the sperm from males for years. She rations the sperm out to her eggs, allowing some of them to be fertilized and become females. They all have the same DNA, but only one of them will become a queen. It will be the one who gets the right food and care from the external environment. Each female is diploid. They all have a full set of queen genes and a full set of worker genes. The

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ones that get activated depend on the external environment. There is structural coupling. Networks are important [5].

Whether they are natural or made by humans, networks have similar properties. Living systems and the internet are examples scale-free networks [5]. There are many levels of organization in the scale-free networks in living cells. There is a network of genes, a metabolic network, a regulatory network and a cellular network. The metabolic network is the network of metabolic reactions. The regulatory network controls metabolism from development to metabolism to cell death. The cellular network is a network of cellular components and organelles, connected by biochemical reactions and physical links. The web of life contains these networks, as well as all protein-protein, protein-DNA, DNA-RNA, protein-lipid, sugar-lipid (lipopolysaccharide) and sugar-protein (glycoprotein) interactions. Ecosystems are networks of eukaryotic organisms, Bacteria, Archaea and viruses [5].

The properties of a network depend on its topology or structure [5]. There are scale-free and random networks. One example of a random network is a party in which 100 people attend, and none of them know each other. At first, they will form a random network as they meet the people around them. If you serve some fruit juice and food, soon they will divide themselves into small groups. Suppose the juice comes in red and yellow bottles. Unknown to the guests, the yellow bottles contain a sweeter juice. If one of the guests is told the secret, but is asked to only tell their new acquaintances about it, only a few minutes are needed for the guests to break up into smaller groups. Soon, almost everyone who is not diabetic or on a strict diet will start drinking only the juice that is in the yellow bottle. A giant cluster is likely to emerge as the initially random network changes until it becomes a non-random network [5].

When connections between nodes in a network are not directed but are made at random, there are an average number of connections for a node [5]. Few nodes have much more or less than the average number of connections or links. A graph of the number of nodes, N, having a particular number of connections, k, would look like a bell curve. An example would be a plot of the height of all the adult men in the world. Very few of them are under 120 cm or over 250 cm tall. Most are a little less than 180 tall. However, if connections are not made at random, a scale-free network can emerge. Examples include the internet and World Wide Web. Computers, routers, cables and web pages are not connected randomly. On the World Wide Web, some websites like Google are hubs, with many connections or links. Most websites and Facebook pages,

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though, have only a few links to other websites and Facebook pages. They follow a power law [5].

Scale-free networks have some other common properties [5]. First, they tend to grow. As they grow, new nodes are added and new links are formed between nodes. Nodes with the most links are called hubs. Scale-free networks are rugged and not very vulnerable to random attacks. For example, if a toxin or pathogen were to randomly inhibit a reaction in the metabolic network that is not a hub, it would have little or no adverse effects. In contrast, if a toxin inactivates a few key hubs in cellular metabolism, it can kill a cell. Scale-free networks may be resilient and resistant to random attacks, but they are vulnerable to attacks that are directed at hubs [5].

Another property of scale-free networks is that nodes are attached non-randomly [5]. If a node (such as a website) has been around for a long time it will have more connections. Sometimes, though, a new node can attract many links. For example, there are many nodes in the human brain that don't exist in other animals, because we evolved more recently. However, they make us more fit and better able to reproduce and form productive societies. So, the number of links that a node has depends on its fitness, as well as the number of nodes it already has and the length of the time it has existed. Every network has its own fitness distribution, which is a measure of how similar or different the nodes are. It is also useful to measure the path length. This is the number of links that need to be traversed in order to travel between the nodes. There can be many alternative paths between nodes. The shortest path is the path with the smallest number of links between the selected nodes. The mean path length, $<l>$, is the average of the shortest paths between all pairs of nodes. It is a measure of a network's overall navigability. The degree of a node is the number of edges that are connected to it. The average degree $<k>$, average path length and average clustering coefficient all depend on the number of nodes and links in the network. The cost, κ, of a network is equal to the number of edges present in the network divided by the number of total possible edges $(N(N-1)/2)$, where N is the total number of nodes in the network. This cost is sometimes referred to as the sparsity of the network. The efficiency, E, of a network is equal to the inverse of that network's path-length L. The modularity, m, of a network is a measure of the degree to which the network is made up of modules. A minimum spanning tree is a subgraph of the network that contains the smallest subset of edges of the graph in which all nodes are still connected together. [5].

Networks are also characterized by their clustering coefficient [5]. That is, if one node (A) is connected to another (B), and it is connected to a

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third node (C), there is a chance that A is also directly connected to C. This can be described by the clustering coefficient, CI. It is calculated by the equation: CI = 2nI/k(k-1), where nI is the number of links connecting the kI neighbors of node I to each other. That is, CI equals the number of triangles that go through node I. Moreover, kI(kI –1)/2 is the total number of triangles that pass through node I, if all of node I's neighbors are connected to each other. The average clustering coefficient, <C>, is also important. It is the overall tendency of nodes to form clusters or groups. Another important measure of the network's structure is the function C(k), which is defined as the average clustering coefficient of all nodes with k links. Networks in which C(k) is directly proportional to k^{-1} are hierarchical. So, there is a hierarchy parameter, β. It is defined as the slope on a log-log plot of the clustering coefficients and the degrees (k) across all nodes in the network. There are also small world networks, in which there is much clustering and a short path length between any two nodes in the network. They are characterized by a small-worldness scalar, σ. It is the clustering coefficient of a network, normalized C, (Cr,) divided by the path-length of a network, L, normalized by the path length of a random graph, Lr. That is, $\sigma=(C/Cr)/(L/Lr) = \gamma/\lambda$ where the normalized clustering coefficient $\gamma = C/Cr$ and the normalized path-length $\lambda = L/Lr$. The human brain has this small world characteristic [5].

So, the scale-free network in living cells has many levels of organization that are organized into networks [5]. There is a network of genes, a metabolic network, a regulatory network, a network of amino acid residues in proteins, a cellular network and a network of tissues. The web of life contains these networks, with protein-protein, protein-DNA, DNA-RNA, protein-lipid, sugar-lipid (lipopolysaccharide) and sugar-protein (glycoprotein) interactions. The human ecosystem contains links between not just our human eukaryotic cells, but also viruses, Bacteria, Archaea and other Eukarya (protists) [5].

When hubs are attacked selectively, communication deteriorates rapidly in scale-free networks [8]. At first thought, this should make hubs attractive therapeutic targets for drugs. However, some of the hubs are essential for life. So, attacking them can cause toxicity and even death. This is useful when the targets are pathogenic Bacteria, other infectious organisms or cancer cells. So, major hubs can be good therapeutic targets for antibiotics, drugs for curing infections and anticancer therapies. So, on average, targets of FDA-approved drugs that are not in these categories tend to have more links than peripheral nodes, but fewer links than hubs. So, targeting hubs can produce two opposite effects. First, some hubs are so well connected that their attack leads to cascading effects that damage

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or destroy the function of a major segment of the network. At the same time, edges or nodes that connect major hubs also offer very interesting drug targeting options. In contrast, modulating some nodes that have just a few links often has minor effects. However, there are also peripheral nodes called choke points, which uniquely produce or consume an important metabolite. They are the only link between network modules. When choke points are blocked, the result can be lethal. Moreover, interdependent networks made up of at least two interconnected networks are much more vulnerable to attacks than single network structures. For example, our cells have networks of signaling proteins and transcription factors, as well as membrane proteins that interact with nuclear, plasma, mitochondrial and endoplasmic reticulum membranes. They are interdependent networks. They are quite vulnerable and seldom make good therapeutic targets. In contrast edgetic drugs, multi-target drugs and allo-network drugs are emerging as more promising therapeutic targets that will have fewer adverse side effects [8].

Many networks contain groups of nodes that are well-connected within a module [8]. The nodes in a module are more densely connected with each other than with nodes outside the module. Modules of protein and RNA networks often perform cellular functions. Many modules of molecular networks overlap with each other. Some modules can be connected to other nodes and modules with more than one link. Such connections are called bridges. Bridges usually have fewer direct links than hubs, and are regulated independently from the nodes belonging to the modules which they connect. Many of the proteins that are involved in aging are bridges. This makes bridges attractive therapeutic drug targets. If all the bridges are blocked except one, the remaining bridge becomes a bottleneck [8].

Many inter-modular hubs have a regulatory role and are can be mutated in cancer cells [8]. There are different types or classes of inter-modular hubs. Some are called date hubs. They have one or just a few binding sites for other proteins and frequently changing protein partners. They are found between modules, as opposed to party hubs that mostly reside in the center of a module. Party hubs tend to have binding surfaces with higher affinities for other proteins than date hubs. There are also some nodes that occupy a unique and monopolistic inter-modular position. They are called bottlenecks, because almost all information flowing through the network must pass through them. This makes bottlenecks potentially more effective therapeutic targets than bridges. So, hub-bottlenecks are the preferred targets of microRNAs and play an important role in cellular re-programming. However, inhibiting bottlenecks can

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compromise the integrity of the network. As a result, they are limited to antibiotics, anti-infectious agents and anti-cancer therapies. That is, cancer-causing proteins (oncoproteins) tend to be inter-modular hubs of cancer-specific networks. They can be good therapeutic targets. Nodes connecting more than two modules are in the part of the network where modules overlap. Overlapping nodes can be regulated more subtly than bridges or bottlenecks. Modular overlaps are the most important transmitters of perturbations in a network. They are key determinants of network cooperation and play a crucial role in how cells adapt to stress. So, changes in the ways that network modules overlap affect the ways that complex systems adapt to changes. Modular overlaps enable communication or cross-talk between signaling pathways. As a result, modular overlaps are often very good therapeutic targets. There are also creative nodes that are found in the parts of the cellular network where several modules overlap. These nodes regulate the ability of complex networks to adapt, and are also promising targets [8].

Network centrality is another factor that is related to importance [8]. There are different ways to measure or define centrality. There is the network degree, which is the number of neighbors (or links). It is a local measure of centrality. Some measures are related to modular structures. This includes bridging centrality and community centrality, or subgraph centrality. Centrality measures, like betweenness centrality (the number of shortest paths traversing through the node), random walk related centralities (like Google's PageRank algorithm) and network salience are based on more global network properties. Global network centrality only measures network segments and uses network compression to calculate it. Centralities based on network modules are based on the determination of bridges and overlaps. Betweenness centrality is used to define bottlenecks. In addition, centrality hubs with high betweenness dominate the drug target network for treating myocardial infarctions [8].

It is also useful to construct network skeletons that are interconnected subnetwork of high centrality nodes [8]. If a network skeleton contains many hubs, it is called a rich-club network. Network skeletons can also consist of high betweenness centrality nodes or be made up of inter-connected centers of network modules. Some network skeletons are densely inter-connected and form an inner core of the overall network. Other network skeletons can traverse the network like a highway. In both cases, the nodes that make up the skeleton are the elite nodes in the network. Network skeleton nodes are also attractive therapeutic targets. There is also a subset of nodes that can give any prescribed set of centrality values to all other nodes by cooperatively tuning the weights of

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their out-going edges. Moreover, there is a rich-club of prescription drugs that are in the core of the drug–therapy network that contains drugs and established classes of medical therapies [8].

It is also important to consider the network assortativity [8]. It is a measure of the preferential attachment of nodes having similar degrees to each other. Network cores like rich-clubs may or may not be part of an assortative network. If peripheral network nodes are connected to the network core and not to each other, they are disassortative networks with a low degree. Such core-periphery networks have a nested structure. If peripheral nodes are linked with each other and form a border around the network, it is called an onion network (Figure 3) [8].

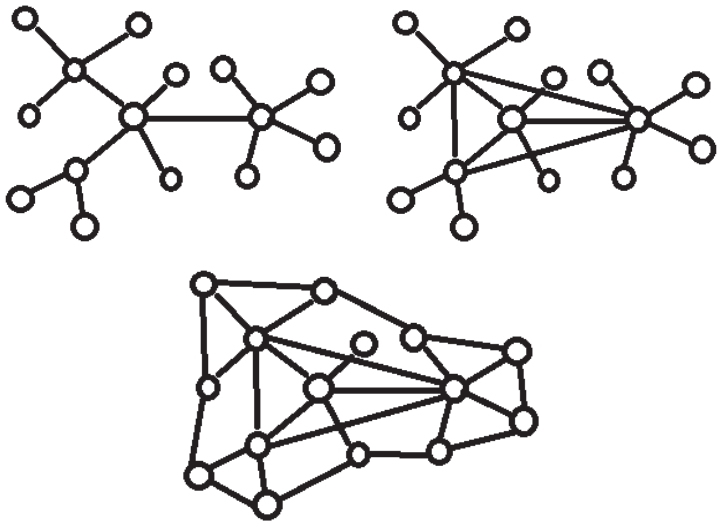

Figure 3. Depictions of rich-club, nested and onion networks, adapted from Figure 9 in reference [8].

The global topology of most real-world networks has a small-world property in which the nodes are well-connected [8]. For example, the concept of six degrees of separation in the small-world social network means that most members of the human social network are separated from each other by no more than six connections, or edges [2, 8].

It is also important to consider the connectedness of nodes in a network [8]. That is, some networks have a connected, giant component. This is related to the integrity and reliability of a network. That is, how much of the network remains intact and functional if a node fails or and edge or

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connection is broken? As mentioned before, an attack on the most connected hub can destroy many non-random networks. However, such attacks can be fatal. So, it is interesting to note that the effects of a single attack of the largest hub in biological networks can be replicated by a surprisingly low number of partial attacks on nodes that are not as well-connected as hubs. This makes drugs that target several nodes in a metabolic network very desirable. They are potentially quite effective and would have far fewer (if any) adverse side effects. So, the effect of a drug on the integrity of the metabolic and regulatory networks is an important measure of a drug's effectiveness. That is, the number of links in a protein-protein interaction network is an important measure of how essential a network is. In addition, the weights (or importance) and directions of edges are important factors in determining essentiality. It is also important to determine the effects that damaging a node will affect the properties of the network. For example, network centrality is related to the influence of nodes on each other. Network controllability and systems dynamics are also important. That is, measuring the ability of a network to shift from one state to another is important in network analyses [8].

So, the description of network dynamics is a fast-developing field of network science [8]. It is inspiring systems-based thinking in drug design. It involves concentrating on transient changes in networks that play major roles in communication, signaling and in the development of diseases. That is, the actions of drugs should be viewed as a network perturbation that pushes pathophysiological networks back into their normal, healthy states. So, studies addressing perturbation dynamics are very important in developing new drugs. This includes measuring the ability of cellular networks to maintain their healthy functions even when they are being perturbed. Cellular networks both in health and in disease have enhanced robustness. Drugs that are designed to cure infectious diseases often fail due to the robustness of the Bacteria or other infectious organisms. In contrast, adverse side effects can appear when the drug affects an unexpected point of fragility of the affected networks. So, an analysis of robustness is important in identifying the primary therapeutic targets and to characterize the mechanisms of action of a new drug [8].

Protein–protein interaction networks are very promising therapeutic targets [8]. That is, network proteomics can be used to understand the mechanisms of actions of many existing drugs and to help identify therapeutic targets for new drug candidates. That is, protein–protein interaction networks (PPI networks) are often called interactomes - especially if they are based on genome-wide data. The nodes in PPI networks are proteins. These nodes are not single points, but are also

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networks of interacting amino acid residues. The edges of PPI networks are the direct, physical interactions between proteins. PPI interaction networks include an indication of probability. That is, the weights of the edge weights are based on the probability of the interaction between protein nodes. The weights of edges in an interactome are often calculated as confidence scores. This includes the abundance of the proteins, their affinity for each other (binding energy), co-expression levels and co-localization in subcellular compartments [8]. Reference [8] lists several major PPI interaction datasets on the human interactome that are publicly available.

Many human diseases are due to changes or mutations in more than one gene [8]. So, there are several genome-wide datasets that characterize disease related mechanisms of pathology. They contain information on the patterns of mRNA expression, the results of genome-wide association studies (GWAS) of disease-associated single-nucleotide polymorphisms (SNPs) and disease-related changes in posttranslational modifications (such as the phospho-proteome). They also include system-wide changes of subcellular localization of key proteins. All this information can be incorporated in PPI networks as changes in the configurations and weights of edges. Still, disease-associated proteins are not usually hubs in the interactome. Somatic mutations, such as those occurring in cancer, are important exceptions. So, multi-interface hubs that are associated with a disease form an inter-connected rich club. Disease-related protein nodes have a smaller clustering coefficient than average nodes. They can be used to predict and find previously unidentified disease-related genes [8].

PPI interaction networks are being used in modern drug design [8]. There were about 650 000 interactions in the human interactome by 2013. Databases like ChemProt: (http://www.cbs.dtu.dk/services/ChemProt) contain about 700 000 chemicals and two million interactions with their target proteins in various species. However, the complexity of an interactome involves much more that an inventory of contacts and binding partners. It also includes variations in the expression levels and posttranslational modifications, as well as cellular environment-induced (such as Ca^{2+}-dependent) and protein domain-dependent variations. Therapeutic drug targets usually have more links than the average nodes. They are mostly linked to middle-degree nodes, and not hubs. Drug targets in cancer are exceptions, since they have a more defined hub structure. Proteins that are therapeutic targets have a lower clustering coefficient than other proteins. Drug targets often occupy a central position in the human interactome and serve as bridges between two or more modules. Nodes that have an intermediate number of links tend to have an extensive

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contact structure. By targeting these non-hub nodes (with the exception of infectious diseases and cancer) adverse side effects can be avoided or minimized. Successful new drugs that have few or no adverse side effects perturb the interactome in a controlled manner. In contrast, the therapeutic targets of many drugs that have been withdrawn due to adverse side effects have too large of an influence on the metabolic network [8].

Signaling pathways are an important part of the metabolic network [8]. They transmit or transduce extracellular information from primary messengers (ligands) through membrane-bound receptors and mediators to transcription factors, which induce specific changes in the expression of genes. Signaling pathways are considered to be the upstream part of signaling networks. Signaling pathways are highly structured and are engaged in considerable cross-talk as a result of the directed physical interaction between pathways. Since input signals (ligands/receptors) and output components (transcription factors) are limited, communication (cross-talk) between pathways creates new combinations of input and output. This contributes to the functional diversity and plasticity of signaling networks. However, this crosstalk must be precisely regulated to maintain the specific meaning of the output and the fidelity of the input. Thus, inputs preferentially activate their own output and outputs preferentially respond to their own input. Crosstalk and communication must be controlled to prevent leaking or unwanted communication with other networks. This can be done by controlling scaffolding proteins, inhibiting unwanted pathways and by regulating the spatial and temporal expression patterns of proteins [8].

The regulatory subnetwork (gene regulatory network) is the downstream part of signaling pathways and networks [8]. The gene regulatory network includes both transcriptional and post-transcriptional sub-networks. That is, protein transcription factors bind to specific regions of DNA sequences (called transcription factor binding sites, or response elements) and regulate their transcription into mRNA. Horizontal contacts of middle-level regulators also play important roles in gene regulatory networks in human cells. The human transcription factor regulatory network has a basic architecture in almost all types of cells and is complemented by specific segments for different types of cells. For example, microRNAs can suspend or permanently repress the expression of genes by binding to complementary sequences on target mRNAs. The expression of almost every human gene is controlled by one or more microRNAs. Moreover, interactome hubs, bottleneck proteins and downstream signaling components are regulated by more microRNAs than are other nodes. So, mutations in genes coding for microRNAs are often pathological [8].

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Drug discovery is also being assisted by a better understanding of the structure and dynamics of signaling networks [8]. Drugs having a similar pharmacological profile affect similar parts of signaling networks. There are critical nodes in the junctions of signaling pathways of healthy cells. Examples in insulin signaling include the phosphatidyl inositol 3-kinase (PI3 kinase), protein kinase B (also known as AKT) and insulin receptor substrate (IRS). Proteins that form a bridge between signaling modules have been popular therapeutic targets. By studying pathologically altered signaling networks, possible drug targets whose malfunction is involved in the etiology of the disease can be identified. For example, driver mutations of tumorigenesis only affect a few central metabolic pathways. Targeting these specific pathways may prevent tumors from growing. However, aggressive tumor cells can emerge and cause a systems-level change of the signaling network resulting in the emergence of angiogenesis and metastasis. Moreover, cellular metabolism can become deregulated, enabling cancer cells to avoid elimination by the immune system. This includes changes in multi-pathway proteins involved in crosstalk that cause signaling networks to be rewired or reconnected. One example is the transformation of a death signal into a survival signal [8].

There are also more than 500 types of post-translational modifications that help in cell signaling [8]. This includes phosphorylation by protein kinases which are the most targeted proteins in the cellular signaling network. However, the similar structures of ATP-binding pockets make many drugs bind to unintended targets. However, associations of kinase regulatory domains, kinase-associated scaffold proteins and multisite docking proteins often control the subcellular localizations of protein kinases. They play key roles in determining signaling kinetics and substrate specificity. Scaffolding proteins are flexible. Proteins that can bind to many different sites can increase the flexibility of networks by enabling integrative cross-talk. So, drugs that disrupt kinase-centered sub-interactomes and/or remodel kinase-centered protein complexes are being designed [8].

The transcription of genes is also controlled, in part, by genetic interaction and chromatin networks [8]. Genetic interaction networks are part of gene regulatory networks. However, many interactions between genes on chromosomes are indirect. The structure of chromatin is flexible, so genes that are far apart in one state can be brought close together in another state of chromatin structure and function. Chromatin networks that encode 3D interactions between distant segments of DNA are specific representations of genetic interaction networks. While an understanding of genetic interaction networks has already helped design new drugs,

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chromatin interaction networks are recent developments that offer much potential for future drugs [8].

Genetic interaction networks have been characterized by comparing the phenotypes produced by a single mutation with the phenotype of the double mutant [8]. If double mutants are not as fit as expected from the additive effect of the two single mutants, then the genes have a negative interaction. On the other hand, if double mutants are more fit than expected, the two genes interact positively. The extreme form of negative interactions is synthetic lethality, when the two single mutants are viable, but their double mutant becomes lethal. However, there can be perturbations in which a debilitating effect can be compensated by another inhibitory effect [8].

One way that genetic interactions can occur is through the network of long-range interactions of the 3D chromatin structure [8]. The chromatin network contains functional modules, and has a rich club of interactions between hubs. The chromatin network can affect changes in the structure of chromosomes that occur in various forms of cancer. For example, the chromatin network configuration is altered considerably by the overexpression of an oncogenic transcription factor (ERG) that is activated in prostate cancers. ERG stands for ETS-related gene, where ETS is the erythroblast transformation-specific family of transcription factors [8].

The chromatin network is controlled mostly by posttranslational modifications of histones, DNA silencers and nascent RNA scaffolds [8]. Our understanding of chromatin networks is improving rapidly. Hopefully, this will help to predict interactions between two or more drugs, as well as their effects on metabolism and adverse side effects [8].

So, metabolic networks are also being studied [8]. In a metabolic network, each metabolite is a node. Two nodes are linked if there is a biochemical reaction that can transform one of the metabolites into the other. The reactions and the enzymes that catalyze them are the edges. Because of their complexity, metabolic processes are often represented as hypergraphs, in which edges connect several nodes. An edge may represent many reactions - both in the forward and in the opposite direction. Moreover, some reactions occur spontaneously, so they have no enzymes associated with them. Metabolic networks are reconstructed in a highly integrative, nonlinear process. This includes looking at genome sequences and enzyme databases, while specifying the network using data from the transcriptome and proteome. Finally, metabolic networks have a small-world character, have hubs, and display a hierarchical modular structure similar [8].

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So, many types of networks permeate all living systems – including humans [5]. They are especially important in network medicine and new drug development.

1.3.8 Genetics and systems thinking

In reductionist thinking, DNA is the blueprint or book of life. It encodes all the instructions needed to make an organism. Information is thought to flow from DNA to mRNA to proteins, which were thought to be the machinery of life. Only proteins were thought to catalyze the many biochemical reactions needed to support life. The human genome was thought to be a fixed set of instructions that could not be cut up and rearranged. The instructions are written in a triplet code of purine and pyrimidine bases bound to deoxyribose phosphate, as part of nucleotides. However, the third base is variable. That is, a mutation in the third base of a triplet usually causes no important changes. For example, all four of the following triplets code for glycine: GGA, GGG, GGC, GGU (where G, A and C are guanine, adenine and cytosine). The first two bases must be G, but the third base can be any of the four possible bases. So, a mutation at the third nucleotide will cause no change in the amino acid produced by the mRNA. Some amino acids have redundancies in all three nucleotides. For example, all of the following base sequences code for serine: UCA, UCG, UCC, UCU, AGU, AGC. There are changes in the first, second and third bases. Thus, there are five possible silent mutations of the one wild-type triplet that codes for serine.

There are also triplet bases in mRNA that are recognized by ribosomes as the places where translation starts and stops. For example, the start codon is usually AUG, which codes for methionine in eukaryotes and a modified methionine (formyl methionine) in prokaryotes. The start and stop codons are used by researchers and geneticists to identify the parts of chromosomes that code for proteins. That is, some parts of DNA can code for different proteins, depending on how they are read. That is, there is a reading frame for all genes [1]. Consider the following stretch of bases on a piece of DNA: AGAGGATGAT. When an RNA polymerase reads that stretch of DNA, it might start reading it from the beginning and read the first nine of the bases, AGAGGATGA. This would code for the following bases in mRNA: UCUCCUACU. This would code for the following three amino acids: Serine-Proline-Threonine. However, if this piece of DNA is read in a different reading frame, different mRNA and amino acids would be made. If the RNA polymerase started reading from the second base and ended one base later, it would read the following base sequence in the

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DNA: GAGGATGAT. It would code for the following piece of mRNA: CUCCUACUA. This would code for the following three amino acids: Leucine-Leucine-Leucine. Thus, two different reading frames can produce two different peptides. So, geneticists look for open reading frames (ORFs) that begin with a start codon and end with a stop codon [1].

However, some of these ideas are now known to be over-simplifications [4]. That is, we now know that the mutated third base in the triplet code can make a difference. For example, in a rare condition called the Hutchinson-Gilford progeria syndrome, a change in the codon for glycine 608 (coding for the Lamin A protein) changes from a C to a T. Even though the same glycine is produced by what would be a silent mutation in many other genes, a very different protein is produced, with tragic consequences for the patient. This is partly due to the fact that genes coding for proteins in humans and other eukaryotes contain exons that must be spliced together to make a functioning gene. In Hutchinson-Gilford progeria syndrome, the Lamin A gene (*LMNA*) is spliced improperly, producing a truncated protein with 50 amino acids missing [4].

There was another misconception in reductionist thinking. One gene was thought to code for one protein, and genes had fixed locations on chromosomes. Only a small fraction of the human genome coded for proteins. The rest was thought to be "junk DNA". Attempts were made to determine the functions of every protein coded for by every gene. The idea was to find the dysfunctional or mutated protein that caused a disease and the gene coding for it. The hope was that the gene could be replaced by the functional, wild-type gene and the disease would be cured. This idea persists to this day, as some people are trying to make super-humans and designer babies using CRISPR technology that can edit DNA and replace it with the desired gene.

This is part of biotechnology, which is the use of biological tools in many fields of technology to study biology and develop new drugs. For example, genetic engineering was used to solve the human genome and is leading to the production of new types of Bacteria, algae and other life forms that will make many of the products that are now made from fossil fuels. Biotechnology is used to identify DNA at crime scenes and analyze DNA from extinct species. It has been and continues to be used to identify genes, new therapeutic targets and to enable personalized medicine based on an individual's genome and/or the genome of his or her tumor cells.

That is, DNA, RNA, proteins and even whole cells are being made and used for therapeutic purposes [4]. Biotechnology can also be used to help predict susceptibilities to diseases, to suggest therapies once a disease

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starts and to predict outcomes when a drug is administered and when changes in diet and behavior are added to the therapy. The tools of genomics, transcriptomics, metabolomics and others can be used not just for prediction, but also to help prevent diseases and expensive, toxic side effects caused by therapies that are not very effective. The data are being stored in personalized data clouds and accessed through mobile communications. Patients are participating in their own therapies and following the progress of their recoveries [4].

DNA can be cut using specific enzymes and inserted as recombinant DNA (rDNA) into a vector [4]. The viral or bacterial vector can be inserted in living cells that are grown under the conditions needed to make the proteins that are coded for by the rDNA. This includes enzymes needed to catalyze the biosynthesis of large quantities of therapeutic proteins, such as hormones, cytokines and monoclonal antibodies. Antibiotics, vitamins, vaccines and natural products can also be made. The DNA can be modified by inserting genes that code for the desired products. For example, the restriction endonuclease Eco R1 can be used to catalyze the hydrolytic cleavage of DNA and a human gene can be inserted into it. Specific genes can be cut out and isolated from a human (or other organism) and inserted into small, circular DNA plasmids in Bacteria. Genes can also be made by chemical synthesis and inserted into plasmids that can replicate outside the larger chromosomal DNA in the Bacteria. These plasmids are used as vehicles or vectors to carry and amplify genes in the host Bacteria. Genes can be inserted into Bacteria, algae, plants, animal and even human cells. They are modified and used to make a variety of products. Even transgenic goats are used to make a prescription drug. The DNA can be genomic DNA, recombinant DNA (rDNA) or complementary DNA (cDNA). That is, genomic DNA can be modified and inserted into a vector as rDNA. Also, one can find out which genes are translated into mRNA by forming cDNA that is complementary to the mRNA, insert it in a vector, amplify it in Bacteria, and then sequence it. This was done as part of the Human Genome Project [4].

However, it has not always been easy. In 1975, shortly after developing ways to make rDNA, the U.S. National Institutes of Health (NIH) sponsored a meeting at the Asilomar Conference Grounds in Pacific Grove, California to discuss the ethics of using this ‘God-like power’ to insert genes from one organism into another. Even though biologists knew that rDNA could potentially save millions of lives, they feared that it could have some terrible, unforeseen consequences. At the end of the meeting, a consensus statement was written. It described ways of determining whether or not a planned experiment was too dangerous to be allowed.

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This included cloning dangerous pathogens. Some people also feared that modifying the human germ line would cause changes that would be passed on to future generations. However, it was thought that this would be so difficult that it was not important to deal with it at that time.

In the mid-1970s and early 1980s, DNA sequencing was one of the most boring jobs a person could have in a biology lab. Compared to modern sequencing, it was slow and inefficient. So, when the NIH convened a subsequent conference to decide if it was worthwhile to fund a Human Genome Project, the consensus was no – it would take way too long and be far too expensive, based on foreseeable technologies. In contrast, physicists at the Department of Energy (DOE) decided that they could do it. So, biologists decided that they did not want to leave this up to a bunch of physicists and they decided they should join in the effort.

The result was the beginnings of systems thinking in this field. That is, an international, interdisciplinary collaboration was formed. It developed the tools to determine the nucleic acid sequence of the entire genome of a mosaic of different people. The project was completed three years ahead of schedule, partly due to the clever decision to use shotgun sequencing. The idea was to break up (hydrolyze) each individual chromosome into DNA fragments of 200-500 base pairs. These short fragments could be easily sequenced. It was hoped that computer algorithms would be developed to determine how all of these fragments overlapped with each other in the original chromosomes.

Originally, this was a very controversial idea. It was doubted that such an algorithm would ever be developed and implemented. Instead, popular reductionist thinking led to the idea that the human genome should be sequenced carefully in a well-organized manner. That is, the sequencing would start at the 5' end of each chromosome and proceed slowly towards the 3'-terminus. Even though it would be much slower than shotgun sequencing, it would eventually work. However, this was far too slow to satisfy J. Craig Venter and The Institute for Genomic Research (TIGR).

They used restriction endonucleases like EcoR1 and BamH1 (from *Bacillus amyloliquefaciens*) to hydrolyze the DNA into fragments, each containing 200-500 base pairs [4]. Computational tools were used to find fragments with overlapping sequences, to build larger contiguous pieces of chromosomes. This led to the complete human genome being solved much faster than expected. DNA sequencing has been used to identify and characterize potential therapeutic targets. Signal transduction pathways and important links between them have been identified. This helped and continues to help to produce new prescription drugs and identify biomarkers for many diseases. Therapeutic targets identified by genetics

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have been used to predict whether or not drugs directed against them could correct the biochemical defects, before the launch of lengthy and expensive clinical trials. Genetic analyses can also identify subsets of patients who are most likely to be effectively treated by new therapies [4].

1.3.8.1 The importance of bioinformatics in genetics and systems thinking

In the process, biotechnology, molecular biology, and genetic engineering produced huge amounts of data about individual cellular and subcellular components. However, these disciplines are limited. By themselves, they can't describe whole systems or predict their emergent properties. This requires bioinformatics and the interdisciplinary efforts that are inherent in systems thinking. Biologists are working with engineers, computational scientists and mathematicians to learn about systems biology [3]. Bioinformatics is the use of science and math to get more information from biological data and generate the computational tools to obtain that data. It includes not just writing algorithms, but also designing and using databases, information systems, web technologies, artificial intelligence and soft computing, together with information theory and computation theory. It also includes structural biology, software engineering, data mining, image processing, modeling and simulation, signal processing, discrete mathematics, control and system theory, circuit theory, and statistics. It is at the forefront of the fusion of math, hard science, biochemistry, biology, engineering and systems thinking.

The causes of almost all diseases can NOT be reduced to one or two defective genes and the proteins they encode. Through a combination of systems biology, bioinformatics, information technology and graph theory, many advances have been made. Moreover, the US National Center of Biotechnology Information (NCBI) maintains databases and provides easily downloadable programs to analyze genomes from humans and other organisms and any new data that labs may produce [74]. However, systems biology is not just trying to measure as many things as possible [74]. It also needs mathematical and computational modeling to make sense of it all and make predictions about living systems. Originally, most studies of systems biology focused on generating models at the cellular level to provide a better understanding of intracellular processes. Now, attempts are being made to study the interactions of many cells and to predict their behavior. This is important because there are substantial differences between different types of cells. For example, there are many different types of cells in the stromal environment of tumors. Tumors

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make up an ecosystem consisting of many cells and types of cells. This includes normal cells such as infiltrating immune cells, endothelial cells, and adipose cells. Physical and chemical interactions between these cells and cancer cells occur over short and long distances. They modulate the disease process and the response to therapy [74].

For example, mechanisms of drug action and resistance have been identified by looking at the transcriptome [75], which is the set of all DNA transcripts, including all types of RNA. They established a cell line that was sensitive to the cytotoxic anticancer drugs BI 2536 and bortezomib. The transcriptomes of drug-resistant and normal clones were compared. Mutations that caused the drug resistance were identified. Mutations common to more than one clone were used to reveal the drugs' physiological targets and indirect resistance mechanisms [75, 76]. In another study, a library of over 10 000 different compounds was screened in a drug-sensitive fission yeast model [70]. The physiological target was Cutin-1, an inhibitor of mitosis and chromosome segregation [70].

This work is assisted by The Cancer Genome Atlas (TCGA), a project that collects and integrates vast datasets from multiple cancer types. The TCGA has collected data from 33 different primary tumor types from various organs. Samples were analyzed for DNA, RNA, epigenetics, copy number variation, microRNA and protein content. Also, clinical data were included. The comprehensive comparison of different types of cancer is helping to improve our understanding of molecular processes that are involved in the onset and progression of cancer. This includes a better understanding of the immune response to cancer. The immune cells that are present in malignant tumors have been identified and characterized. This includes immune modulators and mechanisms, such as the programmed cell death-1 (PD-1 or PD-L1) checkpoint inhibitors that could be targeted therapeutically [74, 77].

The PD-1 (or PD-L1) receptor (also known as CD279) is on the surface of activated T-cells [77]. It is an important part of our natural protection against cancer through a cancer immunity cycle. This cycle enables the immune system to have an anti-cancer response and kill cancer cells. First, dendritic cells capture tumor cells that have mutated cell surface antigens. Second, the dendritic cells prime T cells with the tumor antigen, thus activating cytotoxic T cells. Third, the activated T cells travel to the tumor and infiltrate its environment. Fourth, the activated T cells recognize and bind to the cancer cells. Fifth, the bound effector T cells release cytotoxins, which induce apoptosis in the cancer cells that they target. Finally, dying cancer cells release more tumor-associated antigens which propagate the immunity cycle further and kills more cancer cells.

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However, this response must be properly regulated to maintain the balance between appropriate recognition and destruction of tumors and the inappropriate overstimulation of immune responses, that can lead to damage to normal, healthy cells and tissues. The PD-1 (or PD-L1) signaling pathway is an essential part of this regulation [77].

The PD-L1 receptor is on the surface of activated T cells [77]. Its ligands, PD-L1 and PD-L2, are widely expressed on the surface of dendritic cells or macrophages. PD-L1 and PD-L2 are immune checkpoint proteins that act as co-inhibitory factors. They can halt or limit the development of the T cell response. The interaction between PD-L1 and PD-L2 with their cognate receptor ensures that the immune system is activated only at the appropriate time. This minimizes the probability of chronic autoimmune inflammation. However, tumor cells can exploit this immune-checkpoint pathway so they can evade detection and inhibit the immune response. The PD-L1 is frequently over-expressed on tumor cells or on non-transformed cells in the tumor microenvironment. PD-L1 on tumor cells can bind to PD-1 receptors on the activated T cells. This inhibits the cytotoxic T cells. These deactivated T cells remain inhibited in the tumor microenvironment [77].

Research on cancer immunotherapy aims to overcome the ability of cancer cells to resist the immune responses and to stimulate the body's own mechanisms that enable it to remain effective against cancer [78, 79]. Recently, monoclonal antibody therapies against PD1 and PD-L1 have shown promising results. This includes Nivolumab (Opdivo®), which is approved for previously treated metastatic melanoma and squamous non-small cell lung cancer. Another anti-PD1 drug, Pembrolizumab (Keytruda®) has been approved for previously treated metastatic melanoma. Similar strategies are being explored for treating other types of cancer, including non-squamous NSCLC, renal cell carcinoma and bladder cancer. MEDI4736 has been shown to be effective in early clinical trials. Another anti-PD-L1 drug, Atezolizumab (Tecentriq®), went through clinical trials and is now approved for treating chronic lymphocytic leukemia [78, 79]. Note that the names of drugs that are monoclonal antibodies end in mab, as in Nivolumab, Pembrolizumab and Atezolizumab. However, the immune response is not the only factor that is important in treating and curing diseases. Gene networks are important in other parts of our bodies.

Note that monoclonal antibodies (mAbs) are produced *in vitro* by B cells [4]. *In vivo*, most B cells (immune cells made in the bone marrow) produce a mixture of antibodies in response to a single toxic antigen. These are polyclonal antibodies. They can bind specifically to antigenic proteins on the surface of foreign cells in the blood, such as Bacteria and

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cancer cells. This specificity and activity is used when a nearly immortal cell line of myeloma B cells is fused with antibody-producing spleen cells from a mouse. These hybridomas produce large amounts of a single mAb because they can survive and grow in cell cultures. These mAbs can be directed against specific sites (epitopes) on antigens that are produced by cancer cells. They are also useful in diagnosing diseases. Some of them are also used in home pregnancy tests, while many others become therapeutic drugs and/or research tools [4].

Systems thinking has led to the understanding that genes exist in networks that resemble social networks [80-82]. The mechanisms by which multiple genetic variants that are associated with diseases contribute to their progression are being determined. However, it is not just the genes that are directly involved in a particular disease. Transcription factors, enhancers, promoters and epigenetics are also involved. So, researchers at the Swiss Institute of Bioinformatics used a network-centric view to perform genome-wide association studies (GWAS). They used innovative software tools, such as the *Pa*thway *Sc*oring *Al*gorithm (Pascal) to construct maps of gene networks for 394 types of cells. These networks described hundreds of thousands of regulatory interactions between thousands of genes. This provided the first global view of the control systems acting in a very diverse collection of cells and tissues. The Swiss researchers worked with researchers from the Broad Institute of MIT and Harvard. They discovered that genetic variants can disrupt components of regulatory networks in disease that are specific to different tissues. They also mapped perturbed molecular circuits that underlie complex diseases. These studies provided new insights into disease mechanisms that may lead to targeted treatments that are more effective and have fewer side effects for each individual patient. However, there were and still are important complications. First, over 90% of disease variants lie outside of protein-coding genes, in regions of the genome that are still poorly understood. Second, regulatory relationships may vary between different types of tissue, such as the heart, brain and liver. Still, they were able to build the largest collection of such networks to date, describing the regulatory interactions between over 19 000 genes [80-82].

The parts of the human genome that lie outside of genes coding for proteins were once known as junk DNA, with no known function [4]. However, we are now learning about its function. For example, it was found that some of this supposedly junk DNA plays an important role in preventing breast cancer [83]. The transcription of a strand or fragment of a long non-coding RNA (lncRNA) called GNG12-AS1 was shown to impair cell cycle progression and metastasis. Moreover, lncRNAs play

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pivotal roles in gene regulation during early development and in the pathogenesis of diseases. They can regulate gene expression through transcriptional interference or through the RNA product itself. Transcriptional interference occurs when the transcription of one gene directly interferes with the transcription of an adjacent gene, either at its initiation site or at an essential cis-regulatory element. Transcription of lncRNAs can interfere with the transcription of adjacent genes by altering the binding of transcription factors and/or RNA polymerase II. They can also alter nucleosome remodeling or induce changes in histone modifications [83].

The part of the genome that lies outside the nucleus is also important. That is, a mother's metabolic syndrome (obesity) can be passed down to future generations through her mitochondrial DNA (mtDNA) [84]. This can happen even if the offspring consume a normal, healthy diet. The mother's mtDNA can cause some proteins to be expressed incorrectly, leading to mitochondrial defects. These studies were done in mice, but this may also apply to humans. Moreover, human infants usually learn their eating habits from their mothers. So, not only nature, but also nurture can work together to pass metabolic syndrome and its associated pathologies on to future generations [5]. However, unlike mice, humans can learn to modify their diets and engage in regular exercise.

1.3.8.2 RNA-based genes in living systems

Systems thinking has changed the way we think about genes. In reductionist thinking, genes were pieces of DNA that coded for proteins, which were the machinery of life. We now know that only about 2% of the nuclear human genome codes for mRNA (or its precursor, hnRNA, or heterogeneous nuclear RNA) that codes for proteins [4]. To make human proteins, first the DNA is transcribed into hnRNA, which is converted to mRNA, which can be translated into polypeptides or proteins. Proteins provide structural support, catalyze many different reactions in cells, and assist in intercellular communication. When RNA molecules act as catalysts, they are called RNA enzymes, ribozymes or catalytic RNA [4]. On the other hand, bacterial DNA is transcribed directly into mRNA (without any hnRNA intermediate). The central dogma of molecular biology in the 20th century was that information could not flow from proteins to RNA to DNA and that DNA codes for mRNA, which is translated into proteins. Genes were said to code only for proteins. There was even a hypothesis called the one gene one polypeptide hypothesis. It suggested that each gene codes for a single polypeptide or protein. Now,

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we know that there are important exceptions to this. Millions ($>10^6$) of different types of proteins are made in humans from only about $2x10^4$ genes. This is done by splicing portions of gene segments, such as for genes that code the variable, diversity and joining regions of antibodies in the human immune system [4].

Also, some genes code for small pieces of RNA, such as miRNA, rather than proteins. A miRNA is single stranded and contains about 22 nucleotides. Many stay within cells, but others (extracellular miRNAs) can circulate throughout the extracellular environment. Both types can bind to mRNA and keep it from being translated into a polypeptide or protein. First, though, a longer, primary miRNA (pri-miRNA) is transcribed from DNA. The pri-miRNA has double-stranded hairpin structures that are recognized by a protein in the nucleus of the cell. This complex between the nuclear protein and the pri-mRNA associates with the Drosha protein to form a Microprocessor complex. It hydrolyzes the pri-miRNA to form a precursor miRNA (pre-miRNA). The pre-miRNA is exported out of the cell nucleus and into the cytosol. Here, it is hydrolyzed further in a reaction catalyzed by the RNase enzyme called Dicer to make miRNA. The miRNAs can then bind to select mRNAs to stop them from being translated into proteins. This is an important part of post-transcriptional regulation of gene expression.

Also, miRNAs play an important role in chromosome segregation, differentiation of developing cells, metabolism and programmed cell death (apoptosis). Defects in miRNA have been implicated in cancer and diabetes. For example, miRNA-802 is upregulated in type II diabetes. Also, double-stranded miRNA can bind to complementary portions of DNA, turning off its transcription into mRNA. Other types of RNA, called noncoding RNA-activating (ncRNA-a), can activate transcription by interacting with the Mediator protein complex to stabilize the structure of chromatin [4, 85].

Of the approximately 2650 mature miRNAs in the human body, only a few of them (35-40) are very abundant in the CNS [86, 87]. This strongly suggests that there was highly selective evolutionary pressure for the CNS to use only these single-stranded ncRNAs for productive miRNA–mRNA interactions and the down-regulation of gene expression. They are involved in intercellular signaling and help regulate embryonic development, adult neurogenesis and responses to environmental stimuli. Moreover, DNA methylation and histone modification regulate miRNA expression. In addition, mitochondria store many miRNAs and affect their activities. At the same time, miRNAs are enriched in dendrites and synaptosomes. Six of them are up-regulated in Alzheimer's disease. They

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can account for much of the neuropathology of this common, age-related inflammatory neurodegenerative disease. In addition, miRNAs are involved in Fragile X syndrome, Rett syndrome, autism spectrum disorders, major depression disorders, schizophrenia and Parkinson's disease [87]. Six inducible, pro-inflammatory miRNAs that are abundant in the CNS are up-regulated in sporadic AD. They appear to be key contributors to the sporadic AD process and can explain much of its neuropathology [86, 87].

In contrast, the DNA sequences that miRNAs target are being used together with oncolytic viruses to express genes for a variety of therapies [88]. That is, some viruses can be genetically modified so that they contain genes that can kill cancer cells (so they are called oncolytic). This strategy has been developed well enough so that it can make potent oncolytic viruses with much lower toxicities than conventional anti-cancer drugs. They improve the delivery, duration and therapeutic efficacy of miRNA-based therapeutics that either restore or inhibit the function of dysregulated miRNAs in cancer cells. The result is increasingly potent and safe cancer therapeutics [88].

Like hnRNA, precursors of rRNA (ribosomal RNA) and tRNA are hydrolyzed and then ligated to make rRNA or tRNA [4]. Sometimes, the precursor RNA molecules act as their own catalysts, accelerating the process. So, not just mRNA, but also other forms of RNA are important. In fact, rRNA, which is produced in the nucleolus in eukaryotic cells, accounts for most of the RNA in eukaryotic cells. Ribosomes are assembled in the nucleolus. Also, other forms of RNA can regulate gene expression and the translation of mRNA into many different kinds of proteins. There are also crucial parts of the human genome that code for the microRNAs or miRNAs. They cause some mRNAs to be translated into proteins and prevent others from being translated. Many miRNAs have important roles in cardiovascular health, Alzheimer's disease (AD) amyotrophic lateral sclerosis (ALS), autoimmune diseases, inflammatory responses, insulin sensitivity, and antidepressant sensitivity [4].

At the same time, miRNAs regulate gene expression in other mammalian cells and expressed at different levels in different tissues [89]. Researchers are using miRNA to target sequences that control the expression of exogenous genes as well as which tissues will support viral growth. This is being used to enhance the therapeutic indices of oncolytic viruses that express therapeutic transgenes. Their efforts have produced exciting new therapies and possible cures for many types of cancer. These oncolytic viruses are more potent, can infect many species and have lower toxicities than conventional anti-cancer drugs. Furthermore, oncolytic

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viruses have been used to enhance the delivery, duration and therapeutic efficacy of therapies based on miRNAs that are designed to either restore or inhibit the function of dysregulated miRNAs in cancer cells. Recent efforts focused on combining oncolytic viral therapy and miRNA regulation have generated much interest and investment of resources in developing potent and safe cancer therapies and cures [89].

The miRNAs are usually encoded within introns [4]. First they are transcribed from DNA to make a long RNA, called primary RNA, or pri-RNA, which may contain many mi-RNAs, including short hairpin RNAs (shRNAs). They are partly broken down in the nucleus of the cell to make shorter (about 70 nucleotides) precursor mi-RNAs (pre-mi-RNAs). This is catalyzed by a protein complex called Microprocessor. It contains an RNA hydrolase (RNase III) called Drosha. The pre-mi-RNA is exported out of the nucleus and is hydrolyzed in a reaction catalyzed by another RNase III enzyme called Dicer, producing the mature mi-RNA. The imperfectly complementary miRNA duplexes contain a passenger and guide strand. This binds to an RNA-induced silencing complex (RISC). The RISC is guided to its mRNA target by the miRNA strand. The passenger strand is removed and the guide strand binds to its mRNA target. The RISC complex can silence genes by either inhibiting the initiation of translation or by transporting the complex to cytoplasmic processing bodies (p-bodies) where the mRNA is deadenylated and destroyed [4].

One type of miRNA, called mirtrons is not cleaved by Drosha [90]. Instead, splicing and debranching produces a pre-miRNA substrate for Dicer. Once they are hydrolyzed by Dicer, they function the same as Dicer-dependent miRNA. There are also some non-coding RNAs that are come from introns, but aren't hydrolyzed by Dicer. They are called agotrons. Their mature structure resembles pre-miRNA. They are highly conserved in mammals and are stabilized by Argonaute proteins. They induce repression the transcription of mRNA targets in the way that miRNA does. This is another way that short introns can regulate the expression of genes [90].

After many introns are removed from hnRNA, they are broken down by hydrolysis [91]. However, when an RNA gene is embedded within an intron, it is expressed to form miRNAs, small nucleolar RNAs (snoRNAs), small-interfering RNAs (siRNAs), and various long non-coding RNAs (lncRNAs). Some lncRNAs can function as oncogenes [85]. It is thought that many genes self-regulate their expression through the ncRNAs within their introns [91]. It should be noted that when introns were first discovered, it was thought that they were only present in eukaryotes and not in prokaryotes [92]. However, it is now known that viruses, Bacteria

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and Archaea, as well as the mitochondria and chloroplasts in lower eukaryotes and plants have introns that perform a variety of functions [93, 94]. Bacterial Group I introns are self-splicing and act as ribozymes [94]. They are mobile RNA catalysts that can move within and between genomes [94]. Group II introns are mobile metalloribozymes that also self-splice [93]. They encode a reverse transcriptase that stabilizes the structure of the RNA and changes it to DNA that can be inserted into the specific regions of the genome. This enables Group II introns to be used in biotechnology [93].

So, introns have been assigned to four different groups [92]. Group I is made up of introns that are mRNA precursors in the nuclei of eukaryotes. Group II introns are spliced by a reaction that is catalyzed by RNA. Group III introns are in fungi and Group IV are introns of genes coding for tRNA [92]. In eukaryotes, the presence of introns in genes can increase the abundance of proteins coded for by those genes [91]. They can help initiate transcription and termination as well as the organization of the genome. They can also slow down the transcription of RNA. Many introns take hours or even days to be fully transcribed. This enables them to cause delays between gene activation and transcription. This can cause oscillations that are essential for proper embryological development [91].

Genes can also be silenced by small interfering (siRNAs) in plants and some invertebrates [4]. They are being used in clinical trials for treating a variety of diseases. Some non-coding RNAs are processed by Dicer and Drosha to form Dicer and Drosha-dependent RNAs (DDRNAs), which play an essential role in the DNA-damage response of cells. Dicer catalyzes the hydrolytic cleavage of long double-stranded DNA into shorter fragments of siRNA containing about 20 nucleotides. They enter the cytoplasm and interact with the RISC. They target mRNA by binding to as little as 6-8 nucleotides in the seed region at the 5' end of miRNA. Argonaute proteins in the RISC can bind miRNAs, siRNAs and piwi-interacting RNAs (piRNAs) to prevent the translation of mRNA into proteins. The piRNAs form complexes with piwi proteins that have been linked to epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, especially during spermatogenesis. The piRNAs bind to piwi proteins to repress transposons and protect the genome from this type of mutation. They trigger the generation of another class of small RNAs, called 22G-RNAs, which silence the transposons. So, the piRNAs act as part of the immune system that distinguishes mRNA from exogenous and deleterious RNAs. The genes coding for piwi proteins were first described as P-element induced wimpy (piwi) testis in *Drosophila*. They are now known to

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encode regulatory proteins that help maintain incomplete differentiation in stem cells and the rate of cell division in germ line cells. High throughput sequencing of cross linked immunoprecipitation (HITS-CLIP) of Argonaute can be used to identify miRNA targets for drug development [4].

A much longer RNA molecule (16 000 nucleotides) called XIST (X-inactive specific transcript) binds to one of the two X chromosomes in female mammalian diploid cells and inactivates it [4]. There are also ribozymes that can catalyze their own hydrolysis (self-cleaving ribozymes) and are converted to a form that can control the expression (transcription) of genes [4].

1.3.8.3 Encyclopedia of DNA elements

Even though only about 2% of the nuclear human genome codes for hnRNA, the rest is not junk [4]. About 76% of human DNA is transcribed into different types of RNA. People working on the ENCODE project have constructed an Encyclopedia of DNA elements (thus the name ENCODE) [4, 95]. It found that at least 80% of human DNA has a biochemical purpose. Not only does a small portion of our DNA code for pre-mRNA, which will become mRNA and proteins (from a little over 20 000 protein-coding genes), but other parts of DNA provide binding sites for regulatory proteins (which influence gene activity). Some of the DNA also codes for different types of RNA that have many diverse roles. Also, some parts of DNA can be modified by the addition of methyl groups or acetyls, causing genes to be silenced. Thus, the regulation of gene transcription and mRNA translationinto proteins in humans is more complicated than previously thought. Gene transcription can be controlled by several stretches of DNA located both near and far (*cis* and long-range regulatory elements) and by ncRNA [4, 95]. Moreover, histone proteins and ribonucleoproteins are integral parts of chromosomes that can be modified and affect gene transcription [4].

Some genes code for siRNAs [4]. They ensure genomic stability by silencing endogenous selfish genetic elements such as retrotransposons and repetitive sequences. The siRNAs contain 21-25 base pairs. They are used in research to knockdown specific proteins. There are also some pieces of DNA code for other types of RNA molecules, including some that are catalysts and others that regulate transcription and translation (such as siRNA). Some of these RNAs can bind to DNA or mRNA, turning them off or on. During the life of a cell, different genes need to be turned on or off at just the right times, in response to environmental

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stimuli. The RNA and proteins that are needed are made at the right times and in the right locations in the cell. On the other hand, in cancer, transcription of some of the DNA (oncogenes) can be left on when they should have been turned off. Other portions of DNA can be translated into precursors of ribosomal RNA (rRNA) and transfer RNA (tRNA) [4].

1.3.8.4 Genes, selfishness and cooperativity

System thinking has changed our concept of genes, selfishness and cooperativity [4]. Genes can code for proteins or RNA. There is a division of labor in cells. DNA and RNA are used to store and process genetic information, while proteins, lipids and carbohydrates primarily perform regulatory, metabolic and structural roles. Some proteins also help control the expression of genes and work with self-assembled ribosomal RNAs to make themselves and other proteins in ribosomes. Even though enzymes catalyze many of the reactions in cells, some can be catalyzed by catalytic RNAs, also known as ribozymes. One ribozyme, from the bacterium *Azoarcus* can even assemble itself after being fragmented [96]. It has also been shown that similar RNA fragments can self-assemble and form cooperative hypercycles [92]. Through such cooperative hypercycles, RNAs are able to form ribozymes, which may have pre-dated DNA or proteins. So, the original genes are thought to have been cooperative, not selfish, and may have been made from RNA, not DNA [96, 97].

1.3.8.5 Long noncoding RNAs, lncRNAs

There are also thousands of long noncoding RNAs called lncRNAs that were not predicted by reductionist thinking. They are RNA genes that orchestrate and regulate other genes and are regulated by some key proteins [98]. At least one of them, lncRNA-p21, is directly regulated by the oncoprotein p53. It forms an lncRNA-ribonucleoprotein (RNP) by combining with a nuclear factor. It is a transcriptional repressor that facilitates p53-mediated apoptosis. So, some lncRNAs play important roles in cancer etiology. There are also long intergenic RNAs (lincRNAs) that do not overlap protein coding genes. They have highly conserved promoter regions that recruit the binding and direct regulation of essential transcription factors. There are also antisense lncRNAs that overlap known genes that code for proteins and intronic lncRNAs that are encoded within introns of protein coding genes. The lncRNAs are a cryptic, but critical, layer in the genetic regulatory code. About one third of them are linked

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with chromatin-modifying complexes that modulate key cellular pathways. Moreover, purified chromatin contains twice as much RNA as DNA [98].

Noncoding RNA (ncRNA) is also important in regulating the expression of genes involved in circadian rhythms [99]. The transcriptomes of 12 mouse organs over time were analyzed and quantified. Researchers found that 43% of all protein coding genes are transcribed in harmony with circadian rhythms, mostly in an organ-specific manner. In most organs, the transcription of many oscillating genes peaked during transcriptional rush hours preceding dawn and dusk. The rhythmic genes clustered together, were longer, and had more splice forms than non-oscillating genes. A systems-level analysis showed that there was an intricate rhythmic orchestration of gene pathways throughout the body. There were also oscillations in the expression of more than 1000 known and novel ncRNAs that exist (are conserved) in both mice and humans. This supports their potential role in mediating the function of biological clocks. Also, ncRNAs were expressed rhythmically and in similar proportions as genes that code for proteins. It is important to note that most of the best-selling drugs and World Health Organization list of essential medicines directly target the products of rhythmic genes. Since many of these prescription drugs have short half-lives, patients may benefit from timed dosage. These results highlight critical, systemic, and surprising roles of the human circadian clock and provide a way to advance chronotherapy [99].

1.3.9 Nonlinearity and rhythms in living systems

1.3.9.1 Circadian clock and sleep

In contrast to reductionist thinking and its obsession with linear phenomena, systems thinking is quite compatible with circular organization and life's many rhythms. The human circadian clock interacts with sleep-wake cycles and metabolic networks to affect nearly every physiological process in our brains and bodies [100, 101]. They work together to guide physiological and behavioral processes through the approximately 24 hr cycle of night and day, such as the sleep-wake cycle. Energy is used rhythmically, while sleep plays a crucial role in maintaining metabolic homeostasis. Circadian rhythms, sleep homeostasis, redox cycles, and other cellular metabolic networks are interdependent. Autonomous redox oscillations in cells work with transcription/translation feedback loops to alter cellular activity at specific times of day. So, there are important non-transcriptional timekeeping mechanisms. This includes antioxidant peroxiredoxin proteins that are markers of 24-hr rhythms.

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Moreover, circadian dysfunction and sleep disruption can contribute to the etiology (cause) of many types of cancers, heart disease and diabetes, as well as metabolic, vascular, and mental disorders. In addition, changing concentrations of hormones lose their rhythm and their amplitude decreases with increasing age until they finally taper off [100-102].

The interactions between human molecular clockwork, health and disease can be understood better when the genome, metabolome, proteome and microbiome are monitored as people live their lives [102]. Our temperature and blood pressure, along with concentrations of melatonin and many other hormones, oscillate in a diurnal fashion in both human eukaryotic cells and in the gut microbiome. For now, remote sensing technologies on smart phones can provide important information on how one's phenotype varies during the day. Current apps can record one's exercise, sleep, mood, pain, dietary content and frequency, as well as body temperature, blood glucose and cytokines. In research settings, the oscillatory metabolites in exhaled breath, urine and plasma can be monitored. In the clinic, the concentrations of about 2/3 of the top 100 selling drugs oscillate in a diurnal fashion. Most drugs have half-lives of six hours or less. Moreover, drug metabolizing enzymes and transporters are high amplitude oscillators in the liver and pharmacokinetics varies as a function of time of drug dosing. Hypertension is a good example, due to the phenomenon of nocturnal hypertension or 'non-dipping' which complicates high blood pressure in about 40% of the people who take them. In a mouse model, controlled, but not rapidly released melatonin administered in the morning attenuated nocturnal hypertension [102].

Intracellular circadian rhythms exist across all phyla, but mammals have their own additional interneuronal system [103]. The suprachiasmatic nucleus (SCN) is the master regulator. It has been studied *in vitro* as a 'clock in a dish'. This has shown how intra-neuronal signals are translated by SCN neurons into circuit-level emergent properties and how the circuit decodes and responds to light. There is an intracellular transcription-translation feedback loop (TTFL) in SCN neurons. The TTFL is controlled by the properly timed transcription of *period* (*per*) and *cryptochrome* (*cry*) genes. This is activated by BMAL1/CLOCK dimers. The subsequent accumulation of Per and Cry proteins progressively inhibit BMAL1/CLOCK activity. The timely degradation of Per and Cry proteins subsequently removes the inhibition from BMAL1/CLOCK and starts a new 24-hour cycle [103].

However, these cycles and the output of proteins from the circadian clock decreases in the aging brain [104]. There is probably a link between circadian clock dysfunction, oxidative stress, and age-related

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neurodegeneration. In this clock, there are interacting transcriptional activators and repressors. The activators (or 'positive limb') regulate the transcription of many diverse genes. These positive limb proteins drive the transcription of circadian repressors, or 'negative limb' components, which in turn inhibit the transcriptional activity of the positive limb. This cell-autonomous feedback system synchronizes intracellular gene expression with external cues such as light and aligns physiologic oscillations in cells and tissues throughout the body. In peripheral tissues, clock genes regulate cellular metabolism and redox homeostasis. They are also involved in the aging process. That is, the expression of positive-limb clock genes decreases during aging. Also, impaired circadian oscillation and oxidative injury are associated with brain aging and age-related neurodegenerative diseases. So, there is a possible link between circadian clock dysfunction, oxidative stress, and age-related neurodegeneration [104].

Dysregulated circadian rhythms and sleep deprivation also occur during adolescence [105]. This is not simply due to adolescents being over-stimulated or under much stress, since it also occurs in animals. It is partly due to changes in the homeostatic and circadian regulation of sleep during puberty. These changes promote a delayed sleep phase that can be made worse by exposure to light in the evening and when adolescents must awaken before sunrise to get ready for school. At the same time, the homeostatic and circadian systems that regulate sleep are affected by gonadal hormones. All these factors, together with social pressures can lead to dysregulated cycles, sleep deprivation, accidents and diseases [105].

The amount and quality of sleep that we receive impacts our immune systems [106]. There are two separate processes. Process C (circadian rhythmicity) controls the timing of sleep, while process S (for sleep homeostasis) controls the duration. Some people experience advanced sleep phase. They feel sleepy in the late afternoon and wake up before sunrise, even though they sleep as long as others. They have an unusual process C. Others experience short sleep, which is due to changes in process S. Process C is associated with the molecular clock, which allows cells to maintain circadian rhythmicity. Mutations in the *PER2* gene result in advanced sleep phase. Mutations in the *DEC2* gene cause shortened sleep duration. The protein products coded for by these genes affect the immune system simultaneously, but through separate mechanisms. The effects are seen by clinical evidence. For example, the risk of dying from sepsis increases from 2 AM to 6 AM. Many people with rheumatoid arthritis or

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asthma experience daily cyclical variations in the severity of their symptoms [106].

Circadian rhythms also affect daily eating patterns, which can affect one's health and susceptibility to disease [107]. Dysregulation of these rhythms contributes to obesity and metabolic syndrome. One way to counter this is through time-restricted feeding, in which access to food is restricted to a few hours, without caloric restriction. This supports robust metabolic cycles and protects against nutritional challenges that predispose to obesity and metabolic syndrome [107].

In contrast, a high-fat diet can lead to these conditions by reprogramming the circadian clock [108]. Several metabolic pathways are reorganized, which leads to widespread remodeling of the liver clock. A high-fat diet can also cause the large-scale production of new oscillating transcripts. This, in turn reorganizes the coordinated oscillations between coherent transcripts and metabolites. Moreover, it is specifically the nutritional challenge, and not the development of obesity that causes the reprogramming of the clock. Fortunately, the effects of the diet on the clock are reversible [108].

In conclusion, circadian clocks along with metabolic and cellular rhythms are important in maintaining health. When they are dysregulated, diseases can emerge. This is part of the system that makes us human.

1.3.9.2 Retrotransposons

Retrotransposons are also very important in making us human [4]. They are DNA sequences that share a homology with retroviruses that have RNA that is reverse transcribed into DNA when the virus is replicated. So, they are an example of how information can flow from RNA to DNA, in contrast with reductionist thinking. About half of the nucleotides in the human genome are parts of retrotransposons [109]. They can disrupt the expression of specific genes and are biased towards regions where DNA is hypomethylated. L1 retrotransposons are active in the hippocampus and caudate nucleus in the human brain [109] and may account for much of the differences that are seen in so-called identical twins (actually, monozygotic twins). Retrotransposons are also important in generating new neurons throughout life in the hippocampus. L1 retrotransposons are also used in the developing human brain, in which new neurons are constantly being made. On the other hand, L1 insertions occur in genes that are commonly mutated in cancer [4].

There are also Alu retrotransposons [4]. They are members of the class of RNAs called short interspersed nuclear elements (SINEs). Alu repeat

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sequences are the most common non-coding repeat sequences in the human genome. Some of them are active as retrotransposons. Some are found in the human brain [109]. They contain about 300 nucleotides, 280 of which comprise a central core [110]. The human genome has about 1.1 million copies of the *Alu* retrotransposons [110]. They are divided into lineages, based on how old they are. The *AluJ* lineage is about 65 million years old and is not transcribed. The *AluS* lineage is 30 million years old and some of them are transcribed. The youngest lineage, *AluY* has the largest number of functionally active sequences. One new Alu insertion is estimated to occur for every 20 live human births. The mobility of the *Alu* DNA depends on its sequence of nucleotides and the ability of the RNA that it codes for to interact with a host protein called SRP9/14 [111]. *Alu* repeats were once thought to be junk DNA, with no known function. We now know that they are not only retrotransposons, but some of them can bind to pieces of DNA called hormone responsive elements, to which nuclear hormone receptors bind and initiate transcription of DNA into RNA. So, the regulation of gene translation and mRNA transcription in humans is quite complicated. Gene transcription can be controlled by several stretches of DNA located both near and far from the genes (cis and long-range regulatory elements) [112] and by non-coding RNA [4].

1.3.9.3 Circular RNA and DNA

There are also circular RNAs and DNA with covalently linked ends [4]. They are in Bacteria and viroids (virus-like infectious particles) or circular satellite viruses. One of them, the hepatitis delta virus, causes severe liver disease in people who are infected with it. More recently, data from high throughput sequencing has shown that there are circular RNAs in species ranging from Archaea to humans. This is in contrast with reductionist thinking that only looked at human genes in the 23 pairs of chromosomes that make up our karyotype. For example, over 25 000 circular RNAs have been found in human fibroblasts and they are derived from about 15% of the actively translated genes. Moreover, circular RNAs accumulate in the cytoplasm where they can bind miRNAs. One that was found in mouse and human brains had over 70 binding sites for miR-7, a micro-RNA. So, circular RNA might act like a sponge, absorbing miRNAs and keeping them from inhibiting the translation of mRNA into proteins [4].

Circular DNA is also present in Bacteria as plasmids. They are mobile genetic elements that are transferred between bacterial cells of the same or different genera, through the process of conjugation [113]. Many of them

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contain DNA that codes for genes that make Bacteria resistant to antibiotics. Such resistance to most antibiotics has increased – especially in Gram-negative Bacteria. To help address this problem, a method (fluorescence microscopy) was developed to identify rapidly those plasmids that confer resistance to antibiotics. This produces barcodes that reflect their DNA sequence [113].

More recently, extrachromosomal circular DNA (ecDNA) has been found to be quite common in cancer cells, but rare in normal, healthy cells [114]. This discovery was made by an interdisciplinary team that used genomics, bioinformatics and classical cytogenetics to detect, quantify, and analyze ecDNA. This type of ecDNA codes for multiple copies of oncogenes that drive tumor evolution and genetic heterogeneity. So, it is becoming just as important to know where oncogenes are located than to just simply show that they exist [114].

1.3.9.4 The importance of mobile genetic elements

In 1948, Barbara McClintock published a manuscript that showed that some genes could move around (or jump) in maize [4]. This paper was ignored by men for many years, but we now know that there are jumping genes, more commonly known as transposable elements, transposons or mobile genetic elements. For example, when some of our highly differentiated white blood cells make antibodies, they have variable, diversity and joining genes that are rearranged in chromosomes before coding for the antibody. Transposons can also cause mutations and can change the amount of DNA in cells. Most transposons in human cells copy themselves through retrotransposons [4]. This is in contrast to reductionist thinking which preached that the genetic code was like the book of life or a blueprint of life that remained intact. That is, nobody tears out a piece of a book or a piece of a blueprint and then tapes it into a different part of the book or blueprint. Books and blueprints are linear, but life is not.

Mobile genetic elements are ‘selfish’ because their only function is to make more copies of themselves, with no benefit to the organism (including humans) in which they are found [4]. For example, 10.6% of the total human DNA contains *Alu* elements. This is the most abundant form of transposons. There are also long interspersed elements (LINES), which are up to 9000 base pairs long. The human genome contains about 868 000 LINES and they are 20.4% of the total genome. Transposons can be mutagens if they are inserted into a functional gene. So, there is a big difference in how the term “selfish genes” is used [4]. Richard Dawkin’s book [13] used the term as a metaphor, while others use the term to

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indicate an actual scientific concept concerning transposons and a few genes [4].

Graph theory was used to analyze inclusive similarity networks from data on over 3000 viral genomes [115]. There is a large, complex network of mobile genetic elements (mobilome) that remodels genetic material and moves base sequences throughout different kinds of organisms in the biosphere. The vast majority of these mobile genetic elements are transposons and retrotransposons that were probably derived from DNA and RNA viruses, respectively [115, 116]. They are especially important in human embryology, neurology and carcinogenesis [117, 118]. It is also quite likely that there are ancient viral origins of cognition [114]. Moreover, viruses were extremely important in evolution [119]. They enable non-gradual or punctuated evolutionary changes [120]. Moreover, horizontal gene transfer from viruses and Bacteria to human cells is just as significant as vertical gene transfer from parents to their children. So, viruses and Bacteria in the environment had and continue to have important effects on the human body. We are part of the environment – not separate from it [4].

1.3.9.5 Epigenetics and post-translational modifications of proteins

Systems thinking has shown that the human genome is not the blueprint or book of life. Genetics does not control everything. There is a layer of control on top of genetics [4]. It is called epigenetics. So, the prefix epi- means on top of and epigenetics is a layer of control on top of the classical DNA-based genetic control. One form of epigenetics is the modification of histone proteins. That is, DNA in the cell nucleus is wrapped around histone proteins making it inaccessible to DNA and RNA polymerases. The ε-amino group of lysine residues on histones can be acetylated, neutralizing its positive charge. This reduces the affinity of the histone tail that protrudes from the nucleosome core to negatively charged DNA. As a result, chromatin adopts a more relaxed structure, giving RNA polymerase and other key enzymes access so they can activate transcription of DNA [4].

One set of such proteins contain bromodomians that bind to specific acetylated lysines as they co-activate transcriptions [4]. So, histone acetylation is a dynamic process that is controlled by histone acetylases (HATs) and deacetylases (HDACs). They can control the access of the transcription network to DNA by tagging or erasing acetyls on specific lysine residues. HDACs are classified into four groups or classes that depend on their amino acid sequences, domain organization and function.

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Groups I and II are inhibited by trichostatin A, an antifungal agent. Group III is a family of NAD^+-dependent enzymes. NAD^+ is the oxidized form of nicotinamide adenine dinucleotide, while NADH is the reduced form. Human HDACs in group III are homologues of some types of Sir proteins, so they are called SIRTs. They are also known as Sirtuins. They are best known for their ability to catalyze the deacetylation of lysyl residues on histones and other proteins in reactions that require NAD^+. This enables them to regulate transcription, DNA repair, metabolism, fat mobilization, neurodegeneration, aging and lifespan [4, 121-124].

In addition, methyl groups can be added to arginines or lysines in histones in reactions catalyzed by histone methylases [4, 124]. Histone lysine methylation is regulated dynamically at individual genes by recruiting methyltransferases and demethylases, similar to acetylation. Lysine residues in histones can be methylated with one, two or three methyls, without affecting their charge. So, methylation does not affect the ionic bonds between histones and DNA, but creates tags that can be identified by co-regulatory proteins. In differentiated cells, inactive portions of the genome are partitioned between different forms of repressive chromatin. Repressive structures with a compact organization that are resistant to regulatory activity can affect large regions of chromosomes. Typical repressive states include heterochromatin marked by histone H3 lysine 9 trimethylation (H3K9me3), broad regions repressed by Polycomb proteins, regions enriched in trimethyl Lys 27 on histone 3 (H3K27me3), and highly condensed regions associated with the nuclear lamina and chromatin remodeling the dimethyl form, H3K9me2. On the other hand, there are characteristic chromatin states for active genes and regulatory elements. Enhancers and promoters are marked by varying degree of H3K4 methylation and histone acetylation [4, 124].

Also, DNA is super-coiled, so it must be unwound before it can be copied into more DNA or transcribed into RNA [4]. There are enzymes (topoisomerases) that catalyze the unwinding of DNA. There are other epigenetic factors that affect gene transcription. For example, when cytosine (C) is next to guanosine (CpG) in eukaryotic genes, a methyl group is usually attached to the C. This prevents the transcription of the gene, but it does not prevent it from being copied when a cell grows and divides. So, it is not just our genes that are copied from one generation to the next, but also our epigenetics, including the degree of methylation [4].

Almost all the enzymes that modify chromatin structure require cofactors or substrates like acetyl coenzyme A (acetyl-CoA), or are also crucial metabolites in essential metabolic pathways, like α-ketoglutarate [4]. So, some biochemicals in the human body have very different

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functions, depending on their location. When in the mitochondria, α-ketoglutarate helps generate energy as part of the in the tricarboxylic acid cycle (also known as the Kreb's cycle). This cycle is fed by the transfer of two carbons from acetyl-CoA to oxaloacetate to make citrate, which goes through a series of reactions in which it loses two of its carboxylates as CO_2. One of the enzymes in this cycle, isocitrate dehydrogenase (IDH), catalyzes the production of α-ketoglutarate, which can not only continue through the cycle, but also act as a substrate for a class of enzymes called α-ketoglutarate dioxygenases. Some of them act as histone demethylases, catalyzing the removal of methyl groups from histones and others are 5-methylcytosine hydroxylases that add a hydroxyl to 5-methylcytosine. Both enzyme activities affect epigenetics. When IDH is mutated, it can lead to cancer. The covalent modifications of DNA and histones have major influences on the control of gene transcription during embryonic development and in differentiated tissues in adults. This affects the types of proteins that are produced [4].

Other epigenetic modifications can be made as well [4]. For example, the sirtuin enzyme SIRT6 catalyzes the removal of fatty acyls such as myristoyl from lysine residues from histones and an important protein involved in inflammation, tumor necrosis factor-α, or TNF-α, The fatty acyl from TNF-α is removed in the endoplasmic reticulum, enabling it to be secreted [4, 106]. Moreover, SIRT6 may play a key role in preventing cancer since its loss can cause changes in glucose metabolism that lead to cancer [99]. These changes were first discovered by a scientist named Otto Heinrich Warburg and they are called the Warburg effect [4, 124].

More recently, a new physiological role was found for SIRT6 [4, 125]. It regulates the secretion of TNF-α by catalyzing the hydrolysis of long chain fatty acyls (such as myristoyl) from lysine residues [112]. That is, it can catalyze the hydrolysis of long chain fatty acyls that are attached to specific lysine groups in a specific region of TNF-α. So, the post-translational modification of proteins by attaching fatty acyl groups to lysine residues plays an important role in protein secretion. Moreover, there may be important connections with this and the addition of a palmitoyl group to the amino acid cysteine and a myristoyl to N-terminal lysines [110].

The epigenetic modifications of the DNA packaging are different in different types of cells and tissues. Thus, epigenetics is part of the foundation of physiology [4]. Moreover, the epigenetic state can be changed. It proceeds in an orderly manner during development and differentiation. It can also change in response to the environment [125].

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Gene transcription must be carefully regulated to maintain the normal and dynamic functions of all eukaryotic human cells [126]. There are intricate and extensive links between metabolism, epigenetics and the circadian cycle that regulate gene activity. This enables cells to respond to environmental changes and physiological demands at exactly the right time. For example, stem and progenitor cells continuously produce specialized cells during development and organogenesis while maintaining tissue homeostasis throughout one's lifetime. Moreover, various stem cells have a cell-specific metabolism (metabotype) and metabolic pathways that are modulated during differentiation. This suggests that metabolism may control the fate of stem cells by regulating signaling pathways and gene expression. Thus, metabolism and stemness are closely linked processes. Many chromatin-modifying enzymes use the products of cellular metabolism to modify both histones and DNA. This is a direct link between nutritional changes, metabolism and gene expression [126].

There are also post-translational modifications (PTMs) of proteins. They help regulate protein function. Some act as on-off switches, while others mark a protein for destruction. The most common PTM is phosphorylation. Moreover, acetyls, methyls, hydroxyls, sulfate, nitrate, sugars, lipids (especially palmitoyl), and even other proteins (ubiquitin and SUMO, or small ubiquitin-like modifier) can be added to a variety of proteins. The Uniprot Database [127] includes at least 435 types of PTMs. Another database, PTMcode has an interactive web interface [128]. "It provides the context of the co-regulation of nearly 75,000 amino acids in >10,000 modifiable proteins. This enables the exploration, retrieval and analysis of predicted and known functional association between PTMs within proteins" [128]. That is, there is a global network of types of PTMs that are associated by function. They are enriched in membrane-associated and secreted proteins. Phosphorylation, acetylation and O-linked glycosylation are central to the PTM network. The phosphoproteome is the collection of all phosphorylation states of proteins in cells. Enzymes that catalyze the phosphorylation of other proteins are called protein kinases. The collection of kinases is the kinome. One especially important protein kinase is activated by adenosine monophosphate, or AMP (not to be confused with anti-microbial peptides, abbreviated as AMPs) [128, 129]. AMP-activated protein kinase (AMPK) responds to the cellular energy state, which affects the relative concentrations of AMP, adenosine diphosphate (ADP) and adenosine triphosphate (ATP). AMPK regulates metabolism and is involved in non-metabolic processes and diseases. Interestingly, many substrates of AMPK are proteins that are involved in cell motility, adhesion, and invasion. Since AMPK is now known to be a

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critical node in aging and pathological processes, potential targets for therapeutic strategies have been identified [129].

There is a PTM code, or a combination of individual PTM states. PTM code also refers to a database that has been constructed to compile known and predicted PTM associations [130]. Different types of PTMs form an interconnected network that contains 450 000 amino acid residues in about 6000 proteins in humans [131]. Many of these modifications affect the interactions between proteins and lipid membranes.

So, epigenetic mechanisms have important roles in diseases related to diet, lifestyle, early life experience and exposure to environmental toxins [125]. The epigenetic protein families include histone deacetylases, protein methyltransferases, lysine demethylases, bromodomain-containing proteins and proteins that bind to methylated histones. These protein families and their protein–protein interaction domains are emerging as potential drug targets. Epigenetic regulation includes covalent modification of DNA and the proteins that package it [4, 125].

That is, for transcription to occur, the underlying organization of chromatin, higher-order DNA structures, RNA, histones, and regulatory proteins must be correct [4]. Transcription factors (TFs) recruit chromatin regulators (CRs) that modulate the accessibility of target DNA. However, because TF binding is dependent on chromatin accessibility, CRs and chromatin states also function as gatekeepers that modulate the regulatory activities of TFs. A network of TFs, cooperating CRs, and coordinated chromatin states guide successive differentiation and commitment events during early developmental and regeneration. During development, cell fate is specified in a dynamic process. Diverse phenotypes are established in precise patterns at the right place and time. Beginning from a single totipotent cell, successive waves of self-renewal, differentiation, and commitment ultimately produce the intricate network of cell types, tissues, and organs in a baby. So, epigenetics is important in cancer, inflammation, metabolic disease and neuropsychiatric disorders, as well as in regenerative medicine [4, 125].

It is also important in regulating homeostasis in stem cells [125]. There are direct links between nutrition, metabolism and the expression of genes. For example, acetyl-CoA is used by HATs use as a substrate and is a key metabolite in energy metabolism. At the same time, deacetylation catalyzed by HDACs is linked to intracellular energy level and its redox state. HDACs use NAD^+ as a substrate. The concentration of NAD^+ is controlled in part by the amount of energy in the cell and the ratio of NADH to NAD^+. Histone methylation and demethylation use cellular metabolites to change chromatin. Enzymes that catalyze the methylation of

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DNA and histones use S-adenosylhomocysteine (SAH) as a substrate. It is recycled back to S-adenosylmethionine (SAM) by cyclic one-carbon metabolism from the amino acid methionine and ATP. Methionine, the direct precursor of SAM, is made in a reaction with the methyl donor tetrahydrofolate (THF) with glycine. THF is a derivative of folate. SAH is a potent inhibitor of DNA methyltransferases and histone methyltransferases. Its clearance determines the rate of methylation reactions in cells. SAM and some amino acids can affect DNA and histone methylation patterns and the expression of genes. The circadian clock is linked to metabolic transcriptional regulation by epigenetic mechanisms and is essential to stem cell homeostasis [125].

Epigenetics can be either transitory or stable. They can persist as transcriptional memory, through mitosis and meiosis [126]. Substrates that are used in chromatin modification can move easily from the cytosol to the cell nucleus. As a result, information can be transferred to the transcriptional network. This means that metabolism affects chromatin structure and the expression of genes. Changes in nutrition and metabolism can lead to many types of cancer, which experience metabolic and epigenetic drift [126].

So, folate (or folic acid) is an important nutrient, especially for pregnant women [132]. It is essential for proper fetal development. It is added to food (especially bread) to prevent neural tube defects. By using Illumina's HumanMethyl450 Beadchip, 1988 newborns from two European cohorts were examined. Four-hundred forty-three CpGs (320 genes) were found to be associated with maternal plasma folate levels during pregnancy. Many of the genes were not previously known to be involved in folate metabolism. So, the concentration of folate in pregnant women affects the epigenome of the developing fetus [132]. It should be noted that folic acid is converted to folate when it enters the blood. The structure of folic acid is shown in Figure 4. Each H in the two –COOH groups are acidic, so they become ionized ($-COO^-$) at the pH of blood.

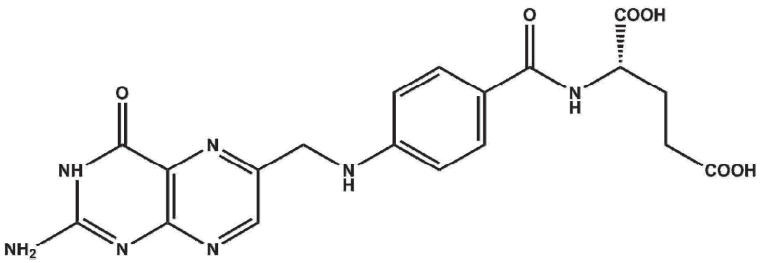

Figure 4. 2D structure of folic acid, with two ionizable hydrogens on the two –COOH groups.

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Like the genome, the epigenome is passed from parents to children and grandchildren. An example of this is the transgenerational transmission of Holocaust trauma [133]. It caused some children to become more vulnerable to stress while others became more resilient. The stress was previously thought to be caused primarily by environmental factors, such as the way that some parents raised their children. Instead, it appears that epigenetic changes in the way that the glucocorticoid receptor gene is controlled were the main cause. So, the diagnosis called transgenerational stress disorder has emerged in the scientific literature [133].

The role of epigenetic dynamics in psychiatric disorder has been known for several years [134]. These dynamics are important during embryological development, since they have profound impacts on neurodevelopment and may increase the risk to psychiatric disorders. In the zygote, most of the methyl groups attached to DNA are removed immediately after fertilization. The genome of the father's pronucleus is demethylated before the first cell division, while the mother's remains highly methylated. It is gradually demethylated during several cycles of cleavage division. Some epigenetic marks are protected from being reprogrammed. This includes imprinted genes and active retrotransposons. This extensive demethylation restores the totipotency of the zygote, enabling it to divide and differentiate into all types of human eukaryotic cells. After implantation, the inner cell mass of the blastocyst undergoes extensive demethylation, enabling it to make embryonic tissue. These and other epigenetic changes control the fate of the differentiated cells. However, adverse environmental factors can alter the delicate balance of these dynamics and lead to neurodevelopmental disturbances that are related to psychiatric disorders [134].

These factors include prenatal nutrition, exposure to alcohol and other drugs, maternal stress and care, traumatic experiences and environmental enrichment [134]. Love from parents, siblings and other caregivers can provide positive mental stimulation. This has many healthy effects on neural and cognitive development that can endure for a lifetime. The epigenetic changes link genetics and environment. So, the old question of nature vs nurture is reductionist [134]. Nature (genetics) can't be separated from the internal ecosystem or the external ecosystems in which we are all embedded. This means that cause can't be separated from effect and that unexpected, nonlinear behavior can emerge.

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1.3.9.6 The role of epigenetics in hormesis

One of the most interesting and formerly controversial aspects of systems thinking is the concept of hormesis – especially when it applies to ionizing radiation. As mentioned in section 1.2, reductionist thinking was once popular with toxicologists. They assumed that there a linear dose-response 'curve' described the risk of dying from solid cancer as the dose of ionizing radiation increased. The results of studies on the risk of dying vs the dose of radiation were fit to a straight line that did not pass through the origin. That is, when the straight line was extrapolated to a very low dose (near zero), there was still some excess mortality. This led to the conclusion that there is no safe dose of radiation [4].

In contrast, non-linear dose-response curves have been documented and accepted when biochemical mechanisms have been discovered to explain them. The classical case in modern science is selenium. Before the advent of an analytical method called inductively coupled plasma spectroscopy (ICP), selenium (Se) was thought to be toxic at all the doses that could be measured. Agricultural scientists used a specific reagent, 2,3-diaminonaphthalene, to react with Se and produce fluorescence, the absorbance of UV light or turbidity, depending on the concentration of Se in the sample. These methods are still being used to quantify Se in grass and cattle feed. However, in the 1970s ICP lowered the concentrations of Se that could be detected, due to its much better sensitivity. Se was found in humans and is now known to be an essential nutrient. It is frequently present in multivitamins. This is because Se is absolutely necessary for many enzymes to function properly. So, hormesis in the case of Se has been widely accepted by even the most conservative scientists as having a hormetic dose-response curve.

More recently, arsenic has also attracted much more attention. Popular media highlighted the fact that the concentration of arsenic in most apple juice samples is higher than that which is acceptable in drinking water. This was partly explained by the fact that we drink much more water than apple juice. The FDA is reviewing its regulations on how much should be allowed in apple and other fruit juices, but the answers are not simple. The situation is complicated by reports of hormesis in the *in vitro* neurotoxicity of arsenic, which depends on its oxidation state [135]. At low concentrations (0.1 μM), As^{+5} stimulated the proliferation of human neurons, but at higher concentrations (up to 400 μM), mitochondria were damaged and the neurons died [135].

It should be noted that arsenic and selenium are similar in that they can both cause and cure cancer [136]. Arsenic has been used to cure acute

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promyelocytic leukemia in humans [137]. On the other hand, arsenic in the environment causes many adverse health effects, including many types of cancers. It has been estimated that millions of people worldwide suffer from chronic arsenic poisoning mainly due to consumption of arsenic-contaminated water and food. In contrast, Se is an essential nutrient for humans, animals, and Bacteria. Furthermore, low doses of Se can prevent many types of cancer in animals and humans. However, it is toxic at levels slightly above what is needed to maintain proper homeostatic. It should be noted that As^{+5} is less toxic than As^{+3} and Se^{6+} is less toxic than Se^{+4} in eukaryotes and prokaryotes. Moreover, inorganic arsenic is more toxic than organic arsenic (in organometallic compounds like rosaxerone). Toxicity involves the production of ROS, but other mechanisms are also important. Arsenic decreases DNA methylation and alters the expression of the p53 protein, which has been called the guardian of the genome. On the other hand, Se modifies thioredoxin reductase. However, arsenic and Se can react with glutathione and S-adenosylmethionine by forming an As–Se complex, which can be secreted. So, at low concentrations, Se can decrease arsenic toxicity by excreting the As–Se compound. At high concentrations, Se can increase arsenic toxicity by reacting with the biochemical S–adenosylmethionine and glutathione, and by changing the structure and activity of arsenite methyltransferase [137].

Others have studied bimodal (hormetic) dose-response curves with other trace metals, including copper (Cu) [138]. They found that As^{+5} and Cu^{2+} induce bimodal dose-response effects on human cells *in vitro*. Still, they admitted that it is difficult to evaluate the potential health risks of exposure to low doses of metals and other chemicals. This is especially true when toxicities are only studied in rodents that are given much higher doses than what people are ever exposed to. In previous decades, it was assumed that direct acting carcinogens always produce linear dose-response curves. For non-carcinogens, it was assumed that they must be above a threshold concentration to have a toxic effect. In contrast, modern hormesis theory suggests that most chemicals stimulate cell growth at low doses and only become toxic at higher doses [138].

However, hormesis is not applicable to all metal poisoning – especially lead. Still, systems thinking is useful. It has shown that there are large social and economic benefits from controlling and limiting childhood exposure to lead. It was estimated that each dollar invested in household paint control in children ≤ 6 years of age saved $17–$22, with a net savings of $181–269 billion [139]. So, it is unfortunate and potentially catastrophic when cities are forced to use acidic, polluted drinking water that corrodes lead pipes. This leads to dangerously high concentrations of

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lead in children's blood, with subsequent neurological and other health problems. So, the amount of money that taxpayers saved by forcing a city to use cheap, polluted drinking water is much less than what they will have to pay in health insurance as healthcare costs increase.

One thing that can lower healthcare costs is diet. This is especially true of phytochemicals (antioxidants) that are present in most fruits and vegetables. Still, their effects on cellular stress and neuroprotection are hormetic [140]. Dietary antioxidants, such as polyphenols and L-carnitine/acetyl-L-carnitine, are neuroprotective through the activation of hormetic pathways, including vitagenes. Vitagenes are genes that are involved in preserving cellular homeostasis during stressful conditions. Hormesis provides the central underpinning of the neuroprotective responses of dietary antioxidants. Hormesis explains the concept of biological plasticity and provides important insights into how to improve the accuracy of the therapeutic dose of pharmaceutical agents within the highly heterogeneous human population [140].

There is another example of hormesis that we have almost all experienced. It is exercise, which can make you smarter, happier and generate more neurons in the hippocampus [141]. When we exercise, ROS are produced and there is some temporary inflammation. Also, exercise causes thermal, metabolic, hypoxic, and mechanical stress. In the long term, though, regular exercise protects neurons in every part of the brain in which this has been studied. It also improves different parameters related to neurodegenerative diseases (age of onset, progression, and severity of symptoms). Moreover, exercise can help patients recover from the symptoms of some neurodegenerative diseases and from different types of brain damages and insults. Vigorous exercise increases blood flow and oxygen consumption, while modulating the growth factor signaling cascade and increasing the availability and function of neurotransmitters. On the other hand, endurance exercises can represent another part of the hormetic response. It can be harmful when done to extremes. That is, a low dose of a stressful stimulus (moderate exercise) can activate an adaptive response that increases the resistance to a severe level of stress. A high dose can be harmful. For example, Pheidippides, the first man to run a marathon in Ancient Greece, collapsed and died just after saying "we have won" to the Athenians who had just defeated the ancient Persions. Also, performance curves on cognition after exercise in humans have shown inverted-U shapes. The relationship is not simple because it depends on the duration of the exercise and on the individual's fitness level. On the other hand, lengthy aerobic endurance exercises can temporarily impair information processing and cognition depending on the

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level of dehydration. The biphasic hormetic response depends strongly on oxidative stress, oxygen consumption and mitochondrial metabolism. This affects the whole body, but mainly skeletal muscles and the cardiovascular system. Also, exercise induces increased production of not just ROS but also antioxidant enzymes and redox signaling in the brain and the rest of the body. There is an important balance between exercise-induced generation of systemic ROS-related factors and brain antioxidant enzymes. They mediate the effects of exercise on the brain in a hormetic, bell-shaped curve, where high levels of ROS cause oxidative damage, while moderate amounts induce an adaptive response to oxidative challenge. Moreover, intense exercise that leads to anaerobic production of lactate can lead to temporary mitochondrial dysfunction in the brain. Finally, one should remember that the degree of harm caused by excess endurance exercise varies from one person to the next. This depends mostly on how fit a person is before running a marathon or doing other extreme endurance exercises [141].

Still, it is widely accepted that moderate exercise can help limit age-related cognitive impairment [142]. Human cognition requires interconnections between neurons that form complex information processing networks. Experience-based changes in the activities of these information processing networks lead to neuroadaptation and synaptic plasticity. Synaptic plasticity is affected by aging. Astrocytes supply energy that is needed to support glutamatergic synaptic activity (that uses glutamate as the neurotransmitter). At the same time, a functional astrocyte-neuron metabolic shuttle is essential for cognitive function. However, a lack of proper coordination between astrocytes and neurons may contribute to cognitive impairment. So, it is significant that exercise increases the effectiveness of the astrocyte-neuron metabolic shuttle by upregulating lactate transporter levels in astrocytes [142].

Another study found that physical activity increases the concentrations of the neurotransmitters, glutamate and gamma-amino butyric acid (GABA) in the visual cortex and glutamate in the anterior cingulated cortex [143]. It also leads to a global increase in brain nonoxidative metabolism of carbohydrates. This nonoxidative consumption of carbohydrates increases *de novo* biosynthesis of glutamate and GABA [143].

With all these examples of hormesis and with additional recent evidence, it is also quite possible that it also applies to ionizing radiation. Radiation hormesis (also called radiation homeostasis) is the biphasic dose-response of radiation. That is, there may be a beneficial stimulation after receiving a low dose of ionizing radiation (LDIR), but high doses are

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harmful or lethal. The beneficial effects are thought to include increased longevity, improved growth, increased embryo production, increased immune response and enhanced repair of cytogenetic damage. These effects would protect against disease. These effects don't occur in the absence of levels of ionizing radiation that are above natural background. Specific signal transduction pathways have been identified as being involved in genomic changes caused by LDIR. They include Ataxia telangiectasia-mutated (ATM), extracellular signal-related kinase (ERK), mitogen-activated protein kinase (MAPK), phospho-c-Jun NH2-terminal kinase (JNK) and p53. Also, ATM, p53, phosphor-JNK, ERK and cyclooxygenase-2 (COX-2) are responsible for radioresistance [144].

In traditional medicine, hormesis has been widely accepted for thousands of years, almost as a dogma. No mechanisms were needed to justify the belief in it. This was rejected by many physicists and chemists who usually need a clear mathematical explanation for such phenomena. Still, even they recognize that hormesis occurs with nanoparticles [144]. It is widely recognized that the physical properties of metals, carbon and other materials change dramatically when they become much smaller nanoparticles. Opaque substances can become transparent (copper), inert materials can become catalysts (platinum), stable materials can turn combustible (aluminum), solids can turn into liquids at room temperature (gold) and insulators can become conductors (silicon). Moreover, the solubilities and bioavailabilities of many drugs increase when they are put into nanoparticles [4]. So, hormesis is increasingly understood as being a dynamic adaptive response. It involves the biological plasticity of a complex human ecosystem when exposed to intermittent mild stressors. This includes physical, chemical, biological, and/or psychological factors [144].

Changes in epigenetics occur when people and animals are exposed to LDIR from environmental and medical sources [145]. It increases DNA methylation at the viable yellow agouti (*Avy*) locus in a sex-specific manner in a mouse model. Exposure to LDIR during gestation caused epigenetic changes that resulted in positive adaptive phenotypic changes that were negated with antioxidants. This indicated they are mediated at least in in part by oxidative stress. These findings provide evidence that in the isogenic *Avy* mouse model, epigenetic changes caused by LDIR help explain how radiation hormesis occurs. This brings into question the assumption that every dose of radiation is harmful [145].

Hormesis is also important in homeopathic medicine, which teaches that a disease can be cured by a substance that produces similar symptoms of the disease in healthy people [146]. Even though homeopathic remedies

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are regulated in the USA by the FDA, they are not evaluated for safety or effectiveness [146]. Moreover, the FDA website expressed skepticism when it wrote, "Several key concepts of homeopathy are inconsistent with fundamental concepts of chemistry and physics" [146]. However, an expert panel sponsored by the NIH recently recommended that peanut allergies might be preventable if infants are fed foods containing small amounts of peanuts at around six months of age [147]. So, at least in this case, the NIH has acknowledged the ability of a low dose of a toxic allergen to prevent (not cure) a disease that is caused by higher doses of that substance. Still, it is extremely important to note that whole peanuts and even very small particles of peanuts can be a serious choke hazard for children up to about four years of age.

Also, another tenant of homeopathy is absolutely wrong, even though it doesn't violate any fundamental concepts of chemistry or physics. That is, homeopathy teaches that it is possible to prepare effective treatments (and cures) by preparing nearly infinite dilutions of a toxic substance. Supposedly, the process of dilution (sometimes with alcohol) will extract the 'essence' of the substance. Any analytical chemist who has analyzed highly purified water (deionized or distilled) for very low concentrations (picograms per gram or pg/g) of salt (sodium chloride, NaCl) or any environmental sample for pg/g concentrations of polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs or just dioxins) will know that infinite dilutions can't be made. That is, when an analyst takes a stock solution of about 10 pg/g of NaCl in water or a PCB in hexane and tries to dilute it 1/10, the resulting solution is almost never 1 pg/g. Such ultra-trace analyses must be done in a clean room equivalent to rooms used to manufacture printed circuit boards and other electronic devices. Even under such stringently clean conditions, some contamination occurs. The water or hexane that is used to do the dilution is never completely pure. So, the US FDA and the regulatory agencies of other countries require all drug manufacturers to show that they can detect any low dose of their drug that they want to get approved. So, homeopathic 'medicines' that claim to have less than one molecule of the active ingredient in them are false. Neither single molecules nor 1 pg/g or 1 pg/mL solutions of a molecule can be quantified accurately. It might be possible to dilute a molecule so much that it produces a signal on a detector (such as a mass spectrometer, or MS) that is only three or ten times the signal to noise ratio (S/N). If so, this is often defined as the detection limit. However, it is a practical (but not theoretical) impossibility to prepare a solution that gives no detectable signal using a sensitive

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technique like MS. There is always noise in a detector response. This limits the concentration of an analyte that can be quantified accurately.

1.3.9.7 Clinical applications of epigenetics

Epigenetics are inconsistent with the reductionist idea that information can only flow from RNA to DNA to proteins. Epigenetic modifications of proteins create information that flows back to DNA, affecting how it's transcribed into mRNA and translated into proteins. Epigenetics are extremely important in exploring development, differentiation, homeostasis, and diseases that impact nearly every clinical discipline [148]. We now realize that previous attempts to understand the causes of human diseases based solely on DNA sequencing can't succeed. At least half of all illnesses are based on environmental causes and epigenetic changes that don't alter the DNA sequence. For example, exposure to the sun leads to large blocks of the human genome being hypomethylated in the epidermis. This is an important risk factor for skin cancer. By using epigenetic analyses, both genetic factors and environmental exposures can be evaluated. Another study found that there are SNPs on human chromosome 11, located 100 kb apart. They are involved in different aspects of lipid metabolism that are controlled by DNA methylation at two CpG sites in a bidirectional promoter. It is located between two genes that encode fatty acid desaturase enzymes. GWAS alone would not have captured the link between these two SNPs because they regulate DNA methylation in the shared promoter region [148].

There is an important difference between GWAS and epigenetic studies [148]. To analyze the genome in GWAS, a sample can be taken from almost anywhere in the body. The genome is the same in most human eukaryotic cells. However, the epigenome is not. It varies from one tissue to another, between different types of cells in the same tissue and can vary over time. So, it is important to identify metastable epialleles, which are genomic loci with differential epigenetic regulation that are expressed in different ways in genetically identical people. The epigenetic state is established stochastically in the very early embryo, before gastrulation, and is subsequently kept constant. This produces systemic (non-tissue-specific) inter-individual epigenetic differences that are not mediated genetically. DNA methylation at metastable epialleles is especially sensitive to environmental factors. This makes these loci valuable in exploring the mechanisms behind developmental origins. It is quite likely that adverse environmental exposures during critical periods of

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prenatal and early postnatal development can have long-term effects on the baby's health and the risk of disease [148].

Researchers identified changes in DNA methylation that occur in the cleavage-stage embryo (shortly after the time of conception) [148]. They identified *VTRNA2-1* as the likely key indicator of early environmental influences on the epigenome. This environmentally-responsive epiallele is a genomically imprinted small ncRNA and a likely tumor suppressor gene. It is preferentially methylated on the maternally inherited allele. Loss of imprinting at this locus links early embryonic environment to epigenetic changes that affect the risk for disease later in life. Besides *VTRNA2-1*, over 100 other metastable epialleles have been found. So, DNA methylation can be assayed in peripheral blood or in any easily accessible tissue. The results can provide important information about epigenetic regulation throughout the body [148].

Heterochromatin domains are also being mapped [148]. The results have shown that a histone chaperone helps to organize chromatin on a large scale. Histone variants are being identified and characterized to help understand chromatin organization and dynamics. One of the most interesting histone variants is H3.3. Even though 96% of the amino acids in H3.3 are the same as those in histone H3.1, histones H3.3 and H3.1 are different. Histone H3.3 is expressed throughout the cell cycle. It is enriched in transcriptionally active chromatin and in certain types of post-translational modifications. One of the proteins associated with histone H3.3, the death domain-associated protein DAXX, is its chaperone. When DAXX is lost, it leads to a global structural change in the chromatin landscape, characterized by genomic regions enriched in the trimethylated H3K9 epigenetic mark (K is the abbreviation for lysine). These changes were placed close together in large chromatin domains devoid of this modification, with contrasting effects. They probably occur because the boundaries between heterochromatin domains and other regions were not being respected. This leads to the inappropriate insertions of histone H3.3, with profound effects. The loss of DAXX leads to the uncoupling of the epigenetic marks from the global chromatin architecture with a major global reorganization of the chromatin structure [148].

Previously, it was thought that the well-known epigenetic modifications make the structure of heterochromatin compact [148]. We now know that this is not always the case. For example, if a heterochromatin domain is marked with H3K9 trimethylation, it is in a structurally open state, similar to euchromatin. This indicates that the boundaries between heterochromatin and euchromatin are much more dynamic than previously thought. This concept is crucial for understanding

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factors that dynamically shape the three-dimensional interactions that occur after epigenetic modifications. A key corollary to these findings is that the epigenetic marks at a specific genomic locus depend on both the local environment factors and the three-dimensional structure [148]. Euchromatin is tightly packed chromatin that is enriched in genes that are frequently transcribed, while heterochromatin is not.

So, researchers are trying to find out which loci come together in specific regions of the cell nucleus and how they affect each other [148]. As we learn more about the biology of epigenetics, it will become easier to learn how to make small molecule probes or drugs that will be effective in the clinic. It is important to integrate biological and chemical perspectives. For example, researchers have shown that many epigenetic processes use protein complexes. In the past, efforts to understand the roles of specific genes have relied on knocking down or knocking out a single gene and its protein product and observing the resulting phenotype. More recently, researchers have learned how to knock down a protein that is part of a protein-protein complex. This can change that complex drastically. As a result, the phenotype is frequently quite different from the one that can be seen with a small molecule inhibitor of a single protein in of the complex. Medicinal chemists are identifying small molecules that can selectively affect and alter just one particular property of these protein complexes. They are focusing on identifying therapeutic agents that could target epigenetic processes. For example, several bromodomain inhibitors have been found to have powerful antitumor activity in human osteosarcoma cell lines and in osteosarcoma primary cells from mouse models of this disease. One of the bromodomain inhibitors tested, JQ1, exerted its anti-proliferative activity by inducing apoptosis, and not by stopping the cell cycle, as was previously thought. Moreover, even though previous studies identified the oncogene MYC as an important causative factor in osteosarcoma, JQ1 was active, independent of MYC downregulation. It was also shown that downregulation of *FOSL1*, a gene previously thought to be involved in osteoblast differentiation, is an important contributor to the effects of JQ1. This was the first time that this gene was found to be important in osteosarcoma [148].

Epigenetic drugs have also been shown to work not only on protein-coding genes, but also on the noncoding part of the genome [148]. Several such drugs have entered clinical trials for malignancies, atherosclerosis, and type-2 diabetes. More recently, BET bromodomain inhibitors were found to inhibit glioblastoma cell proliferation by inducing a cyclin-dependent kinase inhibitor. Next, long lncRNAs that are differentially expressed in glioblastoma multiforme were studied. A set of transcripts

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that are specific for this malignancy were identified. They might be regulated by bromodomain inhibitors. In glioblastoma multiforme cells, the I-BET151 bromodomain inhibitor is near to the promoter of HOTAIR. This is a tumor-promoting lncRNA that is as an epigenetic silencer and has been implicated in several cancers. Its expression decreased, while the expression of several other lncRNAs was downregulated in this type of cancer. A semi-high-throughput gene-expression-based screen identified small molecules that could increase the expression of the gene *C9ORF72*, the most common genetic cause for amyotrophic lateral sclerosis. It has more GGGGCC hexanucleotide repeats in the noncoding region than the wild-type gene. People who don't have this condition have two to 25 of these repeats, but their number can reach up to several hundred in ALS patients, reducing *C9ORF72* expression. This has been implicated in the pathogenesis of ALS. The gene-expression-based screen in fibroblasts from affected and unaffected individuals identified siRNAs against the BRD3 bromodomain protein and several small molecule bromodomain inhibitors that were able to increase *C9ORF72* expression. This happened even without changes in promoter CpG hypermethylation and trimethylated H3K9 marks. These are heterochromatin markers of the expanded *C9ORF72* alleles. So, the mechanism of action of these bromodomain inhibitors is probably broader than originally thought [148].

RNA-based therapeutic methods are also being developed [148]. It is possible to manipulate gene expression in two directions. Medically relevant genes can be either knocked down with siRNA or upregulated with mRNA. So, collaborations between at least three companies have succeeded in developing and patenting UNA Oligomer™ and LUNAR™ delivery technologies. By combining UNA chemistry with LUNAR delivery, they are able to design effective drugs that can be used against many diseases [148].

So, the epigenetics market is growing [149]. In an effort to measure the growth in this market, 53 846 PubMed summaries of papers in epigenetics were gathered. To find the most current trends, the analysis focused its efforts on 30 848 manuscripts that were published between January 2012 and May 2016. The PubMed titles and were analyzed using keyword-based algorithms to extract terms and trends that were related to research and the market. Results showed that the field is expanding rapidly with a compound annual growth rate for the period 2012–2015 of 12.2%. About 76% of the marketplace was due to DNA methylation. Acetylation was second. DNA was the main biopolymer mentioned in the epigenetics database, but RNA was a close second. Both mRNA and miRNA applications grew significantly, while an increase in applications using

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ncRNA was a small, but significant trend. There is also a growing trend in research on lncRNAs that act as mediators and/or modulators of epigenetic modifications. There is also an increasing interest in sequencing different types of RNA. The study also found 273 human miRNAs in their epigenetics database. Research on some of these miRNAs are showing exceptional growth rates. The ones showing the largest growth in interest were MIR21 and MIR 155. They are frequently associated with cancer and other diseases. Cancer is the most widely reported disease in their database. The strong focus on cancer also means that other areas are being neglected and represent untapped market potential for growth in epigenetics in the future [149]. Still, epigenetics is becoming an essential part of modern drug development in oncology, diabetes, inflammation, developmental and metabolic disorders, as well as in cardiovascular and autoimmune diseases, pain, and neurological disorders [150]. Moreover, a search of ClinicalTrials.gov showed that over 40 different epigenetics-related drugs are undergoing clinical trials [150]. So, both epigenetics and genetics are important field in new drug development. The epigenomes and genomes of many cells and tissues are being evaluated.

1.3.10 The –omics, from genomics to personal –omics

1.3.10.1 Overview

Systems thinking has taught us that our metabolism and health depend on much more than just our genome, or collection of all of our human eukaryotic genes. We have learned much from genomics, which compares the genomes of humans and other organisms. However, there are also many other important –omics, including epigenomics, transcriptomics, proteomics, lipidomics, metabolomics and glycomics, as well as exposomics [4, 6, 151, 152]. They are the collections of all the epigenetic modifications, transcription products, proteins, lipids, metabolites and glycans (carbohydrates) in a cell or organism, as well as the effects of exposure to environmental chemicals. There is some overlap. There are some lipoproteins in both the lipidome and proteome, as well as lipopolysaccharides in the lipidome and glycose. There are also glycoproteins in the glycome and proteome. There is also a Human Microbiome Project (HMP), which aims to sample, determine and quantify all human-associated microbial life. There is also a diseaseome that is the collection of all the diseases that humans experience. Few diseases occur in isolation. Most are linked to other diseases. For example, diabetes increases the probability of cardiovascular diseases, CKD and

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neurological damage. Heart disease is often part of a multi-organ failure [4, 6, 151, 152]. For example, Alzheimer's disease is frequently not given as the cause of death on a death certificate, heart failure is. That is, when the brain degenerates too much in patients with Alzheimer's disease, it can no longer send signals to the heart and it stops beating, causing death. In addition, many diseases share one or more genes and metabolic pathways, as well as have co-morbidities [8]. Diseases can also be affected by the same protein-protein interactions, microRNAs and tissue-specificity. So, disease similarity networks have been constructed [8].

There is also an etiome, which is the set of all causes or etiologies of diseases. Moreover, there are websites with data on interactions (interactome, integrome) and even an omnisciome that aims to be all the knowledge about a cell, organism or system [4, 6, 151, 152]. Cognitive genomics is the study of the relations between cognitive processes and the genome. Comparative genomics compares the genomes of different organisms. Functional genomics studies the interactions between gene and protein functions. Metagenomics is the study of the genes obtained in environmental samples and is especially useful in studying microorganisms that can't be grown in isolated conditions *in vitro*. This includes all the viruses (the virome) and Bacteria (bacteriome). Prior to the introduction of metagenomics and shotgun sequencing, it was thought that the main type of Bacteria in the gut was *Escherichia coli* (*E. coli*), because it can be kept alive and easily grown in the lab. It is now known that the primary Bacteria in the gut are in the phyla Firmicutes and Bacteroidetes, which are difficult to keep alive in the lab. Nutritional genomics studies the many relationships that exist between genetics, nutrition and health. Pharmacogenomics evaluates the effects of genetics on effects of druge, as well as their metabolism and toxicity. Toxicogenomics investigates the effects of genes on the responses of cells and organisms to toxins. Stem cell genomics is the study of the genetics of stem cells. The epigenetic changes are the epigenome. Phosphoproteomics is the collection of all the phosphorylated proteins in the proteome. The kinome is the collection of all the protein kinases that catalyze the phosphorylation of other proteins. Since proteins are continuously being broken down and re-made, some consider proteomics to be a susbset of metabolomics [4, 6, 151, 152].

1.3.10.2 Metabolomics

Metabolomics is the study of metabolites and metabolism and is at the nexus of interactions between genes and the environment [153]. Metabolism is how all organisms interact with the environment and exhibit

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the emergent property of cognition [154]. It is "the biochemical rendering of cognition at the cellular level" [154]. Metabolism has also been called metabonomics, targeted metabolomics, untargeted metabolomics, metabolic footprinting, metabolic fingerprinting, fluxomics, lipidomics, metallomics and exposomics. It has emerged as an important tool in learning how to predict disease susceptibility, how to prevent diseases, how to treat them once they emerge and how to monitor the patient's recovery. Unlike genomics and GWAS that predict what might happen, metabolomics shows what is happening and provides fundamental insight into the causes of diseases. These new insights "are leading to a paradigm shift in how drugs are being discovered, developed, delivered and dosed" [153]. It is enabling the personalized phenotyping of metabolites. When combined with genomic and epigenomic data, it can advance the field of precision medicine [153, 155]. The goal is "to use advanced diagnostic testing to customize an individual's medical treatment according to their specific omic profiles" [153]. On a population level, drug discovery and development are improving. On the level of each individual, metabolomics is advancing the field of precision medicine. At the same time, several initiatives have started that look at genomic, epigenomic, transcriptomic, proteomic, phosphoproteomic, glycomic, lipidomic and metabolomic data of hundreds of thousands of volunteers. This includes integrated personal -omics profiling (iPOP) as well as predictive, preventive, personalized and participatory medicine (P4 medicine) [153].

Moreover, the U.S. National Institute of Health (NIH) has launched a Precision Medicine Cohort Initiative Program, which was announced by President Obama in his State of the Union address on January 20, 2015 [156, 157]. The goal is to build a cohort of over a million volunteers that "will broadly reflect the diversity of the U.S. population by including participants from diverse social, racial/ethnic, and ancestral populations living in a variety of geographies, social environments, and economic circumstances, and from all age groups and health statuses" [157].

It should be noted that the word precision is used much differently in this context than in analytical chemistry. When the Precision Medicine Initiative (PMI) uses the word precision, it implies accuracy. That is, the precisely proper medicines will be used to target the disease accurately for each individual and each disease. In contrast, an analytical chemist uses the term precision to mean repeatability. If an analytical method produces nearly the same result every time it is used, it is considered to be precise, but not necessarily accurate. Precision to an analytical chemist does not imply accuracy. A broken clock is very precise (it tells the exact same time all the time, such as 1:00 PM), but accurate only once a day. In analytical

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chemistry, the completely accurate concentration of an analyte is never known. It can be defined by a regulatory agency, such as the U.S. Environmental Protection Agency (EPA) when it prepares samples for round robin studies. The purity of the analytes that are added to such a sample might be as high as 99.9%, but they are never 100%. Moreover, they can only be weighed with an accuracy of ±0.1 mg. Still, this is quite sufficient for almost all applications. In contrast, precision medicine will very accurately target the disease, such as cancer and kill only the cancer cells and not healthy cells. It also means that precisely the right medicine is given to the precisely the right patient.

In earlier metabolomic studies, quantitation was seldom a priority [158]. Now that governments, pharmaceutical companies, universities and research institutes have gained access to modern instruments like liquid chromatography coupled to mass spectrometry (LC-MS), quantitation is being done. Modern LC-MS instruments are equipped with autosamplers and computers that can analyze the data. So, many samples can be analyzed in untargeted metabolomic studies. This also allows scientists and analysts to use systems thinking, rather than reductionist thinking. Instead of looking for individual analytes, untargeted analyses can detect hundreds of metabolites that can act synergistically to exert physiological effects. Hopefully, this will enable truly personalized clinical trials while improving diagnosis, treatment and prognosis. Since personalized health has been the basis of traditional medicine, a unified view of medicine and biology is emerging in which both Western and Eastern knowledge is used [158].

This new way of thinking is emerging that demands a new way of communicating about life [159]. As a result, the term Metabolic Field – Schrödinger has been proposed. It describes metabolism as a field, similar to the Field theories that are the foundation of the physical sciences as described by Erwin Schrödinger and others. Reductionist techniques produced good descriptions of gravity, electromagnetism, the strong and weak nuclear forces and quantum fields. Field theories use vectors and scalars to describe how the conditions of any point in space will affect matter or another field. “The success of the reductionist method which led to the Field Theories gave rise to the hope that a grand unified Theory of Everything would derive and allow penetrance into the nature of uni- and multi-cellular life by generating a set of equations capable of describing all phenomena ever observed and ever to be observed” [159]. However, this Theory of Everything is probably not applicable to living systems. As described by Schrödinger, “all life is an inevitable by-product of energy

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flow on matter, over time". Metabolism involves the transduction of energy flows with respect to time, space, and matter [159].

In contrast, time is excluded from the domains of gravity, magnetism, the strong and weak molecular forces, and quantum fields [159]. Moreover, a fixation on genes, genome-wide association and proteomic studies led to a reductionist search for single causative agents. Dynamic, ever-changing systems are equally important in life. That is, life is not a static experience in which metabolites are kept constant through homeostasis. Instead, homodynamics might be a better term. It accounts life's many polyrhythms and the fact that life is almost always on a trajectory, from conception, to birth, growth, development, puberty, aging and menopause. On the other hand, the basic properties of inorganic matter and the five field theories that describe its properties do not change over time. They ignore the openness and irreversibility of human life [159].

1.3.10.3 Heterogeneity and mosaicism in cellular DNA

Reductionist thinking described DNA as if it were a book of life that was written the same way in all nucleated human cells. The genetic code was supposed to be the same on all 23 pairs of human chromosomes. There was no heterogeneity and it was not like a mosaic. However, recent advances in DNA sequencing of single cells have shown that there is some heterogeneity or mosaicism in cellular DNA [4, 160]. This was already well known in cancer cells, which can become polyploid, and in healthy Kupffer cells in the liver [6]. However, mosaicism is now known to occur in healthy skin, brain and white blood cells, as well as in induced pluripotent stem cells (iPSCs) [161]. There are also important differences in the transcriptome, epigenome, metabolome and exposome of cells [4, 161]. Recently, the whole genome, transcriptome, sites of DNA methylation, protein-DNA interactions, three-dimensional genome structure and B and T cell repertoires of a single person were determined. Also, the expression ratio of viral infection to healthy states was measured. Polysomal mRNAs were purified and the functional consequences of genetic variations were determined. This produced an integrated personal –omics profile. It found a link between elevated blood glucose concentrations and viral infections. The subject changed his lifestyle to lower his blood glucose. Subsequently, his whole genome methylation pattern was evaluated at several time points, his microbiome was analyzed and biosensors were used to monitor his physical activity and heart rhythm. Finally, he found members of his extended family who had

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smoldering inflammation and unrecognized glucose intolerance. This caused several of them to change their medical care [4, 161]. So, much more than just the base sequence of one's DNA affects one's health.

1.3.11 A multidimensional, non-linear view of diseases

Before the emergence of all this data on -omics, drugs were developed based on their effect on a disease phenotype or their ability to affect a single molecular target [4, 159]. This was based on a linear cause and effect model for many diseases and the drugs that affected them. However, this is being replaced by a multidimensional, non-linear view of diseases. For example, we now know that types 1 & 2 diabetes, coronary artery disease, and glioblastoma all result from small defects in many genes, rather than large defects in a few genes [162]. Instead of measuring the effects of a test compound on a single gene or gene product, the effect of a perturbagen on the entire network system should be evaluated. This would allow researchers to probe the pattern of interactions in a disease model and identify hubs that could be novel drug targets. The effects of test compounds on different types of cells should be evaluated. One should look for the changes in gene networks that define disease, toxicity or other relevant phenotypes. This will lead to predictive models that define networks for a particular disease subtype and help predict toxicity. These models can be used to develop gene expression-based assays for high-throughput screening. The effects of test compounds on the relevant networks can be assayed in cell-based systems that reflect their states. Such assays can be developed by identifying nodes and hubs in the networks of interest that reflect network states. High-throughput screening of compounds that affect such nodes can identify drugs that affect disease networks favorably and identify toxins that affect networks adversely and cause adverse side effects. Compounds can be identified that target specific subtypes of diseases without affecting networks that can lead to toxicity or adverse side effects. Drugs specific to a particular disease subtype can be matched with the right patients so that the right dose of every drug is given to the right person at the proper time [162].

Ideally, the network-based drug discovery process will identify all the molecular nodes that affect a disease network [150, 162]. For example, the variations in DNA or genes in a population are perturbations that affect RNA and/or protein concentrations. Those changes can affect disease prognosis and the response to drugs. Instead of identifying a single target in the web of genes and proteins, large classes of compounds should be screened to identify those that perturb the disease networks in ways that

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return them to its pre-disease states. Test compounds that have favorable effects on disease networks can be selected and improved, so that effects on metabolism and toxicity can also be optimized [150, 162]. This has been described in more detail [162].

A major advantage of the disease network approach is the construction of networks that predict specific forms of diseases that represent different phenotypes [150, 151]. Drugs can be screened against these phenotypes by monitoring thousands of variables in thousands of contexts, such as the presence of different perturbagens. New combinations of drugs that treat different cancers have been developed by screening compounds against the expression of many genes [150, 151]. Combination therapy has also proven successful in anti-malaria and anti-HIV therapies.

1.3.12 Drugs that bind to multiple targets

Even drugs that were approved based on their binding to one target are now known to bind to multiple targets [150, 163, 164]. This includes Gleevec, which was initially developed to specifically inhibit the abnormal tyrosine kinase BCR-ABL and is used to treat chronic myeloid leukemia. It is now known to target several other kinases simultaneously, including c-KIT and platelet-dependent growth factor receptor (PDGFR). Similarly, celecoxid was developed based on its ability to inhibit cyclooxygenase, but it is now known to inhibit carbonic anhydrase [150, 165]. The atypical antipsychotic drug clozapine binds to many targets in the CNS at nanomolar concentrations. So, computational chemogenomics is being used to predict the biological activities of test compounds against many diverse biomolecular targets [150, 165].

As a result, researchers are looking for similarities in drug binding sites [150, 159]. There is a database, Cavbase, which stores the descriptors of three-dimensional properties and physicochemical characteristics of each of the protein cavities obtained from structures in the protein databank (PDB). It also ranks the similarity according to the match of the property descriptors [150, 159]. In addition to facilitating the prediction of unknown protein function, Cavbase can also be used to discover protein targets that have similar ligand binding properties.

Drugs that bind to multiple targets will be discussed in more detail in Chapter 3.

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1.3.13 Modularity of biological networks

It is also important to consider the modularity of biological networks [140, 140]. Many previous studies have dealt with network modules as if they were static. These studies focused on the topological properties of the networks. However, many biological networks are dynamic. Expression levels of different types of RNA and proteins can depend on internal and external environmental factors and signals. Dynamic modules are closely regulated, whereas static modules are not [166]. So, the variance in expression levels, or expression variance (EV) of proteins, was determined in a recent study [167]. An EV of zero indicates that the system is static, while an EV of one is for genes with the largest variance. They are the most dynamic. Genes with low EV values are expressed at lower levels that genes with high EV values. There was a high correlation between the EVs of proteins and their neighbors in a module. That is, the protein interaction network is enriched with sub-networks that are primarily composed of either static proteins or dynamic proteins, but not both. Proteins that have more interacting partners in the network are segregated into distinct network neighborhoods that are characterized by low and high EVs. Also, dynamic proteins, in contrast to static proteins, are more functionally similar to their neighbors when they are in dynamic neighborhoods. They also have higher Pearson correlation coefficients, which reflect how well proteins in a neighborhood are co-expressed with each other [150, 167].

Also, it was found that the hubs that had been called date hubs are found within static modules, where there is also no statistical correlation of expression among neighbors [150, 167]. That is, static hubs that interact with static proteins within static modules should not be considered to be date hubs. Instead should be called family hubs, since they are always present in the network and interact constitutively with their neighbors. Thus, family and party hubs form static and dynamic modules, respectively, whereas date hubs organize the network. Date hubs are mostly proteins used to transduce and regulate signals. Examples include protein kinases, phosphatases, small G-proteins and molecular chaperones [9, 150, 167].

Dynamic modules can be modified, so they are mainly responsible for regulating cell behavior, depending on environmental conditions [150, 168]. They tend to be co-expressed together. On the other hand, static modules help make cells robust and resistant to genetic changes and differences in the levels of protein expression. Static modules helped develop the concept of robustness-related module criticality. That is, perturbations of self-organized systems are neither dampened nor

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amplified, but are propagated over long temporal or spatial scales. This enables information to propagate over time from one part of the system to another with a high degree of specificity and sensitivity. Criticality enables the coordination of network behavior to maintain an optimal balance between stability and adaptability. For example, the human brain has broadband criticality that helps to optimize information processing and balance excitation and inhibition [150, 168]. Moreover, diseases have modified modules.

A cascade of network modules can define cancer progression and help influence its diagnosis, prognosis and treatment [150]. Some modules show significant changes in cancer-related signaling through the tumor suppressor proteins, p53, as well as retinoblastoma protein (pRb), phosphatidyl inositol 3-kinases (PI3Ks) and receptor protein kinases. Module maps help to understand gene expression profiles in different tumors. Specific network modules consisting of sets of genes that act together to perform specific functions can be evaluated. Some of the modules may be activated in particular types of cancer [150, 166]. Also, specific treatments have been found by examining the network of genes that are expressed in a disease. For example, the Human Genome Atlas project found that breast cancer is actually four different diseases that are treatable by different approaches [150, 169]. Primary breast cancers were analyzed for genomic DNA copy number, DNA methylation, exome base sequences, mRNA, miRNA sequences and proteins. They used a software package called mutual exclusivity modules in cancer (MEMo) to analyze the data and identify the different disease network modules [150, 169].

Another software package, called Pascal (Pathway scoring algorithm) has been produced that can compute gene and pathway scores from SNP-phenotype association summary statistics [81, 170]. It was used to analyze summary statistics from dozens of large meta-studies for various traits. Extensive testing indicated that Pascal not only excels in controlling type I errors (the incorrect rejection of a true null hypothesis or false positives), but also results in more biologically meaningful discoveries [81, 170]. This should not be confused with the outdated computer language called Pascal.

1.3.14 Going beyond genomics and looking for hidden connections

One of the key features of systems thinking is looking for hidden connections [7]. In order to develop new drugs for personalized medicine, it is important to go beyond just genomics [171]. So, the term

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environmentome has been coined. It is "the entire complement of elements external to the human host, from microbiome, ambient temperature and weather conditions to government innovation policies, stock market dynamics, human values, political power and social norms that collectively shape the human host spatially and temporally. The ''social proteome'' is the subset of the environtome that influences the transition of proteomics technology to innovative applications in society. The social proteome includes new reimbursement schemes and business innovation models for proteomics diagnostics that depart from the ''once-a-life-time'' genotypic tests and the anticipated hype attendant to context and time sensitive proteomics tests" [171].

It is also important to look for hidden connections [7]. That is, the virome consists of not just the obvious viruses in the human body, but also the silent virome [172]. In the past, viruses were thought to be just pathogens that caused a variety of diseases. However, recent advances in unbiased mass sequencing along with increasing epidemiological evidence have changed our understanding of the virome. That is, the ecosystem that is the human body contains many non-pathological viruses. For example, viruses that infect Bacteria (bacteriophages) help control the composition of the human microbiome [172].

Even more –omics are emerging. One of them is called chinmedomics, which studies of all the traditional Chinese medicines [173]. This type of medicine uses many herbal remedies simultaneously without trying to isolate individual active ingredients. There is little or no information about what is in these herbs. So, blood serum analysis has been integrated with high-resolution metabolomics to enable chinmedomics to identify the bioactive compounds in traditional Chinese medicines. Researchers look for correlations between biomarkers of diseases and the compounds that are present in traditional remedies. This enables them to understand the effects of active ingredients and how they might interact synergistically. This is leading to the discovery of lead compounds from natural sources. It is advancing the development of new drugs from traditional medicine [173].

1.3.15 Tools for understanding metabolism better

Important tools are also being developed to enable researchers to understand metabolism better. When metabolic pathways like glycolysis and the tricarboxylic acid were first discovered, they were described as isolated pathways that were presented in just two-dimensions. Now, metabolic pathways can be shown in three dimensions [174]. Graphical

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representations of them should be informative and not overloaded. They should be recognized and unified easily. Three-dimensional virtual reality visualizations are helping researchers understand better the intricate topology of metabolic and regulatory networks. So, a new way to visualize metabolic networks as graphs in three-dimensional space by means of Virtual Reality Modeling Language (VRML) has been developed. It is based on an XML-compliant Metabolic Network Visualizer (MNV) language, with an MNV language standard and parser, MNV to VRML translator, and interactive pathway constructor, all unified by the HTML graphic user interface. It is available in viewer mode at http://www.patronov.net/sciencevr/mnv/indexview.html or in constructor mode at http://www.patronov.net/sciencevr/mnv/indexmake.html [174].

This software package can be used to analyze metabolomics data so we can find biomarkers for diseases [4, 5]. For example, such data has been used to study diabetes, which is characterized by altered metabolism of key molecules and regulatory pathways [175]. Recent advances in liquid chromatography (LC) coupled to mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR) have enabled scientists to identify and quantitate small molecule metabolites from a variety of biological systems. Continued development of bioinformatics has led to the discovery of causal links between the pathophysiology of diabetes and its complications. Two groups of biomarkers, amino acids and lipids, have been found to be able to predict one's susceptibility to becoming diabetic. Increased concentrations of leucine, isoleucine, valine, tyrosine and phenylalanine were able to predict risk up to 12 years prior to the onset of diabetes. In addition, 2-aminoadipic acid was found to be a biomarker and promote insulin secretion. A phospholipid, lysophosphatidyl choline, has been found to be a biomarker for impaired glucose tolerance and type-2 diabetes. Metabolomics has also been used to predict the progression of diabetetic nephropathy. Fifty-six features were found to be down- or upregulated. Thirty-two were identified by searching databases. Treatment with a standard therapy, rosiglitazone, reversed the concentrations of 9 of these 32 compounds (including indoxyl sulfate) back to baseline. Since exercise and diet can also reverse some of the symptoms of diabetes, their effects on the diabetic metabolome have been studied. They change the pattern of triacylglycerides (triglycerides) from those containing mostly saturated fatty acyls to an enrichment of unsaturated, long chain fatty acyls [175].

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1.3.16 Evidence based medicine and the Critical Path Initiative

One of the features of systems thinking is to look at all of the data available and integrate it into a systems-based model. So, the data from many –omics studies are providing important evidence that can be used to predict a person's susceptibility to diseases and the probability that a particular therapy will be beneficial to them. The data also helps people find out how to prevent diseases, especially when it turns out that they are susceptible to certain ones. Moreover, the –omics data will enable personalized, or precision treatments. Finally, -omics and physiological data will be stored on data clouds and be available to patients and caregivers who want to participate in their own therapy and/or help find a cure for their disease. So, -omics and physiological data are improving predictive, preventive, personalized and participatory (P4) medicine. The goal is for healthy people, people with diseases and the entire medical system to help the whole person – from preconception to birth, through development and the rest his or her life.

P4 medicine is consistent with evidence based medicine that is used to help prescribe appropriate, individualized drugs and medical devices [6, 176]. A patient's blood or tissues can be analyzed for different types of enzymes (isozyme like cytochrome P450s or CYPs) that catalyze reactions that can metabolize drugs differently and affect their bioavailability. Analyses can also be done to test the ability of drugs to bind to different receptors that are slightly different in various groups of people. This approach led to the development and rapid approval of tratuzumab, or Herceptin®, for treating and curing breast cancer patients who have the HER-2 gene that is involved in many cancer signaling pathways. More recently, it led to four anticancer drugs being approved by the FDA and other governments' regulatory agencies for use in patients who have specific genetic characteristics that can be identified by a companion diagnostic test [177].

The North American Menopausal Society (NAMS), American College of Obstetricians and Gynecologists (ACOG), U.S. Preventative Services Task Force (USPSTF), NIH and FDA use evidence based medicine to develop guidelines for therapeutic practices [178]. "Evidence based medicine uses individual clinical expertise with the strongest available external clinical evidence that is obtained from systematic research. It has also been described as the conscientious, explicit, and judicious use of current best evidence in making decisions about the care of individual patients" [178]. Systems thinking is needed when physicians use their clinical experience to make an effective diagnosis and to consider carefully every individual patient's predicaments, rights, and preferences

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in making clinical decisions about his or her care. So, evidence based medicine is not a ‘cookbook’ approach, in which diagnoses and treatments are dictated from above. Instead, it is a bottom up approach, in which the best evidence is combined with individual clinical expertise and patients' choices [178].

The FDA and other governments’ regulatory agencies evaluate applications for new medical devices, biological products and drugs. Before the Critical Path Initiative (CPI) was launched in 2004, the FDA was facing cuts in resources, while industry was facing technical challenges and a set of partly outdated regulations [179]. Also, there had been an unacceptable decline in the number of new drugs getting through all four stages of clinical trials and subsequently being approved, despite the enormous breakthroughs being made in biomedical science. So, the FDA established the Office of Critical Path Programs (OCPP) in the Office of the Commissioner to support the initiative. Since globalization, rapidly evolving technologies and emerging areas of science were having an increasing impact on all FDA-regulated products, the CPI gradually expanded its scope. They used knowledge gained from new scientific discoveries to improve the tools used in human and animal medicine and food safety. It included rapid analytical methods to detect biological and chemical contaminants in foods. The CPI continues to support many projects related to streamlining information management systems for all Agency-related information about medical products. This includes encouraging electronic submissions and reporting negative side effects while analyzing human and animal food [180]. For detailed information on CPI, the FDA was a website for it [181].

The FDA’s Center of Drug Evaluation and Research (CDER) has funded research that has led to many excellent publications [181]. This includes developing process analytical technologies, doing root cause analyses for drug product recalls, standardizing spectral libraries for rapid screening of pharmaceuticals, improving knowledge management tools for creating a cross-disciplinary multiple-NDA (New Drug Applications) disease database. CDER also encourages quality by design, systems biology, computational modeling, streamlining clinical trials and much more [181].

So, the following goals were described in a recent report from the FDA [179]. “Personalized medicine seeks to reduce the burden of disease by targeting prevention and treatment more effectively. With the help of personalized medicine, the healthcare management paradigm will focus on prevention, moving from illness to wellness, and from treating disease to maintaining health. By improving our ability to predict and account for

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individual differences in disease diagnosis, experience, and therapy response, personalized medicine offers hope for diminishing the duration and severity of illness, shortening product development timelines, and improving success rates. At the same time, it may reduce healthcare costs by improving our ability to quickly and reliably select the effective therapy for each patient while minimizing the costs associated with ineffective treatment and avoidable adverse events" [179].

Since its inception in 2004, the CPI has established several collaborations, such as the Predictive Safety Testing Consortium [181]. It is a partnership between public and private organizations. It is led by the non-profit Critical Path Institute (C-Path). It facilitates information sharing and developing novel tools for new drug development. This is being done under the advisement of FDA and the European Medicines Evaluation Agency (EMEA). They have reviewed and accepted seven new biomarkers for kidney injury. These new tests are being used to predict the safety of experimental drugs, while enabling them to reach the market faster and with greater confidence in their safety. There is also a Clinical Trials Transformation Initiative (CTTI) between the FDA and Duke University. Their goal is to modernize clinical trials in the USA. They are working with representatives from academia, professional societies, patient and consumer groups, industry, and Federal agencies to explore new methods and tools to make the current system more efficient. The FDA is also working with Harvard University on a Sentinel Initiative. Its goal is to develop a miniature Sentinel System that could pilot a coordinating center with scientific operations and methodologies that will be needed for the ultimate Sentinel. The FDA also has projects with the Centers for Medicare & Medicaid Services (CMS), Assistant Secretary for Planning and Evaluation (ASPE), the Veteran's Administration and Department of Defense to develop active surveillance methods and conduct studies about negative side effects of prescription drugs and other medical products. This effort is contributing to the broader Sentinel Initiative. The CMS, ASPE and FDA are also collaborating on *SafeRx*. It aims to enhance existing safety surveillance capacities and provide fast monitoring of safety for seasonal and H1N1 influenza vaccines. The FDA and NIH increased their collaborations in 2010. They have provided additional funding to develop and apply the new tools, standards, and approaches that are needed to properly assess the safety, effectiveness, and quality of products currently in development. The collaboration is leveraging the NIH's breadth of experience as a leader in biomedical sciences to help make the regulatory review process at the FDA as seamless as possible. This collaboration is using the best minds in

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colleges, universities, research institutions and the pharmaceutical industry to help develop and apply new, 21st-century tools, standards, and approaches [181].

The FDA launched the Advancing Regulatory Science Initiative in 2010 [182]. It is building on the achievements of the CPI to transform the way medical products are developed, evaluated, and manufactured. They have a strategic plan for regulatory science [183]. It identified eight priority areas where new or enhanced engagement in regulatory science research is essential to advancing its regulatory mission. They are:

(1) Modernize Toxicology to Enhance Product Safety
(2) Stimulate Innovation in Clinical Evaluations and Personalized Medicine to Improve Product Development and Patient Outcomes
(3) Support New Approaches to Improve Product Manufacturing and Quality
(4) Ensure FDA Readiness to Evaluate Innovative Emerging Technologies
(5) Harness Diverse Data through Information Sciences to Improve Health Outcomes
(6) Implement a New Prevention-Focused Food Safety System to Protect Public Health
(7) Facilitate Development of Medical Countermeasures to Protect Against Threats to U.S. and Global Health and Security
(8) Strengthen Social and Behavioral Science to Help Consumers and Professionals Make Informed Decisions about Regulated Product.

In 2013, they added a ninth strategic priority, strengthening the global product safety net. All of these efforts use systems thinking [183].

At the same time, the NIH supports the Community Child Health Network (CCHN) [184]. They have found that the preconception period strongly influences the outcomes of pregnancy and the health of the child. It is a multidisciplinary effort that uses community-based participatory research. They are developing a novel, integrative theoretical framework to design future investigations, integrate new findings, and identify promising, evidence-based interventions to improve intergenerational health and reduce disparities. The CCHN uses a Preconception Stress and Resiliency Pathways (PSRP) model. It builds local and multi-site partnerships between the community and academia. They are establishing guidelines for planning research and making decisions. They review relevant findings from diverse disciplinary and community perspectives to find out where stress and resilience exist within families and communities.

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"The PSRP model focuses on inter-relating the multiple, complex, and dynamic biosocial influences theoretically linked to family health disparities. The PSRP model borrowed from and then added original constructs relating to developmental origins of lifelong health, epigenetics, and neighborhood and community influences on pregnancy outcome and family functioning" [184].

The PSRP model demonstrated the centrality of the preconception and inter-conception periods, the importance of fathers and the parental relationship, maternal allostatic load (a composite biomarker index of the cumulative wear-and-tear of stress), resilience resources for parents, as well as influences by the local neighborhood and larger community (such as employment, housing, education, healthcare, and availability of basic necessities) [184]. CCHN's integrative approach encourages new ways of thinking about how to improve outcomes for future generations. Their approach starts before conception, includes all family members and vigorously engages the community at many levels. This promotes resiliency, reduces chronic and acute stressors, and expands individualized healthcare. It integrates proactive strategies with disease prevention. The PSRP model is engaging communities, health and social services providers, as well as scientists so they can overcome the problems that emerge. The goal is to reduce family health disparities. Model limitations include the difficulty in measuring all elements with sufficient precision and sensitivity. The CCHN looks at health and well-being from both positive and ecological perspectives. It recognizes that the impact of particular stressors or facilitators on individuals or groups may depend partly on whether these occur in environments that have low, moderate, or high amounts of resources. The network endorses the idea that the psychological or subjective interpretation of events does vary among individuals within the same community. This happens because of the many different factors in their current lives and their previous experiences in life history [184].

So, all parts of society are involved in improving our health. It starts with each individual, their families and their support groups. It extends into research institutes, universities and government agencies. So, systems thinking also means that every part of society is being used to leverage their assets to improve the health of every individual and overall public health.

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1.3.17 Cancer Genome Characterization Initiative, CGCI

The NCI has a Cancer Genome Characterization Initiative (CGCI) that supports cutting-edge genomics research on adult and pediatric cancers [5, 185]. Advanced sequencing and other genome-based methods are being developed and used to identify new genetic abnormalities in tumors. The extensive genetic profiles produced by CGCI are making it easier to improve cancer diagnosis and treatment. They completed a pilot lung cancer project by using next generation transcriptome sequencing, as well as gene expression and epigenome profiling to study early-stage lung cancer. They discovered several changes in early-stage lung epithelial tumor cells and tested them to learn their roles in cancer development. They also compared possible changes in the many pathologically-determined epithelial phenotypes (normal, dysplastic, neoplastic, and malignant) to identify alterations that are associated with those phenotypes. Some were found to correlate with early-stage phenotypes (dysplastic and neoplastic) that are important in the initiation of lung cancer. This comparative analysis is being used to identify a new set of regulatory pathways in lung carcinogenesis. This is being pursued in studies to find prospective biomarkers for risk assessment, early diagnosis, and targeted therapies [5, 185].

The CGCI also has a Cancer Genome Anatomy Project (CGAP) that is producing extensive genomics data on cancerous cells that are accessible through the CGAP website (www.cgap.org) [5]. Part of this is the SNP500 database that aims to re-sequence 102 reference samples to find known or newly discovered SNPs. These SNPs are very important to molecular epidemiology studies of cancer. The CGCI, together with the Initiative for Chemical Genetics (ICG) established Chembank, an interactive database for small molecules. It contains data on the evaluations of hundreds of biomedically relevant small molecules. Chembank also provides analytical tools to facilitate data mining. They also have a Childhood Cancer Center that has important information about childhood cancers, including current treatments, clinical trials, prevention, genetics, testing and more. There is also a Cancer Genetic Markers of Susceptibility (CGEMS) initiative that aims to identify commonly inherited genetic variations that are associated with a number of cancers, including breast and prostate. Data are available at the Division of Cancer Epidemiology & Genetics website. (http://dceg.cancer.gov/research/how-we-study/genomic-studies/cgems-summary). The CGCI is also providing validated, full open reading frame (ORF) cDNA clones for all of the currently defined human genes. Moreover, there is a mammalian cDNA Library from the NIH, called the Mammalian Gene

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Collection (MGC). It provides full-length clones for most of the human and mouse genes, along with selected clones of cow and rat genes [5].

There have also been important advances in how toxicity tests are being done [186]. In the past, the toxicity of a new drug had to be tested on animals – usually rodents [4]. Even though new drugs were tested at doses to which patients might be exposed, this was not the case with the evaluation of industrial chemicals and chemicals to which the public is exposed. Such chemicals were given to rodents at doses much higher than those that any human would ever consume in an entire lifetime. The idea was that a small fraction of the human population might be especially sensitive to some chemicals. It is not practical to test the toxicity on thousands or millions of rodents. Instead, doses were given that were thousands or millions times higher than those to which humans would ever be exposed. This not only contradicts the idea of hormesis, but often leads to false conclusions. Data from rodents and other animals frequently do not reflect what happens in humans. So, many new drug candidates passed the initial animal toxicity testing, but failed in clinical trials when unexpected human toxicities emerge [4]. So, a new paradigm is emerging that does not require animal testing. Instead, 3-D printing can make human tissues that can be used in toxicity testing [187, 188].

An important part of this paradigm is that actual mechanisms must be identified to show that something is toxic – as opposed to just seeing how many rodents die under the old paradigm. That is, "The US National Research Council blueprint for change, entitled Toxicity Testing in the 21st Century: A Vision and Strategy, called for a transformation of toxicity testing from a system based on high-dose studies in laboratory animals to one founded primarily on *in vitro* methods that evaluate changes in normal cellular signaling pathways using human-relevant cells or tissues" [185]. This paradigm has expanded by including the concept of Adverse Outcome Pathways (AOPs). That is, the human metabolic pathways that are adversely affected by a potential toxin must be identified. Even though toxicity testing is required when developing new prescription drugs, it is not required for dietary supplements. The different ways that new prescription drugs and dietary supplements are developed will be discussed in length in Chapter 3. For now, let's look at the various ways that systems-based thinking is used to educate the public about prescription drugs. This is quite different than the way that reductionist thinking is used in advertisements and propaganda.

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1.3.18 Systems-based thinking in education, in contrast with reductionist thinking inadvertisements and propaganda

To begin with, there are several acronyms and abbreviations that can be quite confusing [51]. For example, EPA usually means the US Environmental Protection Agency, but its also the abbreviation for an important omega-3 polyunsaturated fat, eicosapentaenoic acid. It has 20 carbons and five carbon-carbon double bonds (C=C), including one that is three carbons from the omega end of the fat (the terminal methyl, abbreviated as $-CH_3$). It is quite similar to DHA, which stands for docosahexaenoic acid. It has 22 carbons, six C=C bonds and is also an omega-3 fat. This is not to be confused with DHEA, or 5-dehydroepiandrosterone. It is a steroid hormone that has been called the mother of all hormones. Some people take it to help them build muscle mass and to help handle stress. It has nothing to do with DHA. There is also an acronym, DSHEA, which stands for the Dietary Supplement Health and Education Act, passed by the US Congress in 1994. It made dietary supplement manufacturers responsible for ensuring that their dietary supplements are safe before they are marketed. However, the FDA is responsible for taking action against any unsafe dietary supplement after it reaches the market. The FDA has contracts with the Association of Official Analytical Chemists (AOAC) to develop standardized methods for analyzing supplements [51].

There is also some confusion about anise and licorice [51]. Licorice is can be either a plant (*Glycyrrhiza glabra*) or a type of candy made from licorice extract, sugar and a binder. Licorice extract is sweet because it contains glycyrrhizin, which is sweeter than sucrose, glucose and fructose. Licorice also contains a similar compound, glycyrrhizic acid. In Japan, it is used to treat viral hepatitis and in China, it is used to treat tuberculosis. Some types of licorice candy also have anise oil, which contains the fragrant chemical, anethole. So, the smell of licorice (or liquorice) is due to anise or anise seed which comes from a flowering leguminous plant, *Pimpinella anisum*. The same fragrance is in the spices, fennel and tarragon [51].

On the other hand, these are quite different than Chinese and Japanese star anise [51]. Chinese star anise is used to prepare a tea that is given to infants to treat colic. Chinese star anise is an entirely different genus and species (*Illicium verum*) than Japanese star anise (*I. anisatum*). Chinese star anise is often sold as a dehydrated powder. As a result, it is difficult to distinguish between it and Japanese star anise, which is to be used only as incense and should never be used to make a tea. It contains several

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potentially deadly neurotoxins, including anisatin. Several years ago, there were cases of seizures in infants who were given tea made from mislabeled star anise. Since then, analytical methods have been validated to identify anisatin and distinguish Chinese from Japanese star anise [51]. So, it is important, but difficult to clearly identify the contents of a dietary supplement. However, that is not always done. Many of them are proprietary mixtures.

In contrast, when an NDA is filed with the FDA, the public is told what the drug is, how it is made and what its potential benefits are. Pharmaceutical companies and research institutes can patent their new drug. This protects their intellectual property and ensures that another company doesn't manufacture the same drug after the original developers spent millions of dollars to do the initial research. So, the composition of the new drug or mixture of substances is not proprietary. Frequently, this is not the case with dietary supplements. Some are made from a proprietary mixture of extracts from fruits and/or vegetables. Others are simply mislabeled. What is in the bottle is not always what's on the label.

When developing a new drug, many manuscripts are published in peer-reviewed journals that show that the new drug and the lead compound used in its development are safe and effective. Moreover, the studies are repeated several times as the new drug goes through several clinical studies. This is an extremely important part of the scientific method. However, it is elusive in many cases. That is, unless a company, research institute or university has vast resources, it is not practical. Much excellent original research is done and published in journals. Unless there is an exceptional reason (like developing a new drug), few journals or funding agencies will accept research articles or proposals that repeat a previous study. So, much of the research on dietary supplements is not repeated – even though the initial publications can generate tremendous interest. At the same time, many dietary supplements are based on traditional remedies that emerged long before the invention of the printing press and the widespread availability of books and journals. Also, new prescription drugs can make claims about their health benefits that manufacturers of dietary supplements can't make. For example, manufacturers of dietary supplements can write on the label that their products help improve your mood, but not that it reduces depression [189]. They can say that it maintains a healthy circulatory system, but not that it prevents cardiovascular disease. They can say that it supports the immune system but not that it helps to prevent colds and flu [190].

However, the makers of all prescription drugs and most dietary supplements monitor the effectiveness of their products [4]. After a

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prescription drug gets past all four stages of clinical trials and is approved, the law requires that all adverse effects are monitored. This is not true for dietary supplements. In contrast, their manufacturers and sales force often maintain websites that document personal testimonials of efficacy by people who consumed the supplements. People who buy the dietary supplements often are encouraged to talk to their friends and post their personal testimonials on social media. In contrast, there is a report of at least one type of dietary supplement being sold about which no customer would ever describe openly. That is, one dietary supplement was sold for weight loss and performance enhancement for athletes [191]. It was sold on the black market by calling a friend or a cell phone number provided on internet message boards. Most customers probably understood that the supplement was actually the illegal, deadly drug – amphetamine. Analysis by mass spectrometry (actually UPLC-QTOF-MS/MS) found 120 mg of it per capsule in one case and 100 mg per capsule in another. Such products are not available in stores or even on freely accessible websites. Also, they are sold as capsules with no imprint on them and no list of ingredients on the label [191].

In contrast, prescription drugs are sold openly in well-regulated pharmacies. Their contents are known and indicated on the label. Moreover, their effectiveness and mechanisms of action are studied even after they are made available and approved by the FDA and other governments' regulatory agencies. For example, statins were developed based on their ability to inhibit the rate-limiting step in the biosynthesis of cholesterol [4]. At the time, it was thought that the human circulatory system was like indoor plumbing. If it became clogged up with too much cholesterol, it would cause high blood pressure – an important risk factor for cardiovascular diseases and stroke. Further studies have shown that other new drugs that lower cholesterol don't increase the median lifespan of those who take them. In contrast, statins do. This is because they have many more beneficial health effects than just lowering cholesterol. They lower the concentration of triglycerides in the blood. They also inhibit angiogenesis and they do it in a critical tissue – the lymph nodes [5, 160]. Lymphangiogenesis is important because the lymph nodes are the first places where metastasis occurs in most epithelial cancers and malignant melanomas. In addition, malignant tumors cause lymphatic vessels to grow [4, 192].

Statins were identified as inhibitors of lymphangiogenesis by doing high content screening of the Library of Pharmacologically Active Compounds database, or LOPAC® [192]. They used individual human lymphatic endothelial cells along with automated image acquisition and

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analysis to look for a specific phenotype – the formation of lymphatic vessel sprouts [5, 192]. Statins also improve endothelial function, increase the stability of atherosclerotic plaques, decrease oxidative stress and inflammation and inhibit the thrombogenic response [193]. They also have beneficial effects on the immune system, CNS, and bones. Many of these pleiotropic effects are mediated by inhibiting the activities of isoprenoids, which are attached to several intracellular signaling molecules. For example, statins inhibit the small GTP-binding proteins Rho, Ras and Rac. For them to function, they must be in the right location. This depends on isoprenyl groups being attached to them. Statins inhibit this [193].

Still, adverse effects can occur to some people who take statins. The most serious is myopathy, which is muscle pain or weakness combined with large increases in the concentration of creatine kinase in the blood [194]. It has been estimated that treating 10 000 patients for five years with an effective regimen (such as 40 mg of atorvastatin daily) can cause about five cases of myopathy, 50–100 new cases of diabetes, and 5–10 hemorrhagic strokes. Still, data from numerous randomized clinical trials show that statins reduce the risk of major vascular events (including coronary deaths or myocardial infarctions, strokes, and coronary revascularization procedures) by about 1/4 for each mmol/L reduction in LDL cholesterol during each year (after the first) that they continue to be taken. “It is, therefore, of concern that exaggerated claims about side-effect rates with statin therapy may be responsible for its under-use among individuals at increased risk of cardiovascular events. For, whereas the rare cases of myopathy and any muscle-related symptoms that are attributed to statin therapy generally resolve rapidly when treatment is stopped, the heart attacks or strokes that may occur if statin therapy is stopped unnecessarily can be devastating” [194].

However, the muscle pain can be severe enough to make some seek a natural alternative – red yeast rice [195]. However, the amounts of statins in different supplements that contain red yeast rice are not well controlled and can vary considerably [196]. So, one should exercise caution when buying or consuming red yeast rice or any other supplement that is not well controlled. More importantly, you should always tell your primary care physician and any other doctors that you see if and when you take any dietary supplement.

At the same time, many dietary supplements are legitimate and well-controlled. Some, like vitamin D3 are recommended by many physicians and are well controlled. The dose in each bottle containing gelatin capsules or pills is written clearly on the label. It can prevent bone fractures and many diseases – especially in older people [197]. The Biochemical

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Efficacy and Safety Trial of vitamin D (BEST) is trying to establish the optimum dose of vitamin D3 by giving people with a median age of 72 years either 50 or 100 µg or placebo daily for 12 months [197].

It is also important for children [198]. The NIH and Care Excellence recommends that vitamin D should to be available to all pregnant and breastfeeding women and children under five years of age. Vitamin D receptors have been found in many different cells in the human body. A deficiency in vitamin D has been linked to hypertension, cardiovascular diseases, bowel cancer and insulin resistance. A deficiency is defined as a concentration of <20 ng per mL (50 nmol per L), and insufficiency is defined as a serum 25-hydroxyvitamin D concentration of 20 to 30 ng per mL (50 to 75 nmol per L) in the blood serum [198]. To prevent vitamin D deficiency, the American Academy of Pediatrics recommends that infants and children receive at least 400 IU per day from diet and supplements. Vitamin D supplementation of at least 700 to 800 IU per day can reduce bone fractures and fall rates in adults. People with vitamin D deficiency can be given 50 000 IU per week ergocalciferol (vitamin D2) by mouth for eight weeks. After the serum concentration of vitamin D normalizes, it is recommended that a maintenance dose of cholecalciferol (vitamin D3) at 800 to 1000 IU be consumed per day from dietary and supplemental sources [199].

Many recent studies have shown that vitamin D has many more beneficial health effects than its classical actions on mineral homeostasis and bone metabolism [200]. It also modulates immune function and has important antibacterial properties. It also has beneficial effects on CKD [200].

In total contrast, there are many dietary supplements that are sold as weight loss products [201]. Despite much advertising to the contrary, there are no dietary supplements that safely and effectively cause significant weight loss. In addition, some of them are adulterated with banned prescription drugs as well as narcotics, stimulants, antidepressants, anorectics, laxatives and diuretics [201]. It is unfortunate that many people become frustrated when they lose just a few kg, because even a modest weight loss through counseling, diet and exercise can reduce the incidence of disease by 40-60% over 3-4 years [202]. It is even more unfortunate for those people who are not satisfied with a modest loss of weight when they decide to take dietary supplements that may contain narcotics or prescription drugs that were once approved by the FDA and other countries' regulatory agencies, but were pulled from the market due to harmful and sometimes deadly side effects [203-211]. Also, deadly narcotics such as cocaine, codeine, ketamine, 3,4-methylenedioxy-methamphetamine

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(MDMA), methaqualone and 1-(1-phenyl cyclohexyl)piperidine (PCP) have been found in some supplements [207, 212]. Even those supplements that contain only natural ingredients like the bark of the yohimbe plant (*Pausinystalia johimbe*) [213] or the dried, immature fruits of bitter orange (*Citrus aurantium* L.) [207] can be toxic. So, it is important for the public to be well educated about the value of losing just a few kg and avoiding potentially toxic dietary supplements.

Even the usually quite healthy ingredients in green tea can be toxic when consumed at too high of a dose as green tea extracts [214-220], even though it may be quite healthy at moderate doses [220]. Caffeine can also be toxic at high doses [221], so some green tea extracts are decaffeinated [222]. It is usually safe when consumed in moderation (500 mg or less daily), and may even protect against some diseases, including Parkinson's disease [223]. However, it can be toxic at doses over 10 grams per day for adults. Even though energy drinks contain much less than this, they can still be quite harmful – especially to children and adolescents [224]. In addition, caffeine is an adenosine receptor antagonist. This is important because there are several drugs in clinical trials that target these receptors. That is, all four types of adenosine receptors are elevated in autoimmune diseases and cancer, so they are being investigated as therapeutic targets. So, it will be important in these studies to determine the effects of caffeine consumption on the efficacy of these drugs [225].

The phenolic compounds (especially EGCG) in green tea may help prevent cancer when consumed in moderation [224, 226]. Meta-analyses of previous publications found that consumption of green tea was associated with a 20% and 18% reduction of the risks of breast cancer and colorectal cancers, respectively. A prospective cohort study of over 40 000 Japanese adults found that green tea was inversely associated with cardiovascular mortality. Moreover, participants who consumed more than five cups (about 237 mL) per day had a significantly reduced incidence of stroke [227].

However, EGCG is like fresh air, fresh water or any other substance. All substances are toxic if given at a high enough dose to the most vulnerable part of the body [5]. That is, the dose is the poison [228]. Still, physiologically tolerable doses of EGCG activate antioxidant signaling pathways that have neuroprotective and anticancer effects [229, 230]. However, at higher doses, EGCG suppresses not only the major antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) but also the antioxidant rescue pathway that it activates at lower doses [225]. So, if one is not careful, attempts to 'cure' obesity with weight loss supplements can be dangerous. They can not only cause illness

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and death, but also cause consumers of such products to lose their jobs when they fail a urine test because they consumed a supplement that contained an illegal drug, even though its presence was not indicated on the label of the supplement [231]. Moreover, even though the FDA recalled many dietary supplements because they contained banned ingredients, many of them still contained those toxic ingredients in supplements that were purchased at least six months after they were recalled [232]. Perhaps the most concerning weight loss supplements are those that are sold over the black market by calling a friend or a cell phone number provided on internet message boards [204]. Such products are not available in stores or even on freely accessible websites. They are sold as capsules with no imprint on them and no list of ingredients on the label [204]. So, the FDA has issued a warning about weight loss dietary supplements [202].

Moreover, the idea that the FDA and other governments' regulatory agencies are in a conspiracy with pharmaceutical companies to keep people sick, so they can continue to sell them prescription drugs that don't cure any diseases is wrong. This thinking can lead people to believe that dietary supplements are always safe and effective – especially if they are labeled 'natural'. It can also lead people into thinking that the FDA and other governments' regulatory agencies are trying to keep dietary supplements out of the hands of sick people. In contrast, there has been a long-term collaboration to get people to take at least one of them – folic acid. Still, much is being done with limited resources to warn people about the dangers of some supplements.

Even the premise that pharmaceutical companies are only interested in developing drugs that have a huge profit potential is wrong. There is little or no potential for profit in curing malaria, but there is a broad international effort to eradicate it. This includes the Bill & Melinda Gates Foundation, National Institutes of Health, UK Medical Research Council, UK Department for International Development and the Wellcome Trust [233], among others. Even though malaria has been almost completely eliminated in over 50 countries, it can still enter into those countries through international travel from countries in which it is endemic [233]. Worldwide, there were an estimated 429 000 malaria deaths in 2015 [234]. This is an improvement. The rate of incidences of new cases of malaria fell by 21% globally from 2010-2015. The worldwide mortality rate fell about 29% during this period [234]. Still, malaria takes a huge toll on humanity, and many very talented people are looking for a cure - even though it is not profitable.

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Using systems thinking, international collaborators have realized that drug discovery thrives in an open environment [235, 236]. This includes open science, open innovation, open access to data and open sources of published reports. Systems thinkers know that most scientific discoveries emerge from cycles of thesis and antithesis. At the same time, true innovation depends on intensive peer review. That is, it is much easier to see other peoples' mistakes than to see our own. This is not new. In the early years of new drug development, academia, industry and governments collaborated openly. This changed somewhat in the 1970s, when many large pharmaceutical companies became secretive organizations. It was thought that sharing results obtained in the first stages of drug discovery would compromise a company's competitive edge. Academic research also became more secretive, as researchers needed to publish in high-impact journals to increase their chances of getting their research proposals funded. Moreover, being the first author (or sole author) on publications was very important when building one's scientific reputation. Also, there were unrealistic expectations of financial benefits that could be obtained through technology transfer [235].

In time, many academic researchers published hundreds of peer-reviewed articles and obtained tenure. Fortunately, it became not so important to publish more papers or to be the first author. It became more important to publish truly significant research that benefits all of humanity. Similarly, once a person has more money could possibly be spent in a lifetime, he or she often becomes very interested in using that money to help save lives and relieve human suffering. Especially as many people age, they often become much more concerned about what they will leave behind for the next generation, rather than worry about earning a few more million dollars or publishing a few more journal articles. At the same time, many politicians and government workers who have spent their entire adult lives in public service want to help humanity. Such attitudes led to the formation of the Medicines for Malaria Venture (MMV) [235].

The MMV and similar collaborative organizations realize that open science starts with depositing data such as base sequences in DNA and protein structures in databanks that are available to everybody [235]. However, it is essential to ensure that the data are accurate. In the case of genomics, this was done by repeating the DNA sequencing several times and by making three-dimensional models of protein structures. As more data was entered into the public domain, it became essential to avoid allowing inaccurate data that can be very misleading to future investigators. So, the MMV has established a Quality Control system to validate primary data [235]. They test sets of known standards, blinded to

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enable cross-validation of results. They also use standardized *in vivo* pharmacology assays when making decisions. This enables them to make direct comparisons of the pharmacological and potentially toxic properties of molecules being evaluated. This is especially important in developing new drugs to treat and cure malaria, since most assays are phenotypic screens using different platforms. As the process of development proceeds, many ideas and results are so important that they become intellectual property. Using systems thinking, intellectual property is no longer a right but a responsibility. That is, MMV partners who develop intellectual property must spend resources to advance the work or pass it on to others who do have the resources to do so. Since 2010, the MMV has coordinated sharing data on over seven million compounds that were obtained from phenotypic screening of the mosquitoes that carries malaria. They are in the genus *Plasmodium*. However, all this information can only provide wisdom if the data are used properly. So, the MMV launched the Malaria Box. It is an informatics and pragmatics approach to select, purchase and re-test about 400 compounds for distribution to partners who retested them to evaluate reproducibility and reliability. From this, many publications and a high-quality database have emerged. Moreover, the data were used to launch a pathogen Box 400 program. About 400 compounds are being evaluated for their effects on other pathogenic organisms, based on phenotypic screens. The MMV consortium plans to launch similar programs to evaluate the effects of test compounds on viruses and pathogenic Bacteria. This will all be done with open sourcing, which is even more collaborative than open access. In open sourcing, all project data and structures of test compounds are available to anyone with access to the Internet. At the same time, the entire scientific and healthcare communities have been invited to fully engage with MMV. They want their advice. They also want help in synthesizing new compounds, as well as in testing them and providing in-kind technical support. More importantly, the MMV is inspiring more groups to work together. For example, collaborations on data generated in the development of the bromodomain inhibitor JQ1 inspired further fruitful efforts to discover new drugs in this group of molecular targets [235].

Frequently, academia, research institutes and small biotech companies do important scientific research and generate hits in drug screens [236]. Then, pharmaceutical companies and other well-funded organizations develop the hits into drug candidates. For example, the Manchester Collaborative Centre for Inflammation Research (MCCIR) is developing new drugs for inflammatory diseases. GlaxoSmithKline (GSK), AstraZeneca, and the University of Manchester formed the MCCIR [236].

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In addition, pharmaceutical companies are using the crowdsourcing model which utilizes the combined expertise of a group (or crowd) of external scientists [236]. For example, Lilly has initiated some open innovation projects that are supported by the web-based solution providers InnoCentive and YourEncore. There is also a growing interest in polypharmacology, or the use of several drugs that interact with multiple targets. It uses the tools of chemogenomics to find several potentially useful ligands that will interact with the most probable and useful targets. This can be used to identify multifunctional peptides that can deliver personalized therapies to diverse groups of patients. This includes the approved drug Tanzeum™ (albiglutide) that is a GLP-1 (glucagon-like peptide-1) agonist [236].

There are other important collaborations that are developing new drugs for rare diseases, despite the lack of profit potential [237]. A team of professionals working for Collaborative Drug Discovery Inc. (CDD), the National Brain Tumor Society, the Institute for Rare and Neglected Diseases Drug Discovery (iRND3), Collaborations in Chemistry, Phoenix Nest, Inc., the Hereditary Neuropathy Foundation and Hannah's Hope Fund described some important collaborations in rare disease drug discovery [237]. The CDD provides drug discovery informatics to many very prestigious clients [238]. This includes Columbia University, the Swiss Federal Institute of Technology (or Eidgenössiche Technische Hochschule, ETH), Johns Hopkins University, Sanofi, AstraZeneca, Pfizer, Lilly, Acetylon and many others [238]. The CDD has created software packages that enable researchers to have their own private vaults for storing chemical and biological data, which can be shared and mined securely. CDD has also hosted large-scale collaborations such as More Medicines for Tuberculosis (MM4TB), Bill and Melinda Gates Foundation, TB Accelerator and the NIH Blueprint for Neuroscience Research (BPN). The CDD also has datasets that contain lists of drugs and compounds that have been identified for possible new uses (repurposing), based on *in vitro* screens. This includes drugs that have already been approved by the FDA as well as compounds identified by the National Center for Advancing Translational Sciences (NCATS). The CDD recently added the NIH's Molecular Libraries Probe Production Centers Network (MLPCN) probe compounds to their databases and included a scoring of these compounds by medicinal chemists [237].

There are even mobile apps that are being used to share data and inspire new collaborations [237]. This includes an app called the Open Drug Discovery Teams (ODDT) that collected Twitter feeds on multiple scientific hashtags (such as #huntingtons) and created an open database for

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rare diseases. The app can be used as well to share data on molecular structures and biological activities. These can be used with already existing databases that relate genes to biological pathways with known pharmacological impact. Hopefully, they can help identify treatment options for some FDA-approved drugs, nutraceuticals or other compounds [237].

So, even the highly profitable pharmaceutical industry is realizing that the previous reductionist and competitive drug discovery process is no longer viable [237]. They are linking their considerable assets with very productive colleges, universities, research institutes and hospitals to translate important scientific discoveries into new, life-saving drugs and devices. Not only is their research more collaborative, but it also encourages active participation by patients, caregivers, advocates and foundations. There is more data sharing as networks and social networking tools (social media) enable more patients and their caregivers to connect with each other, as well as with other physicians and researchers. This is especially important for people who have rare diseases, since they will seldom meet anyone else in person who has the same disease. So, social media are very useful. Patients can now find each other on websites and social media, including Facebook pages set up by many groups of people who have or are affected by rare diseases. Patient networks can also lead to important breakthroughs. This includes learning about the genetic and epigenetic origins as well as natural histories of their diseases. They can also help find biomarkers for diseases and recruit appropriate patients for clinical trials. Maybe even more important, they raise public awareness of their diseases and symptoms. This can even affect a pharmaceutical company's profit potential as new blockbuster drugs such as Kalydeco™ for cystic fibrosis make headlines and improve the company's reputation. At the same time, patient advocacy groups such as the Michael J. Fox Foundation for Parkinson's Disease Research and the Cystic Fibrosis Foundation are providing funds for the early, most expensive phase of new drug development. Similarly, patient advocacy groups for brain tumors not only provided funds for research, but also helped recruit patients for clinical trials and to help design them and establish reasonable endpoints. For example, the FDA has pointed to the Jumpstarting Brain Tumor Drug Discovery Coalition as an example of how patient advocacy groups and sponsors of clinical trials can collaborate successfully [237]. At the same time physicians are able to use network sites such as Sermo [239] and Doximity [240] to crowdsource their diagnoses of patients' diseases [237, 239, 240].

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Open collaborations that discover new science and develop new drugs for one rare disease can lead to treatments and cures for other diseases [237], once the hidden connections [7] are discovered. For example, imatinib (Gleevec®) was developed and subsequently approved for treating Philadelphia chromosome positive chronic myelogenous leukemia (CML), in which the tyrosine kinase BCR-ABL is mutated [237]. It was subsequently found that it also inhibits other tyrosine kinases that are important in other diseases. So, imatinib is now known to be effective in treating gastrointestinal stromal tumors, many other cancers, and steroid refractory Graft-versus-Host disease. Finally, many people hope that a united patient population with a well-established genetic and epigenetic etiology can work with patient advocacy groups to accelerate new drug discovery [237].

Governmental organizations are also finding ways to collaborate – even in the currently divisive political environment. For example, at the end of 2016, the U.S. Congress overwhelmingly approved a bill to speed the Federal approval of new pharmaceuticals and medical devices. In a rare show of bipartisan fellowship, Republicans and Democrats focused on the similarities in their human condition and temporarily forget about their differences. After all, everyone wants to avoid getting sick, suffering or dying. So, while Vice President Joe Biden presided, the Senate passed the $6.3 billion biomedical bill that included $1.8 billion for cancer research. Moreover, President Obama put Vice President Biden in charge of a 'moonshot' to find ways to cure and treat cancers, such as the type that killed his son Beau. The bill also included $4.8 billion over the next 10 years for research by the NIH, including work on brain diseases and developing personalized medical treatments. So, there is not a conspiracy to keep people sick, but in an open collaboration to prevent, treat and cure diseases.

The conspiracy theory is especially harmful when it applies to immunizations and vaccines that prevent many formerly deadly diseases (such as influenza) and even eliminated one (smallpox). The conspiracy theory and general distrust of governments has led some to believe that responsible parents should not let their children be vaccinated – despite the devastating history of communicable diseases such as the measles, mumps and rubella (MMR). One reason for this confusion is that autism spectrum disorders have been and continue to be misunderstood by many people [241]. Originally, autism was thought to be caused by bad parenting and possibly even exposure to the cold and "refrigerator mothers". Children with autism were sent to dank institutions. Fortunately, these mistakes were corrected. We now know that autism is not just a single disease, but a

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spectrum of disorders. It is not the parents' fault if they have a child who suffers from autism spectrum disorder (ASD). In addition, physicians are now much better at diagnosing it. Most of the increase in the incidence of ASD is due to better diagnosis, though some is due to the increased average age of fathers and the fact that more premature babies survive infancy. That is, paternal age and premature birth are risk factors for ASD. So, there isn't an epidemic of ASD and vaccines don't cause it [241].

Unfortunately, the legal system in the USA and in many other countries has a much lower standard for 'proof beyond a reasonable doubt' than do scientists who realize that science can't prove anything [3]. Math can prove things, such as the fact that 1+2+3+ … 99 = 4950. You can prove this yourself either by carefully entering each number into a calculator or Excel spreadsheet. Instead, you can realize that 1+99=100, 2+98=100 … 49+51=100 for the first 4900 and then add the 50 in the middle to get 4950. There is no doubt in a proof. Unfortunately, much of the public confuses the terms evidence and proof. So, some families with autistic children have pursued legal channels to try to 'prove beyond a reasonable doubt' that vaccines were responsible for their children's condition [242]. Most of them alleged that the cause was the mercury-containing preservative thimerosal, which was formerly used in many vaccines in the USA and elsewhere. Others argued that the culprit was the MMR vaccine itself or perhaps the vaccine in combination with thimerosal. Although most experts have concluded that there is no evidence of a causal tie between autism and thimerosal or the MMR vaccine, some doctors and scientists, some groups representing families with autistic children, and many parents fervently believed there was a connection. Claimants not only wanted to 'prove' that the Federal government, the Institute of Medicine, vaccine makers, and mainstream science were wrong. They also wanted money. A child with autism requires very expensive services and will have very limited employment opportunities when they become adults. Besides, many parents of autistic children felt better psychologically when they blamed profit-seeking drug companies for their children's problems. Over 5000 such families filed claims with the federal Vaccine Injury Compensation Program (VICP). This was created by legislation that was adopted by the United States Congress in 1988 in response to a somewhat similar scare over the pertussis portion of the diphtheria–pertussis–tetanus (DPT) vaccine in the United Kingdom.

At least one British researcher fed fears that the vaccine was causing some children grave neurologic harm - claims that were later widely discredited. Yet the alarm was so great that many British families refused

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the pertussis vaccine, while many children became ill with pertussis (whooping cough), and about 70 of them died. In the USA, several parents sued the manufacturers of DPT vaccines. Even though most public health officials thought that the claims of side effects were unfounded, some families won large awards from sympathetic, scientifically illiterate juries who were convinced otherwise. As a result, most companies making the DPT vaccine stopped production, and the remaining major manufacturer threatened to do so. Health officials feared the loss of herd immunity, so the US Congress created the VICP. This program provided compensation to children who had serious adverse effects from any childhood vaccine. The compensation covered medical and related expenses, lost future income, and up to $250 000 for pain and suffering. The funding for paying successful claims based on vaccines that were administered before 1988 came from the U.S. Treasury. For claims regarding later vaccinations, funding came from a patient fee of 75 cents per vaccination. To win a VICP award, the claimant did not need to do everything that is required to hold a vaccine maker liable in a product liability lawsuit. But a causal connection had to be shown. This has become much more difficult. An advisory committee helped to amend the list of adverse effects that are recognized because the consensus view changed as the results from new studies were reported. If families try to claim that a vaccine caused an adverse effect that is not on the list, the burden of proof rests with them. ASDs are not on the list of adverse side effects for any vaccine, and the VICP has rejected about 300 such claims outright [242].

Recently, the ‘controversy’ re-emerged due to an award-winning film, *VAXXED from Cover-up to Catastrophe*. It was released recently and made national news due to its subject and primary thesis. It seemed to be trying to support the idea that vaccines have caused autism and that the U.S. Centers for Disease Control (CDC) was guilty of hiding the truth and withholding important Classified data. It did this through a bait-and-switch technique. The audience was baited with the idea that the public has been vexed by a government cover-up. Then the movie switched to the ideas that vaccines and antibiotics are causing a catastrophe in healthcare. It did this by misusing terms like ‘classified’ and ‘linked’ to imply that the CDC and the Federal government are causing a catastrophic increase in autism, but did not accuse them outright. They had a pediatrician give her personal testimony that she had seen many more children with autism that she did when she first started practicing medicine. It was hoped that the audience would assume that this meant that many more children were becoming autistic and not that physicians were getting much better at diagnosing ASDs. In contrast, it is well established that the incidence of ASDs is not

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increasing – only our ability to diagnose them [241]. The vast array of data supporting the idea that we are getting much better at diagnosing autism spectrum disorders was not mentioned.

Instead, the movie presented powerful, emotional personal testimonials from several people that were believable and somewhat convincing. One of the most emotional ones came from a woman with relatively dark skin who was labeled as an African-American. She had two so-called identical twins that scientists would describe as monozygotic twins. Such twins are born with the same genes, but rapidly develop different patterns of epigenomic changes and different types and amounts of Bacteria in the gut (the gut microbiome). So, 'identical' twins are not identical. In the personal testimonial, one of the woman's infants received at least one dose of the MMR vaccine under poorly controlled conditions, but the other did not. The one who received the vaccine quickly developed symptoms of autism, while the other did not. It was stated that the CDC did a study on the possible link between the age at which an African-American child is immunized and the likelihood that they would become autistic. The study supposedly showed that African-American children who were immunized at age 18 months were more likely to be diagnosed with autism than those who were immunized at age four or five (which is required for entering Kindergarten in a public school). Instead, most of the audience probably got the impression that the MMR vaccine caused autism in that unfortunate child. Many were sympathetic towards this formerly brilliant child, and felt that the physician who was about to administer the vaccine was going to do something terrible. So, the child became very upset and cried, making the mother unsure of how many doses the child received.

To make things much worse, we were told that the CDC classified the results of the study and didn't mention it in subsequent publications. For the results to reach the public, a whistle blower at the CDC told an investigative reporter where to go on the CDC website to find the classified studies. To anyone in the military or any civilian who works in the defense industry, the words classified and whistle blower mean something very different than this. As we all know from the truly Classified documents released by Wikileaks, not just Classified, but also Secret information was released. It was not available on any government agency's website. Only people with a security clearance had access to the computers that contained Classified data. Copying the data and sending it to Wikileaks led to the prosecution and conviction of the whistle blower, Bradley (now Chelsea) Manning. Unlike the so-called whistle blower at the CDC, Chelsea Manning did not tell anyone where to find the Classified data. That would have been pointless. It was kept on a

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Classified computer that required a username and password. Access to the facility containing the Classified computer required a government-issued badge and security clearance. In complete contrast, the studies on autism and the MMR vaccine in African Americans may have been classified as misleading or insignificant by the CDC, but it was neither Classified, Secret nor Top Secret. No laws were broken, national defense was not threatened and nobody was sent to prison. Anybody who knew where to look on the CDC website could freely download it. The problem was that there was so much data collected during the studies that it would take years to find the information about African-Americans in it.

This is not unique. Almost every scientific study that is published in a journal, magazine or book is based on hundreds or thousands of pages of data. For example, the study data needed to get most prescription drugs approved by the FDA could fill a room full of boxes. So, it can be quite challenging to decide what small fraction of the data should be incorporated in the final report. The portions of the data that are not published are kept on a government agency's website, but can be very difficult to find. A recent example of this occurred a few years ago. Doctor Oz reported on national television in the USA that he sent some apple juice to an analytical lab and they found that it had more arsenic in it than what was (and still is) allowed in drinking water (EPA regulations). Supposedly, this was completely new. However, the FDA had been analyzing apple juice and hundreds of other foods and beverages for not just arsenic, but also lead, other toxic metals, pesticides, mycotoxins and industrial chemicals (like benzene) for many years. The results of the analyses were not Classified. They were and still are freely available to the public on the FDA website – but they can be difficult to find.

Still, may people might wonder why something as important as a government-backed vaccine possibly causing autism in African Americans would not be highlighted and published. Actually, there are several reasons. First, to a scientist there is only one race – the human race. Second, the entire human race originated in Africa, so we are all African. Those of us who were born anywhere from the southern tip of Argentina to the northern parts of Alaska or Canada are Americans, too. So, everybody who was in the CDC study on vaccines could have been classified as African-American. Third, the CDC studies were done before the Affordable Care Act was passed. So, many African-Americans (white, black and in between) did not have health insurance. This is why their ASDs were not diagnosed at age 18 months. Also, Congress (not President Obama) passed the Affordable Care Act. The fact that many opponents of this renamed it after a person who did not even have a vote (Presidents in

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the USA do not have a vote in Congress), simply because they hate him tells us something about the emotional impact of being a so-called African American. So, it would have been quite controversial for the CDC to ask if there was a link between whether or not a child was covered by health insurance and the age at which they were diagnosed with autism.

For some reason, it was considered acceptable to arbitrarily classify people as being African-American or not African-American, even though everyone in the study was African-American. This comes to the main point of disagreement between those who think that vaccines cause autism and those who don't. Are more children becoming autistic or are we just getting much better at diagnosing it? Are younger children more susceptible to being adversely affected by vaccines? The answer is no. It is much more likely that an African-American child of any skin color who is covered by health insurance will see a pediatrician early and often. Such children are much more likely to be vaccinated early and are more likely to be diagnosed with an ASD, because they see a doctor early and often. So, vaccines don't cause autism any more than health insurance does. Just because there is a link between having health insurance and being diagnosed with ASD at an earlier age does not mean that health insurance causes it.

The movie was also confusing and even contradicted itself about the issues of two things being linked and one thing causing another. It also jumped from the MMR vaccine to an implication that all vaccines were dangerous and could cause autism. The first personal testimonial used the term linked, but later in the movie, the term caused was used. Throughout the movie, the audience was reminded of how the British and American (USA) governments supposedly harassed and destroyed the reputation of Dr. Andrew Wakefield, the primary author of the controversial article, "Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children" [243]. It was published in the very prestigious British medical journal *The Lancet*. His interest in autism was based on observations that there were changes in the types and amount of Bacteria in the lower digestive tract of autistic children (from the terminal ileum; ascending, transverse, descending, and sigmoid colons, and rectum). The changes in the microbiome that occur in ASD have been described earlier in this book, but it is important to stress a short quote from Dr. Wakefield that most of the audience probably missed. It came directly from his article in *The Lancet*, **"We did not prove an association between measles, mumps, and rubella vaccine and the syndrome described"** [243]. So, despite the suggestions, personal testimonials and insinuations in the movie, the supposed victim of government harassment

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admitted that he and his coworkers did NOT establish a link between the MMR vaccine and autism. Also, no other vaccine was mentioned in the controversial article.

So, if Dr. Wakefield did not write that there was a link, why did he have to retract the article and why was his license to practice medicine in England was revoked? The reasons are explained on several websites including Exposed: Andrew Wakefield and the MMR-autism fraud [244]. In this website, the investigational reporter Brian Deer discovered that Dr. Wakefield had been paid by an anti-MMR lawyer, Richard Barr, "to create evidence against the shot and, while planning extraordinary business schemes meant to profit from the scare". He was paid two years before writing the controversial article. "The goal was to find evidence of what the two men claimed to be a "new syndrome", intended to be the centrepiece of (later failed) litigation on behalf of an eventual 1600 British families, recruited through media stories. This publicly undisclosed role for Wakefield created the grossest conflict of interest, and the exposure of it by Deer, in February 2004, led to public uproar in Britain, the retraction of the *Lancet* report's conclusions section, and, from July 2007 to May 2010, the longest-ever professional misconduct hearing by the UK's General Medical Council (GMC)" [244].

In addition, the children in the *Lancet* study were not what they appeared to be. According to the website [244]: "In the *Lancet*, the 12 children (11 boys and one girl) had been held out as merely a routine series of kids with developmental disorders and digestive symptoms, needing care from the London hospital. That so many of their parents blamed problems on one common vaccine, understandably, caused public concern. But Deer discovered that nearly all the children (aged between 2½ and 9½) had been pre-selected through MMR campaign groups, and that at the time of their admission, most of their parents were clients and had contacted a lawyer, Barr. None of the 12 lived in London. Two were brothers. Two attended the same doctor's office, 280 miles from the Royal Free. Three were patients at another clinic. One was flown in from the USA. The investigation revealed, moreover, that the paper's incredible purported finding - of a sudden onset of autism within days of vaccination - was a sham: laundering into medical literature, as apparent facts, the unverified, vague - and sometimes altered - memories and assertions of a group of unnamed parents who, unknown to the journal and its readers, were bound to blame MMR when they came to the hospital because that was why they had been brought there. Wakefield, a former surgeon, denied this. But the true number of families accusing MMR wasn't eight, as the paper said: it was 11 of the 12 (later all 12) and in most cases

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records noted parents' compensation claims before the children were referred. "If my son really is Patient 11, then the *Lancet* article is simply an outright fabrication," said the father of the penultimate child in the series - admitted to the Royal Free, at age 5, from northern California and whose history was falsely reported in the paper" [244]. In conclusion, the MMR and other vaccines are safe and effective. They prevent a multitude of diseases and have saved many lives.

Another popular method of selling dietary supplements is to try to make people believe that the physicians and the rest of the healthcare industry are in a conspiracy with governmental regulatory agencies (like the FDA) to keep us sick or even kill us. According to the author of one book, "Medicine only works as a palliative. The cure never cames [sic] because it is focused on DISEASE INDUSTRY" [245]. The author wrote the following: "The FDA supports the claim that the smell of soursop fruit is very similar to that of chemotherapy drugs and there are several reports that it is a powerful weapon against cancer whereby it kills the damaging cells up to 10,000 times more efficiently than chemotherapy drugs because graviola is known to attack cancer cells without harm to the healthy cells". The author cited reference 41 and said that it was the FDA website on Inspections, Compliance, Enforcement, and Criminal Investigations. If you search the website for graviola and cancer, you will find two warning letters to organizations that sell a graviola extract. These letters do NOT support the claim that the FDA thinks that graviola is known to attack cancer cells without harm to the healthy cells. They support just the opposite – that such claims are illegal. This author goes on to claim that physicians don't want people to know that a class of compounds called acetogenins (that are in the popular fruit called soursop, graviola or guanábana) can cure cancer and are 1000 times more effective than the first line treatment for many types of cancer (Adriamycin) [245], which has saved hundreds of millions of lives [4]. Such claims could lead uninformed readers to believe that they should eat fruits that contain these acetogenins or take dietary supplements that contain fruits, seeds, roots, stems and/or leaves of graviola (*Annona muricata*) or other plants in the same family (Annonaceae). This could have disastrous consequences for two reasons. First, it could influence people to avoid talking to a physician or take the well-established prescription drugs that could cure them. Second, when graviola was over-consumed on the Pacific island of Guam, New Guinea, the Caribbean island of Guadeloupe, in parts of the Afro-Caribbean and Indian population in London and New Caledonia it caused an atypical form of Parkinson's disease that doesn't respond to the

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standard, first-line treatment (L-3,4-dihydroxyphenylalanine, or L-DOPA) [246-249].

There is another important example of how many dietary supplements are marketed in a way that is very different than the way that prescription drugs are marketed. Prescription drugs are marketed by publishing peer-reviewed articles in reputable journals and by talks given at scientific and medical conferences. Moreover, sales representatives from pharmaceutical companies communicate regularly with physicians to help explain the science behind the prescription drugs that they are selling. This includes the results of many clinical trials. At no time does a sales representative try to sell a proprietary mixture or talk about personal testimonials given by individuals. Moreover, pharmaceutical companies do monitor any reports of adverse side effects that may occur – even if there is no clear evidence to suggest that their prescription drug caused the adverse effect. In contrast, many dietary supplements are sold through multi-level marketing. Such supplements are usually not be available in stores (retail or wholesale). They are often sold through people who have already bought them and can give emotional and often convincing personal testimonials. Customers are encouraged and often given financial incentives to tell their friends and family members about the many alleged benefits of the supplements that they are trying to sell. Often, these customers are encouraged to talk to their friends about how they can become part of the 'family' that takes the supplements and encourages others to do so, too. The result is that many people decide to take a dietary supplement based on the recommendation of a trusted friend or family member. In contrast, the decision to give prescription drugs is often made by a physician and not a close friend. Moreover, if a patient is unconscious and in need of immediate care, an emergency medical technician or a physician in a hospital may make the decision to give the patient the prescription drugs that save his or her life. So, much of medical care is based on trust. One of the main goals of this book is to encourage people to trust science, medicine and systems thinking instead of hearsay evidence from a friend or family member who is actually trying to sell a dietary supplement.

1.4 More systems thinking is needed

Even though incredible advances have been made in implementing systems thinking into modern medicine, more is needed. This need is being addressed by many people and different organizations. To begin with, the USA's Medicare physician payment system for Part B (fee for

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service) went into effect in 2017 [250]. It is predicted to affect patient care and reform in the healthcare system for many years. It is predicted to be as significant a change as the initial creation of Medicare itself. In a rare sign of unity, the US Congress overwhelmingly passed the Medicare Access and CHIP Reauthorization Act of 2015 (MACRA). It repealed the Sustainable Growth Rate formula that had serious problems. MACRA created a new way of paying physicians who treat patients on Medicare. Instead of rewarding volume, it values results in helping patients through a Quality Payment Program. The goals are to improve the quality of patient care, along with better coordination and efficiencies. In order to minimize stress and maximize choice for already over-burdened physicians, the Centers for Medicare & Medicaid Services (CMS) established a "Pick Your Pace" path that will keep physicians from being penalized in the first year of implementing MACRA. They can choose to be paid per service under the Merit Based Incentive Program (MIPS) or move to an Advanced Alternative Payment Model (APM). The APM will give successful physicians a 5% annual bonus from 2019-2022. On the other hand, physicians who choose not to participate in either MIPs or APMs will be penalized starting with 4% in 2019 to 9% in 2022. Physicians' performances will be evaluated for quality, clinical practice improvement activities (CPIA) and their use of resources. They are encouraged to use electronic health records. The Quality Payment Plan is similar to the Medicare's previous Physician Quality Reporting System (PQRS), Value-Based Modifier, and Meaningful Use (MU) of an electronic health record. However, there are some important improvements. There is no longer an "all or nothing" approach (to which the Academy of Opthamology objected). It has been eliminated and replaced with giving partial credit for trying to participate and for meeting many of the PQRS and MU requirements by only one measure, even though they were successful in all other ways. Moreover, the new system reduces the number of quality measures that physicians must report. The CMS also decided not to punish clinicians for not fulfilling all the previous requirements on resource use and cost, since they were not relevant or fair to subspecialists. Finally, the CMS will continue to publish adjustments to the program in the future [250]. This is an excellent example of TQM and systems thinking.

Another important feature of TQM and systems thinking is interdisciplinary collaboration. For example, behavioral economics can be used to help physicians deliver quality and high-value care [251]. Behavioral economics is part of the science of human motivation, which has improved in the last 30 years. Previous economic theory suggested that rational people use accurate information to make choices that they think

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will help their well-being. So, the main economic incentives were thought to be bonuses and penalties that were linked to performance. That is, people considered the potential benefits and losses, while comparing these to the amount of effort needed to acquire a bonus or pay a penalty. However, many people make decisions that seem to be irrational and inconsistent with getting the maximum reward for the amount of effort exerted [251].

Some of this can be due to health problems, such as addiction [251]. Many people continue to smoke tobacco even though they know it is deadly. Most alcoholics know that they are going to die prematurely, but they are afraid that they might run out of alcohol before it happens. There is also inertia and the status quo. This is evident in the debates that occur in the USA over healthcare and environmental concerns. There is also choice overload. Many people are confused and find it difficult to decide which of many options are best for their health. There are many different dietary supplements and many websites and blogs, as well as non-scientific articles and books that tell people that pharmaceutical companies and government agencies are in a conspiracy to keep us sick so they can sell their deadly prescription drugs. There is also a general mistrust of science that they can't understand, as well as government agencies and the news media. There is also a problem with immediacy. This is an extremely difficult problem in the USA. It is the only country in which a major political party denies that climate change is occurring or that humans can or should do anything to stop it. There seems to be no immediate danger, while widespread misinformation questions environmental science. The dangers of Global Climate Change are often not thought to be immediate and are only occurring in distant places and to people who are not 'Americans' (as if the USA was the only country in the Americas) [251].

There is also an aversion to loss [251]. People are afraid that they will lose their jobs, their health and even their freedom. This affects resistance to meaningful changes in healthcare systems and in reducing pollution and the production of greenhouse gases that are quite deadly. There is also the issue of relative social ranking. People are often more concerned with others who look and think like them and have the same socio-economic status. This can make many people indifferent to the suffering of others who seem to be different. Such reductionist thinking ignores the fact that people who don't have health insurance increase the overall cost of healthcare by not receiving preventative care from physicians. Many people feel that they have to postpone treatment due to a fear of bankruptcy. Others have to choose between being able to feed their families or buy health insurance. They wait until their illnesses or

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symptoms are too severe to endure before going to an Emergency Room, where healthcare is very expensive. It costs much less to prevent illnesses than to cure them once they become extreme. There are also goal gradients and threshold effects. That is, people tend to work harder when they are close to achieving their goal and don't try as hard when they are far from their goal. For instance, physicians who are close to a target threshold (such as performance) will try hard to achieve their goals, while those who are far from their goals often just give up or stop trying so hard. There can also be a limit to willpower. The more that many people need to exert willpower and self-discipline to achieve one goal, the less they will tend to have sufficient willpower for other important activities. So, it can be easier and more productive for physicians to set up a prescription as a default on electronic records than to constantly remember which generic drug to prescribe every time they see a patient. Finally, mental accounting and salience can affect people. Payment of bonuses in a separate check often is more noticeable and rewarding than the same payment that is added to one's regular paycheck [251].

These principles of economic behavior have been applied successfully in the past [251]. They helped predict consumer savings behavior and how people plan for retirement planning, as well as decisions made on personal health and whether or not to buy health insurance. However, these principles have not yet been systematically used when designing physician incentive programs. It has been proposed that there are at least four ways to design these incentives. First, physicians should be informed about the use of costly interventions to diagnose and treat patients' illnesses. This is because physicians don't want to be statistical outliers or be accused of malpractice because they didn't do what most others do. This might include requiring their elderly male patients to have the concentration of their prostate specific antigen (PSA) measured in a blood test – even though it frequently produces false positives that can lead to unnecessary surgery with tragic side effects. So, physicians might be more likely to write orders for expensive procedures and stop ordering unnecessary procedures when they learn that many others are doing the same thing – with support from the scientific and medical literature [251].

Second, the principles of mental accounting, loss aversion, and relative social ranking should be applied to decisions about financial rewards [251]. For example, Advocate Physician Partners tells all the physicians in their group of practices about each other's performance. This is a network of about 5000 physicians that have a partnership with the Advocate Health Care System. They hold an annual public awards ceremony and give paper checks to deserving physicians. This is also done by the Massachusetts

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General Hospital. Third, incentive payments should be given when the money is particularly important and noteworthy (or salient). So, the Advocate Physician Partners pays incentives just before April 15, when taxes are due. Fourth, to stop or limit inertia and the limits of willpower, additional checks should be paid for select practices that are especially effective or when physicians make high quality care and improved performance their default metrics. One example is to stop depending on physicians to remember to do some important practices during office visits. Instead, nurses should review electronic health records and let patients and their physicians know about gaps in effective cancer screening tests, immunizations and care that is needed for chronic conditions [251]. This is consistent with TQM and systems thinking. In previous reductionist thinking, nurses were often afraid of the physicians for whom they worked. They seldom (if ever) questioned a physician's decision or made recommendations to a physician or a patient. In TQM and systems thinking, every member of the team, including patients, caregivers, physicians, nurses and all members of the medical profession are important. They should all feel free to express their opinions and do what they know is right without fear of retribution or being accused of insubordination.

However, there are still some important outstanding issues [251]. Too many performance metrics and complex financial incentives can be problematic. There can be a conflict of interests between having enough metrics to accurately assess the quality of healthcare provided and choice overload. More work is needed to determine the proper balance, which can vary from one group of physicians to another. Second, we must learn more about how to balance incentives for individual physicians and groups of physicians. That is, incentives that improve individual performance at the expense of the group should be avoided. So, Advocate Physician Partners provides 70% of their incentives to individuals and 30% to the performance of the entire physician group. Third, it is important that incentives to control costs don't sacrifice the patients' needs. Sometimes, costly tests and treatments are the best option. We must remember that human lives are worth more than money. Finally, the amount of incentive that is needed to improve physicians' behaviors is still not known. This needs to be studied better [251].

More importantly, the healthcare system in the USA is undergoing a change away from payment based on the number of services and patients seen to payment based on providing quality care [251]. Some systems have contracts that link incentive payments to improved quality and lower costs. Still, it is important to use systems thinking to remember that

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healthcare systems are embedded in a variety of societies. So, it will be important to test financial and non-financial incentives and combinations of infrastructure improvements across different settings, societies and the socio-economic status of the patients. Changes that may increase public recognition, feelings of accomplishment, immediate feedback and cost-effective procedures should be tested to see what works and what doesn't [251]. In the spirit of TQM and systems thinking, dogma should be avoided. Societies and people change, while basic health science and medical knowledge increase. So, healthcare systems should be flexible and constantly listen to the voice of the patients and their caregivers.

One voice that is heard across all societies is that our children, grandchildren and great grandchildren are of utmost importance. Moreover, the needs of children vary with the different communities in which they live [252]. So, evidence-based prevention of childhood illnesses is being used to improve their health and well-being. This requires strong relationships between researchers, program developers, communities, and other stakeholders. Community engagement is essential in this process. Communities should have productive partnerships with researchers and physicians. As in TQM and systems thinking, communication and feedback are crucial. At the same time, evidence-based interventions occur in real-world settings. Potential conflicts of interest between communities and researchers should be identified and resolved when possible. Even though communities often want to target select populations, researchers are also interested in publishing their results, obtaining funding, teaching students and earning approval from the communities in which they exist. Unfortunately, few people (especially patients who are not scientists) read scientific publications. Many get their information from social media, select news agencies, talk shows and by talking to friends. Individual testimonials about the efficacy of a dietary supplement or a weight loss diet are meaningless to researchers and physicians, but are extremely important to the layman. So, education and open, non-judgmental communication is essential. Even though researchers and physicians may know what is best for most people, they need to remember that every individual is different and needs to be respected – even if they are uneducated and judgmental themselves. It is also important to become aware of the important differences that can exist between the community in which a physician was born and educated and the community in which he or she serves. It is also important to include nurses in this. There is a Nurse-Family Partnership and an Invest in Kids program that provide essential links between communities and healthcare systems. It can also be important to be open-minded and flexible. Some

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communities may want to focus on bullying, while others focus on violent crime and drug abuse. Others may focus on obesity and improving diet and behavior (such as exercising). Others may focus on creating jobs and alleviating poverty. Instead of becoming defensive when a program doesn't work well in a particular setting, it's important to be flexible and understanding, so changes that are needed by that particular community are made. It is important to encourage productive, flexible programs instead of counter-productive, inflexible dogmatic ideas that can reduce and trivialize some communities [252]. Good programs can evolve and continuously improve – as in TQM. So, systems thinking and TQM are important in helping our children.

1.5 Reductionist thinking is still useful

Even though systems thinking has become extremely important, reductionist thinking still has its uses. In the early 1900s, before modern medicine and systems thinking, there were no cures for any infectious diseases [4]. Traditional remedies, grandmother's cures, and traveling salesmen were the only options for treatment. People died of diphtheria, pertussis, tetanus, Black Death, influenza, tuberculosis, Yellow Fever, typhoid fever, syphilis, rheumatic fever and cholera - all caused by Bacteria. Viruses killed and maimed people by causing smallpox, polio, measles, mumps, pertussis and other diseases. Appendicitis was frequently fatal, as doctors were unable to operate under sterile conditions. A simple puncture wound could cause death by tetanus. Men often had to marry several times, when one wife after another died giving birth to a child. Women who were lucky enough to survive the dangers of pregnancy and childbirth often had many children, in hope that one or two might survive infancy. However, many died in the first year of life. Children who did survive often suffered from common diseases [4].

The problem was made worse because food and drug production was not regulated [4]. Meat packing plants were notoriously bad [253]. Food was often contaminated and caused food poisoning. To treat the sick, anybody could sell anything to an uneducated, desperate public. If the 'snake oil' had enough alcohol, opium or cocaine in it, the patient might feel better, but the disease would usually get worse. Producers of such 'elixirs' or 'snake oils' did not have to list the ingredients on the bottle if they filed for a patent, so such things were called patent medicine. Eventually people started to realize that cocaine was bad, not good. So, in 1906, the US Congress passed the Pure Food and Drug Act, which paved the way for the formation of the FDA. In 1938, Congress passed the more comprehensive Food, Drug and Cosmetic Act. It gave the FDA the

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authority to oversee the safety of food, drugs and cosmetics [4]. In time, controls (like cGMP and GLP) were established to ensure that potential drugs are tested in a scientific manner and that approved drugs are manufactured in a clean, safe, and well-documented way (cGMP) [4].

This helped change the world, as scientists discovered antibiotics that kill Bacteria and developed vaccines to prevent viral infections and to prevent chronic inflammation, stroke, cancer and cardiovascular diseases [4]. As the 20th century progressed, agricultural revolutions occurred and many more people were fed. Many people would not be alive today if it weren't for these some of these discoveries. These successes led many to believe strongly in the power of modern western medicine and reductionist thinking [4]. So, just as quantum mechanics did not invalidate classical Newtonian physics, systems thinking does not invalidate reductionist thinking. Both are important. As will be described in the Appendix, reductionist thinking is an essential part of analytical chemistry, which is needed for cGMP, pharmacokinetics, toxicokinetic and metabolomics.

References

1. Capra F. *The Tao of Physics*. Shambala Publications, Boulder, CO, **1975**.
2. Capra F. *The Web of Life*. Anchor Books. New York, **1997**.
3. Capra F, Luisi PL. *A Systems View of Life: A Unifying Vision*. Cambridge University Press, Cambridge, UK, **2014**.
4. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 2nd ed*. Bentham Science, Sharjah, U.A.E. **2014**.
5. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 3rd ed*. Bentham Science, Sharjah, U.A.E. **2015**.
6. Smith RE, Tran K, Richards KM. Systems thinking for medicinal chemists. *Jacob's J. Med. Chem*. **2016**, *1*, 004, 24 pp.
7. Capra F. *The Hidden Connections*. Doubleday, New York, **2002**.
8. Csermely P, Korcsmáros T, Kiss HJM, London G, Nussinov R. Structure and dynamics of molecular networks: A novel paradigm of drug discovery. A comprehensive review. *Pharmacol. Therapeut.* **2013**, *138,* 333-408.
9. Smith RE, Tran K, Vocque R. Network medicine and high throughput screening. *Curr. Drug Disc. Technol*. **2013**, *10*, 182-194.
10. Yourtee D, Emery J, Smith RE, Hodgson B. Stereolithographic models of biopolymers. *J. Mol. Graph. Model*. **2000**, *18*, 26-28.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

11. Hamburg MA, Paving the Way for Personalized Medicine: FDA's Role in a New Era of Medical Product Development, US FDA, Bethesda, MD, **2013**.
12. Jambekar AB. Frameworks for integration of systems thinking with the total quality management practices. Conference Proceedings The 1994 International Systems Dynamics, Sterling, Scotland **1994**.
13. Starfield B. Is patient-centered care the same as person-focused care? *Perm J*. **2011**, *15*, 63-69.
14. Darwin C. *On the Origin of Species by Means of Natural Selection. Preservation of Favoured Races in the Struggle for Life*. John Murray. London, **1859**.
15. Dawkins R. *The Selfish Gene*. Oxford Press, Oxford, **1976**.
16. Lederman L. The God Particle, Delta; Reprint edition, McHenry, IL, **1994**.
17. The Bible, Genesis, chapter 7, verse 21.
18. Richmond B. Systems dynamics/systems thinking: let's just get on with it, in *International Systems Dynamics Conference*. Sterling, Scotland, **1994**.
19. Arnold RD, Wade JP. A definition of systems thinking: A systems approach. *ProcediaComp. Sci*. **2015**, *44*, 669 – 678.
20. Cassidy S, Thoma C, Houghton D, Trenell TI. High-intensity interval training: a review of its impact on glucose control and cardiometabolic health. *Diabetologia* **2017**, *60*, 7-23.
21. Salvucci, E. Microbiome, holobiont and the net of life. *Crit. Rev. Microbiol*. **2016**, *42*, 485–494.
22. Metcalf SS, Northridge ME, Lanster IB. A systems perspective for dental health in older adults. *Am. J. Pub. Health* **2011**, *101*, 1820-1821.
23. Chen D, Liu Z, Yang Y, Yang H, Lu P. Systematic synergy modeling: understanding drug synergy from a systems biology perspective. *BMC Systems Biol*. **2015**, *9*, DOI 10.1186/s12918-015-0202-y.
24. Astrom KJ. *Feedback Systems. An Introduction for Scientists and Engineers*. Princeton University Press, Princeton, NJ, **2009**.
25. Chialvo DR. Emergent complex neural dynamics. *Nature Phys*. **2010**, *6*, 744-748.
26. Zhigalov A, Kaplan A, Palva JM. Modulation of critical brain dynamics using closed-loop neurofeedback stimulation. *Clin. Neurophysiol*. **2016**, *127*, 2882-2889. 27. Trefois T, Antony PMA, Goncalves J, Skupin A, Balling R. Critical transitions in chronic

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

disease: transferring concepts from ecology to systems medicine. *Curr. Oipn. Biotechnol*. **2015**, *34*, 48-55.
28. Barabási A-L, Bonabeau E. Scale free networks. *Sci. Amer*. **2003**, *292 (May)*, 50-59.
29. Barabási A-L. *Linked. The New Science of Networks*. Perseus Publishing, Cambridge, Mass **2002**.
30. Ma H, Yang Y. Turritopsis nutricola. *Nature Sci*. **2010**, *8*, 15-20.
31. Smith GR. Angiotensin and systems thinking: wrapping your mind around the big picture. *Oschner J*. **2013**, *13*, 11-25.
32. Kipnis J. Multifaceted interactions between adaptive immunity and the central nervous system. *Sci*. **2016**, *353*, 766-771.
33. Marin IA, Kipnis J. Central nervous system: (immunological) ivory tower or not? *Neuropsychopharmacol. Rev*. **2017**, *42*, 28-35.
34. Walker JA, Barlow JL, McKenzie ANJ. Innate lymphoid cells – how did we miss them? *Nature Rev. Immunol*. **2013**, *13*, 75-87.
35. Cording S, Medvedovic J, Aychek T, Eberl G. Innate lymphoid cells in defense, immunopathology and immunotherapy. *Nature Immun*. **2016**, *17*, 755-757.
36. Trevaskis NL, Kaminskas LM, Porter CJH. From sewer to saviour - targeting the lymphatic system to promote drug exposure and activity. *Nature Rev. Drug Disc*. **2015**, *14*, 781-803.
37. Jilliff J, Goldman S, Nedergaard M. Implications of the discovery of brain lymphatic pathways. *Lancet* **2015**, *14*, 977-979.
38. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ et al. Structural and functional features of central nervous system lymphatic vessels. *Nature* **2015**, *523*, 337–341.
39. Solomos AC, Rall GF. Get it through your thick head: emerging principles in neuroimmunology and neurovirology redefine central nervous system “immune privilege”. *ACS Chem. Neurosci*. **2016**, *7*, 435−441.
40. Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J. Exp. Med*. **2015**, *212*, 991−9.
41. Ransohoff RM, Schafer D, Vincent A, Blachère NE, Bar-Or A. Neuroinflammation: ways in which the immune system affects the brain. *Neurotherapeut*. **2015**, *12*, 896-909.
42. Tracey KJ. Reflexes in immunity. *Cell* **2016**, *164*, 343–344.
43. Schwartz M, Kipnis J, Rivest S, Prat A. How do immune cells support and shape the brain in health, disease, and aging? *J. Neurosci*. **2013**, *33*, 17587–17596.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

44. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S et al. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res*. **2002**, *62*, 3347-3350.
45. Quinn M, Erkes DA, Snyder CM. Cytomegalovirus and immunotherapy: opportunistic pathogen, novel target for cancer and a promising vaccine vector. *Immunother*. **2016**, *8*, 211-221. 46. Ahmed T, Soare C, Fluckiger A-C, Ontsouka B, Diress A et al. IMST-31. CMV gB/pp65 eVLPs formulated with GM-CSF as a therapeutic vaccine against GBM and medulloblastoma. *Neurooncol*. **2016**, *18 (S6)*, vi93.
47. Coffey JC, Culligan K, Walsh LG, Sehgal R, Dunne C, McGrath D et al. An appraisal of the computed axial tomographic appearance of the human mesentery based on mesenteric contiguity from the duodenojejunal flexure to the mesorectal level. *Eur. Radiol*. **2016**, DOI 10.1007/s00330-015-3883-0.
48. Coffey JC, O'Leary DP. The mesentery: structure, function, and role in disease. *Lancet Gastroenterol. Hepatol*. **2016**, *1*, 238–47.
49. U.S. National Library of Medicine. The Visible Human Project®. **2015**.
https://www.nlm.nih.gov/research/visible/visible_human.html
50. Capra F. *Learning from Leonardo. Decoding the Notebooks of a Genius*. Berrett-Koehler Publishers, Oakland, CA, **2013**.
51. Furness JB, Rivera LR, Cho H-J, Bravo DM, Callaghan B. The gut as a sensory organ. *Nat. Rev. Gastroenterol. Hepatol*. **2013**, *10*, 729–740.
52. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann. Gastroent*. **2015**, *28*, 203-209.
53. Huffman MA. Self-medicative behavior in the African great apes. *Biosci*. **2001**, *51*, 651.
54. De Roode JC, Lefèvre T, Hunter MD. Self-medication in animals. Sci 2013; 340: 150-151.
55. Janzen DH, in *The Ecology of Arboreal Folivores,* G. C. Montgomerie, Ed. Smithsonian Institution Press, Washington, DC, **1978**, pp. 73–84.
56. Meltzer SM, Monk BJ, Tewari KS. Green tea catechins for treatment of external genital warts. *Am. J. Obstet. Gynecol*. **2009**, *200*, 233.e1-233.e7.
57. U.S. Department of Health and Human Services, Guidance for Industry. *E6 Good Clinical Practice: Consolidated Guidance*,

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Center for Drug Evaluation and Research (CDER), Rockville, MD, **1996**.
58. Razeto-Barry P. Autopoiesis 40 years later. A review and a reformulation. *Orig. Life Evol. Biosph*. **2012**, *42*, 543-567.
59. Libertini G, Ferrara N. Aging of perennial cells and organ parts according to the programmed aging paradigm. *Age* **2016**, *38*, Article 35, DOI 10.1007/s11357-016-9895-0.
60. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 1st ed*. Bentham Science, Sharjah, U.A.E. **2013**.
61. Jantsch E. Cycles and Hypercycles, in Zeleny, M., ed. *Autopoeisis, A Theory of Living Organizations*, North-Holland/Elsevier, New York, **1981**.
62. Lovelock J. Gaia as seen through the atmosphere. *Atmospheric Environment* **1972**, *6*, 579.
63. Lovelock, J. and Margulis, L. Biological modulation of the Earth's atmosphere. *Icarus* **1974**, *21*, 471.
64. Furusawa C, Kaneko K. A dynamical-systems view of stem cell biology. *Sci*. **2012**, *338*, 215-217.
65. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **2006**, *126*, 663–676.
66. Takebe T, Sekine K, Enomura M, Koike H, Kimura M et al. Vascularized and functional human liver from and iPSC-derived organ bud transplant. *Nature* **2013**, *499*, 481-484.
67. Robinton DA, Daley GQ. The promise of induced pluripotent stem cells in research and therapy. *Nature* **2012**, *481*, 295-305.
68. Ramalho-Santos M. Stem cells as probabilistic self-producing entities. *BioEssays* **2004**, *26*, 1013-1016.
69. Giri S, Bader A. A low cost, high-quality new drug discovery process using patient-derived induced pluripotent stem cells. *Drug Discov. Today* **2015**, *20*, 37-49.
70. Fuchs Y, Steller H. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. *Nat. Rev. Mol. Cell. Biol*. **2015**, *16*, 329–344.
71. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri V et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Diff*. **2009**, *16*, 3-11.
72. Galluzzi L, Pedro JMBS, Vitale I, Aaronson AA, Abrams JM, Adam D et al. Essential versus accessory aspects of cell death:

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

recommendations of the NCCD 2015. *Cell Death Diff*. **2015**, *22*, 58-73.
73. Yamaguchi Y, Miura M. Programmed cell death in neurodevelopment. *Dev. Cell* **2015**, *32*, 478-490.
74. Sayers EW et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res*. **2014**, *42*, D7–D17.
75. Wacker SA, Houghtaling BR, Elemento O, Kapoor TM. Using transcriptome sequencing to identify mechanisms of drug action and resistance. *Nature Chem. Biol*. **2012**, *8*, 235-237.
76. Stein RA. Systems biology digs deep, aims high. The interconnected systems behind disease processes are being unraveled by systems biology. *Gen. Eng. Biotech. News* **2016**, *36*, June 1.
77. Topalian, SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. *New Engl. J. Med*. **2012**, *366*, 2443-2454.
78. Sgambato A, Casaluce F, Sacco PC, Palazzolo G, Maione P et al. Anti PD-1 and PDL-1 immunotherapy in the treatment of advanced non-small cell lung cancer (NSCLC): A review on toxicity profile and its management. *Curr. Drug Saf*. **2016**, *11,* 62-68.
79. Pardoll D. The blockade of immune checkpoints in cancer immunotherapy. *Nature Rev. Cancer* **2012**, *12*, 252-264.
80. Kitsukawa T, Yagi T. The transfer and transformation of collective network information in gene-matched networks. *Sci. Rep*. **2015**, *5*, article 14984.
81. Lamparter D, Marbach D, Rueedi R, Kutalik Z, Bergmann S. Fast and rigorous computation of gene and pathway scores from SNP-based summary statistics. *PLoS Comp. Biol*. **2016**, *12*, article e1004714.
82. Marbach D, Lamparter D, Quon G, Kellis M, Kutalik Z et al. Tissue-specific regulatory circuits reveal variable modular perturbations across complex diseases. *Nature Methods* **2016**, *13*, 366-370.
83. Stojic L, Niemczyk M, Orjalo A, Ito Y, Ruijter AEM et al. Transcriptional silencing of long noncoding RNA GNG12-AS1 uncouples its transcriptional and product-related functions. *Nature Commun*. **2016**, *7*, 10406-10420.
84. Saben JL, Boudoures AL, Asghar Z, Thompson A et al. Maternal metabolic syndrome programs mitochondrial dysfunction via germline changes across three generations. *Cell Rep*. **2016**, *16*, 1-8.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

85. Tang Y, Cheung BB, Atmadibrata B, Marshall GM, Dinger ME et al. The regulatory role of long noncoding RNAs in cancer. *Cancer Lett*. **2017**, *391*, 12-19.
86. Cao DD, Li L, Chan W-Y. MicroRNAs: Key regulators in the central nervous system and their implication in neurological diseases. *Intl. J. Mol.Sci*. **2015**, *17*, article 842.
87. Zhao Y, Pogue AI, Lukiw WJ. MicroRNA (miRNA) signaling in the human CNS in sporadic Alzheimer's disease (AD)-novel and unique pathological features. *Intl. J. Mol. Sci*. **2015**, *16*, 30105-30116.
88. Ruiz AJ, J Russell SJ. MicroRNAs and oncolytic viruses. *Curr. Opin. Virol*. **2015**, *15*, 40-48.
89. Lawler SE, Speranza M-C, Cho C-F, Chiocca A. Oncolytic viruses in cancer treatment. A review. *J. Am. Med. Assoc*. **2017**, *3*, 841-849.
90. Hansen TB, Veno MT, Jensen TI, Schaefer A, Damgaard CK et al. Argonaute-associated short introns are a novel class of gene regulators. *Nat. Commun*. **2016**, *7*, Article 11538.
91. Chorev M, Carmel L. The function of introns. *Front. Genet*. **2012**, *3*, Article 55.
92. Sharp PA. On the origin of RNA splicing and introns. *Cell* **1985**, *42*, 397-400.
93. Molina-Sánchez MD, Nisa-Martínez R, García- Rodriguez FM, Martínez-Abarca F, Toto N. Intron Biology, Focusing on Group II Introns, the Ancestors of Spliceosomal Introns, in *Genomic Elements in Health, Disease and Evolution. Junk DNA*. Felekkis K, Voskarides K, eds. Springer, **2015**, New York.
94. Hausner G, Hafez M, Edgell DR. Bacterial group I introns: mobile RNA catalysts. *Mobile DNA* **2014**, *5*, Article 8.
95. Pennisi E. ENCODE project writes eulogy for junk DNA. *Sci*. **2012**, *337*, 1159-1161.
96. Vaidya N, Manapat ML, Chen IA, Xulvi-Brunet R, Hayden EJ et al. Spontaneous network formation among cooperative RNA replicators. *Nature* **2012**, *491*, 72-77.
97. Lai F. Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature* **2013**, *494*, 497-501.
98. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Ann. Rev. Biochem*. **2012**, *81*, 145-166.
99. Zhang R, Nicholas F. Lahens NF, Ballance HI, Hughes ME, Hogenesch JB. A circadian gene expression atlas in mammals:

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Implications for biology and medicine. *Proc. Natl. Acad. Sci*. **2014**, *111*, 16219-16224.
100. Ray S, Reddy AB. Cross-talk between circadian clocks, sleep-wake cycles, and metabolic networks: Dispelling the darkness. *Bioessays* **2016**, *38*, 394–405.
101. McClung CA. Mind your rhythms: an important role for circadian genes in neuroprotection. *J. Clin. Invest*. **2013**, *123*, 4994-4996.
102. Fitzgerald GA, Yang G, Paschos GK, Liang X, Skarke C. Molecular clocks and the human condition: approaching their characterization in human physiology and disease. *Diabetes Obes. Metab*. **2015**, *17*, 139–142.
103. Brancaccio M, Enoki R, Mazuski CN, Jones J, Evans JA et al. Network-mediated encoding of circadian time: The suprachiasmatic nucleus (SCN) from genes to neurons to circuits, and back. *J. Neurosci*, **2014**, *34*, 15192-15199.
104. Sollars PJ, Pickard GE. The neurobiology of circadian rhythms. *Psychiatr. Clin. North Am*. **2015**, *38*, 645-665.
105. Hagenauer MH, Perryman JI, Lee TM, Carskadon MA. Adolescent changes in the homeostatic and circadian regulation of sleep. *Dev. Neurosci*. **2009**, *31*, 276–284.
106. Kurien PA, Chong SYC, Ptáček LJ, Fu Y-H. Sick and tired: How molecular regulators of human sleep schedules and duration impact immune function. *Curr. Opin. Neurobiol*. **2013**, *23*, 873–879.
107. Zarrinpar A, Chaix A, Panda S. Daily eating patterns and their impact on health and disease. *Trend. Endocrin. Metab*. **2016**, *27*, 69-83.
108. Eckel-Mahan KL, Patel VR, de Mateo S, Orozco-Solis R, Ceglia NJ et al. Reprogramming of the circadian clock by nutritional challenge. *Cell* **2013**, *155*, 1464–1478.
109. Singer T, McConnel MJ, Marchetto MCN, Coufal NG, Gage FH. LINE-1 retrotransposons: mediators of somatic variation in neuronal genomes? *Trends Neurosci*. **2010**, *33*, 345-354.
110. Lee G, Isco R, Yang L, Gokcumen O, Haseley P et al. Landscape of somatic retrotransposition in human cancers. *Sci*. **2012**, *337*, 967-971.
111. Bennett EA, Keller H, Mills RE, Schmist S, Moran JV, Weichenrieder O, Devine SE. Active Alu retrotransposons in the human genome. *Genome Res*. **2008**, *18*, 1875-1883.
112. De Laat W, Duboule D. Topology of mammalian developmental enhancers and their regulatory landscapes. *Nature*, **2013**, *502*, 499-506.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

113. Gage FH, Muotri AR. What makes each brain unique. *Sci. Amer*. **2012**, *306 (5)*, 26-31.
114. Löwer R, Löwer J, Kurth R. The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. *Proc. Natl. Acad. Sci*. **1996**, *93*, 5177-5184.
115. Baille JK, Barnett MW, Upton KR, Gerhardt DJ, Richmond TA et al. *Trends Neurosci*. **2010**, *33*, 345-354.
116. Campioni MR, Finkbeiner S. Going retro: ancient viral origins of cognition. *Neuron* **2015**, *86*, 346-348.
117. Babatz TD, Burns KH. Functional impact of the human mobilome. *Curr. Opinion Genet. Develop*. **2013**, *23*, 264-270.
118. Grabundjiza I, Wang J, Sebe A, Erdai Z, Kajdi R et al. *Sleeping Beauty* transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. *Nucl. Acid Res*. **2013**, *41*, 1829-1847.
119. Jaciet P-A, Colson P, Lopez P, Bapteste E. Extensive gene remodeling in the viral world: new evidence for nongradual evolution in the mobileome network. *Genome Biol. Evol*. **2014**, *6*, 2195-2205.
120. Forterre P, Prnagishvili D. The major role of viruses in cellular evolution: facts and hypotheses. *Curr. Opinion Virol*. **2013**, *3*, 558-565.
121. Bheda P, Wolberger C. Sirtuin on a high-fat diet. *Nature* **2013**, *496*, 41-42.
122. Jiang H, Khan S, Wang Y, Charron G, He B et al. SIRT6 regulates TNF-α secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* **2013**, *496*, 110-113.
123. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: A new frontier for drug discovery. *Nat. Rev. Drug Disc*. **2012**, *11*, 384-400.
124. Hofeldt JW, Agger K, Helin K. Histone lysine demethylases as targets for anticancer therapy. *Nature Rev. Drug Disc*. **2013**, *12*, 917-930.
125. Suvà ML, Riggi N, Bernstein BE. Epigenetic reprogramming in cancer. *Sci*. **2013**, *339*, 1567-1571.
126. Avitabile D, Magenta A, Lauri A, Gambini E, Spaltro G. Metaboloepigenetics: The emerging network in stem cell homeostasis regulation. *Curr. Stem Cell Res. Ther*, **2016**, *11*, 6 pp.
127. The UniProt Consortium. The Universal Protein Resource (UniProt) in 2010. *Nucleic Acids Res*. **2010**, *38*, D142–D148.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

128. Minguez P, Letunic I, Parca L, Bork P. PTMcode: a database of known and predicted functional associations between post-translational modifications in proteins. *Nucl. Acid Res*. **2013**, *41*, D306-D311.
129. Schaffer BE, Levin RS, Hertz NT, Maures TJ, Schoof ML et al. Identification of AMPK phosphorylation sites reveals a network of proteins involved in cell invasion and facilitates large-scale substrate prediction. *Cell Metab*. **2015**, *22*, 907–921.
130. Minguez P, Parca L, Diella F, Mende DR, Kumar R et al. Deciphering a global network of functionally associated post-translational modifications. *Mol. System Biol*. **2013**, *8*, 1-14.
131. IUPS Physiome Project, website: http://www.physiome.org.nz/
132. Joubert BR, den Dekker HT, Felix JF, Bohlin J, Ligthart S. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nature Commun*. **2016**, *7*, article 10577.
133. Kellermann NPF. Epigenetic transgenerational transmission of Holocaust trauma: A review. DOI: 10.13140/RG.2.1.4960.7128.
134. Kofink D, Boks MPM, Timmers HTM, Kasa MJ. Epigenetic dynamics in psychiatric disorders: Environmental programming of neurodevelopmental processes. *Neurosci. Gehav. Rev*. **2013**, *37*, 831-845.
135. Kharroubi W, Haj Ahmed SH, Nury T, Andreoletti P, Haouas Z. Evidence of hormesis on human neuronal SK-N-BE cells treated with sodium arsenate: impact at the mitochondrial level. *Environ. Sci. Pollut. Res*. **2016**, *23*, 8441–8452.
136. Sun H-J, Rathinasabapathi B, Wu B, Jun Luo J, Pu L-P et al. Arsenic and selenium toxicity and their interactive effects in humans. *Env. Int*. **2014**, *69*, 148-158.
137. Miller WH, Schipper HM, Lee JS, Singer J, Waxman S. Mechanisms of action of arsenic trioxide. *Cancer Res*. **2002**, *62*, 3893–903.
138. Llabjani V, Hoti V, Pouran HM, Martin FL, Zhang H.Bimodal responses of cells to trace elements: Insights into their mechanism of action using a biospectroscopy approach. *Chemosphere* **2014**, *112*, 377-384.
139. Gould E. Childhood lead poisoning: conservative estimates of the social and economic benefits of lead hazard control. *Environ. Health Perspect*. **2009**, *117*, 1162–1167.
140. Calabrese V, Cornelius C, Dinkova-Kostova AT, Iavicoli I, Di Paola R. Cellular stress responses, hormetic phytochemicals and

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

vitagenes in aging and longevity. *Biochim. Biophys. Acta* **2012**, *1822*, 753-783.
141. Gradari S, Pallé A, McGreevy KR, Fontán-Lozano A, Trejo JL. Can exercise make you smarter, happier, and have more neurons? A hormetic perspective. *Front. Neurosci*. **2016**, *10* Article 93.
142. Tsai S-F, Chen P-C, Calkins MJ, Wu S-Y, and Yu-Min Kuo Y-M. Exercise counteracts aging-related memory impairment: A potential role for the astrocytic metabolic shuttle. *Front. Aging Neurosci*. **2016**, *8*, article 57.
143. Maddock RJ, Casazza GA, Fernandez DH, Maddock MI. Acute modulation of cortical glutamate and GABA content by physical activity. *J. Neurosci*. **2016**, *36*, 2449 –2457.
144. Tang FR, Loke WK. Molecular mechanisms of low dose ionizing radiation-induced hormesis, adaptive responses, radioresistance, bystander effects, and genomic instability. *Intl. J. Radiation Biol*. **2015**, *91*, 13-27.
145. Bernal AJ, Dolinoy DC, Huang D, Skaar DA, Weinhouse C, Jirtle RL. Adaptive radiation-induced epigenetic alterations mitigated by antioxidants. *FASEB J*. **2013**, *27*, 665-671.
146. NIH National Center for Complementary and Integrative Health. Homeopathy. **2016**, https://nccih.nih.gov/health/homeopathy
147. NIH National Institute of Allergy and Infectious Diseases. NIH-Sponsored Expert Panel Issues Clinical Guidelines to Prevent Peanut Allergy. **2016**, https://www.niaid.nih.gov/news-events/nih-sponsored-expert-panel-issues-clinical-guidelines-prevent-peanut-allergy
148. Stein RA. Epigenetics research reveals a range of clinical possibilities. *Genet. Eng. Biotechnol. News* **2016**, *36(2)*, http://www.genengnews.com/gen-articles/epigenetics-research-reveals-a-range-of-clinical-possibilities/5650/
149. Razvi E, Oosta G. Epigenetics market landscape: A qualitative and quantitative picture. *Genet. Eng. Biotechnol. News* **2016**, June 20.
150. DePalma A. Progress toward therapeutic epigenetics. *Genet. Eng. Biotechnol. News* **2016**, *36*, http://www.genengnews.com/gen-articles/progress-toward-therapeutic-epigenetics/5664/176
151. Baker M. The ‘omes puzzle. *Nature* **2013**, *494*, 416-419.
152. Fahrmann JF, Grapov D, Wanichthanarak K, De Felice BC, Salemi MR et al. Integrated metabolomics and proteomics highlight altered nicotinamide and polyamine pathways in lung adenocarcinoma. *Carcinogenesis*, **2017**, *38*, 271-280.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

153. Wishart DS. Emerging applications of metabolomics in drug discovery and precision medicine. *Nature Rev. Drug Disc*. **2016**, *15*, 473-484.
154. Luisi PL. *The Emergence of Life: From Chemical Origins to Synthetic Biology*. Cambridge University Press, Cambridge, UK, **2016**.
155. Everett JR. Pharmacometabonomics in humans: a new tool for personalized medicine. *Pharmacogenomics* **2015**, *16*, 737–754.
156. Collins FS, Varmus H. A new initiative on precision medicine. *N. Eng. J. Med*. **2015**, *372*, 793-795.
157. NIH, Precision Medicine Cohort Initiative Program, **2016**. https://www.nih.gov/precision-medicine-initiative-cohort-program
158. van der Greef J, Hankemeier Y, McBurney RN. Metabolomics-based systems biology and personalized medicine; moving towards n=1 clinical trials? *Pharmacogenomics* **2006**, *7*, 1087-1094.
159. Bortz WM. Metabolism-Schrodinger the Sixth Physical Field. *Metabolomics* **2016**, *6*, 169. doi:10.4172/2153-0769.1000169
160. Topol EJ. Individualized medicine from prewomb to tomb. *Cell* **2014**, *157*, 241-253.
161. McConnell MJ, Lindberg MR, Brennand KJ et al. Mosaic copy number variation in human neurons. *Sci*. **2013**, *342*, 632-637.
162. Schadt EE, Friend SH, Shaywitz DA. A network view of disease and compound screening. *Nature Rev. Drug Disc*. **2009**, *8*, 286-295.
163. Hieronymus H, Lamb J, Ross KN, Peng XP, Price ND et al. Gene expression signature based chemical genomic prediction identifies a novel class of HSP90 pathway modulators. *Cancer Cell* **2006**, *10*, 321–330.
164. Peng S, Lin W, Guo Z, Huang N. Identifying multiple-target ligands *via* computational chemogenomics approaches. *Curr. Top. Med. Chem*. **2012**, *12*, 1363-1375.
165. Durrant JD, Amaro RE, Xie L, Urbaniak MD, Ferguson MAJ et al. A multidimensional strategy to detect polypharmacological targets in the absence of structural and sequence homology. *PLoS Comp. Biol*. **2010**, *6*, Article e1000648.
166. Komurov K, White M. Revealing static and dynamic modular architecture of the eukaryotic protein interaction network. *Mol. Sys. Biol*. **2007**, *3*, article number 110, 11 pp.
167. Wang Z, Liu J, Yu Y, Chen Y, Wang Y. Molecular pharmacology: the next paradigm in drug discovery. *Exp. Opin. Drug Disc*. **2012**, *7*, 667-677.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

168. Poil S-S, Hardstone R, Mansvelder HD, Linkenkaer-Hansen K. Critical-state dynamics of avalanches and oscillations jointly emerge from balanced excitation/inhibition in neuronal networks. *J. Neurosci*. **2012**, *32*, 9817-9823.
169. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* **2012**, *490*, 61-70.
170. Zhang H, Wheeler W, Hyland PL, Yang Y, Shi J et al. A powerful procedure for pathway-based meta-analysis using summary statistics identifies 43 pathways associated with type II diabetes in European populations. *PLoS Genet*. **2016**, *12 (6)*, Article e1006122.
171. Özdemir V, Dove ES, Gursoy UK, Sardas S, Yildirim A. Personalized medicine beyond genomics: alternative futures in big data - proteomics, environtome and the social proteome. *J. Neural Transm*. **2015**, *124*, 25-32.
172. Popgeorgiev N, Temmam S, Raoult D, Desnues C. Describing the silent human virome with an emphasis on giant viruses. *Intervirol*. **2013**, *56*, 395-412.
173. Wang X, Zhang A, Sun H, Han Y, Yan G. Discovery and development of innovative drug from traditional medicine by integrated chinmedomics strategies in the post-genomic era. *Trend. Anal. Chem*. **2016**, *76*, 86-94.
174. Rojdestvenski I. Metabolic pathways in three dimensions. *Bioinform*. **2003**, *19*, 2436-2441.
175. Sas KM, Karnovsky A, Michailidis G, Pennathur S. Metabolomics and diabetes: Analytical and computational approaches. *Diabetes* **2015**, *64*, 718-732.
176. Flores M, Glusman G, Brogaard K, Price ND, Hood L. P4 medicine: how systems medicine will transform the healthcare sector and society. *Per. Med*. **2013**, *10*, 565-576.
177. Hamburg MA, Paving the Way for Personalized Medicine: FDA's Role in a New Era of Medical Product Development, US FDA, Bethesda, MD, **2013**.
178. Jubb H, Blundell TL, Ascher DB. Flexibility and small pockets at protein-protein interfaces: New insights into druggability. *Progr. Biophys. Mol. Biol*. **2015**, *119*, 2-9.
179. Woodcock J, Woosley R. The FDA critical path initiative and its influence on new drug development, *Ann. Rev. Med*. **2008**, *59*, 1-12.
180. FDA, Critical Path 2010 Update. http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/ucm204289.htm

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

181. FDA, Critical Path Initiative. http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/default.htm
182. FDA, Advancing Regulatory Science Initiative. http://www.fda.gov/ScienceResearch/SpecialTopics/RegulatoryScience/
183. FDA, Strategic Plan for Regulatory Science. http://www.fda.gov/ScienceResearch/SpecialTopics/RegulatoryScience/ucm267719.htm
184. Ramey SL, Schafer P, DeClerque JL, Lanzi RG, Hobel C et al. The Community Child Health Network. The preconception stress and resiliency pathways model: A multi-level framework on maternal, paternal, and child health. Disparities Derived by Community-Based participatory research. *Matern. Child Health J*. **2014**, *19*, 707-19.
185. National Cancer Institute, Office of Cancer Genomics, http://ocg.cancer.gov/
186. Adeleye Y, Melvin Andersen M, Clewell R, Davies M, Dent M et al. Implementing toxicity testing in the 21st Century (TT21C): Making safety decisions using toxicity pathways, and progress in aprototype risk assessment. *Toxicol*. **2015**, *332*, 102-111.
187. Bale SS, Vernetti L, Senutovitch N, Jindal R, Hegde M. In vitro platforms for evaluating liver toxicity. *Exp. Biol. Med*. **2014**, *239*, 1180–1191.
188. 3-D Printed Lifelike Liver Tissue for Drug Screening. http://www.technologynetworks.com/HTS/news.aspx?ID=188213
189. Berkeley Wellness. Supplement Claims: What's Allowed. http://www.berkeleywellness.com/supplements/other-supplements/article/supplement-claims-whats-allowed
190. Schulz MMP, Reisen F, Zgraggen S, Fischer S, Yuen D et al. Phenotype-based high-content chemical library screening identifies statins as inhibitors of in vivo lymphangiogenesis. *Proc. Natl. Acad. Sci*. **2013**, *40*, E2665-E2674.
191. Roelen CCJ, van Riel A, de Vries I. Serious health problems after use of a dietary supplement for weight-loss and sports enhancement. *Clin. Toxicol*. **2014**, *52*, 78–79.
192. SigmaAldrich LOPAC® Library of Pharmacologically Active Compounds. http://www.sigmaaldrich.com/catalog/product/sigma/sc001?lang=en&region=US

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

193. Liao JK, Laufs U. Pleiotropic effects of statins. *Ann. Rev. Pharmacol. Toxicol*. **2005**, *45*, 89–118.
194. Collins R, Reith C, Emberson J, Armitage J, Baigent C et al. Interpretation of the evidence for the efficacy and safety of statin therapy. *Lancet* **2016**, *388*, 19-25.
195. Kalaivani M, Sabitha R, Kalaiselvan V, Rajasekaran A. Health benefits and clinical impact of major nutrient, red yeast rice: A review. *Food Bioprocess. Technol*. **2010**, *3*, 333–339.
196. Huang H-N, Hua Y-Y, Bao G-R, Xie L-H. The quantification of monacolin K in some red yeast rice from Fujian province and the comparison of the other product. *Chem. Pharm. Bull*. **2006**, *54*, 687—689.
197. Clarke R, Newman C, Tomson J, Hin H, Kurien R. Estimation of the optimum dose of vitamin D for disease prevention in older people: Rationale, design and baseline characteristics of the BEST-D trial. *Maturitas* **2015**, *80*, 426–431.
198. Musson P, Collin J. Management of vitamin D deficiency in childhood and adolescence. *Nurs. Child. Young People* **2015**, *27*, 27-35.
199. Bordelon P, Ghetu MV, Langan R. Recognition and management of vitamin D deficiency. *Am. Fam. Physician*. **2009**, *80*, 841-846.
200. Bacchetta J, Pelletier S, Jean G, Fouque D. Immune, metabolic and epidemiological aspects of vitamin D in chronic kidney disease and transplant patients. *Clin. Biochem*. **2014**, *47*, 509-515.
201. Tran K, Richards KM, Smith RE. Prescription drugs and dietary supplements for weight loss. *Adv. Obes. Weight Manag. Control* **2015**, *3*, 00045.
202. Williamson DF, Vinicor F, Bowman BA. Primary prevention of type 2 diabetes mellitus by lifestyle intervention: implications for health policy. *Ann. Intern. Med*. **2004**, *140*, 951-7.
203. US Food and Drug Administration. Safety: recalls, market withdrawals, & safety alerts, **2015**. http://www.fda.gov/Safety/Recalls/ucm165546.htm.
204. US Food and Drug Administration. Tainted_supplements_CDER. http://www.accessdata.fda.gov/scripts/sda/sdnavigation.cfm?sd=tainted_supplements_cder
205. Roelen CCJ, van Riel A, de Vries I. Serious health problems after use of a dietarysupplement for weight-loss and sports enhancement. *Clin. Toxicol*. **2014**, *52*, 78–79.
206. Reeuwijk N, Venhuis BJ, de Kaste D, Hoogenboom RLAP, Ivonne MCM et al. Active pharmaceutical ingredients detected in herbal

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food supplements for weight loss sampled on the Dutch market. *Food Add. Contam*. A **2014**, *31*, 1783-1793.
207. Molinari M, Watt, KDS, Kruszyna T, Nelson R, Huang W-Y et al. Acute liver failure induced by green tea extracts: Case report and review of the literature. *Liver Transpl*. **2006**, *12*, 1892-1895.
208. Khazan M, Hedayati M, Askari S, Azizi F. Adulteration of products sold as Chinese herbal medicines for weight loss with thyroid hormones and PCP. *J. Herbal Med*. **2013**, 3, 39-43.
209. Cohen PA. Hazards of hindsight - monitoring the safety of nutritional supplements. *New Eng. J. Med*. **2014**, *370*, 1277-1281.
210. Stickel F, Shouval D. Hepatotoxicity of herbal and dietary supplements: an update. *Arch. Toxicol*. **2015**, *89*, 851–865.
211. Araujo JL, Worman HJ. Acute liver injury associated with a newer formulation of the herbal weight loss supplement Hydroxycut. *BMJ Case Reports* **2015**, *2015*, doi:10.1136/bcr-2015-210303
212. Choi JY, Heo S, Yoo GJ, Park S-K, Yoon C-Y et al. Development and validation of an LC-MS/MS method for the simultaneous analysis of 28 specific narcotic adulterants used in dietary supplements. *Food Addit. Contam*. **2015**, *32*, 1029-1039.
213. Lucas D, Neal-Kababick J, Zweigenbaum. Characterization and quantitation of yohimbine and its analogs in botanicals and dietary supplements using LC/QTOF-MS and LC/QQQ-MS for determination of the presence of bark extract and yohimbine adulteration. *J. AOAC Intl*. **2015**, *98*, 330-336.
214. Rossato L, Costa VM, Limberger RP, Bastos ML, Remião F. Synephrine: From trace concentrations to massive consumption in weight-loss. *Food Chem. Toxicol*. **2011**, *49*, 8–16.
215. Schöntal AH. Adverse effects of green tea extracts. *Mol. Nutr. Food Res*. **2011**, *55*, 874-885.
216. Wang D, Wang Y, Wan X, Yang CS, Zhang Z. Green tea polyphenol (−)-epigallocatechin-3-gallate triggered hepatotoxicity in mice: Responses of major antioxidant enzymes and the Nrf2 rescue pathway. *Toxicol. Appl. Pharmacol*. **2015**, *283*, 65-74.
217. Mazzanti G, Menniti-Ippolito F, Moro PA, Cassetti F, Raschetti R et al. Hepatotoxicity from green tea: a review of the literature and two unpublished cases. *Eur. J. Clin.Pharmacol*. **2009**, *65*, 331–341.
218. Mazzanti G, Di Sotto A, Vitalone A. Hepatotoxicity of green tea: an update. *Arch. Toxicol*. **2015**, *89*, 1175-1191.
219. Sarma DN, Barrett ML, Chavez ML, Gardiner P, Ko R et al. Safety of green tea extracts. A systematic review by the US Pharmacopeia. *Drug Safety* **2008**, *31*, 469-484.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

220. Pisters KMW, Newman RA, Coldman B, Shin DM, Khuri FR et al. Phase I trial of oral green tea extract in adult patients with solid tumors. *J. Clin. Oncol*. **2001,** *19*, 1830-1838.
221. USP. Powdered decaffeinated green tea extract. USP Monograph, **2015**.
222. Jankun J, Selman SH, Swiercz R, Skrzypczak-Jankun E. Why drinking green tea could prevent cancer. *Nature* **1997**, *387*, 561.
223. Ascherio A, Zhang SM, Hermán MA, Kawachi I, Colditz GA et al. Prospective study of caffeine consumption and risk of Parkinson's diseasein men and women. *Ann. Neurol*. **2001**, *50*, 56-63.
224. Reissig CJ, Strain EC, Griffiths RR. Caffeinated energy drinks - A growing problem. *Drug Alcohol Depend*. **2009**, *99*, 1-10.
225. Chen J-F, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets - what are the challenges? *Nature Rev. Drug Disc*. **2013**, *12*, 265-286.
226. Fujiki H, Sueoka E, Watanabe T, Suganuma M. Primary cancer prevention by green tea, and tertiary cancer prevention by the combination of green tea catechins and anticancer compounds. *J. Cancer Prev*. **2015**, *20*, 1-4.
227. Schneider C, Segre T. Green tea: Potential health benefits. *Am. Fam. Physician* **2009**, *79*, 591-594.
228. Eaton DL, Gilbert SG. *Principles of Toxicology, in Casarett & Doull's Toxicology: The Basic Science of Poisons, 8th edn*. Klaasen CD, ed. McGraw-Hill, New York, **2013**.
229. Qu Z, Meng F, Zhou H, Li J, Wang Q et al. NitroDIGE analysis reveals inhibition of protein S-nitrosylation by epigallocatechin gallates in lipopolysaccharide-stimulated microglial cells. *J. Neuroinflammation*. **2014**, *11*, 2447-2655.
230. Li Y, Go VLW, Sarkar FH. The role of nutraceuticals in pancreatic cancer prevention and therapy: Targeting cellular signaling, microRNAs, and epigenome. *Pancreas* **2015**, 44, 1-10.
231. Cohen PA. American roulette – Contaminated dietary supplements. *New Eng. J. Med*. **2009**, *361*, 1523-1525.
232. Cohen PA, Maller G, DeSouza R, Neal-Kababick J. Presence of banned drugs in dietary supplements following FDA recalls. *J. Am. Med. Assoc*. **2014**, *312*, 1691-1693.
233. Tatem AJ, Ordanovich D, Falkner M, Huang Z, Howes R et al. The geography of imported malaria to non-endemic countries: a meta-analysis of nationally reported statistics. *Lancet Infect. Dis*. **2017**, *17*, 98–107.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

234. World Health Organization. Fact sheet: world malaria report 2016. **2016**. http://www.who.int/malaria/media/world-malaria-report-2016/en/
235. Wells TNC, Willis P, Burrows JN, van Huijsduijnen RH. Open data in drug discovery and development: lessons from malaria. *Nature Rev. Drug Disc*. **2016**, *15*, 661-662.
236. Kamal A, Nekkanti S, Shankaraiah N, Sathish M. Future of drug discovery, in *Drug Resistance in Bacteria, Fungi, Malaria, and Cancer*, Arora G, Andaleeb S, Chandra V, eds. Springer International Publishing, Cham, Switzerland, **2017**.
237. Litterman NK, Rhee M, Swinney DC, Sean Ekins S. Collaboration for rare disease drug discovery research. *F1000 Res*. **2014**, *3*, Article 261, 15 pp.
238. Collaborative Drug Discovery, CCD, **2017**. https://www.collaborativedrug.com/
239. SERMO. Sermo is the #1 social network for doctors in the US and now globally, **2017**. http://www.sermo.com/
240. Doximity. The Medical Network, **2017**. https://www.doximity.com/
241. Donovan J, Zucker C. In a Different Key: *The Story of Autism*. New York: Crown Publishers, **2016**.
242. Sugarman SD. Cases in vaccine court - legal battles over vaccines and autism. *N. Engl. J. Med*. **2007**, *357*, 1275-1276.
243. Wakefield A. Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. *Lancet* **1998**, *251*, 637-641.
244. Exposed: Andrew Wakefield and the MMR-autism fraud (http://briandeer.com/mmr/lancet-summary.htm)
245. Vianna N. *Acetogenins: A New Vision Against Cancer. DIGITAL; FIRST EDITION REVISION 1*, **2015**.
246. Caparros-Lefebvre D, Elbaz A. Possible relation of atypical parkinsonism in the French West Indies with consumption of tropical plants: a case-control study. *Lancet* **1999**, *354*, 281-286.
247. Badrie N, Schauss AG. Soursop (*Annona muricata* L.): composition, nutritional value, medicinal uses, and toxicology, in *Bioactive Foods in Promoting Health. Fruits and Vegetables*. Watson, RR, Preddy VR, eds. Oxford: Academic Press, **2009**, pp. 621-643.
248. Escobar-Khondiker M, Höllerhage M, Muriel M-P, Champy P, Bach A, Depienne C, et al. Annonacin, a natural mitochondrial complex I inhibitor, causes tau pathology in cultured neurons. *J. Neurosci*. **2007**, *27*, 7827–7837.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

249. Höllerhage M, Rösler TW, Berjas M, Luo R, Tran K et al. Neurotoxicity of dietary supplements from Annonaceae Species. *Int. J. Toxicol*. **2015**, *34*, 543-550.
250. Cohen CG, Repka MX, Hancock R. Medicare's new quality payment program for physicians. *Opthamol*. **2017**, *124*, 7-8.
251. Emanuel EJ, Ubel PA, Kessler JB, Meyer G, Muller RW et al. Using behavioral economics to design physician incentives that deliver high-value care. *Ann. Int. Med*. **2016**, *164*, 114-119.
252. Olson S. *Implementing Evidence-Based Prevention by Communities to Promote Cognitive, Affective, and Behavioral Health in Children: Proceedings of a Workshop*. The National Academies Press, Washington, D.C. **2017**.
253. Sinclair U. *The Jungle*. Doubleday, New York, **1906**.

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CHAPTER TWO

THE DEEP ECOLOGY OF THE HUMAN BIOLOGY

2.1 The human ecosystem, holobionts, hologenome and viruses

The human body is an ecosystem, consisting of not just human cells, but also viruses, Bacteria, Archaea and Eukarya (protozoa, yeasts, fungi and worms). The ecosystem that comprises the gut microbiome is nested within it. The gut microbiome can be thought of as an organ in the neuroendocrine immune system. The roles it plays in various diseases are essential parts of the pillars of P4 medicine – prediction, prevention, personalized and participatory activities. The goal is for researchers to be able to predict which group of people (cohort) is most likely to benefit from treatment with new drugs in clinical trials – based in part on the composition of their microbiomes. Modern methods for sequencing DNA enable microbiologists to determine the genetic composition of microbes in environmental samples and fecal material. It has led to the discovery of many previously unknown Bacteria in ocean waters and a better understanding of the human gut microbiome. Prior to the introduction of these sequencing methods, only the Bacteria that could be easily cultured *in vitro* (like *E. coli*) were identified. Now, the genes in the entire fecal microbiome can be identified by shotgun sequencing. It is hoped that physicians will use the results of metagenomics analyses to let patients know how to modify their diets to correct dysbiosis (an imbalance) in their microbiomes and discuss the possible risks and benefits of antibiotic therapy. The advice will be personalized to fit the overall needs of the patient. It will be participatory so patients and their caregivers will be actively involved in the changes to diet, therapies and/or behavior that will be needed to either maintain good health, or correct any health problems that emerge.

Systems thinking was used to describe a deep ecology, in which humans are viewed as just one of many equal parts of the global ecosystem [1]. This can be expanded by modern medicine to define a deep ecology in the human body. That is, human eukaryotic cells are just one of

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the many equally important types of cells in the body and provide only a small portion of the DNA in the human body. Viruses and Bacteria provide much more DNA than the eukaryotic cells and have essential roles in the human body, which is an ecosystem in itself. Like all plants and animals, we are just a small part of Gaia, which is mostly a viral and bacterial world [2]. Plants, animals, microbes and viruses are nested within communities of other organisms that, in turn, coexist in and influence successively larger communities.

Biologists have understood for quite some time that microbes are extremely important in pathogenesis and recycling in ecosystems. The germ theory of disease stated that many diseases were caused by germs that became known as microorganisms or Bacteria [3]. Reductionist thinking led scientists in the 20th century to believe that every organism contained its own genome (one genome-one organism). However, we now know that microbial symbiosis is an essential part of evolution, development and physiology [3]. Symbiosis is the close association of organisms. It can be either beneficial to just one organism (commensalism), both organisms (mutualism) or harmful to one (parasitism). Note that symbiosis should not be confused with synbiotics, which is a mixture of prebiotic and probiotic Bacteria. In any case, the many diverse interactions between trillions of microbes and the human host that exists today are the result of a long evolutionary history. This is supported by DNA sequencing [2]. In the early 1970s, phylogenic relationships were discovered based on similarities in the DNA base sequence [4]. Biologists soon realized that all organisms share a deep evolutionary history that was described by the metaphor called the tree of life [2, 4].

However, the rhizome of life is probably a better metaphor than the tree of life [5]. That is, the DNA in all species is actually a mosaic of gene sequences with a variety of origins. Genomes are collections of genes with different evolutionary histories that are not well-represented by a single tree of life. At the same time, many genes have several different origins due to recombination. Bacteria and Archaea routinely transfer genes laterally from one species to another. Pieces of viral DNA and RNA may be the sources of retrotransposons that make our human brains much different than that of other primates [6, 7]. So, we are changing the way we think about the origins of life and its diversity.

We now know that there is a strong interdependence between complex multicellular organisms and their associated microbes [2, 8]. Most life forms share about one-third of their genes, many of which encode enzymes that are part of central metabolic pathways [2]. In addition, many animal genes are homologs of bacterial genes. Most were derived by

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descent, but there was also gene transfer from Bacteria, Archaea and even other Eukarya [2, 9]. That is, about 37% of the approximately 23 000 protein-coding genes in human eukaryotic cells have homologs in Bacteria and Archaea, while another 28% originated in unicellular eukaryotes. Some of these homologous genes code for proteins that enable signaling between animals and Bacteria. At the same time, Bacteria communicate with each other to form biofilms and plaques while balancing their abundance by quorum sensing.

Moreover, a host can expand its metabolism by using part of the vast collection of bacterial genes within it. Bacteria in the gut can metabolize complex carbohydrates to produce short chain fatty acids (SCFAs), such as acetic and butyric acids. SCFAs affect the amount of abdominal fat that is in the host, as well as the concentrations of hormones that help control the appetite. So, the gut microbiome responds to the diet of the host over both daily and evolutionary time scales. This gives the host enough flexibility to digest many more biomolecules and cope with changes in their diet. For example, the gut microbiome of people who consume the typical American fast food diet is quite different than that of people who don't consume so much saturated and *trans* fats or simple sugars [2].

When we think of ourselves as ecosystems, new insights and terminology emerge. We can be thought of as "super organisms" or holobionts with a hologenome [10]. A holobiont is the whole organism, with not just human cells, but also with a much larger collection of viruses and microorganisms. The hologenome is the sum of all the genetic information of the host, its viruses and all its microorganisms [10]. The skin, mouth, mucosal surfaces, anus, genitals, lungs and even human milk contain their own microbiota [11-16]. There is also much data that show that maternal transmission of some microbes occurs *in utero*, which contradicts the former sterile-womb paradigm [15]. Even though viruses are usually thought of as being bad for human health, those that infect Bacteria (bacteriophages) help control the populations of various species of potentially harmful Bacteria. This is reflected in the decision by the U.S. Department of Agriculture to approve the use of a combination of six bacteriophages to eliminate *Listeria* in food products. Moreover, viral infections can alter human immunity in subtle ways and have an indelible impact on the immune network [16]. So, the holistic view of health that comes from systems thinking must include the microbiome.

Viruses and remnants from ancient viral genomes may be even more important than Bacteria in making us human [10-29]. By applying graph theory to inclusive similarity networks from data on over 3000 viral genomes, it was shown that there is a large, complex network of mobile

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genetic elements (mobilome) that remodels genetic material and moves base sequences throughout different kinds of organisms in the biosphere [22]. The vast majority of these mobile genetic elements are transposons and retrotransposons that were probably derived from DNA and RNA viruses, respectively [22, 23]. They appear to be especially important in human embryology, neurology and carcinogenesis [22-29]. It is quite likely that there are ancient viral origins of cognition [25]. One transposon called *Sleeping Beauty* is not only important in human embryology but can be used to reprogram cells and do targeted insertions into induced pluripotent stem cells (iPSCs) [27]. Moreover, viruses may have been extremely important in evolution [24, 25]. They enable non-gradual or punctuated evolutionary changes [14]. Moreover, horizontal gene transfer from viruses and Bacteria to human cells is just as significant as vertical gene transfer from parents to children. So, viruses and Bacteria in the environment had and continue to have important effects on the human body. We are part of the environment – not separate from it.

2.2 The widespread existence of microbes in the human body

Systems thinking was used by many organizations when they collaborated in the Human Microbiome Project, or HMP, as well as the European MetaHIT and the Eldermet Project [15, 30-32]. The goals of the HMP were to sample, determine and quantify all human-associated microbial life. The European MetaHIT is determining the metagenomics of the human intestinal tract. The Eldermet Project is defining the microbial composition that is associated with aging. These three projects found that human-associated microbiota contains at least 40 000 bacterial strains in 1800 genera [31]. They contain at least 9.9 million non-human genes and about 500 times the number of protein-coding genes than human eukaryotic cells. Moreover, the approximately 100 trillion bacterial cells in the GIT have a mass of 1–2 kg in an adult body. This is comparable to the weight of the adult human brain [15, 31]. Even though the lungs and human milk were not part of the original HMP because they were thought to be germ-free, we now know that they do contain many Bacteria (or germs) [13, 33]. The HMP has produced microbial maps of the human body [15, 30-32]. It also hopes to discover how changes in the microbiome are associated with health and disease. Major findings include discovering important links between oral (dental) health, the gut microbiome, atherosclerosis, cardiovascular and neurodegenerative diseases, as well as many types of cancer. The European HMP-counterpart MetaHIT

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focuses on microbiota in the intestinal tract. The Eldermet project focuses on the microbial composition associated with aging. More recently, the Earth Microbiome Project (EMP) has begun with the goal "to attempt to characterize the global microbial taxonomic and functional diversity for the benefit of the planet and mankind" [32].

For many decades, it was thought that a person's first exposure to microbes was during birth – either through the mother's birth canal or in the hospital room after a Caesarian section (C-section) [15]. However, recent data indicate that this sterile-womb hypothesis is false. Mothers do transmit Bacteria *in utero*. At the same time, a woman's gut microbiome changes during pregnancy, as does her baby's. After a baby is born, its microbiota becomes more complex and abundant. These changes continue during breastfeeding and as new microbes enter the baby from the environment. Moreover, the composition of the microbiota strongly influences pre-, peri- and postnatal development. That is, changes that occur early in life can cause important changes in the adult phenotype. These changes continue as childhood development progresses and are influenced by diet, lifestyle, socio-economic conditions, stress and one's habitat. The emergence of colonies of microbes can depend on the genetics of the host. So, and there is much interest in determining the relative contributions of the environment and host's eukaryotic genes on the microbiome. For example, the host may allow microbes to enter specific tissues, while environmental microbes may have genotypes and phenotypes that allow them to occupy those tissues [15].

Microbes enter human tissues even before birth and affect brain development, behavior and mental health later in life [34]. Bacterial DNA has been found in the amniotic fluid, umbilical cord blood, meconium, placenta and fetal membranes in pregnant women who have no infections or inflammation [35]. Bacterial DNA in full-term placenta came from maternal commensal intestinal Bacteria even after elective C-section when membranes had not been ruptured [36]. Also, a unique microbiome has been found in the human placenta [37]. Even though the amount of Bacteria is relatively low, they are diverse. The placental microbiome contains Firmicutes, Tenericutes, Proteobacteria, Bacteriodetes, and Fusobacteria phyla that provide a variety of important metabolites [37]. The placental microbiome resembles the adult oral microbiome, but its exact composition depends on the health of the mother [36]. It can be affected by the maternal history of infections – even before conception, as well as exposure to antibiotics and preterm birth [36].

The placental microbiome enters the fetal gut and establishes a gut microbiome that is important in the development of the brain [34]. A

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healthy developing gut microbiome is important in establishing an effective blood-brain barrier (BBB). The BBB is essential for providing a proper microenvironment for neural development that begins early in fetal life and continues after birth and into the early stages of infancy. In addition, the composition and diversity of the infant's microbiome is affected by the mode of delivery. Babies delivered vaginally have a gut microbiome that resembles their mother's vagina (e.g. *Lactobacillus, Prevotella* and *Senathia* species). In contrast, babies born by C-section that doesn't rupture the placental membrane have a microbiome that resembles their mother's skin (e.g. *Staphylococcus*, *Corynebacterium* and *Propionibacterium* species). Subsequently, the colonization of their gut microbiomes by *Bacteroides* and *Bifidobacteium* species is delayed. Moreover, differences in the microbiomes caused by different methods of delivery can persist even after seven years. This can cause an increase in the susceptibility to attention deficit hyperactivity disorder (ADHD), as well as social and gross motor skills and cognitive development. In addition, maternal psychological stress during pregnancy can make their babies more susceptible to ADHD. Once the baby is born, breastfeeding can help the baby develop a healthy microbiome, since mother's milk contains Bacteria (including *Staphylococcus* and *Streptococcus* species) and many important biochemicals, such as human oligosaccharides, that stimulate the growth of beneficial microbial communities, such as *Bifidobacterium* species. Even exposure to antibiotics in early life can disrupt the development of a healthy microbiome [34]. Exposure in the first year of life may have serious adverse effects on subsequent neurocognitive abilities [38]. Moreover, exposure to acetaminophen can increase an infant's susceptibility to allergies [39]. Even the microbiome that exist indoors (the built environment) can affect mental health and one's susceptibility to autoimmune diseases [40]. In many ways, the microorganisms within us (especially in the gut) are just as important as the human eukaryotic cells that we have.

2.3 Gut microbiome and the enteric nervous system (ENS)

2.3.1 Composition of the gut microbiome

So, we are changing the way we think about what it is to be human. Our lives and health depend on much more than just our human eukaryotic cells. For example, the viruses, Bacteria and other microorganisms that go from the esophagus to the lower intestines are part of a microbiota-gut-brain axis. The gut microbiome has been described as a second brain, our

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personal oncologist, and an essential part of our neuroendocrine immune systems (or network) [41-43]. The average adult human intestine contains 10^{13} to 10^{14} Bacteria, the vast majority of which belong to the phyla Firmicutes and Bacteroidetes, even though *E. coli* in the Proteobacteria phylum are the best studied and most easily cultured outside the body [44, 45].

The microbiota of most adults has been assigned to three predominant variants, or enterotypes [44, 46]. They have different amounts of three dominant genera: *Bacteroides*, *Prevotella* and *Ruminococcus*. Most of the prokaryotes in human intestines are in the phyla Firmicutes (including the genera *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus*) and Bacteroidetes (including the genera *Bacteroides* and *Prevotella* in proportions partly determined by diet). The relative abundance of these phyla and genera are the basis for classifying the human gut microbiome into three enterotypes: (1) abundant *Bacteroides*; (2) few *Bacteroides* but abundant *Prevotella*; and (3) an abundance of *Ruminococcus*. Each of these genera has a different function in nutrition and metabolism. Their relative abundance can be affected by diet. The enterotype dominated by *Bacteroides* occurs mostly when people consume relatively large amounts of protein and animal fats. The *Prevotella* enterotype is linked to carbohydrate metabolism and a vegetarian diet [44, 46]. The enterotypes do not correlate with age, sex, nationality or body mass index (BMI) [44]. However, many researchers feel that the concept of enterotypes is an over-simplification. That is, there is a gradient of species' functionality with a core microbiome at the gene level rather than at the organismal lineage level. Instead of a core group of species, we all share a core group of microbiome functions. However, obese people may have different patterns of gut microbes than the non-obese, but share a core group of functions. Changes in this core set of genes can affect one's health. On the other hand, it can be difficult to tell whether changes that occur in one's microbiome when diseases emerge are causes or symptoms of the disease [44]. Moreover, Bacteria produce important nutrients that our eukaryotic cells can't make for themselves.

For example, some species of Firmicutes and Bacteriodetes can ferment dietary fiber to produce butyrate [45]. At the same time, some species of Bacteriodetes can degrade polysaccharides. There are also some Actinobacteria (*Bifidobacterium colinsella*) and Melainabacteria that can produce essential vitamins. Deferrobacteres degrade iron. Verrucomicrobia can degrade mucin and decrease inflammation, while increasing the production of butyrate and making the mucus layer thicker. There are also methanogenic Archaea that convert hydrogen gas to methane [45].

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Methane-producing Archaea are quite abundant in the microbiomes of sponges, termites, mice and cattle, but are present at much lower concentrations in the human fecal microbiome [47]. The average concentration of Archaea in the human fecal microbiome does vary among people. About 0.8% of the genes found in the fecal microbiomes of Europeans were from Archaeae. However, the percentage is smaller for Native Americans (Amerindians) and people from the African country of Malawi (0.2-0.3 %) and non-native North Americans (<0.05%). However, the Archaea in humans are not well-studied. This could be partly due to the fact that no Archaea have been found that are human pathogens. However, it is significant that almost all the Archaea in the human colon are obligate anaerobes that produce methane. Most are in the order Methanobacteriales. The most abundant genera are *Methaonbrevibacter* and *Methanosphaera*. *Methanobrevibacter smithii*. They exist in about 95.7% of human subjects, and are the most abundant methanogens in the human gut. Its abundance appears to be inheritable and stable over time and is not strongly affected by diet. *M. smithii* and *Methanosphaera stadtmanae* have many genes that were acquired by horizontal transfer from other species. These genes helped these Archaea in their initial adaptation to mammalian hosts. Moreover, both of these species of Archaea induce the maturation of monocyte-derived dendritic cells. Also, *M. stadtmanae* can cause pro-inflammatory cytokines to be released from these dendritic cells. It is present at higher concentrations (is more abundant) in patients with inflammatory bowel diseases (IBDs) [47]. Note that IBDs include Crohn's disease and ulcerative colitis. IBD is different than IBS, which is irritable bowel syndrome. IBS does not cause inflammation like IBD does.

The bacterial content of the gastrointestinal tract (GIT) varies along its length [44]. It ranges from a low diversity and number in the stomach to a wide diversity and high number in the large intestine. In the adult distal colon, there are about 1100 prevalent species, with at least 160 such species per individual. In its entirety, the microbiota is estimated to contain 150-fold more genes than the human genome. There is much inter-individual variability in the composition of the gut microbiota. There is a core group of over 50 taxa in nearly half of the human population [44]. There is a bimodal distribution of Bacteria in the gut microbiome [48]. Some people have less biodiversity in their gut microbiomes (known as low gene count (LGC)) than others (known as high gene count (HGC)). The LGC microbiota tends to be dominated by *Bacteroides* species and have fewer butyrate-producing Firmicutes. LGC people tend to have a higher incidence of obesity and metabolic syndrome [48].

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2.3.2 Interactions and communication between the ENS and the rest of the body

As in TQM, there is an extensive communication network among different microbes and between microbes and human (eukaryotic) cells. Commensal and pathogenic Bacteria interact with the central, autonomic and enteric nervous systems (CNS, ANS and ENS, respectively), as well as the hypothalamic-pituitary-adrenal (HPA) axis [49]. These interactions form the gut-brain axis (GBA). The interactions go both ways in a bidirectional network of communication. This network connects the emotional and cognitive centers of the brain with peripheral intestinal functions through neural, endocrine, immune, and humoral links. These interactions contribute to both health and disease [50]. For example, neurotoxins produced by *Clostridium* species can stop neurotransmission to or from neurons, producing the characteristic paralyses of botulism and tetanus. Gut Bacteria produce biochemicals that act on enteric neurons to influence gastrointestinal motility. They also produce metabolites that alter neural circuits and affect autonomic function as well as higher-order brain function and behavior. The intrinsic neurons in the gut make up the ENS, which forms a complete sensorimotor reflex circuit. It contains intrinsic primary afferent neuron, interneurons and motor neurons that are within the wall of the gut. These enteric neurons play critical roles in regulating gut motility and peristalsis. There are also extrinsic neurons that relay sensory information from the gut to the CNS. Neurons in the ganglia of the vagus nerve mediate the sensation of nutrients, nausea, appetite, and satiety. Neurons in the dorsal root ganglia (DRG) detect noxious stimuli and mediate pain. Biochemicals that are produced within the gut lumen influence the intrinsic enteric neurons in the ENS both directly and indirectly. For direct actions to occur, a biochemical must pass through the epithelial barrier to access the nerve endings on enteric sensory neurons. They are in the connective and muscular tissue immediately beneath the intestinal epithelium. In a healthy gut with an intact epithelial barrier, bacterial products can only get through the barrier by active or inactive transport or by transcytosis. In an unhealthy gut in which the epithelial barrier is damaged by inflammation (leaky gut), both Bacteria and their products can pass between cells whose tight junctions are no longer intact [50].

There are also indirect interactions between the Bacteria in the lumen and the ENS [50]. This occurs through endocrine or immune cells in the gut. Enteroendocrine cells in the epithelium can release paracrine signals that are detected by sensory neurons that innervate the gut. One set of

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enteroendocrine cells called enterochromaffin cells (ECs) can transduce signals that convert bacterial stimuli into downstream neuronal responses. Enteric neurons also express toll-like receptors (TLRs) that detect bacterial pathogen-associated molecular patterns (PAMPs). Lipopolysaccharides (LPS) that are produced by gram-negative Bacteria are an important example. They are detected by TLR4. Enteric neurons activated by TLR4 regulate neuronal survival and gut motility. In addition, TLR2 can detect peptidoglycans and lipoproteins from gram-positive Bacteria. TLR2 signaling affects the structure of the ENS, as well as intestinal contractility [50].

Bacteria can also communicate with the brain through neural pathways [50]. There are two types of extrinsic sensory neurons that innervate the gut. There are vagal sensory afferents in the nodose ganglia that project directly to the brainstem, and spinal sensory afferents in the DRG that project to the spinal cord. Sensory afferents from the vagus nerve detect gut nutrients, stretch, and hormones to mediate satiety and nausea. Spinal sensory afferents detect noxious and harmful stimuli and mediate pain. They also detect bacterials in the gut, triggering the CNS to modulate physiological functions through neural circuits that feedback to the gut through the ANS [50].

The gut microbiota send signals to the brain, while the brain sends signals to the gut through neural, endocrine, immune, and humoral links [50]. The immune system is an integral part of the signaling pathways in the gut-brain axis. The ANS receives afferent signals that originate from the lumen and are transmitted though enteric, spinal and vagal pathways to the CNS. The ANS also sends efferent signals from CNS to the intestinal wall. The HPA axis coordinates our adaptive responses to stressors. They activate the HPA by secreting corticotropin-releasing factor (CRF) from the hypothalamus. This stimulates adrenocorticotropic hormone (ACTH) secretion from the pituitary gland. Subsequently, the major stress hormone, cortisol, is released from the adrenal glands. As a result, the neuroendocrine immune system enables the brain to influence the activities of intestinal effector cells. This includes immune and epithelial cells, as well as enteric neurons, smooth muscle cells, interstitial cells of Cajal and ECs. At the same time, these cells are influenced by the gut microbiota [50].

Bacteria in the gut can activate the vagus nerve [51]. It is the tenth cranial nerve. It innervates the pharynx, larynx and visceral organs. It is the main afferent pathway from the gut to the brain. Also, information from the heart, lungs, pancreas, liver, stomach and intestines are delivered tonically to the brain through sensory fibers in the vagus nerve. There are

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an estimated 30 000 to 80 000 vagal afferent nerves that supply the intestines with a 9:1 ratio of afferent to efferent fibers. The endings of visceral afferent endings have receptors for several different hormones and regulatory peptides, such as ghrelin, glucagon-like peptide-1, cholecystokinin and peptide YY. They affect food intake, satiety and energy balance. Butyrate affects vagal afferent nerve terminals. In addition, the vagus nerve can affect health and behavior. This includes lethargy, depression, anxiety, loss of appetite and sleepiness – especially when the vagus nerve is stimulated by pro-inflammatory LPS or interleukin-1β. In contrast, other types of stimulation of the vagus nerve can cause a reduction in anxiety and depression. For example, dietary omega-3 polyunsaturated fats, such as docosahexaenoic acid (DHA) and EPA increase vagal tone [51]. Note that DHA is also an abbreviation for dehydroascorbic acid, the oxidized form of ascorbic acid, or vitamin C. Of course, the abbreviation EPA usually stands for the U.S. Environmental Protection Agency. The structures of docosahexaenoic acid and dehydroascorbic acid are compared in Figure 1. The structure of EPA is shown in Figure 2.

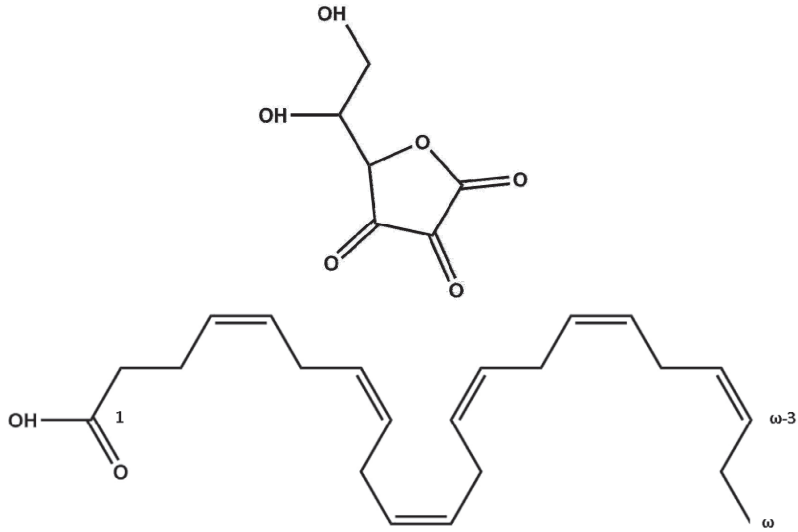

Figure 1. 2D structure of dehydroascorbic acid (left) and docosahexaenoic acid (right), which are both abbreviated as DHA.

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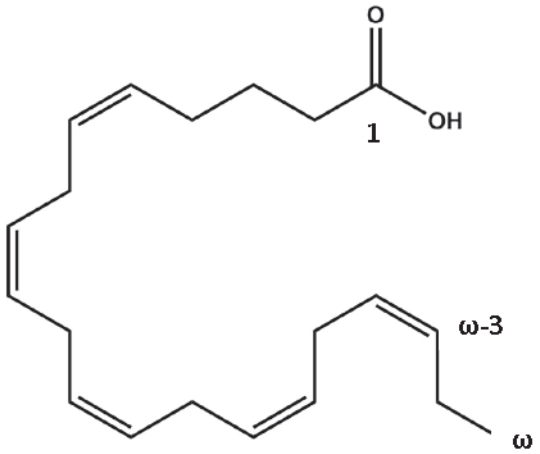

Figure 2. 2D structure of (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid.

Moreover, vagal stimulation is used to treat refractive epilepsy and intractable depression [51]. The vagus nerve also has important functions in the immune system. It senses pro-inflammatory afferent signals sent to the afferent regions of the brain and activates anti-inflammatory efferent responses. Moreover, the gut microbiome affects the ENS, as well as the brain and behavior. It has also been suggested that improper regulation of inflammation that occurs in major depressive disorder may be caused (at least in part) by gut microbiome dysbiosis, or imbalance in the microbial ecosystem. This is an extension of the hygiene hypothesis. It states that the large increases in autoimmune diseases and allergies that are occurring are due in part to the large-scale eradication of Bacteria in clean, modern, indoor environments [51].

Another way that the ENS, gut microbiota and the rest of the body communicate is through the endocannabinoid system [20]. It is widely distributed throughout the brain and most of the rest of the human body. There are two cannabinoid receptors, CB_1 and CB_2. The CB_1 receptor exists in the ENS, while the CB_2 receptor is found mostly in the immune system. Their endogenous ligands are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Their structures are shown in Figures 3 and 4. Anandamide is also known as *N*-arachidonoylethanolamine.

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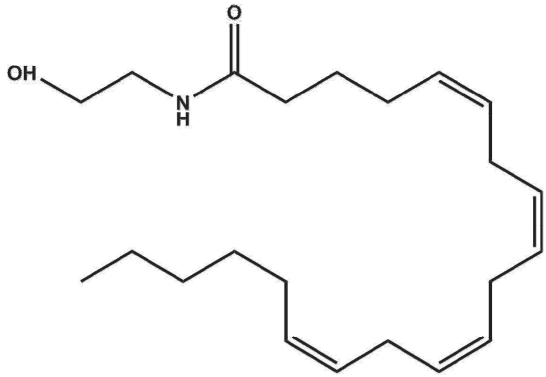

Figure 3. 2D structure of anandamide (AEA, $C_{22}H_{37}NO_2$, MW = 347.53 g/mol), the endogenous ligand for the CB_1 cannabinoid receptor.

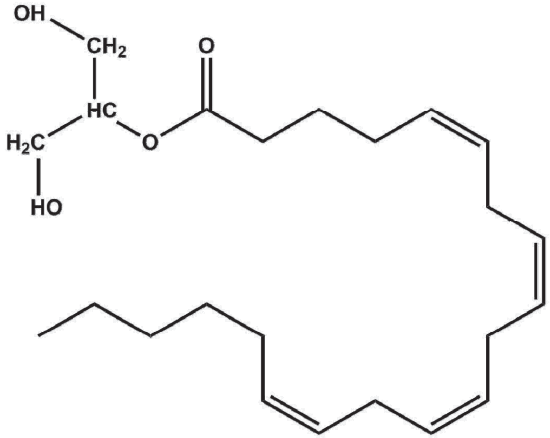

Figure 4. 2D structure of 2-arachidonoylglycerol (2-AG, $C_{23}H_{38}O_4$, MW = 378.3 g/mol), the endogenous ligand for the CB_2 cannabinoid receptor.

Activated CB_1 receptors in the ENS inhibit motility in the GIT [20]. Like CB_2, it is a G protein-coupled receptor (GPCR). Both are coupled to $G_{i/o}$ proteins that inhibit adenylate cyclase, trigger the mitogen activated protein kinase (MAPK) signaling system, inhibit N- and P/Q-type Ca^{2+} channels and activate A-type outward K^+ channels. These activities prevent the influx of Ca^{2+} into the cell, block membrane depolarization and inhibit the release of acetyl choline, which is the fast excitatory neurotransmitter in the ENS. In addition, the gut microbiota can affect the

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endocannabinoid tone [20], which is measured by the amount of mRNA coding for the CB_1 receptor and the fatty acid amide hydrolase (FAAH), as well as the concentration of AEA [52]. The FAAH is the main enzyme that catalyzes the hydrolysis of AEA [52]. That is, EAE and 2-AG can be stored as ligands that are tightly bound to fatty acid binding proteins [53]. They can diffuse in the lipid bilayer to bind to CB receptors. So, they can act as autocrine or paracrine stimulators of CB_1 and CB_2, thus giving the appearance of constitutive activity [53].

Whether activated by EAE that is stored on fatty acid binding proteins or released at synapses, the endocannabinoid system links the gut microbiota to adipogenesis, obesity and diabetes [52, 54]. Obesity can lead to an increased tone in the part of the endocannabinoid system that is located in adipose tissue. Adipocytes produce adipokines like apelin that affect glucose homeostasis, the CNS and the cardiovascular system. Gut Bacteria can produce LPS that are pro-inflammatory and stimulate the synthesis of endocannabinoids. However, during normal, healthy conditions, endocannabinoids down-regulate the expression of apelin and its receptor [54]. On the other hand, endocannabinoid deficiency can lead to anxiety as well as intestinal disorders, such as irritable bowel syndrome (IBS) [52].

2.3.3 Communication between the gut microbiome, ENS, brain and immune system

The gut and brain communicate bidirectionally through anatomic and humoral pathways. Intervening with one system will affect the other [55]. There is an intestinal epithelial layer that forms a very selective barrier that helps maintain homeostasis by coordinating the actions of the different types of cells within the layer. It is important for maintaining good health. There are enteroendocrine cells (EECs) at the base of intestinal crypts. They transduce mechanical and chemical signals from the intestinal lumen to neighbor cells and the local neuronal network. One of the types of EECs, the enterochromaffin (EC) cells, are the main source of 5-hydroxytryptamine (5-HT, also known as serotonin) in the human body. Serotonin that is released from EC cells mediates many functions of the GIT, including secretion, peristalsis, vasodilation, perception of pain and nausea. It is biosynthesized from the essential amino acid, L-tryptophan. First, tryptophan is converted to 5-hydroxy-L-tryptophan, which is converted to 5-HT in a reaction catalyzed by 5-hydroxytryptophan decarboxylase and its coenzyme pyridoxal phosphate (the active form of vitamin B_6). However, L-tryptophan can also be metabolized to kynurenic acid, which an antiexitotoxic and anticonvulsant compound. The structures

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of 5-HT, L-tryptophan and kynurenic acid are shown in Figures 5-7. During stressful conditions, the 5-HT biosynthetic pathway competes with the kynurenic acid pathway for L-tryptophan. This decreases the plasma concentration of 5-HT. So, changes in the 5-HT metabolism may help cause IBS and other disorders of the GIT. Also, IBS patients have lower mucosal and higher plasma concentrations of both kynurenic acid and 5-HT, compared to healthy patients. There is also a positive correlation between signs of depression and the 5-HT concentration in mucosa but not in blood plasma [55].

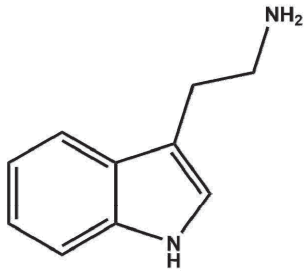

Figure 5. 2D structure of 5-hydroxytryptamine (5-HT, also known as serotonin)

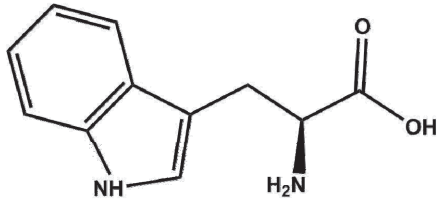

Figure 6. 2D structure of L-tryptophan.

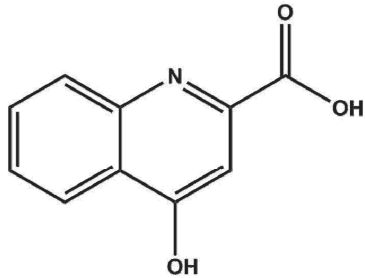

Figure 7. 2D structure of kynurenic acid.

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There is also a mucosal immune system, since the gastrointestinal epithelium is in close proximity to potentially pathogenic Bacteria, viruses, fungi and helminths [55]. This local immune network contains macrophages, lymphocytes and dendritic cells. It is required for the maintenance of homeostasis between the human host and luminal microbiota. Stress hormones that are organized in the HPA axis are part of the humoral communication between the intestinal tract and the CNS. The ENS is highly innervated by intrinsic neurons that are found in groups in the myenteric and submucous plexus. Then there is the vagus nerve, which has essential roles in communication between the gut and the brain. It innervates the thoracic and abdominal tissues. There are left and right cervical pathways. They have afferent and efferent projections that converge in the cranium where either information coming from other organs is processed or chemical signals are sent to peripheral tissues [55]. So, the gut microbiome and the ENS affect the immune system and autoimmune diseases.

For example, microbial metabolism in the gut plays an important role in IBD [56]. The gut microbiomes of infants can also affect their susceptibility to type-1 diabetes (T1D) and distinguish between infants who will be most likely to progress into T1D and those who will not [57]. The gut microbiome is also an important part of the ANS [48, 58]. The ENS influences anxiety and depressive-like behaviors as well as dysbiosis in autism spectrum disorder [58]. Dysbiosis also happens in functional gastrointestinal disorders that can cause mood disorders and are linked to a disruption of GBA. Disruptions in the GBA can cause changes in intestinal motility and secretion. They also cause visceral hypersensitivity and lead to deleterious changes in the entero-endocrine and neuroendocrine immune systems [58].

The ENS is a separate branch of the ANS [59]. It exists throughout the length of the GIT as a series of interconnected ganglionated plexi. The GIT is densely innervated by a network of 200–600 million neurons, which make up the ENS. These neurons innervate all regions of the GIT. The ENS influences the physiology and function of the GIT, while communicating with the CNS by both vagal parasympathetic and sympathetic pathways. At the same time, vagal afferent signals from the ENS, circular muscle layers and mucosa are facilitated by intraganglionic laminar endings, intramuscular arrays and mucosal varicose nerve endings, respectively. There are intrinsic sensory neurons, motor neurons and enteric interneurons. They regulate key functions of the GIT. This includes intestinal muscle activity and gastric peristalsis as well as secreto- and vaso-motor control. The ENS is protected from the contents of the lumen

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by the epithelial barrier and mucous layer, as well as by ion and fluid secretion. Enteric neurons express Toll-like receptors (TLRs) and a large variety of neurochemical receptors. The TLRs are pattern recognition receptors. Microbial components are their ligands. They are sensitive to microbial metabolites. So, the ENS can respond to signals from microorganisms [59].

Often, the ENS is called the second brain because it contains many types of neurons and glia, that are linked in complex circuitry [60]. It autonomously regulates many systems in the bowel while coordinating digestive and defensive functions in the gut. A variety of ions, small molecules, extracellular ligands, proteins (enzymes, receptors and transcription factors) and nutrients support cytoskeletal rearrangements and the development of the ENS. Defects in its development and/or subsequent damage can cause vomiting, abdominal pain, constipation, IBS, Hirschsprung disease, growth failure, and early death. The ENS develops from enteric neural crest–derived cells (ENCDCs) that start primarily from the vagal neural tube. Vagal ENCDCs eventually travel through the paraxial mesoderm and extend throughout the bowel of the developing fetus. ENCDCs differentiate into at least 20 neuronal subtypes, including enteric glia. They form ganglia and extend neurites, while establishing and refining functional neural circuits that control the bowel. Some of the ENDCs migrate inward to form the submucosal plexus. Others migrate outward to form ganglia in the pancreas. Enteric neurons extend processes that enable them to communicate with other enteric neurons, interstitial cells of Cajal, smooth muscle, endothelial cells, mucosal epithelia, and intestinal glands [60].

Enteric glial cells are also important in the ENS [60]. They form an extensive network in the mucosa of the GIT. Like glial cells in the central nervous system (CNS), enteric glia were originally thought to have merely supportive roles, but are now known to be actively involved in the ENS. They link enteric nerves, enteroendocrine cells, immune cells, and epithelial cells. Enteric glial cells also link the nervous and immune systems. They may play important roles in GI disorders, including problems with motility (slow transit constipation). Many biochemicals used in intercellular signaling are made by enteric glial cells. Some of them (including S-nitrosoglutathione), glial cell line-derived neurotrophic factor or GDNF, and tumor growth factor-beta (TGF-β) can increase barrier function by reducing epithelial permeability. Enteric glial cells also play important roles in diseases of the CNS. For example, they are targets of misfolded proteins (prions) that cause transmissible spongiform encephalopathies, including mad cow disease in cattle and Creuzfeldt-

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Jakob disease in humans. Misfolded prion proteins can be transmitted from the brain to the gut. In addition, enteric glia may help cause Parkinson's disease, in which the protein α-synuclein accumulates. Also, inflammation occurs in ENS glial cells, which leads to the constipation that occurs in patients with Parkinson's disease [60].

The ENS can produce new neurons in postnatal and adult lives [61]. Agonists of the 5-HT_4 serotonin receptors increase the survival and development of enteric neurons, while decreasing apoptosis. The development of the ENS depends on the enteric environment. The luminal environment can affect the amount and phenotypes of neurons in the ENS. Dysfunction in the GIT increases in the elderly and may be due in part to age-related loss of neurons. Stimulating the 5-HT_4 serotonin receptor can protect enteric neurons from apoptosis, while inhibiting the degradation of axonal terminals and autophagy that is caused by inflammation [61].

The development of the immune system also depends on a healthy gut microbiome [61]. Serotonin can modulate immune responses and influence intestinal inflammation. The immature immune system of babies and infants has a bias towards the T_H2 version of helper T-cells. The immune system shifts towards a T_H1 bias as the gut is colonized with more Bacteria. Bacteria like *Bifidobacterium* and *Lactobacillus* promote this shift. However, administering broad-spectrum antibiotics can reduce the biodiversity of the gut microbiome and delay its colonization by some strains of *Bifidobacterium* and *Lactobacillus*. Moreover, the decreased diversity and stability of the gut microbiota can cause serotonin-related health problems in the elderly [61].

Dysbiosis in the gut microbiota-gut-brain axis plays important roles in aberrant social behavior as well as the etiology of several neurodegenerative diseases, including anxiety, depression, autism and Parkinson's disease [19, 62-69]. The gut microbiome and ENS also play important roles in maintaining homeostasis and the production of energy as well as being affected by obesity [70-73]. As a result, it is also an important factor in diabetes, inflammatory diseases, colorectal cancer, chronic kidney disease, atherosclerosis and heart disease [74-82]. The human gut microbiota also produces melatonin, which helps cause the gut to exhibit circadian rhythmicity [83].

2.3.4 The gut microbiota and ENS comprise an entero-endocrine organ

The gut microbiota and ENS can be thought of as an entero-endocrine organ that has many more cells and DNA than rest of the human body [41,

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45, 84]. It produces and secretes hormones, responds to hormones produced by human eukaryotic cells and regulates the concentrations of those hormones. Many of the biosynthetic pathways that produce neuroendocrine hormones are found in Bacteria as well as human eukaryotic cells. The gut microbiota produces and responds to neurohormones that are secreted in response to a neuronal input. Microbiota can modulate behavior through neurohormones such as serotonin, dopamine and norepinephrine. At the same time, these catecholamines can alter growth, motility, biofilm formation and/or virulence of Bacteria. Serotonin may be a major neurotransmitter in the brain, but 90% of it is located in the intestines. It is a key signaling molecule in both the gut and the brain. Moreover, the microbiota, hormones and the human host all work together to help maintain a healthy immune system. Diet, exercise, mood, the overall health, stress and gender can change the concentrations of hormones that can affect the gut microbiome. The opposite also occurs. A healthy gut microbiota can help keep a person calm as Bacteria affect the concentrations of stress hormones (corticosterone and adrenocorticotropic hormone, or ACTH). On the other hand, dysbiosis in the gut microbiome can contribute to autoimmune diseases, including type-1 diabetes [41, 45, 84]. This inter-kingdom form of communication has been called microbial endocrinology [84]. Also, many of the biochemical pathways that synthesize hormones in eukaryotic cells also occur in prokaryotes in the gut. Human eukaryotic cells and prokaryotes in the gut recognize and respond to many of the same neurotransmitters. For example, catecholamines stimulate bacterial growth and their virulence. Moreover, the stress-mediated excretion of neurohormones can alter the expression of genes in pathogenic Bacteria in the gut. They can also affect conjugative transfer of DNA between enteric Bacteria [84].

One of the important roles of the gut microbiome is to help control energy production and homeostasis while avoiding obesity and diseases linked to it [45]. Bacteria in the gut can produce or influence afferent signals that directly or indirectly affect energy balance (weight, BMI) and energy stores. As an endocrine organ, it secretes SCFAs and affects signaling due to leptin and other hormones. SCFAs also provide an energy source for the colonic epithelia (butyrate), liver (propionate), and peripheral tissues (acetate). They also bind to two orphan GPCRs, GPR41 and GPR43. GPR41 (also known as the free fatty acid receptor 3 or FFAR3) is mostly activated by propionate and butyrate. Butyrate increases concentrations of gastric inhibitory peptide (GIP), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), insulin, and amylin in the blood. This slows down digestion and the intestinal transit of nutrients, while promoting

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satiety and increasing plasma insulin concentration. Butyrate also inhibits histone deacetylases (HDACs). The hypermethylation of histones leads to changes in the transcription of genes coding for enzymes that are involved in fatty acid oxidation, epithelial integrity, and apoptosis. GPR41 also activates Gi/o proteins in the gut, adipocytes and the peripheral nervous system. Activated GPR41 promotes the growth and activation of the sympathetic nervous system. GPR43 (also known as FFAR2) is more responsive to propionate and acetate than to butyrate. Propionate (but not acetate) has a small effect on GIP, insulin, and amylin. Acetate increases the release of leptin by adipocytes. Butyrate and acetate protect against diet-induced obesity without hypophagia, while propionate has been reported to reduce food intake and increase locomotor activity. So, acetate has little effect on activity or energy intake, while butyrate and propionate have more direct effects on feeding behavior and physical activity. GPR43 also activates the G-protein subunits Gi/o (inhibits adenylate cyclase and opens potassium, or K^+ channels) and Gq (stimulates phospholipase C) in the gut and adipose tissue. When GPR43 is activated in the intestines, it decreases the release of inflammatory cytokines and increases the sensitivity of the hypothalamus to leptin [45].

The gut microbiome is also an essential part of the neuroendocrine immune system [85]. The gut microbiome is part of the GIT, which is the largest endocrine organ in the human body. The GIT is also a hub in the communication network in the human body. It contains the highest concentration of immune cells in the body. It is a network consisting of 200-600 million neurons and the trillions of viruses, Bacteria, Archaea and Eukarya that make up the human gut microbiota [17, 38]. The gut microbiome helps to regulate intestinal function and interacts with the rest of the body to maintain health. The gut microbiota has been called the conductor of communication between the immune and neuroendocrine network [18, 39]. That is, the microbiota produces and secretes hormones, responds to hormones secreted by human eukaryotic cells and regulates their expression [19]. In fact, over 90% of human serotonin is found in the intestines [19].

In addition, the gut microbiome is a major part of the enteric nervous system (ENS) that receives input from the sympathetic and parasympathetic nervous systems, but is also able to function autonomously [20]. So, the ENS has been called our “second brain” that forms a complex, neural network. It has intrinsic, primary efferent interneurons and motor neurons, arranged in two ganglionic plexuses. Interoceptive and nociceptive signals from the gut send information to the brain about the state of digestion, the luminal environment and mucosal defense. At the same time, the brain

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affects GI function through the ANS. There is a dense network of interwoven nerve fibers and their branches and synapses, together with glial filaments in the ENS. It contains unmyelinated axons and dendrites of the enteric neurons and the process of enteric glia, astrocyte-like glial cells that surround enteric neurons. There are more glial cells than neurons in the ENS. There are also enteric glial cells in the mucosa that lies beneath the epithelium. They help regulate the functions of the epithelial barrier. Enteric glial cells and astrocytes also modulate neurotransmission in the ENS. They help regulate the availability, degradation and sequestration of neurotransmitters [21]. The gut microbiome has been called the brain's Gepetto because it controls (or puppeteers) neural function and human behavior [21]. Actually, the brain affects the gut just as much. Still, the expression "thinking with my gut" has taken on a new meaning. Moreover, the expression "you are what you eat" is more significant than once thought. That is, our diet greatly affects the composition of microbes in our gut, which plays a major role in shaping our feelings and behavior. This is starting to change our idea of what it is to be human. Viruses and Bacteria in the intestines are no longer thought of as being outside the human body, but an integral part of it.

2.4 The role of the gut microbiome in preventing diseases

The gut microbiome, GIT and neuroimmune endocrine system are important in cancer. For example, ulcers and other diseases that occur in the GIT are frequently caused by infection with the gut bacterium *Helicobacter pylori* [86]. In addition, *H. pylori* infection may cause gastric carcinoma and may be a cofactor in its pathogenesis [87]. Stress and mental disorders can disrupt proper GI function and the gut microbiome, while leading to ulcers [88, 89]. Even though treatment with antibiotics can cure ulcers in some patients, it does not work for everybody [90]. On the other hand, the same treatment can cause neuropsychiatric symptoms, even when *H. pylori* is eliminated. Moreover, urinary tract infections are associated with neuropsychiatric disorders [90].

Dysbiosis in the gut microbiome can also lead to other types of cancer, as well as arteriosclerosis, atherosclerosis and fatty liver disease [91]. Arteriosclerosis is when blood vessels become thick and stiff [92]. Atherosclerosis is a type of arteriosclerosis, in which the thickening of blood vessels is caused by a buildup of cholesterol and other fats on arterial walls and plaques in those walls. This can restrict blood flow and lead to cardiovascular disease. Sometimes, the terms arteriosclerosis and atherosclerosis are used interchangeably [92]. In a healthy gut, the

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microbiome balances the immune response. Commensal Bacteria inhibit immune responses within their niche, while directing them towards pathogens and uncontrolled growth. This balance is mediated by secreting metabolites and by intercellular signaling through activated toll-like receptors (TLRs) [93]. TLRs deactivate the local immune response [91, 93]. SCFAs (acetic, propionic and butyric acids) suppress inflammation and cancer [91]. In contrast, undesirable Bacteria can produce an excess of secondary bile acids that can increase the concentration of RONS, which cause smoldering inflammation as well as damage to DNA, proteins and lipids. Obesity is a known cause of gut microbiome dysbiosis. The secondary bile acid metabolite deoxycholic acid (DCA) is produced by Bacteria in the phylum Firmicutes. They tend to be present at higher levels in the guts of obese people. A diet rich in red meat tends to increase the levels of *Fusobacterium nucleatum*, which causes DNA damage and genomic instability within developing tumors [93]. Also, *F. nucleatum* stimulates inflammation and can protect tumors from proper immunosurveillance and destruction. This increases the risk of colorectal cancer [94].

In contrast, vegan and vegetarian diets can decrease the risk of not just cardiovascular diseases, but also autoimmune diseases and many types of cancer, as well as metabolic syndrome and diseases linked to it, including neurodegenerative diseases [46, 95]. In a study sponsored by Seventh Day Adventists, who preach the values of a vegetarian diet (the Adventist Study-2), vegetarian diets were found to be healthier than those of omnivores [46, 96-98]. Four types of vegetarian diets were tested: vegan, lacto-ovo vegetarian, pescovegetarian, and semi-vegetarian [95, 96]. Vegan diets had a unique advantage in that they lowered the incidence of type-2 diabetes [96, 97] and decreased overall mortality [98]. Others found that when people were placed on a 20-day vegan diet that they had lower amounts of fecal *Lactobacilli* and *Enterococci*, as well as lower concentrations of bile acids, coprostanol and cholesterol [99]. A subsequent study found that both vegans and vegetarians had a higher proportion of the *Bacterioides-Prevotella* group and a lower level of *Clostridium* cluster XIVa when compared to omnivores [100]. Also, *F. prausnitzii*, the most abundant bacterium in the intestines of healthy adults, was more abundant in vegans than in vegetarians. This species has an especially strong protective role in preventing metabolic disease. Its level is lower in people who have intestinal disorders, inflammation, obesity and type-2 diabetes [100-102]. The gut microbiome of obese diabetics is quite different than that of healthy, lean people [102]. *F. prausnitzii* levels were found to be higher in the lean, healthy people

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[102]. Moreover, consumption of the plant polysaccharide inulin increases the levels of *F. prausnitzii* [100]. At the same time, this species of Bacteria has been associated with an increase in the production of butyrate in the GIT. So, a high-fiber diet can increase the level of *F. prausnitzii*, while reducing the risk of cardiovascular disease, colon cancer, diabetes and obesity [103]. Moreover, dietary fiber inhibits pathogen adhesion and alters the patterns of bacterial fermentation, while increasing stool bulk and decreasing its transit time [46]. The composition of the gut microbiota has been linked to metabolic syndrome, obesity and chronic smoldering inflammation [46]. The microbiota of obese people is less diverse and has a lower ratio of Bacteroidetes to Firmicutes, with an increased abundance of potentially inflammatory Proteobacteria. They also have more local and systemic inflammation [46].

Another study showed that when six obese people who had diabetes and/or hypertension switched to a vegan diet for 60 days that they had lower concentrations of blood glucose, triglycerides, total cholesterol, LDL-cholesterol and hemoglobin A1c, while losing weight and decreasing the abundance of pathobionts, such as Enterobacteriaceae [46, 104, 105]. More recently, a vegan diet was shown to decrease the production of trimethylamine-*N*-oxide (TMAO), which can cause atherosclerotic heart lesions [106].

There is another diet called the extreme raw vegan diet (encouraged by the Living Food movement) [46, 107]. Patients with rheumatoid arthritis (RA) were placed on this diet for one month. The composition of their gut microbiomes changed significantly, while the symptoms of RA improved [107]. So, changing to a vegetarian and/or vegan diet can have very healthy effects that are mediated, at least in part, by the gut microbiome.

The composition of the gut microbiome can affect one's susceptibility to many forms of cancer. However, the difference between healthy and cancer-causing Bacteria can be small. For example, there are two types of *Bacterioides fragilis*, enterotoxic *B. fragilis* (ETBF) and nontoxigenic *B. fragilis* (NBFT) [108]. ETBF secretes a *B. fragilis* toxin, but NBTF does not. Also, there is a strain of *E. coli* that produces an endotoxin called colibactin. Other strains of *E. coli* in the healthy human gut don't produce this toxin. Some Bacteria outside the gut microbiome can cause cancer. *Fusobacterium nucleatum* that are present in the mouths of people with periodontal disease can cause adverse pregnancy outcomes, GI disorders, cardiovascular disease, rheumatoid arthritis, respiratory tract infections and Alzheimer's disease [108].

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While an unhealthy gut microbiome can cause diseases, including cancer, a healthy gut microbiome can act as your personal oncologist and help prevent cancer [109, 110]. Bacteria in the gut produce SCFAs and other metabolites that help prevent cancer. The most abundant SCFAs are acetic, propionic and butyric acids, which exist as the anions acetate, propionate and butyrate at the physiological pH of about 7.3. Their combined concentration in the colon is about 50-150 mM (mmols/L) [109, 110]. They account for about 90% of all the SCFAs and are present in a mole ratio of about 13:4:3 [111]. Butyrate is especially important. It is the main source of energy for colonocytes [112]. It regulates colonocyte differentiation and apoptosis by promoting removal of dysfunctional cells. This helps protect against colorectal cancer [113]. Butyrate is also essential for maintaining mucosal integrity, while modulating intestinal inflammation and promoting genome stability [109, 110]. Non-digestible carbohydrates are fermented by Bacteria in the gut (primarily Firmicutes and Actinobacteria) to produce the SCFAs. Butyrate and propionate are especially important in preventing cancer, since they inhibit histone deacetylases in colon and immune cells. This downregulates the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-12 in colonic macrophages. Butyrate and propionate also induce the differentiation of important immune cells that help to control inflammation. In addition, some gut Bacteria contain enzymes that catalyze the biotransformation of dietary phenolic compounds that act as antioxidants [109, 110]. This includes daidzein (in soybeans), which is metabolized into equol, which has been linked to a decreased risk of breast and prostate cancer in Asian women and men [114]. In addition, anthocyanins, which are found in pomegranates, grapes, blueberries, açaí, elderberries and other fruits, are not absorbed in the upper GI tract, but are in the colon. The structure of an anthocyanin that is in all these fruits, cyanidin-3-*O*-glucoside, is shown in Figure 8. It stimulates the production of healthy *Bifidobacterium* and *Lactobacillus* species and their colonization in the colon [110].

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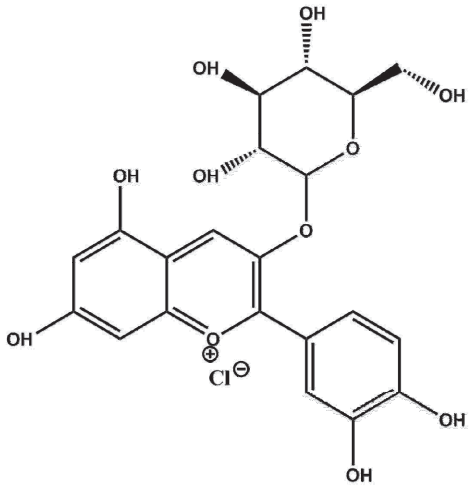

Figure 8. 2D structure of the chloride salt of cyanidin 3-*O*-glucopyranoside, also known as cyanidin 3-*O*-glucoside.

The composition of the gut microbiota can also affect health indirectly by influencing one's state of mind, including happiness, sadness and depression [79]. That is, stress and anxiety can change the function of the gut and its microbiota. Several Bacteria in the gut can influence neural development, complex behaviors and nociception. So, the concept of "state of the gut" has been proposed to take the place of, or augment the concept of state of mind. So, changing the gut microbiota through therapeutic intervention may eventually be used to treat gastrointestinal and affective disorders [79].

2.5 The role of gut microbiome dysbiosis in causing diseases

2.5.1 The importance of biodiversity

Many chronic diseases have been associated with a less diverse microbiome (or microbiota) [67]. This is consistent with the science of ecology. That is, diversity and redundancy are important in maintaining a healthy ecosystem. As in TQM, feedback and communication are essential. So, Bacteria can communicate with each other – mostly through small molecules, peptides and proteins. This communication helps to control the appropriate bacterial movement, gene expression, and

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community structures that are needed for good health. A lack of diversity and robustness can make one more susceptible to diseases, including infection by *Clostridum difficile*. When there is plenty of diversity and redundancy in a robust microbiome, *C. difficile* levels are properly controlled. When diversity and redundancy decreases too much (as can happen after exposure to antibiotics), there is not enough competition to prevent excess expansion of *C. difficile*. This can lead to serious consequences, especially if there are strains of antibiotic-resistant *C. difficile*. There is even a missing microbiota hypothesis that warns that people living in modern industrialized societies often don't sustain a diverse gut microbiome for more reasons than just hygiene. Some of the key Bacteria that provide needed diversity can be lost for other reasons. For example, people who consume fast food, red meat and sugar as well as saturated and *trans* fats, while not consuming fresh fruits and vegetables with their dietary fiber may not properly maintain healthy gut Bacteria that are needed [67].

When the gut microbiome is healthy and functioning properly, there is a tight epithelial junction that forms a colonic, ileal, jejunal and gastric barrier [82, 110]. The intestinal lumen acts as a barrier against Bacteria and food antigens. However, gut microbiome dysbiosis can disrupt the structure and function of the barrier. This can cause pro-inflammatory biochemicals like lipopolysaccharides (LPS) to leak out of the gut and cause smoldering (low-grade) inflammation. The LPS can enter circulation through intracellular transport after binding to TLR4 that can be accesses through the impaired integrity of the tight junction (TJ). This can lead to metabolic diseases and endotoxemia. High-fat diets can also change the TJ, which increases the permeability of the TJ to LPS. Obesity can cause the ratio of gram-negative Bacteroidetes to gram-positive Firmicutes to decrease. In healthy, non-obese people, there is still a low concentration of LPS circulating in the blood. This is needed to help modulate the immune system. However, when the LPS concentration becomes too high, the excess LPS can form a complex with proteins that bind to it and the CD14 co-receptor that is recognized by TLR4. In addition, saturated fatty acids can bind to TLR4. Activated TLR4 triggers intracellular signaling through the protein complex called NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). It is a transcription factor that plays an essential role in the inflammatory response to infection. It is also the master regulator of pro-inflammatory cytokines and chemokines. However, dysegulation of NF-κB can lead to improper development of the neuroendocrine immune system, as well as the emergence of autoimmune diseases, septic shock, inflammatory diseases and cancer [82]. At the same

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time, a dysfunctional gut microbiome can produce other pro-inflammatory toxins, such as indoxyl sulfate, *p*-cresol sulfate and trimethylamine-*N*-oxide that can cause chronic kidney disease [82].

2.5.2 The gut microbiome and inflammation

When the body's rhythms are altered, it can lead to stress, chronic inflammation and dysbiosis in the gut microbiome [115]. When mice were exposed to a reverse light-dark cycle (circadian rhythm disrupted mice), their gut microbiomes were altered in the group that was fed a high-fat diet. The mice fed standard rodent chow did not experience such changes [115].

Inflammation can cause oxidative stress and the production of reactive oxygen and nitrogen species (RONS) by activated immune cells [82]. However, most of the scientific literature uses the term ROS (reactive oxygen species) instead of RONS. Regardless of what they are called, ROS and RONS can initiate polyol and hexosamine flux, help form advanced glycation end products (AGEs), activate protein kinase C (PKC) and increase vascular inflammation mediated by NF-κB. This can lead to chronic kidney disease and cardiovascular diseases. Moreover, obese, hypertrophic and/or insulin resistant adipocytes release saturated fatty acids. They activate the TLR-4/NF-κB signaling pathways on macrophages in adipose tissue. They subsequently release TNF-α, which binds to its cognitive receptors on the adipocytes. This further stimulates fatty acid release and starts a vicious cycle of increasing inflammation and insulin resistance. However, unsaturated and omega-3 fats are anti-inflammatory and can help prevent this [82].

2.5.3 The benefits of vegan and vegetarian diets

As discussed in the previous section, vegetarian and vegan diets can prevent many diseases. One of the reasons for this is that the composition of the gut microbiota changes. So, a high-fat omnivore diet can cause dysbiosis in the gut microbiota, which can lead to many diseases. In addition, over-consumption of red meat can cause some of the gut bacteria to produce trimethylamine (TMA), as they metabolize choline, phosphatidylcholine (PC, also known as lecithin) and/or *L*-carnitine [82, 116, 117]. TMA is transported to the liver, where it is converted to TMAO. The structures of *L*-carnitine, TMA, TMAO, choline and PC are shown in Figures 9-12. Dietary sources of choline, PC and *L*-carnitine include lecithin dietary supplements, red meat, liver, egg yolk and dairy

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products with a relatively high fat content. TMAO is both a cardio- and renal toxin and a biomarker for these diseases [82, 116, 117]. It increases the expression of scavenger receptor proteins (CD-36 and SR-A) that cause atherosclerosis to develop [82]. TMAO also causes macrophage foam cells to develop and aortic root legions to occur. It also decreases the transport of cholesterol in peripheral macrophages [82]. So, not everybody is equally susceptible to TMAO toxicity. People who have a kidney disease or are highly susceptible to cardiovascular disease may want to restrict their consumption of red meat and egg yolks, as well as avoid dietary supplements containing lecithin [118].

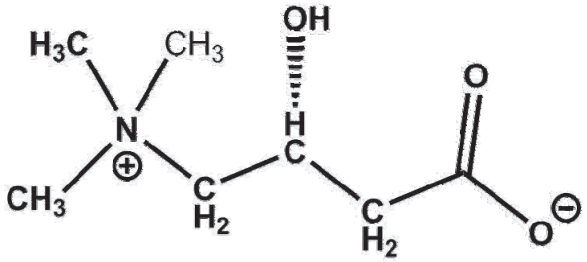

Figure 9. 2D structure of *L*-carnitine, or (3R)-3-hydroxy-4-(trimethylazaniumyl)butanoate

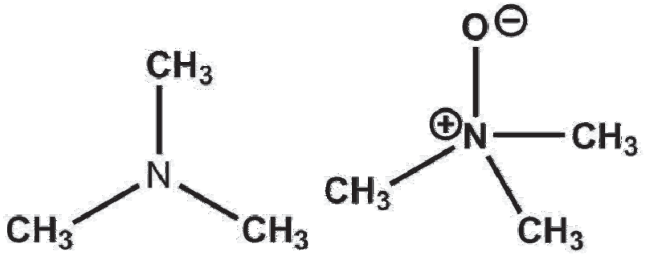

Figure 10. 2D structures of TMA, or trimethylamine (left) and trimethylamine-*N*-oxide, or TMAO (right)

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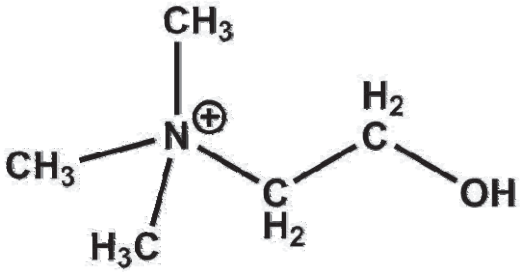

Figure 11. 2D structure of choline

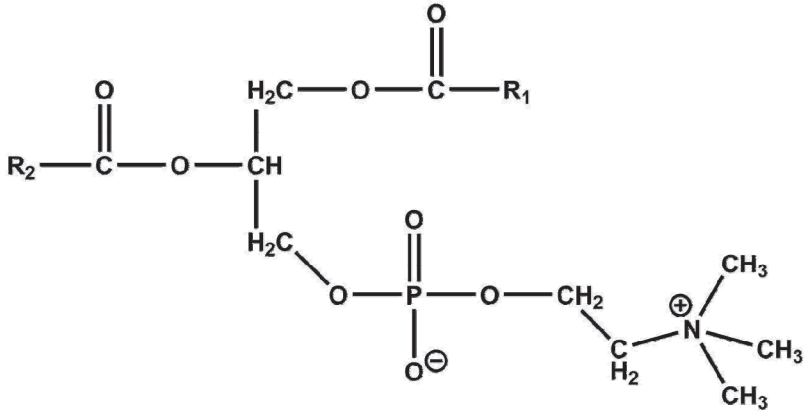

Figure 12. Generalized 2D structure of phosphatidylcholine (PC), also known as lecithin, where R_1 and R_2 are long hydrocarbon chains, such as $CH_3(CH_2)_{14}$ (in palmitoyl, or $CH_3(CH_2)_{14}COO^-$)

Systems thinking was used in a multi-disciplinary effort to show which Bacteria in the gut are responsible for producing TMAO [82]. A combination of shotgun gene sequencing, bioinformatics and microbiology was used. Shotgun gene sequencing of bacterial genes in the gut produced a large data set in a top-down approach. The data produced new, testable hypotheses. These hypotheses were tested using classical microbiological and biochemical methods in a bottom-up approach. This approach used high-throughput screening methods and data mining from datasets that are available to the public. The biochemical pathways and Bacteria that produced TMAO were identified. Choline metabolites and L-carnitine (which is in red meat) were identified as substrates for the biosynthesis of

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TMAO. Gene clusters in the sulfate-reducing bacteria, *Desulfovibrio*, were identified and found to be responsible for the anaerobic degradation of choline to produce TMA. Human gut bacteria in the genera *Acinetobacter* and *Serratia* were found to be able to metabolize *N*-carnitine to produce TMA. Reference genomes from the Human Microbiome Project were searched. Gene clusters that encoded enzymes that catalyzed the production of malate and succinate were identified. This linked *L*-carnitine metabolism with the tricarboxylic acid cycle and the transport of *L*-carnitine. Finally, another microbial pathway was found that was able to convert an intermediate in *L*-carnitine metabolism (γ-butyrobetaine) into TMA. In addition, a diet enriched in *L*-carnitine changed the composition of the gut microbiome in mice. In contrast, when human vegans and vegetarians were fed *L*-carnitine they produced less TMA than the control group. Bacteria in the *Lachnospira* genus were found to be more abundant in the feces of vegetarians and vegans. An abundance of *Lachnospira* species was negatively correlated with TMA production. So, a combination of top-down and bottom-up approaches was able to shed light on how certain Bacteria can produce TMA [82].

2.5.4 The gut microbiome and cardiovascular diseases

In addition, chronic infection by pathogenic Bacteria and viruses can cause cardiovascular diseases [12]. This includes *Helicobacter pylori*, *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*, hepatitis A virus, and various herpesviridae such as cytomegaloviruses. Chronic infections can help cause atherosclerosis directly by invading vascular cells or leukocytes or indirectly by causing inflammation. Some infections can even have systemic effects that help cause atherosclerosis. For example, *C. pneumonia* infection can stimulate the production of pro-inflammatory cytokines such as IL-6 and amyloid A, while decreasing the cytoprotective HDL protein paraoxonase. Gut Bacteria and the immune system affect each other, with serious consequences for human health. For example, infection by pathogenic Bacteria can initiate signaling by inflammasomes, which are protein complexes that recruit caspases and trigger the formation of pro-inflammatory cytokines (IL-1β and IL-18) and RONS. Consumption of too many calories (overnutrition) can cause smoldering inflammation and metabolic syndrome. This is mediated by nutrients that activate TLRs and NOD-like receptors (NLRs), which have been implicated in atherosclerosis. It should be noted that diet affects not only the gut microbiome, but also the structure of the gut mucosa. This can lead to an

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increase in the permeability of the intestines, which can cause systemic inflammation by increasing the release of pro-inflammatory bacterial products into the bloodstream. The gut microbiome can also affect bile acid and systemic lipid metabolism. They can produce the enzyme bile salt hydrolase (BSH), which catalyzes the deconjugation of tauro-muricholic acid (T-MCA). The presence of bacterial strains that express BSH can help cause weight loss as well as lower serum LDL-cholesterol and triglycerides. Moreover, part of the health benefit of bariatric surgery is altered bile metabolism. Finally, consumption of healthy dietary phenolic compounds found in colorful fruits and vegetables can lead to a production of protocatechuic acid by Bacteria, which can reduce the incidence of atherosclerosis [12].

2.5.5 The role of the gut microbiome in stress, anxiety and neurodegenerative diseases

Smoldering inflammation can also lead to stress, anxiety and neurodegenerative diseases. Bacteria in the gut can contribute to this. There is an undeniable link between the brain, the gut and the immune system. When the cerebral cortex decides that an individual is under a stressful situation, it can give that person a ‘nervous stomach’. This can also lead to an imbalanced immune system. So, the gut microbiota can interact with the neuroendocrine system to play important roles in anxiety, depression, IBS, autism spectrum disorders, Parkinson’s disease and multiple sclerosis, many of which are influenced by peripheral immune system homeostasis [21, 64, 65]. The gut microbiota is also important in the development of a healthy, well-balanced neuroendocrine system. When the hypothalamic-pituitary-adrenal (HPA) axis is stimulated, it can change the composition of the gut microbiome. Depression has been associated with a dysreg0075lation in the HPA axis. Resolution of episodes of depression has been associated with normalizing the HPA axis. Microbiota can also activate neuronal stress circuits by activating pathways in the vagal nerve. A combination of exposure to antibiotics and stress caused a decrease in the abundance of *Clostridium* species as well as an increase in Verrucobacteria, Enterobacteria, and Lactobacillus species. Stress can lead to an increase in the expression of corticotropin-releasing hormone (CRH), increased activity of the proto-oncogene c-Fos, higher concentrations of serotonin in the blood and increased colon motility. Stress can be reduced by increasing signaling by gamma aminobutyric acid (GABA), since GABA is an inhibitory neurotransmitter in the brain. Proper serotonin signaling is also important for good brain function. So, it

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is important to remember that over 90% of human serotonin is found in the intestines. Bacterial infections can increase stress and anxiety by decreasing the expression of brain-derived neurotrophic factor (BDNF). Moreover, a high-fat diet increased anxiety and depressive behavior in mice and rats. There are several humoral, neural, and cellular signaling pathways that link microbiota to neuroendocrine and brain function. This link is especially important in autism spectrum disorders (ASD) [64, 65].

Systems thinking can help us understand ASD better, since it teaches that the whole is more than the sum of its parts [67]. When all of our parts (including viruses, Bacteria, Archaea and Eukaryotic cells) function in a harmonious balance that flows with exquisite rhythms, we can maintain our health. When this harmony and synchrony are disrupted, it can lead to ASD and other diseases. For example, the microbiomes of people with ASD are often different from healthy individuals. The levels of *Clostridium bolteae* and *Clostridium* clusters I and XI, as well as *Desulfovibrio*, *Sutterela* and *Ruminococcus* species were higher in people with ASD, when compared to controls [67]. Others have found significant increases in *C. histolyticum* and several species of *Clostridium*, as well as *Bacteroides*, *Prophyromonas*, *Prevotella*, *Pseudomonas*, *Betaproteobactera*, *Aeromonas* and *Enterobacteria* [62]. There have also been reported decreases in *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Staphylococcus* and *Coprococcus* species. However, ASD is not a single disease, but encompasses a wide spectrum of conditions. So, some studies have found a decrease in *Prevotella* and *Bifidobacterium* species [67].

The immune system is another part of one's body that is important for good health. It is so closely linked to the neuroendocrine system that it has been called the immune-neuroendocrine system [42]. So, when authors describe the effects imbalances in the gut microbiome and immune-neuroendocrine system on ASD, they are really describing an imbalance in the gut-immune-neuroendocrine system [119]. This imbalance in the GIT is evident through the higher incidence of acid reflux, constipation and diarrhea in patients who have ASD [78]. In addition, if a mother has an autoimmune disease, some of her IgG antibodies can be transferred to her fetus, making it more susceptible to ASD. Antibiotic treatment to the mother or neonate in the perinatal period, as well as sepsis or infection by pathogenic Bacteria can also increase the baby's susceptibility to ASD [78].

As mentioned previously, an imbalance in the gut microbiome can disrupt the structure and function of the tight epithelial junction that forms a colonic, ileal, jejunal and gastric barrier [82, 110]. There are also reports that there is increased permeability or leaky intestines, as well as an

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imbalance in the immune systems in many patients who have ASD [119]. This includes the peripheral blood and CNS. There is also a decrease in the levels of $CD4^+$ and $CD8^+$ lymphocytes, as well as albumin, gamma globulin and immunoglobulins (IgG, IgG2 and IgG4). Also, the function of the blood-brain barrier is impaired in ASD [119].

Cross-talk between the gut microbiome and the immune-neuroendocrine system can inappropriately over-activate the immune system when one is suffering from an imbalance [120]. When there are too many potentially pathogenic Bacteria in the gut, bacterial toxins like LPS and metabolites such as propionic acid can cause systemic and cellular damage. This can lead to systemic, smoldering inflammation and damage to neurons. When cells from both the innate and adaptive immune system are over-activated, the concentrations of tumor necrosis factor-α (TNF-α) and IL-1 can increase. This, in turn, can lead to inflammatory responses and over-activation of glial cells in the brain. This is consistent with the observation that microglia (the immune cells of the brain) are important mediators of neuroinflammation in ASD [120].

However, there is much more to learn. For example, even though exposure to antibiotics may increase a child's susceptibility to ASD, there is at least one case in which antibiotics seemed to cure ASD [121]. In contrast to other studies that seemed to show a positive relationship between exposure to antibiotics and the risk of developing ASD, some studies showed an improvement in ASD symptoms [121, 122]. Moreover, antibiotics are given routinely to treat symptoms of ASD that are associated with Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections (PANDAS), Pediatric Acute-onset Neuropsychiatric Syndrome (PANS), and chronic Lyme disease [120, 123, 124]. Those antibiotics target pathogenic Bacteria, such as *Streptococcus* and *Borrelia* [120]. However, antibiotics are not usually given to treat other groups of ASD patients if the only goal is to relieve its symptoms and not to clear up a potentially dangerous bacterial infection [120]. Still, vancomycin was used in one clinical trial to manage symptoms of ASD, since it does target just a few strains of Bacteria [120, 125].

Since the effect of antibiotics on ASD is not clear, several physicians, researchers and parents of children with ASD discussed how to design clinical trials to study this [126]. They used one of the pillars of P4 medicine, prediction, to set one of the most important goals. They want to be able to predict which groups of patients would be most likely to benefit from treatment for ASD that would use a specific antibiotic to target

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specific Bacteria. They also discussed using appropriate biomarkers for ASD to monitor the effectiveness of new drugs in clinical trials [126].

ASD is not the only neurodegenerative disease that can be affected by an imbalance in the gut microbiome – Parkinson's disease (PD) is, too [77]. It is the second most common neurodegenerative disorder, affecting 1-2% of all people over 65 years of age. Midbrain dopaminergic neurons are lost. This causes characteristic motor deficits, upon which the clinical diagnosis is currently based. In addition, the protein α-synuclein can form neurotoxic aggregates. There are also several non-motor symptoms (NMS) that occur. Some of them have a greater impact on the quality of life of patients who have PD [77]. Interdisciplinary teams are working on ways to detect early signs of PD, including facial recognition software [127-129]. This could be important because early detection of PD could lead to physicians recommending changes in diet that could help prevent PD from developing any further or even stopping it. This could include recommendations on consuming more sources of dietary fiber, including fresh fruits and vegetables. As mentioned previously, dietary fiber can help improve balance and biodiversity in the gut microbiome. However, it's important to remember one of the hallmarks of systems thinking – avoid dogma. That is, not all fresh fruits are healthy for everybody. Fruits in the Annonaceae family (graviola, soursop, the pawpaw and others) can cause an atypical form of PD when over-consumed [130-132].

One early feature of PD is the appearance of α-synuclein aggregates in the submucosal and myenteric plexuses of the ENS, prior to entering the brain through the vagus nerve [77]. As PD progresses, the gut microbiome becomes more imbalanced, as evidenced by increased intestinal permeability, glial dysregulation in the ENS, more frequent constipation and impaired gastric emptying, as well as infection by *Helicobacter pylori* and an overgrowth of Bacteria in the small intestine. At the same time, the composition of the gut microbiome changes in PD patients. The changes are related to the clinical phenotype. Bacteria that are associated with anti-inflammatory properties tend to be lower in people who have PD. This includes Bacteria from the genera *Blautia*, *Coprococcus* and *Roseburia* that can produce butyrate. At the same time, pro-inflammatory Bacteria in the genus *Ralstonia* are more abundant in people with PD. Gut microbiome dysbiosis and symptoms of PD might be improved in some cases by the consumption of probiotics, such as *Lactobacillus casei* Shirota. In addition, consumption of a Mediterranean diet is recommended for people who are in the early stages of PD, since it can increase biodiversity in the gut microbiome. In contrast, consuming a high-fat diet can cause both intestinal and systemic inflammation by altering the gut

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microbiota composition and increasing intestinal permeability. On the other hand, consuming the Bacteria *Akkermansia muciniphila* may be able to reverse the imbalance in the gut microbiome caused by consuming a high-fat diet [77].

It is also possible that gut microbiome dysbiosis could help cause an especially deadly form of brain cancer, glioblastoma. This is because Bacteria in the gut produce muramyl dipeptide (MDP), which decreases the replication of the human cytomegalovirus (CMV) [133] that is linked to glioblastoma [134]. So, a healthy, well-balanced and diverse gut microbiome may help prevent the replication and spread of CMV and the diseases it can cause – especially in immunocompromised patients and congenitally-infected newborns [133].

The microbiome also affects the brain through epigenetic mechanisms [135]. Biochemicals produced by gut Bacteria can affect chromatin plasticity within their host's brain. This then leads to changes in gene transcription in neurons that can change the behavior of the host. Moreover, the microbiota is an important mediator of interactions between genes and the environment. That is, the microbiota is an epigenetic entity. So, the fields of neuro-epigenetics and microbiology are converging at many levels and leading to important interdisciplinary studies to better understand this interaction [135].

2.5.6 The importance of healthy microbiota in obtaining an effective response to vaccines

Our response to vaccines is improved by a healthy microbiome, which acts as our natural adjuvant to optimize our immune response [136]. Moreover, a healthy microbiome helps to ensure that our immune system is properly regulated at both a steady state and when challenged by infections and/or cancer. It helps to control almost all aspects of our immune system, from hematopoiesis to the function of lymphocytes. Commensal Bacteria lower the threshold required to respond to infection, which enables a rapid and efficient response to pathogenic infectious by microbes. A healthy microbiome helps to control the development of the immune system, the level of innate immune responses and the induction of effector and regulatory responses. It exerts its control by producing metabolites, such as butyric acid (or butyrate at a physiological pH), by activating inflammasomes and by acting on epithelial cells. It ferments polysaccharides in dietary fiber and other parts of plants to produce short chain fatty acids (SCFAs), including butyrate. These SCFAs promote the differentiation of B cells into plasma cells. A healthy microbiome also

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exerts systemic control of the immune system since it constitutively activates the response of our antibodies to the gut microbiome, thus preventing it from spreading throughout the body. Moreover, some of the antibodies against our commensal Bacteria can cross-react with pathogenic viruses, such as HIV. The pre-existing immune response to our microbiome helps to control our response to vaccines – especially those that use 'live' attenuated viruses or organisms. Our microbiome also increases our response to the seasonal trivalent influenza vaccine and the polio vaccine. On the other hand, a dysfunctional gut microbiome can lead to vaccine failure. For example, oral vaccines for Rotavirus, Poliomyelitis, *Vibrio cholerae* and *Shigella* that are given to children in countries that have a relatively low average income fail to protect as many children compared to higher-income countries. In addition, malnutrition and chronic exposure to pathogens can lead to a 'leaky gut' and the systemic leakage of microbes and subsequent inflammation. This increased inflammatory tone can adversely affect one's immune response to vaccines and prevent the establishment of a healthy memory pool [136].

In addition, "systems vaccinology has emerged as a multidisciplinary field that combines modern high-throughput technologies, computational modeling and conventional immunology to provide a holistic view of the molecular mechanisms of vaccine-induced immunity" [137].

The goal is to determine all of the interactions and metabolic pathways that link commensal microbes with their human host. This is leading to new vaccines for yellow fever, influenza, meningococcal infections and malaria. In the process, there are early biomarkers in the immune system that predict the responses of CD8+ T cells and antibodies to vaccines [137].

The nasal microbiome also affects a person's response to the live attenuated influenza vaccine that is administered intra-nasally [138]. This vaccine changes the nasal microbiome to allow operational taxonomic units (OTUs) that are less abundant to establish a new community niche. So, the abundance of several bacterial species is linked to this vaccine. Two of them (*Prevotella melaninogenica* and *Veillonella dispar*) were already known to be related to respiratory health. Hopefully, systems vaccinology will lead to new and better vaccines, as well as more personalized and effective vaccines [138].

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2.5.7 The importance of oral health and preventing periodontal disease

One of the worst examples of how reductionist thinking can contribute to misunderstanding and serious health problems is the way that dental health is frequently ignored. Most physicians simply know that the teeth are located in the one of the filthiest parts of the body (the mouth) and are someone else's business (dentists). Moreover, many health insurance plans (including Medicare) don't cover dental procedures or even routine preventative dental care. The idea is that you can still eat even if you don't have teeth. However, such thinking has led to an epidemic of periodontal disease and malnutrition – especially in the elderly. If this only meant that many people suffer some pain that can be treated with a local anesthetic, it would not be so bad. But it also means that many people are exposed to chronic bacterial infections and the attendant smoldering inflammation that can lead to Crohn's disease, diabetes, atherosclerosis, cardiovascular diseases, many types of cancer and neurodegenerative diseases [139-143].

Periodontal disease was once thought to be due primarily to a triad of oral anaerobic bacteria, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* [140]. However, metagenomics and mechanistic analyses have produced a new systems-based model for the pathogenesis of periodontal disease. It states that there are other important Bacteria that interact with the classical triad. In this model, periodontal disease is not caused by just a few pathogenic Bacteria. It is caused by a synergistic interaction between many Bacteria that lead to an imbalance or dysbiosis. This changes the ecologically balanced biofilm that is associated with the healthy homeostasis that exists in periodontal tissue [140].

As a result, Bacteria that infect the mouths of people with periodontal disease can enter the bloodstream and circulate throughout the body. They can enter the heart and other parts of the body and cause diseases. So, there is a clear interaction between atherosclerotic vascular disease (ASVD) and periodontal disease [139]. Periodontal disease is an all too common chronic inflammatory condition that damages the structures that support the teeth. It affects the epithelial, connective and osseous (bone) tissues that surround the teeth. Bacteria that were attached to the teeth along the gingival margin before the onset of periodontal disease can grow and form biofilms that protect them, but harm the patient. At the same time, these Bacteria can induce an unfavorable immune response in adjacent gingival tissues. If the biofilm and inflammation migrate along the surface of the root, they can affect the structure that supports the tooth and become periodontitis [139].

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ASVD causes more morbidity and mortality in industrialized countries than any other disease and is the leading cause of death in the entire world [139]. In the USA alone, ASVD causes about 700 000 deaths each year (mostly from heart disease and stroke). Several risk factors have been identified, including age, diabetes, smoking tobacco, body mass index, treated and untreated systolic blood pressure, as well as the concentration of cholesterol, high-density lipoproteins (HDL), low density lipoproteins (LDL) and triacylglycerides (more commonly known as triglycerides) in the blood. However, these risk factors account for only about half of all the cases of ASVD. Moreover, about 40% of all cases of ASVD occur in people who have lower concentrations of total cholesterol in the blood than the average population [139]. Much of this could be due to people who have periodontal disease, tooth decay and often no teeth at all. Often, such people can no longer chew red meat and have little appetite due to chronic pain. So, their dietary intake of cholesterol may be very low.

There is much evidence to support the hypothesis that systemic smoldering inflammation caused by periodontal disease can lead to ASVD [139]. First, the concentration of C-reactive protein in the blood has been associated with the 10-year risk of ASVD. This supports the idea that there is systemic inflammation long before the ASVD emerges. Moreover, ASVD and periodontal disease are linked by the systemic release of specific pro- and anti-inflammatory cytokines, small signal molecules and enzymes. They affect the onset and progression of ASVD. Also, periodontal pathogens have been found within atherosclerotic lesions and thrombi isolated from patients who have suffered a myocardial infarction. At the same time, a high concentration of LDL is a risk factor for both periodontal disease and ASVD. On the other hand, statins that are used to treat ASVD can also help to prevent or reduce periodontal disease [139].

However, it is important to use systems thinking and realize that oral biofilms can be quite healthy in people who don't have periodontal disease [143]. One should remember that oral biofilms have evolved symbiotically within a Paleolithic, hunter–gatherer setting. Moreover, the Paleolithic diet was mostly acidic, and acted as a selective force for much of the evolution of the stomatognathic system (teeth, jaws and soft tissues that are associated with them). The relationship between saliva, biofilm and teeth has been called the 'sialo–microbial–dental complex'. It provides benefits to oral health, while offering an important contrast to the old dental paradigm that only associated oral biofilms (plaque) with disease (dental caries). This new paradigm emphasizes that oral biofilms are essential for maintaining the mineral integrity of teeth. Oral biofilms provide physical protection from dietary acids. They also work with bacterial metabolic

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acids to keep the pH of the biofilm slightly acidic. At the same time, a nearly neutral environment is established by chemical interactions mediated by the saliva within the biofilm. Such pH fluctuations lead to a cyclic demineralization and re-mineralization of teeth, which is needed for teeth to mature. However, the ecology of biofilms has changed since the advent of farming and the industrial revolution. These led to a huge increase in the amounts of carbohydrates, refined sugars and acidic drinks that are consumed. This reduced the biodiversity of the biofilm and proliferation of Bacteria that generate SCFAs. This can cause mineral loss and subsequently dental caries (cavities). In addition, consuming acidic beverages can damage or destroy the protective properties of the biofilm, leading to erosion. Erosion and dental caries are modern diseases that are due to an imbalance within the oral biofilm that causes teeth to demineralize [143].

One sign of systems thinking helping with this problem is that transplants of a healthy oral microbiome are being considered as a therapy for periodontitis [145]. This is in contrast to reductionist thinking, in which only a single drug or bioactive substance should be given to solve the root cause of a problematic disease. Before a microbiome transplant could be developed, it was necessary to compare the oral microbiomes of people with good oral health and those who had either periodontal disease or tooth loss. This comparison showed that (unlike the gut microbiome), the oral microbiomes of people with periodontal disease or tooth loss (edentualism) were more diverse than those of healthy people. This is consistent with the rejection of dogma in systems thinking. So, a clinical study was done in which systemically healthy people who had periodontitis, established caries or edentualism were studied, along with people with good oral health (as controls). Their oral plaques (biofilms) were treated with normal saline, followed by 16 mM sodium hypochlorite (NaOCl, or the active ingredient in bleach). Next, they were neutralized by an ascorbate buffer (pH balanced mixture of ascorbic acid and sodium ascorbate) and plated on blood agar. Analysis of rRNA gene abundance showed that the oral microbiomes of people with periodontal disease had more biodiversity. However, people with good oral health did have a higher amount of ten microbial species in their mouths than people with periodontal disease or edentualism [145]. Based on this established difference, it now makes sense to try to develop a healthy oral microbiome transplant.

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2.6 Factors affecting the composition of the microbiome

Birth, development, diet, aging and diseases can affect the composition of the microbiome. As mentioned previously, the womb is not sterile. The placental microbiome resembles the adult oral microbiome, but its exact composition depends on the health of the mother [32]. It can be affected by the maternal history of infections – even before conception, as well as exposure to antibiotics and preterm birth [32]. Consuming a probiotic during the perinatal period had a positive impact on the composition of the microbiota in breast milk [146]. It increased the amounts of both Lactobacilli and Bifidobacteria in the colostrum and mature milk. The probiotic contained 900 billion viable lyophilized Bacteria of four strains of lactobacilli (*L. acidophilus* DSM24735, *L. plantarum* DSM 24730, *L. paracasei* DSM 24733, *L. del-brueckii* subspecies *bulgaricus* DSM 24734), three strains of Bifidobacteria (*B. longum* DSM 24736, *B. breve* DSM 24732, *B. infantis* DSM 24737), and one strain of *Streptococcus thermophilus* (DSM 24731) [146].

Human milk is the best source of nutrients for babies [147]. It contains not just essential nutrients, but also prebiotics, immunoregulatory proteins and beneficial Bacteria. This includes the most abundant genera: *Staphylococcus*, *Streptococcus*, *Serratia* and *Corynebacteria*, and eight other genera with a relative abundance >1%. Recently, one group of researchers did a network analysis of human milk bacterial communities. They found that the milk microbiome consists of two disconnected sub-networks. One sub-network can be described as a fully connected complete graph. It has seven genera as nodes and all of its pair-wise interactions among the Bacteria as edges. The interactions were shown to be either facilitative or cooperative. In contrast, the interactions in the other sub-network of eight nodes are mixed but mostly cooperative. Still, they found that non-cooperative nodes in the second sub-network are mutually cooperative *Staphylococcus* and *Corynebacterium* genera that include some opportunistic pathogens. This was described as a potentially 'evil' alliance. It can be inhibited by the remaining nodes that cooperate with each other in the second sub-network. They postulated that the 'confrontation' between the 'evil' alliance and 'benign' alliance and the shifting balance between them may be responsible for the occasional dysbiosis of the milk's microbiome that can lead to mastitis. Still, in women whose milk has a healthy microbiome, the pioneering colonizers such as *Bifidobacterium longum* carry several gene clusters that enable babies to metabolize human milk oligosaccharides (HMOs). This allows infants to digest breast milk [147].

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Once a baby is born, its gut is colonized by Bacteria that originate either from the mother's intestine and vagina or from other environmental sources in a C-section [148, 149]. The first Bacteria to colonize the gut are mostly facultative anaerobes, such as *Enterobacteria*. They create a favorable niche for more strictly anaerobic Bacteria (*Bifidobacterium*, *Clostridium* and *Bacteroides*) that will dominate the microbiome within a few weeks. During the first three years of life there are significant dynamic changes due to weaning, introducing solid food, possible antibiotic use, and natural modifications in the baby's physiology. The absence of a stable, healthy microbiome increases an infant's susceptibility to infections in the gut. At about five years of age, the gut microbial communities should have a richness and diversity that is characteristic of a healthy adult gut, although this could happen as early as 2-3 years of age [148, 149].

The composition of a baby or young child can also be affected in one way by synbiotic administration of a mixture of prebiotic and probiotic Bacteria or in another way by antibiotics [149]. For example, when probiotic *Bifidobacterium animalis* subspecies, *lactis* BB-12, *Streptococcus thermophiles* and *Lactobacillus bulgaricus* were administered together with the prebiotic carbohydrate inulin, the stability of *Actinobacteria* increased. In contrast, antibiotics decreased the abundance of *Clostridium* cluster XIVa. The bacterial diversity in children one to five years of age did not increase and remained lower than in adults. *Actinobacteria*, *Bacilli* and *Clostridium* cluster IV remained at child-like levels, while some other Bacteria were converted to adult-like profiles. Microbiota stability increased, with Bacteroidetes being the main contributor. As the children increased in age, a more diverse set of Bacteria emerged. The number of highly abundant genus-level taxa increased from about 18 to 25. This included several Bacteria that produce butyrate. However, if the composition of the microbiota is not healthy, it can lead to autism, obesity, Crohn´s disease, celiac disease and food allergies. If the gut microbiome is not diverse enough and if there are not enough Bacteria that produce butyrate, severe atopic eczema in young children can emerge. Moreover, reduced bacterial diversity and a low ratio of Bacteroidetes to Firmicutes have been associated with obesity. Also, a dysfunctional microbiome in early life can harm one's health later in life. For example, low levels of Bifidobacteria in infancy may predispose a child to obesity by the time they are 7 years of age. In healthy adults, Firmicutes and Bacteroidetes, constitute over 90% of the microbiota [149]. Still, there is much diversity. There are at least 40 000 bacterial strains in 1800 genera in about 100 trillion cells that have a total mass of about 1–2 kg in adults [15]. The

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adult human gut contains about 3.3 million prokaryotic genes (sometimes called non-human genes), compared to only 23 285 protein-coding genes in human eukaryotic cells [15].

Before an adult can have a healthy, diverse gut microbiome and brain, he or she must have a healthy fetal, neonatal and infant microbiome [34]. The gut microbiome affects the development of the brain. It is especially important for the formation of synapses that connect neurons and the blood-brain barrier, as well as the proper function of microglia. Many of the metabolites produced by gut Bacteria are important in the development of the young brain. The gut microbiome is also important for priming the innate immune system in both the peripheral and central nervous systems. Breastfeeding can help establish a healthy gut microbiome. The mother's breast milk contains oligosaccharides that stimulate the growth of beneficial communities of Bacteria, including *Bifidobacterium* species. This leads to better cognitive development of the baby. In contrast, prenatal stress can cause dysbiosis in the baby's gut microbiome. Moreover, children with neurodevelopment disorders, such as ASD often have an unbalanced gut microbiome that can lead to atypical patterns of connectivity between the cells in the brain. Moreover, exposure to antibiotics during infancy can disrupt the normal, healthy formation of the gut microbiome [34].

Hygiene is another factor that can affect the gut microbiome and the function of the immune system [150]. During early development, the immune system selectively enhances the growth of certain Bacteria in a process that has been called 'farming'. The portions of the gut microbiome that are farmed influence the development and function of the brain. At the same time, the gut microbiota influences the immune system. This is essential, because inflammation during pregnancy needs to be controlled or limited. One of the factors not yet discussed that can affect the baby's and infant's microbiome is moving from a rural to an urban community. Infants are exposed to more Bacteria in rural environments, while urban environments contain more neurotoxic and immunotoxic industrial chemicals and pollutants. That is, an urban environment can deprive an infant of the immunoregulatory Bacteria with which humans co-evolved, while encouraging exposure to non- immunoregulatory organisms, associated with more recently evolved 'crowd' infections that can't persist in hunter-gather groups. Crowd infections are not immunoregulatory and don't protect people from chronic inflammation and diseases that they can cause. So, there is a hygiene hypothesis. It states that our lower exposure to immunoregulatory Bacteria in rural communities is a major cause of the increased prevalence of autoimmune disorders. However, some prefer the

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terms biodiversity hypothesis or Old Friends mechanism, because it is no longer a hypothesis with limited experimental support and has little to do with hygiene. Instead, it is linked to broad changes in lifestyle that occur in urban communities [150]. Diet can also affect the gut microbiome and one's susceptibility to many diseases.

There are about 500 g of contents in the average adult colon, most of which are Bacteria. About 100 g/day is excreted as stool [151]. Diet and lifestyle can affect the gut microbiota and the health of adults [151]. As mentioned already, consuming red meat and other dietary sources of saturated fatty acids can lead to dysbiosis in the gut microbiome, while unsaturated and omega-3 fats can help prevent it [93]. Furthermore, obese people with an LGC microbiota who switched to a controlled weight-loss diet showed an increase in the diversity of their gut microbiota [47]. It approached that of a HGC community [47]. In addition, consumption of 30 g/days of L-glutamine for 14 days as a dietary supplement reduced the Firmicutes to Bacteroidetes ratio of the gut microbiome in obese adults [152]. This was similar to results obtained from weight loss programs [152]. Thus, diet can have a major impact on the composition of the gut microbiota. For example, changes in carbohydrate intake mostly affect Firmicutes and Actinobacteria. Weight loss diets that involve low carbohydrate consumption can cause a decrease in Firmicutes and Actinobacteria phyla that make butyrate. There are also important changes in the composition of the gut microbiota when one switches between extreme plant-based diets (that contained high levels of fiber and low levels of fat and protein) and animal-based diets (that contained no fiber and had high levels of fat and protein). The abundance of Bacteroidetes (such as *Bacteroides* and *Alistipes* species) and *Bilophila wadsworthia* increased, and several members of the Firmicutes decreased in the animal-based diet. One's long-term dietary habits also affect the composition of the gut microbiota. For example, people with a high proportion of *Prevotella* spp. tend to consume more fiber, while *Bacteroides* species are more abundant in people who consume high amounts of protein and fat. So, within the Bacteroidetes phylum, *Prevotella* species are better able to digest dietary fiber than *Bacteroides* species [47].

The typical western diet provides about 50 g of potentially fermentable substances – mostly dietary fiber [151]. However, the average consumption of dietary fiber is higher in Australians than in the USA. Bacterial growth and fermentation are highest in the proximal colon, where bacterial nutrients are the most abundant. The pH increases from about 5.8 to 7.0-7.5 from the proximal to distal colon. This is due to the progressive depletion of carbohydrates and the absorption of SCFAs that

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are produced by bacterial fermentation. One way that dietary fiber promotes and maintains a healthy bowel is by increasing the mass of digested material which can absorb water. This dilutes toxins, reduces intracolonic pressure, shortens transit time and increases the frequency of defecation. Also, about 5-15 g of protein and 5–10 g of lipid enters the proximal colon daily. About 90% of the approximately 1 g/day of dietary phenolic compounds (like anthocyanins) is not digested or absorbed in the small intestines. Instead, they are metabolized by Bacteria. So, dietary phenolic compounds can significantly affect microbial populations and activities [151].

It is interesting to note that even though long-term dietary patterns tend to produce stable enterotypes, some short-term changes can also occur [153]. For example, the microbiomes of ten people were seen to change changed detectably within 24 hours of starting them on a high-fat/low-fiber or low-fat/high-fiber diet, but the identities of their enterotypes remained stable during the 10-day study. Even though the short-term changes were quick and statistically significant, they were modest. The changes were not large enough to cause a change in the enterotype [153].

In another study, subjects consumed barley kernel-based bread for three days as part of their evening meals [154]. The goals were to see if the dietary fiber in the bread made from barley could improve glucose metabolism and affect the role of the gut microbiome as it enables this improvement. Subjects with the highest ratio of *Prevotella*/*Bacteroides* responded more than those with a lower ratio of these genera. *Prevotella* promoted increased storage of glycogen. This supports the idea that the composition of the gut microbiome may be an important factor in determining one's risk for avoiding or developing metabolic syndrome and diseases that are linked to it [154].

Despite the well-known health benefits of consuming dietary fiber, almost 90% of all the people in the USA don't consume the dietary reference intake amount for fiber (38 g/d for men and 25 g/d for women) [155, 156]. A randomized, double-blind, placebo controlled clinical trial was conducted to determine the effects of having adult men consume either 21 g/d soluble corn fiber or polydextrose for 21 days on the composition of the gut microbiome and its production of fecal fermentation end products [157]. These two sources of dietary fiber changed the composition of the gut microbiome and decreased the formation of fecal putrefaction compounds, including ammonia, phenolic compounds and compounds containing an indole ring [157]. A subsequent whole-genome shotgun 454 pyrosequencing analysis [158] of the fecal samples showed that both soluble corn fiber and polydextrose increased

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the ratio of the amount of Bacteria in the phyla Bacteroidetes to Firmicutes, when compared to placebo (no dietary fiber) [157]. At the same time, there were changes in the abundance of genes coding for enzymes involved in carbohydrate, amino acid, and lipid metabolism, as well as the metabolism of cofactors and vitamins. Dietary fiber also increased the abundance of genes coding for enzymes involved in the degradation of xenobiotics and those involved in energy metabolism as well as cellular processing and signaling [157].

These and most of the other articles that describe the composition of the gut microbiome described the metagenomic analyses of fecal samples [157]. This reflects the bacterial composition of the colon. In contrast, much less is known about the microbial composition of the small intestine because it is accessible only by invasive sampling [149]. It is important since that is where food is digested, nutrients are absorbed and much metabolic signaling occurs. Moreover, Peyer's patches and Paneth cells link the luminal microbiota with the host immune system. The microbiota of the small intestines is less diverse and abundant than the colon. The abundance ranges from about 10^4 bacterial cells per gram in the duodenum to 10^8 cells per gram in the terminal ileum. However, as many as 10^{10} cells can be ingested when fermented foods and beverages are consumed. This temporarily over-crowds the resident Bacteria and influences the host's immune and neuroendocrine systems. Subsequently, ingested Bacteria integrate within the gut microbiome. They can change its composition and become part of the gut's highly dynamic and individualized transient microbiome. For example, probiotic *Lactobacilli* can metabolize plant polymers such as hemi-cellulose and xylans. This can increase the fitness of the gut microbiome [149].

The proper growth and development of a healthy gut microbiome is essential for producing a healthy immune system [151]. Not only diet, but also smoking and a lack of physical activity can adversely affect the gut microbiome. Both can harm the large bowel and lead to colorectal cancer. Smoking not only damages the lungs and heart but also the gut. It can lead to Crohn's disease. Toxic airborne particles can reach the large bowel through mucociliary clearance from the lungs. Air pollution caused by industrial activity can lead to IBS as well. Even stress can be harmful. It can damage colonic motor activity by means of the gut-brain axis, which can decrease the abundance of potentially beneficial *Lactobacillus* species [151].

At the same time, starting a program of moderate exercise increased biodiversity in the Firmicutes phylum in participants of the American Gut Project [159]. Also, the gut microbiome of elite rugby athletes was more

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biodiverse than that of sedentary people. The gut microbiota can serve as an effective biomarker of an athlete's immune function. Finally, consuming probiotics may improve the performance of some athletes [159].

The gut microbiota plays an important role in communications between the immune and neuroendocrine systems [42]. It can stimulate some of the immune cells in the gut. Subsequent cross-talk between the immune cells and enteric neurons has a major impact on human health and disease. The mature neuroendocrine immune system is continuously being stimulated by bacterial cells. It responds by producing lymphocytes and cytokines. As a result, the microbiota produces a chronic state of low-grade activation of the innate immune system. Cytokines that are released create a basal state of immune activation. The adult human gut can contain as much as one gram of LPS. As immune cells are exposed to LPS, mucosal homeostasis is established and maintained. In addition, *Bacterioides fragilis* produces a glycosphingolipid that inhibits the proliferation of natural killer T-cells in the colonic lamina propria and a polysaccharide that induces colonic regulatory T-cells (Tregs). At the same time, the motility of the GIT is regulated by interactions between the ENS, microbiota and macrophages that are near the myenteric plexus and intestinal cells of Cajal. However, when the gut microbiome is unbalanced (dysbiosis), there is less biodiversity and an outgrowth of pathobionts. This can cause inadequate immune function and inflammatory responses due to an imbalance between pro- and anti-inflammatory lymphocytes, GI motility and permeability. This can lead to further changes outside the intestines, including the neuroendocrine system. For example, increased production of cytokines is linked to systemic inflammation and high concentrations of pro- and anti-inflammatory cytokines, as well as cortisol and norepinephrine concentrations in the saliva and blood plasma. Increased concentrations of cytokines and neuropeptides have been linked to disrupted sleep, depression, increased anxiety, impaired long-term memory for emotional stimuli and increased sensitivity to visceral pain. The gut microbiomes of elderly patients in particular can produce more LPS, which can lead to endotoxemia [42].

As in TQM and systems thinking, communication between the gut microbiota and the human eukaryotic cells is bi-directional. That is, human eukaryotic cells send signals and provide important feedback to the gut microbiota [160]. For example, norepinephrine is an important quorum sensing signal in Bacteria such as *E. coli*. So, it activates this intercellular signaling system to regulate bacterial growth and the formation of biofilms. Also, cholic acid, a bile acid, promotes the formation of bacterial

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spores by activating the CspC bile acid receptor on *C. difficile* spores, while chenodeoxycholic acid can bind to this receptor as an antagonist and prevent bacterial growth [160]. The structures of cholic acid and chenodeoxycholic acid are shown in Figures 13 and 14. Note that at a physiological pH of about 7.4 cholic acid and chenodeoxycholic acid exist as their conjugate bases, cholate and chenodeoxycholate.

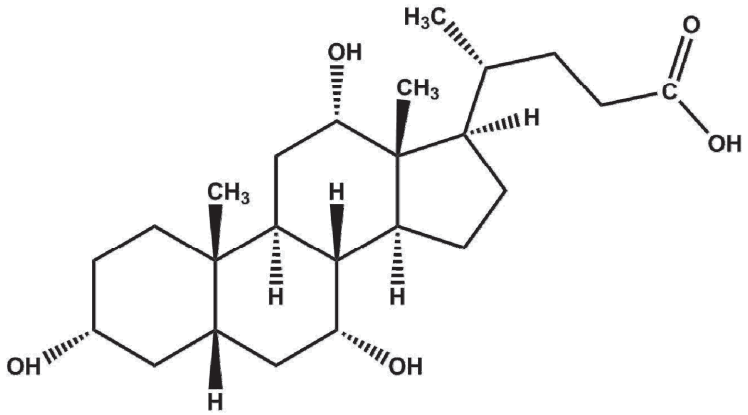

Figure 13. 2D structure of cholic acid, $C_{24}H_{40}O_5$.

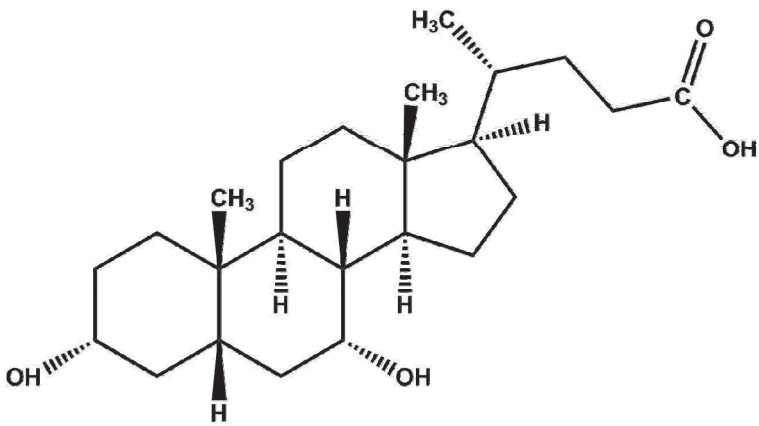

Figure 14. 2D structure of chenodeoxycholic acid, $C_{24}H_{40}O_4$.

Also, inappropriate and unbalanced metabolic activities in the gut microbiota can adversely affect the entire ENS [160]. Epigenetics and

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stressors early in life can have long-term adverse consequences on metabolism, the neuroendocrine immune system and human health. This includes a tendency towards obesity. It is just as important to remember that epigenetic signals from the mother can affect her baby's susceptibility to diseases. Moreover, inappropriate use of antibiotics can cause antibiotic-resistant Bacteria to expand and translocate. One should remember that mass-produced meats (from animals in concentrated animal feeding operations or CAFOs) are the largest source of antibiotic-resistant Bacteria. At the same time, the gut microbiota of patients who receive broad-spectrum antibiotics can be changed. There are fewer Bacteria that can produce SCFAs. This can produce epigenetic signals on enteric neurons and glia. This can lead to harmful changes in immune responses, intestinal motility and luminal pH. Diets that include excess saturated and *trans* fats and foods that have significant amounts of biogenic amines, such as L-glutamine, affect both the composition of the microbiota and the function of the nervous system. At the same time, Bacteria in the gut (such as *E. coli*) produce the gases hydrogen sulfide (H_2S) and nitric oxide (NO). It may also be possible to manipulate the gut microbiota to target intestinal diseases and generate many druggable targets that don't interfere with the patient's nervous system. So, researchers are calling for "carefully controlled prospective clinical studies utilizing state-of-the art longitudinal systems biology analysis" [160].

The genetics of the host can also affect the composition of the gut microbiome [161]. This was demonstrated when the gut microbiota of 110 strains of inbred mice were studied by genome-wide association analysis. Single nucleotide polymorphisms (SNPs) were identified. It was shown that genetic differences accounted for the abundance of most of the Bacteria. A publicly available database that contained the results was posted on the internet [161].

The composition of the gut microbiome can also be affected by infection with strains of *Clostridium difficile* that are resistant to several antibiotics [162]. Fecal microbiota transplants can be given to treat and cure recurrent *C. difficile* infections. The viral and bacterial microbiome of one patient varied for the first seven months, but did not resemble that of the donor. Seven months after treatment, the microbiomes started to resemble each other at the Order level, but were still different at the taxonomic level. Four of the five most prominent genera in both the patient and the donor of the fecal transplant were *Alistipes*, *Bacteroides*, *Dialister* and *Faecalibacterium*. These are known to be important constituents in healthy gut microbiota. Another important change was an increase in Caudovirales bacteriophages (bacterial viruses). So, it is

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important to look for not just Bacteria, but also viruses when analyzing a fecal microbiome [162].

2.7 Viruses are important, too

The importance of ancient viral DNA sequences that have been incorporated into our human eukaryotic cells in making us human was described in Chapter 1. This includes retrotransposons that share a sequence homology with retroviruses that have RNA that is reverse transcribed into DNA when the virus is replicated. About half of the nucleotides in the human genome are retrotransposons [24, 163]. However, these are not considered to be part of the human virome, which is a collection of all the viruses that are found in or on humans [164, 165]. It includes viruses that can infect people – even if they aren't present in everyone at all times [166]. New methods for analyzing the metagenome have enabled scientists to reconstitute entire viral genomes from the genetic material in the human gut [164-166]. However, several viruses have been known to be human pathogens for over a century [165]. They have been well-studied because they can be grown in culture. So, Norwalk, Rotavirus and Enterovirus are known to cause gastroenteritis. They can harm the host in the near term by causing nausea, vomiting, diarrhea and weight loss. When they persist for a long time, they can cause functional dyspepsia and post-infectious IBS. In addition, there is a higher concentration of viruses in the fecal samples of children who have diarrhea. Many of them (such as viruses in the Adenoviridae, Picornaviridae and Reoviridae families) were not previously known to be pathogenic. Viruses that infect amoebae that can enter the human body from cooling towers, rivers, lakes, and seawater have also been found in the gut microbiome. This includes the giant DNA viruses in the Mimiviridae, Mamaviridae and Marseilleviridae families. There are also viruses that infect plants that we eat. This includes the pepper mild mottle virus, oat blue dwarf virus, grapevine asteroid mosaic-associated virus, maize chlorotic mottle virus, oat chlorotic stuntvirus, panicum mosaic virus, and the tobacco mosaic virus. However, viruses that infect Bacteria (the bacteriophages) are by far the most abundant viruses in the gut, accounting for about 90% of its composition. Still, many of the viruses found in the human gut show an unusual temperate behavior. That is, the viral composition of the adult human gut is often quite stable throughout one's lifespan. Also, the gut virome composition mimics the evolution of the infant bacterial microbiota. There are 51 hypervariable loci that have been found in virus-like particles purified from human fecal samples. They

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exist in both viruses and Bacteria. They are implicated in bacterial wall adhesion and the biosynthesis of immunoglobulin receptors. This helps maintain viral and bacterial immune tolerance in the gut. This allows Bacteria and viruses to persist in the gut. They exert many effects on enterocytes and their human hosts. At the same time, they have intrinsic variability. This enables new species to emerge relatively fast and allows them to escape extinction. There are also genes that code for proteins that are involved in harvesting energy. This includes carbohydrate transport and degradation [165].

At the same time, the enteric virome is in a continuous and dynamic equilibrium with other components of the gut microbiome and the host's neuroimmune system [164]. These interactions can influence one's health and susceptibility to diseases [164, 165]. For example, dysbiosis in the human gut that is caused by portions of the virome can trigger IBS, Crohn's disease and cancer [164]. In addition, the human cytomegalovirus (CMV) has been found in about 70% of the patients who have IBD, as well as in their blood and brain tumors of over 80% of the people who have glioblastoma multiforme [167].

As we are learning more about viruses are continuing to re-define life [168]. That is, viruses are not considered to be alive by many biologists because they are totally dependent on their hosts for replication and they don't continually break down and re-make their outer membranes and many of the components inside them. However, the reclassification of a parasite that was once called the bacterium *Bradfordcoccus* as a giant virus has changed the previous concept and definition of viruses. That is, Bacteria and viruses were once distinguished based on their small size and these five criteria [168]:

(1) Bacteria (as well as Archaea and Eukaryotes) have both DNA and RNA, while viruses have only one of these (so there are RNA viruses and DNA viruses).
(2) All Bacteria are reproduced from their inner constituents, while the only part of a virus that is used for reproduction is either their RNA or DNA.
(3) While Bacteria grow, they retain their individuality and eventually undergo binary fission. Viruses don't undergo binary fission.
(4) Viruses don't have the enzymes needed to convert food into energy and necessary biochemicals, but Bacteria do.
(5) Viruses use the ribosomes in their host to translate mRNA into proteins.

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It was also thought that all viruses were smaller than any Bacteria, so viruses should pass through a porcelain filter (the Chamberlain filter). Since giant viruses are larger than than other viruses, they are too big to pass through the filter and were easily seen by a light microscope. So, they were originally classified as Bacteria. Some of the confusion was due to the fact that few people distinguished between a virion and a virus. That is, to most biologists, a virus is a filterable object made of a small number of self-associating proteins that transport the genome. Many small viruses (such as Polyomaviridae, Papillomaviridae, Circoviridae, Geminiviridae, Parvoviridae, Picornaviridae, Hepadneviridae and Retroviridae) fit this paradigm. They are made up of just a few proteins, contain a genome that is just large enough to encode the structural components of the particle plus a few other proteins or non-coding RNA that enable the viruses to interfere with the cellular metabolism of the host. There are also some medium-sized viruses (such as the virus causing smallpox or many bacteriophages which encode from forty to several hundred proteins). However, the giant viruses have a larger genome than many Bacteria. They contain much more than the few genes needed to code for the virus particle. They code for over a thousand proteins. Once the viral inner core particle is delivered into the cytoplasm of the host cell, it is transformed into something that closely resembles an organelle. It produces many more virus particles. It functions independent from the cell nucleus, which is pushed aside. So, it resembles a bacterial infection. So, it has been suggested that this quasi-organelle should be called the virus, while the virion is just a vehicle by which the virus is replicated. So, the inert virion is to the virus what a seed is to a plant. So, the genome of the virus is much larger than the genome of the virion as it codes for the many proteins that the virus must have to be temporarily ‘alive’ in the host cell. The authors proposed that distinguishing between a virion and a virus in its intra-cellular active stage means that all viruses are alive. The actively replicating virus exhibits all the properties of parasitic Bacteria, but the virion does not. In addition, like parasitic Bacteria, Mimiviruses can be infected by much smaller viruses, called virophages by some. These virophages don’t have a host-pathogen relationship with the Acanthamoeba host cells. Their DNA is transcribed and replicated within their Mimivirus host. So, virus particles are no more representative of a giant virus than a seed is to a plant, even though they have the same genome. So, the authors proposed a new paradigm of life as something that has five subsystems: “the cell division apparatus, the genome replication apparatus, the genome transcription (from DNA to mRNA) apparatus, the protein translation apparatus, and a metabolism both in

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charge of providing the above subsystems with biochemical building blocks (amino-acids, nucleotides) and the energy required to perform biosynthesis (e.g. ATP)" [168].

The authors also pointed out that the complete metabolism of many organisms often requires the cooperation of several other organisms [168]. For example, parasitic, endosymbiotic microorganisms can only live within their cellular hosts, most often in their cytoplasm. However, some exotic Bacteria are confined to the nucleus of the host cell. The metabolic pathways in these parasites are limited and have defects in the biosynthesis of amino acids, nucleotides, and/or membranes, as well as in the production of energy as ATP. Photosynthetic microorganisms are an exception because they only need the sun, the atmosphere, water and a few minerals. Still, it is tempting to classify cellular microorganisms based on their level of autonomy. So, some should be considered more 'alive' than others. For example, endosymbiotic, parasitic microorganisms such as the bacterium *Tremblaya princeps* has a genome that only codes for 121 proteins. They are thought to be 'alive' even though they have almost no anabolic or energy-producing capabilities. Moreover, the proteins that are needed to copy their DNA, transcribe it into mRNA or translate it into proteins are not fully encoded in their genomes. In contrast, giant viruses (like the Megaviridae) encode their own DNA replication apparatus and load their particle with the corresponding RNA polymerase and transcription factors. This enables them to initiate their replication cycle without the help of the host cell's nucleus. At the same time, endosymbiotic cellular parasites undergo reductive evolution of their genomes. That is, once a microorganism becomes an endosymbiotic cellular parasite, it starts to lose some of the functions that it needed to be a free-living organism. This slow, cumulative, irreversible loss of function means that no intracellular parasitic organism can ever evolve back toward a more independent life form. In contrast, giant viruses increase the sizes of their genomes over time. That is, they have captured genes from their hosts and their environments. Besides, most of the genes in giant viruses have no traceable ancestry to today's eukaryotes or prokaryotes. Moreover, the four known families of giant viruses are as different from each other as they are different from cellular organisms. So, these different viral lineages have different origins. As a result, the authors wrote "Our inborn fascination for unique causes to which attributing the emergence of complex phenomena, such as life, might constitute the most serious epistemological barrier that remains to be broken to understand the true significance of giant viruses" [168]. Even though the authors used some of the language of reductionist thinking when they described microorganisms

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in their article as machines, they were using systems thinking when they wrote about our inborn fascination with trying to find a single root cause for complex phenomena. It might even be considered reductionist to classify things as being forms of life as opposed to being integral parts of a whole life form – Gaia or Mother Earth. That is, we are all totally dependent on our symbiotic relationships with Bacteria, Archaea, Eukarya and viruses. At the same time, all viruses, be they classified as alive or not, are essential parts of Gaia.

2.8 Eukarya in the human microbiome

2.8.1 Introduction to the microbial eukaryotic cells in the human microbiome

Even though it was once thought that all eukaryotic microbes (protists) residing in the gut must be parasites, we now know that they can also be commensal or even beneficial [169]. The hygiene hypothesis states that the large increases in autoimmune diseases and allergies are due to the large-scale eradication of Bacteria in clean, modern, indoor environments [51]. It can be expanded to include all our co-evolved microbial symbionts [169]. That is, even intestinal worms (helminths) that are usually thought to be parasites were most likely part of the human gut communities that were in our ancestors. However, the transition to modern, hygienic lifestyles has lowered the diversity and prevalence of helminths and other protists. So, some scientists have called them ‘Old Friends’. So, the hygiene hypothesis is also known as the ‘Old Friends Hypothesis’. Instead of always being pathogenic, such ‘Old Friends’ are part of a healthy gut microbiota when they are present at controlled levels. They can improve anti-bacterial defenses by ensuring that the intestines provide a sufficient barrier against undesirable Bacteria and by increasing the biodiversity of the Bacteria in the gut [170-175]. This hypothesis has been expanded to include the widespread use of antibiotics (especially in mass-produced meat from CAFOs), feeding babies with formula instead of breast feeding and the modern diet that includes consuming processed foods, as well as diets high in fat and carbohydrates, and low in fiber [176]. These changes in our environments and diets have led to an increase in food allergies and autoimmune diseases [176]. However, the potential use of helminths as therapeutic agents is quite controversial, given recent failures in placebo-controlled double-blind clinical trials [177, 178].

A recent study determined the composition of microbial eukaryotes in the guts of humans and other mammals [168]. This included healthy

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people from agrarian communities in Malawi as well as Boulder, Colorado and Philadelphia, Pennsylvania who live in modern, more hygienic environments. They found that their guts contained *Blastocystis* (a genus of Stramenopiles that includes algae, diatoms, and water molds), *Entamoeba* (amoebae), trichomonads (in the order Trichomonadida) and yeasts. Yeasts and *Blastocystis* were detected in both populations - the agrarian and modern. They are most likely commensals, even though one species of *Entamoeba* (*E. histolytica*) is the causative agent of deadly amoebic dysentery. The vast majority of the DNA sequences that were found in both populations were the commensals *E. coli*, *E. dispar* and *E. hartmanni*. However, *E. histolytica* was found at a low concentration in two healthy people who also had *E. dispar*. Moreover, *Blastocystis* species were found in many samples, even though it was formerly thought to be a pathogen that has been associated with IBS [168]. In addition, some eukaryotic species such as the yeast *Saccharomyces boulardii* have been used as probiotics [179]. It was originally isolated to combat Cholera, and it now used to treat and often cure diarrhea – especially in children [179, 180]. However, it may be harmful immunocompromised people [181].

In comparison, the eukaryotic communities on human skin mostly contain fungi and have much lower biodiversity than the gut [168]. They resembled the eukaryotic communities in terrestrial samples (dust and soil) [168].

2.8.2 Fungi

Fungi are also important components of the gut microbiome [182]. The collection of all fungi in the microbiome is sometimes called the mycobiome (since mycology is the study of fungi). The type and abundance of fungi does depend on the diet. The gut microbiomes of 96 healthy human subjects were analyzed for fungi and Archaea. The two genera of Archaea that were found in most samples were *Methanobrevibacter* and *Nitrosophaera*, but their occurrence was mostly exclusive. That is, three groups were identified. They contained one of these two genera of Archaea or none of them. A higher dietary intake of carbohydrates was correlated with the group that contained *Methanobrevibacter*. On the other hand, a diet rich in vegetable fat and polyunsaturated fats was correlated with the group that contained either *Nitrosophaera* or no Archaea. The group with no Archaea also correlated with total mono-unsaturated fat in the diet. Researchers also found ribosomal RNA due to 66 genera of fungi and 13 additional lineages that could not be classified to the genus level. Only 12 fungal genera were detected in nine or more samples. The most prevalent were

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Saccharomyces (in 89% of the samples), followed by *Candida* (57%) and *Cladosporium* (42%). Several associations were found among Bacteria. *Bacteroides* occurred frequently with *Parabacteroides*, Lachnospiraceae and Ruminococcaceae. Lachnospiraceae occurred frequently with *Faecalibacterium*, Ruminococcaceae and *Bacteroides*. In contrast, *Methanobrevibacter* and *Nitrososphaera* occurred infrequently with each other. The yeasts *Candida* and *Saccharomyces* occurred frequently with the Bacteria mentioned above. The bacterial genera *Ruminococcus*, *Oxalobacter* and *Papillibacter* occurred frequently with *Methanobrevibacter*. Moreover, the ratio of *Prevotella* to *Bacterioides* correlated with the amounts of fungi present, but not the types. At the same time, *Bacterioides* was negatively correlated with *Methanobrevibacter* but positively correlated with *Nitrososphaera*. There was a negative association between *Candida* and *Bacterioides*. *Candida* was positively correlated with dietary carbohydrates and negatively correlated with total saturated fats. *Aspergillus* was negatively correlated with consuming short chain fatty acyls [182]. So, diet can affect the mycobiome, which can affect the bacterial microbiome.

It should be noted that the authors used the term short chain fatty acids (SCFAs) instead of short chain fatty acyls. Many reputable scientific journals in the field of food science use the term fatty acids instead of fatty acyls. Unfortunately, this can be confusing to people in other fields (such as medicine, biochemistry and organic chemistry) who know that all acids have ionizable hydrogens (like the H in carboxylic acids, or –COOH). Although it was not made clear in the article cited above [182], the authors did state that they obtained dietary information using a standard food frequency questionnaire. People seldom ever consume SCFAs that have two to six carbons (acetic through hexanoic acid). However, they do consume things like coconut oil that do contain short chain fatty acyls that are attached to the glyceride backbone of triacylglycerides. So, the negative correlation was almost certainly due to the consumption of readily available foods like coconut oil, instead of the exotic Polynesian fruit called noni (*Morinda citrifolia*) that does contain SCFAs. The structures of a triacylglyceride that contains three fatty acyls and the free fatty acid, hexanoic acid, are shown in Figures 15-16.

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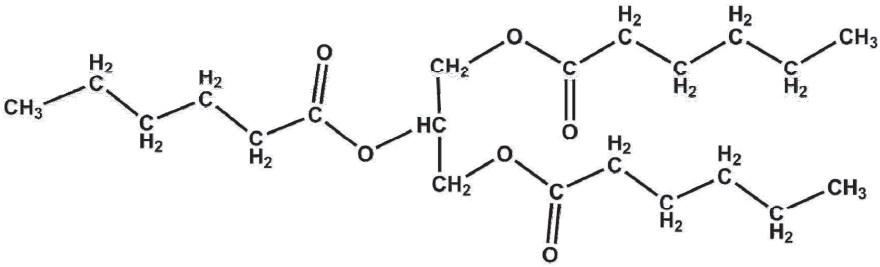

Figure 15. 2D structure of a triacylglyceride in which all three fatty acyls are hexanoyls. It has no ionizable hydrogens and is not an acid.

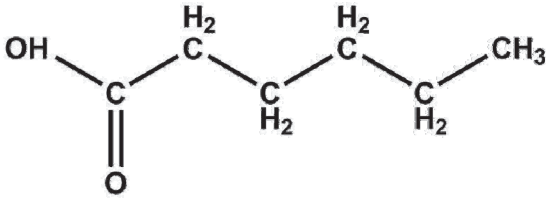

Figure 16. 2D structure of hexanoic acid, a short chain fatty acid. It has an ionizable hydrogen and is an acid.

Fungi also make up an important part of the oral microbiome [183, 184]. As stated in a recent review article, "modern microbiologists are learning to apply 'systems thinking' to take a holistic approach to investigations. Research has transitioned from culture-dependent studies of a single species to complex *in vitro* multispecies communities" [184]. So, the microbiomes of the oral cavities of 20 healthy people were analyzed [183]. They contained 74 culturable and 11 non-culturable fungal genera. *Candida* species were found in 75% of the people, followed by *Cladosporium* (65%), *Aureobasidium*, *Saccharomycetales* (50% for both), *Aspergillus* (35%), *Fusarium* (30%), and *Cryptococcus* (20%). Four of these predominant genera are known to be pathogenic in humans. The minimum number of genera found in one sample was three and the maximum was 16 [183]. Subsequent studies have been made of the mycobiome in people who were HIV positive [184]. An antagonistic relationship was found between two species of fungi in patients who were HIV positive [182].

In addition, a relatively new group of very small Bacteria called candidate phyla radiation group (CPR) has been identified [184]. CPR Bacteria have been found in many niches, including the oral cavity. This

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previously unknown group of organisms contains over 25 phyla and may account for 15% of all Bacteria (by number, not weight). They are characterized by their ultra-small size, the presence of genes that code for 16S rRNA self-splicing introns, archaeal-specific RuBisCO genes, and reduced genomes. Note that Rubisco is an abbreviation for Ribulose-1,5-bisphosphate carboxylase/oxygenase (first step in carbon fixation and the most abundant protein in Gaia). Moreover, they don't contain the genes that code for the CRISPR/Cas bacterial defense-system against bacteriophages. Three CPR phyla, Gracilibacteria, Absconditabacteria and Saccharibacteria (TM7), exist in several parts of the body, including the oral cavity. The TM7 phylum has been associated with several mucosal diseases such as vaginosis, IBD, halitosis, and periodontitis. It is often present at a concentration of about 1% in healthy oral cavities. However, it can increase to as much as 21% in patients who suffer from periodontitis. One species of TM7 (TM7x) has been isolated with its host bacterium, *Actinomyces odontolyticus actinosynbacter*. TM7x is an obligate epiparasite that lives on the surface of its bacterial host. This relationship is dynamic. It can change depending on the availability of nutrients and oxygen (O_2). There could be many more interactions between CPR organisms and other microbes [184].

Even though fungi are a small percentage of the human oral microbiome by number, their relatively large size (compared to Bacteria) means that they are a sizable portion of the microbial biomass [184]. This enables them to interact with Bacteria to form a stable biofilm that is an important structural component of the oral cavity. Moreover, fungi stimulate the host's immune system in a different way than do Bacteria, Archaea and viruses. So, fungi and Bacteria work together synergistically to help their hosts develop a healthy and robust immune system [184].

Intestinal worms (helminths) that were once thought to be capable of only being parasites when they enter the human gut are now also considered to be important for human health.

2.8.3 Protozoa and intestinal worms (helminths)

A wide variety of protozoa can infect the human GIT [185]. Some can invade host cells by entering them (e.g., *Cryptosporidium* spp.), some are specialized for one host (for example, *Entamoeba histolytica*), but many others can infect more than one host (for example, *Giardia duodenalis*). However, few of them do any serious damage except to cause diarrhea [185].

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Intestinal worms (helminths) are also well-known as parasites of the GIT [168, 185-195]. Exposure to relatively low levels of helminths may be important in the development of a fully functional immune system [186]. They can activate the immune system and help prevent further infection by enough helminths to cause diseases of the GIT [190]. They may also improve the function of the mucosal barrier and help to increase the biodiversity of the microbiome of the GIT [188, 189]. The mechanisms by which helminths might exert their potential health effects have been described [190, 191]. Some patients have even been given helminths to treat or prevent allergies [191, 192], multiple sclerosis (MS) [194, 196] and repetitive behaviors that are a symptom of ASD [195].

However, these reports that suggest that helminths can be given safely to effectively treat diseases were not followed up with successful clinical trials [168]. For example, the pig whipworm *Trichuris suis* and the human hookworm *Necator americanus* were used in open-label studies to see if they could effectively treat IBD and celiac disease [196, 197]. These preliminary studies showed that *T. suis* therapy for treating IBD correlated to a remission rate of 70% [196]. In a subsequent placebo-controlled, double-blind, randomized clinical trial, there was an improvement in the severity of ulcerative colitis, but no statistically significant change in the remission rate [198]. Subsequent phase II clinical trials in which 250 patients in the USA [178] and 240 European patients [178] were given *T. suis* eggs to treat moderate-to-severe Crohn's disease were terminated in 2013 because of a lack of efficacy.

So, these two clinical trials are excellent examples of how much more is needed than just a proof of principal and phase I studies to show that a new chemical entity or biologic is safe and might be effective in some people. For such drugs or biologics to be approved by the FDA and other governments' regulatory agencies and paid for by insurance, phase II, II and IV studies must be successfully completed. Moreover, any adverse side effects that occur in patients who receive a prescription drug or biologic is approved must be reported. At the same time, the ability of a new prescription drug or biologic to not only treat the disease that is targeted, but also their effects on further morbidity and mortality is monitored. That is, it is important that a new prescription drug or biologic should not only treat a disease, but also save lives (extend lifespan).

In contrast, dietary supplements only need to demonstrate safety (not efficacy) and this does not have to be done in a clinical trial. The result is that such supplements reach the market much sooner than prescription drugs and biologics and are available to anyone who is willing to buy them. No prescription or any other kind of approval by a physician is

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required. Fortunately, to the best of my knowledge, no intestinal parasites are sold as dietary supplements. However, many 'natural' substances that show no more safety or efficacy than the preliminary studies on intestinal worms are readily available to the public. To be fair, it should also be mentioned that some very popular prescription drugs that were effective on many patients have been withdrawn from the market because a small percentage of the people taking them suffered from very serious harmful side effects.

References

1. Capra F. *The Web of Life*. Anchor Books. New York, **1997**.
2. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T et al. Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci*. **2013**, 110, 3229-3236.
3. Sapp J. The symbiotic self. *Evol. Biol*. **2016**, 1-8, DOI 10.1007/s11692-016-9378-3.
4. Pace NR, Sapp J, Goldenfeld N. Phylogeny and beyond: Scientific, historical, and conceptual significance of the first tree of life. *Proc. Natl. Acad. Sci. USA* **2012**, 109, 1011–1018.
5. Georgiades K, Raoult D. How microbiology helps define the rhizome of life. *Front. Cell. Infect. Microbiol*. **2012**, 2, article 60, 11 pp.
6. Gage FH, Muotri AR. What makes each brain unique. *Sci. Amer*. **2012**, 306 (5), 26-31.
7. Löwer R, Löwer J, Kurth R. The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. *Proc. Natl. Acad. Sci*. **1996**, 93, 5177-5184.
8. Gilbert SF, Sapp J, Tauber AI. A symbiotic view of life: We have never been individuals. Q. Rev. Biol. **2012**, 87, 335–341.
9. Soucy SM, Huang J, Gogarten JP. Horizontal gene transfer: building the web of life. *Nature Rev. Genet*. **2015**, 16, 472-483.
10. Zilber-Rosenberg I, Rosenberg E. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev*. **2008**, 32, 723–735.
11. Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G. The microbiota–gut–brain axis: neurobehavioral correlates, health and sociality. *Front. Int. Neurosci*. **2013**, 7, article 70.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

12. Org E, Mehrabian M, Lusis AJ. Unraveling the environmental and genetic interactions in atherosclerosis: Central role of the gut microbiota. *Atherosclerosis* **2015**, 241, 387-399.
13. Fernández L, Lang S, Martín V, Maldonado A, Jiménez E. The human milk microbiota: origin and potential roles in health and disease. *Pharmacol. Res*. **2013**, 69, 1-10.
14. Cui L, Morris A, Huang L, Beck JM, Twigg III HL, von Mutius E, Ghedin E. The microbiome and the lung. *Ann. Am. Thorac. Soc*. **2014**, 11, S227–S232.
15. Stilling RM, Bordenstein SR, Dinan TG, Cryan JF. Friends with social benefits: host-microbe interactions as a driver of brain evolution and development? *Front. Cell. Infection Microbiol*. **2014**, 4, 147, 17 pp.
16. Hall RJ, Draper JL, Nielsen FGG, Dutilh BE. Beyond research: a primer for considerations on using viral metagenomics in the field and clinic. *Front. Microbiol*. **2015**, 6, article 224.
17. Forsythe P, Kunze W, Bienenstock J. Moody microbes or fecal phrenology: what do we know about the microbiota-gut brain axis? *BMC Med*. **2016**, 14, article 58.
18. Dinan TG et al. Cryan JF. Microbes, immunity and behavior. *Neuropsychopharmacol*. **2016**, doi: 10.1038/npp.2016.103.
19. Maranduba CM, de Castro SB, de Souza GT, Rossato C, da Guia FC et al. Intestinal microbiota as modulators of the immune system and neuroimmune system: impact on the host health and homeostasis. *J. Immunol. Res*. **2015**, 93, 1574.
20. Trautmann SM, Sharkey KA. The endocannabinoid system and its role in the intrinsic neural circuitry of the gastrointestinal tract. *Int. Rev. Neurobiol*. **2015**, 125, 85-126.
21. Stilling RM, Dinan TG, Cryan JF. The brain's Gepetto - microbes as puppeteers of neural function and behaviour? *J. Neurovirol*. **2016**, 22, 14-21.
22. Rutten N, Van der Gugten A, Uiterwaal C, Vlieger A, Rijkers G, Van der Ent K. Maternal use of probiotics during pregnancy and effects on their offspring's health in an unselected population. *Eur. J. Pediatr*. **2016**, 175, 229–235
23. Löwer R, Löwer J, Kurth R. The viruses in all of us: Characteristics and biological significance of human endogenous retrovirus sequences. Proc. Natl. Acad. Sci. **1996**, 93, 5177-5184.
24. Singer T, McConnel MJ, Marchetto MCN, Coufal NG, Gage FH. LINE-1 retrotransposons: mediators of somatic variation in neuronal genomes? *Trends Neurosci*. **2010**, 33, 345-354.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

25. Campioni MR, Finkbeiner S. Going retro: ancient viral origins of cognition. Neuron **2015**, *86*, 346-348.
26. Babatz TD, Burns KH. Functional impact of the human mobilome. *Curr. Opinion Genet. Develop*. **2013**, 23, 264-270.
27. Grabundjiza I, Wang J, Sebe A, Erdai Z, Kajdi R et al. Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. Nucl. Acid Res. **2013**, 41, 1829-1847.
28. Jaciet P-A, Colson P, Lopez P, Bapteste E. Extensive gene remodeling in the viral world: new evidence for nongradual evolution in the mobileome network. *Genome Biol. Evol*. **2014**, 6, 2195-2205.
29. Forterre P, Prnagishvili D. The major role of viruses in cellular evolution: facts and hypotheses. *Curr. Opinion Virol*. **2013**, 3, 558-565.
30. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett C, Knight, R. The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* **2007**, 449, 804–810.
31. Gevers D, Knight R, Petrosino JF, Huang K, McGuire AL et al. The Human Microbiome Project: a community resource for the healthy human microbiome. *PLoS Biol*. **2012**, 10 (8), article e1001377.
32. Earth Microbiome Project. **2017**, http://www.earthmicrobiome.org/
33. Gomez-Gallego C, Garcia-Mantrana I, Salminen S, Collado MC. The human milk microbiome and factors influencing its composition and activity. *Semin. Fetal Neonatal Med*. **2016**, 21, 400-405.
34. Heitjtz RD. Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. *Semin. Fetal Neonatal Med*. **2016**, 21, 410-417.
35. Prince AL, Chu DM, Seferovic MD, Antony KM, Ma J, Aagaard KM. The perinatal microbiome and pregnancy: moving beyond the vaginal microbiome. *Cold Spring Harb. Perspect. Med*. **2015**, 5, Artilce a023051.
36. Satokari R, Gronroos T, Laitinen K, Salminen S, Isolauri E. Bifidobacterium and Lactobacillus DNA in the human placenta. *Lett. Appl. Microbiol*. **2009**, 48, 8-12.
37. Jimenez E, Fernandez L, Marin ML, Martin R, Odriozola JM et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr. Microbiol*. **2005**, 51, 270-274.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

38. Slykerman RF, Thompson J, Waldie KE, Murphy R, Wall C et al. Antibiotics in the first year of life and subsequent neurocognitive outcomes. *Acta Ped*. **2017**, 106, 87-94.
39. Wang J-Y, Liu L-F, Chen C-Y, Huang Y-W, Hsiung CA, Tsai H-J. Acetaminophen and/or antibiotic use in early life and the development of childhood allergic diseases. *Int. J. Epidemiol*. **2013**, 42, 1087-1099.
40. Hoisington AJ, Brenner LA, Kinney KA, Postolache TT, Lowry CA. The microbiome of the built environment and mental health. *Microbiome* **2015**, 3, 60, 12 pp.
41. Neuman H, Debelius JW, Knight K, Koren O. Microbial endocrinology: the interplay between the microbiota and the endocrine system. *FEMS Microbiol. Rev*. **2015**, 39, 509-521.
42. Aidy SL, Dinan TG, Cryan JF. Gut microbiota: The conductor in the orchestra of immune–neuroendocrine communication. *Clin. Therapeut*. **2015**, 37, 954-976.
43. Avetisyan M, Schill EM, Heuckeroth RO. Building a second brain in the bowel. *J. Clin. Invest*. **2015**, 125, 899-907.
44. Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF. Intestinal microbiota, diet and health. *Br. J. Nutr*. **2014**, 111, 387-402.
45. Rosenbaum M, Knight R, Leibel RL. The gut microbiota in human energy homeostasis and obesity. *Trend. Endocrin. Metab*. **2015**, 26, 493-501.
46. Glick-Bauer M, Yeh M-C. The health advantage of a vegan diet: Exploring the gut microbiota connection. *Nutrients* **2014**, 6, 4822-4838.
47. Lurie-Weinberger MN, Gophna U. Archaea in and on the human body: Health implications and future directions. *PLoS Pathog*. **2015**, DOI:10.1371/journal.ppat.1004833 8 pp.
48. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. *Nat. Rev. Microbiol*. **2014**, 12, 661-672.
49. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann. Gastroenterol*. **2015**, 28, 203-209.
50. Yang NJ, Chiu IM. Bacterial signaling to the nervous system through toxins and metabolites. *J. Mol. Biol*. **2017**, 429, 587-605.
51. Forsythe P, Bienenstock J, Kunze WA. Vagal pathways for microbiome-brain-gut axis communication, in M. Lyte and J.F. Cryan (eds.), Microbial Endocrinology: The Microbiota-Gut-Brain

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Axis in Health and Disease, Advances in Experimental Medicine and Biology 817, DOI 10.1007/978-1-4939-0897-4_5, Springer New York **2014**.

52. Muccioli GG, Naslain D, Bäcked F, Reigstad CS, Lambert DM et al. The endocannabinoid system links gut microbiota to adipogenesis. *Mol. Syst. Biol*. **2010**, 6, Article 392.
53. Howlett AC, Reggio PH, Childers SR, Hampson RE, Ulloa NM et al. Endocannabinoid tone versus constitutive activity of cannabinoid receptors. *Br. J. Pharmacol*. **2011**, 163, 1329-1343.
54. Geurts L, Lazarevic V, Derrien M, Everard A, Van Roye M et al. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. *Front. Microbiol*. **2011**, 2, Article 149.
55. González-Arancibia C, Escobar-Luna J, Barrera-Bugueño C, Díaz-Zepeda C, González-Toro MP. What goes around comes around: novel pharmacological targets in the gut–brain axis. *Ther. Adv. Gastroenterol*. **2016**, 9, 339–353.
56. Moco S, Candela M, Chuang E, Draper C, Cominetti O et al. Systems biology approaches for inflammatory bowel disease: Emphasis on gut microbial metabolism. *Inflamm. Bowel Dis*. **2014**, 20, 2104-2114.
57. Kostic AD, Gevers D, Knip M, Xavier RJ. The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. *Cell Host Microbe* **2015**, 17, 1–14.
58. Hao MM, Foong JPP, Bornstein JC, Li ZL, Berghe PV et al. Enteric nervous system assembly: functional integration within the developing gut. *Develop. Biol*. **2016**, 417, 168-181.
59. Hyland MP, Cryan JF. Microbe-host interactions: Influence of the gut microbiota on the enteric nervous system. *Develop. Biol*. **2016**, 417, 182-187.
60. Sharkey KA. Emerging roles for enteric glia in gastrointestinal disorders. J. Clin. Invest. **2015**, 125, 918-925.
61. O'Mahoney SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav. Brain Res*. **2015**, 277, 32-48.
62. Parashar A, Udayabanu M. Gut microbiota regulates key modulators of social behavior. *Europ. Neuropsychophramacol*. **2016**, 26, 78-91.
63. Anderson, G, Maes M. The gut–brain axis: The role of melatonin in linking psychiatric, inflammatory and neurodegenerative conditions. *Adv. Int. Med*. **2015**, 2, 31-37.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

64. Luna RA, Foster JA. Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression. *Curr. Opin. Biotechnol*. **2015**, 32, 35-41.
65. Foster JA, Neufeld KAM. Gut–brain axis: how the microbiome influences anxiety and depression. *Trend. Neurosci*. **2013**, 36, 305-312.
66. Ghaisas S, Maher J, Kanthasamy A. Gut microbiome in health and disease: Linking the microbiome–gut–brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. *Pharmacol. Therapeut*. **2016**, 158, 52-62.
67. Toh MC, Allen-Vercoe EA. The human gut microbiota with reference to autism spectrum disorder: considering the whole as more than a sum of its parts. *Microb. Ecol. Health Dis*. **2015**, 26, 26309.
68. Li Q, Zhou J-M. The microbiota-gut-brain axis and its potential therapeutic role in autism spectrum disorder. *Neurosci*. **2016**, 324, 131-139.
69. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. *Nat. Rev. Gastroenterol*. **2012**, 9, 577–89.
70. Bienenstock J, Kunze W, Forsythe P. Microbiota and the gut–brain axis. *Nutr. Rev*. **2015**, 73, 28-31.
71. Rosenbaum M, Knight R, Leibel RL. The gut microbiota in human energy homeostasis and obesity. *Trend. Endocrin. Metab*. **2015**, 26, 493-501.
72. Bruce-Keller AJ, Salbaum JM, Luo M, Blanchard IVE, Taylor CM. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. *Biol. Psych*. **2015**, 77, 607-615.
73. Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Invest*. **2011**, 121, 2126-2132.
74. Dolpady J, Sorini C, di Pietro C, Cosorich I, Ferrarese R. et al. Oral probiotic VSL#3 prevents autoimmune diabetes by modulating microbiota and promoting indoleamine 2,3-dioxygenase-enriched tolerogenic intestinal environment. *J. Diab. Res*. **2016**, Article ID 7569431, 12 pp.
75. Wu H, Tremaroli V, Bäckhed F. Linking microbiota to human diseases: A systems biology perspective. *Trend. Endocrinol. Metab*. **2015**, 26, 758-770.
76. Petra AI, Panagiotidou S, Hatziagelaki E, Stewart JM, Conti P, Theoharides TC. Gut-microbiota-brain axis and its effect on

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

neuropsychiatric disorders with suspected immune dysregulation. *Clin. Therapeut*. **2015**, 37, 984-995.

77. Felice VD, Quigley EM, Sullivan AM, O'Keefe GW, O'Mahony SM. Microbiota-gut-brain signalling in Parkinson's disease: Implications for non-motor symptoms. *Parkinsonism Relat. Disord*. **2016**, 27, 1-8.
78. Buie T. Potential etiologic factors of microbiome disruption in autism. *Clin. Therapeut*. **2015**, 37, 976-983.
79. Farmer AD, Randall HA, Aziz Q. It's a gut feeling: How the gut microbiota affects the state of mind. *J. Physiol*. **2014**, 592, 2981–2988.
80. Stilling RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? *Neurochem. Int*. **2016**, 99, 110-132.
81. Emoto T, Yamashita T, Sasaki N, Hirota Y, Hayashi T et al. *J. Atheroscler. Thromb*. **2016**, 23, 908-921.
82. Vaziri ND, Zhao Y-Y, Pahl MV. Altered intestinal microbial flora and impaired epithelial barrier structure and function in CKD: the nature, mechanisms, consequences and potential treatment. *Nephrol. Dial. Transplant*. **2015**, 1, 1–10. doi: 10.1093/ndt/gfv095.
83. Paulose JK, Wright JM, Patel AG, Cassone VM. Human gut bacteria are sensitive to melatonin and express endogenous circadian rhythmicity. *PLoS One* **2016**, 11, e0146643.
84. Lyte M. Microbial endocrinology in the microbiome-gut-Brain axis: How bacterial production and utilization of neurochemicals influence behavior. *PLoS Pathog*. **2013**, 9, e1003726. doi:10.1371/journal.ppat.1003726.
85. Moos WH, Faller DV, Harpp DN, Kanara I, Pernokas J. et al. Microbiota and neurological disorders: a gut feeling. *BioRes*. **2016**, 5, 137-145.
86. Rauws EAJ, Tytgat GNJ. Cure of duodenal ulcer associated with eradication of Helicobacter pylori. *Lancet* **1990**, 335, 1233-1235.
87. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N. Eng. J. Med*. **1991**, 325, 1127-1131.
88. Budzyński J, Kłopocka M. Brain-gut axis in the pathogenesis of Helicobacter pylori infection. *World J. Gastroenterol*. **2014**, 20, 5212-5225.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

89. Taha F, Lipsitz JD, Galea S, Demmer RT, Talley NJ et al. Anxiety disorders and self-reported ulcer. *Gen. Hosp. Psychiatry* **2014**, 36, 674-679.
90. Neufeld NH, Mohamed NS, Grujich N, Shilman K. Acute neuropsychiatric symptoms associated with antibiotic treatment of Helicobacter Pylori infections: a review. *J. Psychiat. Pract*. **2017**, 23, 25-35.
91. Ohtani N. Microbiome and cancer. Semin. Immunopathol. **2015**, 37, 65–72.
92. Mayo Clinic. Arteriosclerosis/Atherosclerosis. **2017**, http://www.mayoclinic.org/diseases-conditions/arteriosclerosis-atherosclerosis/home/ovc-20167019
93. Nelson MH, Diven MA, Huff LW, Paulos CM. Harnessing the microbiome to enhance cancer immunotherapy. *J. Immunol. Res*. **2015**, 2015, Article 368736, 12 p.
94. Bultman SJ. The microbiome and its potential as a cancer preventive intervention. *Sem. Oncol*. **2016**, 43, 97-106.
95. Pistollato F, Battino M. Role of plant based diets in the prevention and regression of metabolic syndrome and neurodegenerative diseases. *Trend. Food Sci. Technol*. **2014**, 40, 62-81. 96. Tonstad S, Stewart K, Oda K, Batech M, Herrin RP, Fraser GE Vegetarian diets and the incidence of diabetes in the adventist health styudy-2. *Nutr. Metab. Cardiovasc. Dis*. **2013**, 23, 292–299.
97. Tonstad S, Butler T, Yan R, Fraser GE. Type of vegetarian diet, body weight, and prevalence of type 2 diabetes. *Diabetes Care* **2009**, 32, 791–796.
98. Orlich MJ, Singh PN, Sabaté J, Jaceldo-Siegl K, Fan J et al. Vegetarian dietary patterns and mortality in adventist health study 2. *J. Am. Med. Assoc. Intern. Med*. **2013**, 173, 1230–1238.
99. Van Faasen A, Bol J, van Dokkum W, Pikaar NA, Ockhuizen T, Hermus RJJ. Bile acids, neutral steroids, and bacteria in feces as affected by a mixed, a lacto-ovovegetarian, and a vegan diet. *Am. J. Clin. Nutr*. **1987**, 46, 962–967.
100. Matijašić BB, Obermajer T, Lipoglavšek L, Grabnar I, Avguštin G, Rogelj I. Association of dietary type with fecal microbiota in vegetarians and omnivores in Slovenia. *Eur. J. Nutr*. **2014**, 53, 1051–1064.
101. Miquel S, Martín R, Rossi O, Bermúdez-Humarán LG, Chatel JM et al. Faecalibacterium prausnitzii and human intestinal health. *Curr. Opin. Microbiol*. **2013**, 16, 255–261.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

102. Remely M, Aumueller E, Jahn D, Hippe B, Brath H, Haslberger AG. Microbiota and epigenetic regulation of inflammatory mediators in type 2 diabetes and obesity. *Benef. Microbes* **2014**, 5, 33–43.
103. Benus R, van der Werf TS, Welling GW, Judd PA, Taylor MA et al. Association between faecalibacterium prausnitzii and dietary fibre in colonic fermentation in healthy human subjects. *Br. J. Nutr*. **2010**, 104, 693–700.
104. Kim M-S, Hwang S-S, Park E-J, Bae J-W. Strict vegetarian diet improves the risk factors associated with metabolic diseases by modulating gut microbiota and reducing intestinal inflammation. *Environ. Microbiol. Rep*. **2013**, 5, 765–775.
105. Zimmer J, Lange B, Frick J-S, Sauer H, Zimmermann K et al. A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. *Eur. J. Clin. Nutr*. **2012**, 66, 53–60.
106. Ussher JR, Lopaschuk GD, Arduini A. Gut microbiota metabolism of L-carnitine and cardiovascular risk. *Atherosclerosis* **2013**, 231, 456–461.
107. Peltonen R, Nenonen M, Helve T, Hänninen O, Toivanen P, Eerola E. Faecal microbial flora and disease activity in rheumatoid arthritis during a vegan diet. *Br. J. Rheumatol*. **1997**, 36, 64–68.
108. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. Curr. Opin. Microbiol. **2015**, 23, 141-147.
109. Davies W. The microbiome your inner oncologist. *Ecancer News* **2016**, June 17. http://ecancer.org/news/9611-the-microbiome---your-inner-oncologist.php
110. Morais CA, de Rosso VV, Estadella D, Pisani LP. Anthocyanins as inflammatory modulators and the role of the gut microbiota. *J. Nutr. Biochem*. **2016**, 33, 1-7.
111. Spiller GA, Chernoff MC, Hill RA, Gates JE, Nassar JJ et al. Effect of purified cellulose, pectin, and a low-residue diet on fecal volatile fatty-acids, transit-time, and fecal weight in humans. *Am. J. Clin. Nutr*. **1980**, 33, 754–759.
112. Roediger WEW. Role of anaerobic-bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* **1980**, 21, 793–798.
113. Fung KYC, Cosgrove L, Lockett T, Head R, Topping, D.L. A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate. *Br. J. Nutr*. **2012**, 108, 820–831.
114. Lampe JW. Emerging research on equol and cancer. *J. Nutr*. **2010**, 140, 1369S-1372S.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

115. Voigt RM, Forsyth CB, Green SJ, Mutlu E, Engen P et al. Circadian disorganization alters intestinal microbiota. *PLoS One*, **2013**, 9, e 97500. doi:10.1371/journal.pone.0097500.
116. Falony G, Vieira-Silva S, Raes J. Microbiology meets big data: The case of gut microbiota–derived trimethylamine. *Annu. Rev. Microbiol*. **2015**, 69, 305–21.
117. Quante M. You are what you eat: Metabolites of gut microbiota provide novel insights into diagnosis and development of chronic kidney disease. *Transplantaion* **2015**, 99, 1306-1307.
118. Smith RE, Rouchotas P, Fritz H. Lecithin (phosphatidylcholine): healthy dietary supplement or dangerous toxin? *Nat. Prod. J*. **2016**, 6, 242-249.
119. Samsam M, Ahangari R, Naser S.A. Pathophysiology of autism spectrum disorders: Revisiting gastrointestinal involvement and immune imbalance. World J. Gastroenterol. **2014**, 20, 9942-9951.
120. Liu J, Zhang M, Kong X-J. Gut microbiome and autism: recent advances and future perspectives. *North Am. J. Med. Sci*. **2016**, 9, 104-115.
121. Rodakis J. An n = 1 case report of a child with autism improving on antibiotics and a father's quest to understand what it may mean. *Microb. Ecol. Health Dis*. **2015**, 26, 26382, 6 p.
122. Sandler RH, Finegold SM, Bolte ER, et al. Short-term benefit from oral vancomycin treatment of regressive-onset autism. *J. Child. Neurol*. **2000**, 15, 429-435.
123. Kuhn M, Bransfield R. Divergent opinions of proper Lyme disease diagnosis and implications for children co-morbid with autism spectrum disorder. *Med. Hypotheses* **2014**, 83, 321-325.
124. Kuhn M, Grave S, Bransfield R, Harris S. Long term antibiotic therapy may be an effective treatment for children co-morbid with Lyme disease and autism spectrum disorder. *Med. Hypotheses* **2012**, 78, 606-615.
125. Atladóttir HÓ, Henriksen TB, Schendel DE, Parner ET. Autism after infection, febrile episodes, and antibiotic use during pregnancy: an exploratory study. *Pediatrics* **2012**, 130, e1447-e1454.
126. Frye RE, Slattery J, MacFabe DF, Allen-Vercoe E, Parker W et al. Approaches to studying and manipulating the enteric microbiome to improve autism symptoms. Microb. *Ecol. Health Dis*. **2015**, 26, article 26878.

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127. Chen H-L, Wang G, Ma C, Cai Z-N, Liu W-B. An efficient hybrid kernel extreme learning machine approach for early diagnosis of Parkinson's disease. *Neurocomputing* **2016**, 184, 131-144.
128. Ricciardi L, Visco-Comandini F, Erro R, Morgante F, Bologna M et al. Facial emotion recognition and expression in Parkinson's disease: an emotional mirror mechanism? *PLoS ONE*, **2017**, 12(1), e0169110. doi:10.1371/journal.pone.0169110
129. Lipoff B. Lenexa student catches attention of Michael J. Fox Foundation, **2016**. http://www.kansascity.com/news/local/community/joco-913/article123289099.html
130. Caparros-Lefebvre D, Elbaz A. Possible relation of atypical parkinsonism in the French West Indies with consumption of tropical plants: a case-control study. *Lancet* **1999**, 354, 281-286.
131. Smith, R.E., Tran, K., Shejwalkar, P. and Hara, K. Neurotoxicity of fruits, seeds and leaves of plants in the Annonaceae family. *Austin Neurol. Neurosci*. **2016**, 1, ID1005, 8 pages.
132. Höllerhage M, Rösler TW, Berjas M, Luo R, Tran K et al. Neurotoxicity of dietary supplements from Annonaceae species. *Int. J. Toxicol*. **2015**, 34, 543-550.
133. Kapoor A, Fan Y-H, Arav-Boger R. Bacterial muramyl dipeptide (MDP) restricts human cytomegalovirus replication via an IFN-β-dependent pathway. Sci. Rep. **2015**, 6, 20295, DOI: 10.1038/srep20295.
134. Fornara O, Bartek Jr J, Rahbar A, Odeberg J, Khan Z. Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: prognostic significance and biological impact. *Cell Death Different*. **2016**, 23, 261-269.
135. Stilling RM, Dinan TG, Cryan JF. Microbial genes, brain & behaviour – epigenetic regulation of the gut–brain axis. *Genes Brain Behav*. **2014**, 13, 69–86.
136. Collins N, Belkaid Y. Do the microbiota influence vaccines and protective immunity to pathogens? Engaging our endogenous adjuvants. *Cold Spring Harbor Persp. Biol*. **2017**, Article a028860, doi: 10.1101
137. Nakaya HI, Bruna-Romero O. Is the gut microbiome key to modulating vaccine efficacy? *Expert Rev. Vaccines* **2015**, 14(6), 777–779.
138. Salk HM, Simon WL, Lambert ND, Kennedy RD, Grill DE et al. Taxa of the nasal microbiome are associated with influenza-specific

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IgA response to live attenuated influenza vaccine. *PLoS One*, **2016**, 11 (9), Article e0162803.

139. Metcalf SS, Northridge ME, Lanster IB. A systems perspective for dental health in older adults. *Am. J. Pub. Health* **2011**, 101, 1820-1821.
140. Aarabi G, Eberhard J, Reissmann DR, Heydecke G, Seedorf U. Interaction between periodontal disease and atherosclerotic vascular disease - Fact or fiction? *Atherscl*. **2015**, 241, 555-560.
141. Hajishengal G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat. Rev. Immunol. **2015**, 15, 30–44.
142. Borgnakke WS, Anderson PF, Shannon C, Jivanesc A. Is there a relationship between oral health and diabetic neuropathy? *Curr. Diab. Rep*. **2015**, 15, article ID 93, 9 pp.
143. Kaidonis J, Townsend G. The 'sialo–microbial–dental complex' in oral health and disease. *Ann. Anat*. **2016**, 203, 85-89.
144. Keskin M, Zeidan-Chulia F, Gursoy M, Kononen E, Rautava J. Two cheers for Crohn's disease and periodontitis: Beta-defensin-2 as an actionable target to intervene on two clinically distinct diseases. *OMICS* **2015**, 19, 443-450.
145. Pozhitkov AE, Leroux BG, Randolph TW, Beikler T, Flemmig TF. Towards microbiome transplant as a therapy for periodontitis: an exploratory study of periodontitis microbial signature contrasted by oral health, caries and edentulism. *BMC Oral Health* **2015**, 15, article 125.
146. Mastromarino P, Capobianco D, Miccheli A, Praticò G, Campagna G et al. Administration of a multistrain probiotic product (VSL#3) to women in the perinatal period differentially affects breast milk beneficial microbiota in relation to mode of delivery. *Pharmacol. Rev*. **2015**, 95, 63-70.
147. Ma Z, Guan Q, Ye C, Zhang C, Foster JA. et al. Network analysis suggests a potentially 'evil' alliance of opportunistic pathogens inhibited by a cooperative network in human milk bacterial communities. *Sci. Rep*. **2015**, 5, 8275.
148. Oriach CS, Robertson RC, Stanton C, Cryan JF, Dinan TG. Food for thought: The role of nutrition in the microbiota-gut-brain axis. *Clin. Nutr. Ep*. **2016**, 6, 25-38.
149. Derrien M, Vlieg JETH. Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends Microbiol*. **2015**, 23, 354-366.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

150. Rook GAW, Lowry CA, Raison CL. Hygiene and other early childhood influences on the subsequent function of the immune system. *Brain Res*. **2015**, 1617, 47-62.
151. Conlon MA, Bird AR. The impact of diet and lifestyle on gut microbiota and human health. *Nutrients* **2015**, 7, 17-44.
152. de Souza AZZ, Zambom AZ, Abboud KY, Reis SK, Tanninhão F et al. Oral supplementation with L-glutamine alters gut microbiota of obese and overweight adults: A pilot study. *Nutr*. **2015**, 31, 884-889.
153. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* **2011**, 334, 105-108.
154. Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F et al. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. *Cell. Metab*. **2015**, 22, 971–982.
155. King DE, Mainous AG III, Lambourne CA. Trends in dietary fiber intake in the United States, 1999-2008. *J. Acad. Nutr. Diet*. **2012**, 112, 642–8.
156. Holscher HD, Caporaso JG, Hooda S, Brulc JM, Fahey Jr GC et al. Fiber supplementation influences phylogenetic structure and functional capacity of the human intestinal microbiome: follow-up of a randomized controlled trial. *Am. J. Clin. Nutr*. **2015**, 101, 55–64.
157. Boler BM, Rossoni Serao MC, Bauer LL, Staeger MA, Boileau TW et al. Digestive physiological outcomes related to polydextrose and soluble maize fibre consumption by healthy adult men. *Br. J. Nutr*. **2011**, 106, 1864–71.
158. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **2005**, 437. 376–380.
159. Mach N, Fuster-Botella D. Endurance exercise and gut microbiota: A review. *J. Sport Health Sci*. **2017**, *6*, 179-197.
160. Savidge TC. Epigenetic regulation of enteric neurotransmission by gut bacteria. *Front. Cell. Neurosci*. **2016**, 9, Article 503.
161. Org E, Parks BW, Joo JWJ, Emert B, Schwartzman W. et al. Genetic and environmental control of host-gut microbiota interactions. *Genome Res*. **2016**, 25, 1558–1569.
162. Broecker F, Klumpp J, Schuppler M, Russo G, Biedermann L et al. Long-term changes of bacterial and viral compositions in the intestine of a recovered Clostridium difficile patient after fecal

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

microbiota transplantation. *Cold Spring Harb. Mol. Case Stud*. **2015**, 2, article a000448, 12 pp.
163. Nandi S, Chandramohan D, Fioriti L, Melnick AM, Hébert JM et al. Roles for small noncoding RNAs in silencing of retrotransposons in the mammalian brain. *Proc. Natl. Acad. Sci*. **2016**, 113, 12697-12702.
164. Focà A, Liberto MC, Quirino A, Marascio N, Zicca E. et al. Gut inflammation and immunity: what is the role of the human gut virome? *Mediat. Inflamm*. **2015**, article ID 326032, 7 pp.
165. Scarpellini E, Ianiro G, Attili F, Bassanelli C, De Santis A et al. The human gut microbiota and virome: Potential therapeutic implications. *Digest. Iver Dis*. **2015**, 47, 1007-1012.
166. Xu GJ, Kula T, Xu Q, Li, M.Z, Vernon SD et al. Comprehensive serological profiling of human populations using a synthetic human virome. *Science* **2015**, 348, 1105-1109.
167. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S et al. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res*. **2002**, 62, 3347-3350.
168. Claverie J-M, Abergel C. Giant viruses: The difficult breaking of multiple epistemological barriers. Stud. History Philosophy Biol. Biomed. Sci. **2016**, 59, 89-99.
169. Parfrey LW, Walters WA, Lauber CL, Clemente JC, Berg-Lyons D et al. Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. *Front. Microbiol*. **2014**, 5, article 298.
170. Petersen AM, Stensvold CR, Mirsepasi H, Engberg J, Friis-Moller A et al. Active ulcerative colitis associated with low prevalence of Blastocystis and Dientamoeba fragilis infection. *Scand. J. Gastroenterol*. **2013**, 48, 638–639.
171. Chudnovskiy A, Mortha A, Kana V, Kennard A, Ramirez JD et al. Host-protozoan interactions protect from mucosal infections through activation of the inflammasome. *Cell* **2016**, 167, 444-456.
172. Loke P, Lim YAL. A commensal protozoan strikes a balance in the gut. Cell Host Microbe **2016**, 20, 417-418.
173. Wolff MJ, Broadhurst MJ, Loke P. Helminthic therapy: improving mucosal barrier function. *Trend. Parasitol*. **2012**, 28, 187-194.
174. Leung JM, Loke P. A role for IL-22 in the relationship between intestinal helminths, gut microbiota and mucosal immunity. *Intl. J. Parasitol*. **2013**, 43, 253-257.

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175. Lee SC, Tang MS, Lim YAL, Choy SH, Kurtz ZD. Helminth colonization is associated with increased diversity of the gut microbiota. *PLoS Negl. Trop. Dis*. **2014**, 8(5), e2880.
176. Plunkett CH, Nagler CR. The influence of the microbiome on allergic sensitization to food. *J. Immunol*. **2017**, 198, 581-589.
177. Briggs N, Weatherhead J, Sastry KJ, Hotez PJ. The hygiene hypothesis and its inconvenient truths about helminth infections. *PLoS Negl. Trop. Dis*. **2016**, 10(9), e0004944.
178. Coronado Biosciences. Coronado biosciences announces independent data monitoring committee recommendation to discontinue falk phase 2 trials of TSO in Crohn's disease. https://globenewswire.com/news-release/2013/11/07/587752/10056769/en/Coronado-Biosciences-Announces-Independent-Data-Monitoring-Committee-Recommendation-to-Discontinue-Falk-Phase-2-Trial-of-TSO-in-Crohn-s-Disease.html
179. Parfrey LW, Walters WA, Knight R. Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. *Microbiol*. **2011**, 2, article 153.
180. Dash DK, Dash M, Mohanty MD, Acharya N. Efficacy of probiotic Saccharomyces boulardi as an adjuvant therapy in acute childhood diarrhea. J. Nepal. Paediatr. Soc. **2016**, 36, 250-255.
181. Miller H, Lee R, Trivedi J, Miller H, Lee R et al. *Saccharomyces boulardii* probiotic-associated fungemia: questioning the safety of this preventive probiotic's use. *Diagn. Microbiol. Infect. Dis*. **2017**, *87*, 286–288.
182. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H et al. Archaea and fungi of the human gut microbiome: Correlations with diet and bacterial residents. *PLoS ONE* **2013**, *8 (6)*, Article e66019.
183. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog*. **2010**, *6 (1)*, Articlee1000713.
184. Baker JL, Bor B, Agnello M, Shi W, He X. Ecology of the oral microbiome: beyond bacteria. *Trend. Microbiol*. **2017**, 25, 362-374.
185. Berrilli F, Di Cave D, Cavallero S, D'Amelio S. Interactions between parasites and microbial communities in the human gut. *Front. Cell. Infect. Microbiol*. **2012**, 2, article 141.
186. Spencer HG, Zuk M. For host's sake: The pluses of parasite preservation. *Trend. Ecol. Evol*. **2016**, 31, 341-342.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

187. Glendinning L, Nausch N, Free A, Taylor DW, Mutapi F. The microbiota and helminths: sharing the same niche in the human host. *Parasitol*. **2014**, 141, 1255-1271.
188. Lee SC, Tang MS, Lim YAL, Choy SH, Kurtz ZD. Helminth colonization is associated with increased diversity of the gut microbiota. *PLoS Negl. Trop. Dis*. **2014**, 8(5), e2880.
189. Wolff MJ, Broadhurst MJ, Loke P. Helminthic therapy: improving mucosal barrier function. *Trend. Parasitol*. **2012**, 28, 187-194.
190. Ramanan D, Bowcutt R, Lee SC, Tang MS, Kurtz ZD et al. Helminth infection promotes colonization resistance via type 2 immunity. *Science* **2016**, 352, 608-612.
191. Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. *Science* **2002**, 296, 490-494.
192. Santiago HC, Nutman TB. Human helminths and allergic disease: The hygiene hypothesis and beyond. *Am. J. Trop. Med. Hyg*. **2016**, 95, 746–753.
193. Leung JM, Loke P. A role for IL-22 in the relationship between intestinal helminths, gut microbiota and mucosal immunity. *Intl. J. Parasitol*. **2013**, 43, 253-257.
194. Fleming JO. Helminth therapy and multiple sclerosis. Int. J. Parasitol. **2013**, 43, 259-274.
195. Peon AN, Terrazas LI. Immune-regulatory mechanisms of classical and experimental multiple sclerosis drugs: A special focus on helminth-derived treatments. *Curr. Med. Chem*. **2016**, 23, 1152-1170.
196. Summers RW, Elliott DE, Urban JF, Thompson R, Weinstock J V. Trichuris suis therapy in Crohn's disease. *Gut* **2005**, 54, 87–90.
197. Summers RW, Elliott DE, Qadir K, Urban JF, Thompson R, Weinstock JV. Trichuris suis seems to be safe and possibly effective in the treatment of inflammatory bowel disease. *Am. J. Gastroenterol*. **2003**, 98, 2034–41.
198. Summers RW, Elliott DE, Urban JF, Thompson RA, Weinstock JV. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterol*. **2005**, 128, 825–32.

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CHAPTER THREE

SYSTEMS THINKING IN NEW DRUG DEVELOPMENT

3.1 How new drugs are developed

Drug development using systems thinking is a nonlinear, iterative process that requires feedback and open communication between all stakeholders. Solid science is used throughout the process. To begin with, many basic principles of math, physics, chemistry and biology were established long before the pharmaceutical industry emerged. Many of these principles were established by doing pure research, with no apparent applications at the time. Pure research often occurs in academic surroundings with much less quality assurance than what is legally required to get regulatory approval for new prescription drugs. That is, an individual researcher or small group of collaborators can observe nature, devise a new theory, test it and report the results in peer-reviewed scientific journals and books. Even though this process usually ensures the quality and reliability of the scientific data that is reported, the interpretations of the results can be questionable – especially if there is insufficient theoretical background. For example, Gregor Mendel discovered the basics of heredity by observing pea plants long before anything was known about DNA or RNA. Without that knowledge, his results could be questioned because the characteristics of the pea plants (and all organisms) should be diluted out after a few generations. It wasn't until it was shown that genetic information is transferred from one generation to the next by DNA that his results were understood better. Also, he did not have a quality assurance officer double check his work every day and he did not record his results in an official lab notebook that was approved by any government agency. Still, his work established some basic rules of heredity that are still used today in medicine and new drug development. Even though the significance of his work was not fully appreciated at the time, it eventually led to many important advances. This includes an important tool, On-line Mendelian Inheritance in Man (OMIM). It also led to the Human Genome Project and many others.

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Gregor Mendel could not have envisioned the many practical applications that his work would have for fighting diseases and improving human health.

Even after basic scientific principles are established and the process of new drug development is well under way, pure and applied research continues. So, systems thinking is used to design, develop and test new drugs in a nonlinear manner. As new data emerges, theories may have to be modified or expanded. For example, Charles Darwin expanded on Gregor Mendel's laws of heredity to develop the theory of evolution. As mentioned in Chapter 1, his version of evolution included an explanation of how favored 'races' were preserved in the struggle for life. The misuse of the term race led to the Holocaust in World War II and continues to be misused by racists throughout much of the world. We now know that there is only one race – the human race. In addition, Francis Crick and James D. Watson showed that genetic information was passed from one generation to the next by DNA. This led to an understanding of the genetic triplet code, in which three nucleic acids code for a single amino acid. DNA was shown to be transcribed into mRNA, which was translated into peptides and proteins. As discussed in Chapter 2, we now know that human eukaryotic cells provide only a small portion of the DNA in the human body. Viruses and Bacteria provide much more DNA and have essential roles in the human body, which is an ecosystem in itself. Moreover, evolution is based on the survival of the most cooperative – not the fittest. Species must find an appropriate niche in an ecosystem instead of conquering and eliminating other species. The theory of evolution continues to be expanded and modified as scientists make new discoveries and communicate their results. For example, the rhizome of life is becoming a better metaphor that the tree of life.

Similarly, ideas about how drugs exert their therapeutic effects are being continuously modified. For example, the human body was once thought to be like a machine. The heart was thought to be a pump that delivered blood through arteries and veins that were like pipes in indoor plumbing. If they became clogged with too much cholesterol, blood pressure would increase. If they were clogged up severely, blood would not flow properly. This could lead to a heart attack (myocardial infarction). So, statins were developed as inhibitors in the rate limiting step of cholesterol biosynthesis, catalyzed by HMG-CoA reductase. In the early 1970s, this led to the isolation and characterization of mevastatin. If the drug development process were linear, that would have been the end of the development of statins. Instead, scientists repeated the process from the beginning and produced more statins that are now approved for

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lowering cholesterol. Still, the process did not stop there. Pharmaceutical companies discovered that statins not only reduced cholesterol, but also extended the average lifespans of people who took them. Scientific research continued. It was found that statins have pleiotropic (several) effects. There are many other examples of how new applications are being discovered for prescription drugs that have already been shown to be safe and effective for treating or curing at least one disease.

So, researchers around the world are learning important facts in biochemistry and medicine that are leading to the development of important new drugs and medical devices. For example, pharmaceutical companies have libraries and stores containing millions of compounds that have been tested in previous studies. Such compounds can be screened rapidly by high throughput methods. Other organizations might test thousands of botanicals used in traditional medicine to see if any of them are effective in phenotypic screens. Those botanicals (leaves, roots, fruits and/or seeds) that seem to be effective can be analyzed further. Some of the active ingredients can be separated from other compounds in the botanical by chromatography and tested further by phenotypic screens. Such studies can lead to the identification of a lead compound that may have important pharmacological properties.

Even though the discovery and development of prescription drugs has been described as proceeding in several steps, each step can be repeated as often as necessary until a drug is approved. The process even continues after the approval of the new drug. The steps in new drug development include: select a disease, identify a target, verify the target, find a lead compound, quantify drug-target interactions, determine solubility, pharmacokinetics and toxicity, improve the lead compound, find the best way to deliver the drug to the patient, manufacture the drug, apply to the FDA to do investigational studies, do the clinical studies, apply to the FDA for drug approval and do post-registration studies [1]. Each step can be repeated. Once a drug is approved, its effects on other diseases are often tested. One or more therapeutic targets may be identified at first, but many more can be discovered later – even after the drug is approved. However, the first compound that is found to affect therapeutic targets is seldom the best. It is called a lead compound because it leads to the development of even better compounds that are more effective and have fewer adverse side effects. At first, the interactions between the lead compound and the therapeutic target or targets are quantified. Then, the interactions between improved versions of the lead compound are quantified. However, this is not dogmatic. Many drugs are developed using phenotypic screening, with no knowledge about what therapeutic

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targets are involved. Regardless of whether a drug is developed by phenotypic screening or by its ability to interact with a therapeutic target, its bioavailability and best dosage form must be determined. So, the solubility of the lead compound (or improved versions of it) is determined. This allows the bioavailability and best dosage form to be established. Once this is done, the pharmacokinetics and toxicities are evaluated. Once a lead compound or an improved version has been shown to reach the target organ or organs and has little or no toxicity, the best way to deliver the drug to the patient is determined. Then, the best way to manufacture the drug in its optimal dosage form is established. Finally, the organization that developed the drug must apply to the FDA and/or other countries' regulatory agencies to do clinical trials, that (if successful) lead to approval. After the drug is approved, adverse side effects are monitored and reported as well as the effect on patients' lifespans or survival rate. Feedback and communication are essential at all the steps of new drug development – as are governmental regulations.

That is, once a lead compound is identified, pharmacokinetics and toxicity studies are done in accordance with FDA good laboratory practices, or GLP. Often, the lead compound will have some unacceptable harmful side effects. In that case, test compounds with similar structures can be synthesized and their physical, chemical and/or biological properties can be measured. Quantitative structure-activity relationships (QSARs) can be established between the test chemicals and the desired physical, chemical and/or biological activities. The QSAR can be used to predict the physical, chemical and/or biological properties of chemicals that have not yet been synthesized. Once the best chemical has been made or identified from an existing library, its solubility is determined so the best dosage form can be established. Then, the optimum method for drug delivery is selected. A manufacturing process is developed and documented in accordance with current good manufacturing practices, or cGMP. Eventually the best drug candidate, dosage form and delivery system are chosen. The new drug candidate can be similar to an existing drug or it can be a new molecular entity (NME), also known as a new chemical entity (NCE). That is, a NME (or NCE) is a drug candidate that contains no active portion (or moiety) that is in any drug that the FDA has already approved. Once the safety of an experimental drug or NCE has been demonstrated, a pharmaceutical company can apply to the FDA to ship it across state lines so clinical trials can be conducted. If the application is approved, it becomes an investigational new drug (IND) [1].

So, medicinal chemists try to develop or find chemical compounds that effectively prevent, treat or cure diseases, with little or no harmful side

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effects when taken at the therapeutic dose [1]. After a safe and effective method of manufacturing the NME is established under cGMP, its safety and efficacy are usually tested in at least two test animals (usually rats and mice). However, new tests that don't use animals are becoming more popular and informative. The effective and toxic doses are determined to establish the relative safety of a drug. They are used to calculate a therapeutic index or ratio of the lethal dose to the therapeutically effective dose in 50% of the test animals or in human organs on a chip. They are abbreviated as LD_{50} and ED_{50} for lethal and effective doses, respectively. That is, they are the doses that cause death or are effective in 50% of the test animals or human organs on a chip. The higher the therapeutic index, the greater is the margin of safety of the drug. The therapeutic index for exiting drugs were often determined in test animals before a Phase I trial in humans. Usually, the first step in testing drug safety in humans is to give very low doses to healthy subjects first, before testing it on patients at increasing doses. If it is shown to be safe, it can be tested in patients in Phase II and Phase III trials. Often, a drug fails in these trials. However, the drug can be used as a lead compound to try to find better drugs that are less toxic and/or more effective [1].

On the other hand, some human diseases don't occur in animals so there are no models for those diseases in any animal. Moreover, some human diseases (like many forms of cancer) have a very poor prognosis. Sometimes a promising NME emerges that may help patients who have a low life expectancy and for whom there is no acceptable therapy. In both cases (no animal model or poor prognosis), animal trials are not needed. So, Phase I clinical trials don't have to be done on healthy people. They can be done on people who have a low life expectancy and for whom there is no acceptable therapy. However, even in these cases, lead compounds are usually first tested on possible therapeutic targets *in vitro*.

So, let's look at the different steps in discovering and developing new prescription drugs, starting with selecting a disease to prevent, diagnose, treat and/or cure.

3.1.1 Select a Disease

Systems-based thinking is being used to help researchers and their colleagues select the diseases that they will try to prevent, diagnose, treat and/or cure. If reductionist thinking were used, the choice would be based on profit alone. Unfortunately, many people think that the FDA and other governments' regulatory agencies are in a conspiracy with pharmaceutical companies to keep people sick, so they can continue to sell them

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prescription drugs that don't cure any diseases. Such thinking ignores the fact that people who work for government agencies and pharmaceutical companies and their loved ones can also suffer from the same diseases that they are trying to cure. Many of them started studying pharmaceutical science and medicine because they wanted to find cures for diseases that they and/or their loved ones have or had. Of course, they want to get paid and pharmaceutical companies have to make a profit to stay in business. Still, their desire to help humanity and control the costs of healthcare is paramount. Moreover, many elderly and talented scientists, physicians, pharmacists and business leaders are very concerned about what they will leave behind for the next generation. They are also concerned about their reputations and how they will be remembered by future generations. So, it is very doubtful that very many people would want to conspire with others to maximize their income and profits at the expense of their own health and that of their loved ones. So, reductionist thinking seldom (if ever) is used in selecting a disease to study and cure.

Admittedly, many of the diseases that people work on mostly affect relatively rich people. Examples include heart disease, infectious diseases, neurodegenerative diseases and cancers. They are important, but it leaves out many serious diseases that mostly affect underprivileged people and people with relatively rare diseases. The FDA and regulatory agencies in other countries as well as pharmaceutical companies, hospitals, research institutes, colleges and universities realize that such diseases should also be cured. Even though underprivileged people in poorer countries might be disregarded by some, their importance is recognized by systems thinkers. In addition to a moral responsibility, there are important practical benefits in helping them. When diseases like AIDS, polio or malaria spread through a population, they can lead to loss of productivity, despair, depression and even war. These harmful effects can spread to privileged populations as desperate refugees seek new lives.

So, the FDA and regulatory agencies in other countries provide funding and incentives to researchers and pharmaceutical companies to find treatments and cures that are not profitable. The U.S. Congress passed the Orphan Drug Act of January, 1983 [1, 2]. It gives tax breaks and marketing exclusivity (monopolies) for seven years after the FDA approves drugs that can treat diagnose and/or cure diseases that affect <200 000 people in the USA [2]. There is also a Rare Pediatric Disease Priority Review Voucher Program. It gives vouchers to sponsors who get a new drug or biologic approved for a "rare pediatric disease that can be redeemed to receive a priority review of a subsequent marketing application for a different product" [2]. There is also a Humanitarian Use

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Device (HUD) program that is intended for use by people (or sponsors) who develop a device that will diagnose or treat diseases or conditions that affect <4000 people in the USA. Finally, there are three extramural grant programs for funding research. They include the Orphan Products Clinical Trials Grants Program. It supports clinical research on the safety and efficacy of drugs, biologics, medical devices and medical foods in treating rare diseases or conditions. It also includes the Natural History Grants program that supports the development of medical products for rare diseases by characterizing their natural history. The third program is the Pediatric Device Consortia (PDC). It provides funding to nonprofit consortia so they can develop new pediatric medical devices [2]. Similar orphan drug programs exist in Australia, China, Taiwan, Singapore, South Korea, the EU and Japan [3].

Many new drugs have been developed and approved under this Act [1, 2]. This includes drugs to treat glioma, multiple myeloma, cystic fibrosis, phenylketonuria and venomous snake bites. From January 1983 to May 2010, 2116 different orphan drug designations were granted by the Office of Orphan Products Development (OOPD) and 353 orphan drugs had received marketing authorization in the USA [3, 4]. In contrast, during the decade prior to 1983 less than ten such products were approved and marketed [4]. The FDA has a website for orphan drugs which includes a search engine for all orphan drugs and their approval status [5].

These and other programs also work on diseases that mostly affect people in poorer countries [1]. For example, diarrhea, malaria, leishmaniasis, sleeping sickness, dengue fever and yellow fever can be prevented or cured in richer countries, but not in poorer ones. Not-for profit research institutes, colleges and universities often do this work. One excellent example is the Bill & Melinda Gates Foundation (B&MGF) [6]. "It is the largest transparently operated charitable foundation in the world, founded by Bill and Melinda Gates in 2000 and doubled in size by Warren Buffet in 2006. The primary aims of the foundation are, globally, to enhance healthcare and reduce extreme poverty, and, in the United States, to expand educational opportunities and access to information technology. The foundation, based in Seattle, Washington, is controlled by its three trustees: Bill Gates, Melinda Gates, and Warren Buffett. It had an endowment of $39.6 billion as of July 6, 2015" [6]. One of their projects is to develop improved vaccines for newborn babies (neonates). In the USA and many other countries, there is relatively easy access to doctors for most people. So, parents can wait until their baby is two months old to start most immunizations, such as the DPT, to prevent diphtheria, pertussis (whooping cough) and tetanus. In poorer regions of the world, there aren't

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enough doctors or nurses to do this, so it would be better to immunize a baby the one and only time that it sees a doctor - when it is born [1, 6].

The Wellcome Trust in the UK also funds research and development of orphan drugs. They have a searchable database for rare diseases and orphan drugs. It is called orphanet [7]. They also worked with the European Bioinformatics Institute, the University Medical Centre, Utrecht and the University of Cambridge to develop and support a database called Reactome (http://www.reactome.org) [8]. It is a knowledge base of biological pathways that was built from biological reactions, including binding, phosphorylation, transport and other biochemical reactions and/or events [8].

To help develop new therapies for rare diseases and emerging pathogens, some researchers are trying a new, systems-based collaborative approach. They are looking for new applications for drugs that have already been approved by the FDA and other countries' regulatory agencies for treating other more widespread diseases [9-15]. However, the costs of some of the drugs for rare diseases are so high that they have been called niche-buster drugs [11]. Many databases and computational tools have been created to find new uses for drugs and biologics that have well-documented toxicity profiles and clearly described manufacturing controls [10]. They are applicable to more than just rare diseases. One of these is the Connectivity Map (CMap) project and its ancillary Library of Integrated Network-Based Cellular Signatures (LINCS). The LINCS contains large-scale gene expression profiles from human cancer cell lines that were treated with different drugs under different conditions. The goal of CMap is to make a detailed map of functional associations among diseases, genetic perturbations and drug actions. CMap can be integrated with functional genomics databases to compare drug gene expression profiles to profiles of disease gene expression. Others are looking at miRNAs that regulate a variety of cellular activities. One approach is based on feed forward loops (FFLs) between miRNAs and transcription factors. They found 48 existing drugs that influenced the expression of miRNAs that are part of FFLs and may be repurposed for treating patients who have cystic fibrosis. Another group predicted potentially new uses for existing drugs by constructing small molecule-miRNA networks for different types of cancer. A web server that links miRNA expression and drug function was also developed. It combines data on miRNA targeting and protein–drug interactions. There is a database (SM2miR) that annotates relationships between experimentally validated molecules and miRNAs [10].

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Another approach is to look for mutations in genes and do a multivariate analysis of genetic and gene expression profiles [10]. The profiles of cancer cell lines showed that a few mutated cancer genes are associated with drug sensitivity and may serve as biomarkers that may help predict and monitor drug response. In contrast, others are looking at phenotypes. They have coined the term phenome, which is the collection of all phenotypic information on drugs. Some people do phenome-wide association studies (PheWAS) to find previously unidentified links between genetic markers and human diseases. Moreover, the list of clinical side effects can be used to help discover new therapeutic uses for old drugs. It is also possible to look at nearly complete systems. For example, there is a model that includes the chemical structures of drugs, along with their biological activities and side effects. There is also a drug similarity network that is based on similarities between chemical structures and genes. Another group described a new algorithm called ligENTS. It finds new associations between drugs and their targets by mapping each drug to its global pharmacological space according to its chemical information [10].

It is also important to look for new applications of drug combinations or single drugs that have several therapeutic targets, since many diseases have several causes [10]. Computational methods have been developed to distinguish between safe and unsafe combinations of drugs. Known effects were extracted from the Drug Combination Database (DCDB). Statistical methods were used to that look for side effects, genes or disease pathways that could be affected by combinations of drugs. There are many databases and other sources of data that help with computational drug repositioning. Models based on machine learning can use the data to find underlying or previously hidden systems that can illuminate new links between drugs and diseases. There are also algorithms that use collaborative filtering techniques to predict previously unknown associations between drugs and diseases. The genome, phenome and chemical structures were integrated to extract drug and disease similarity matrices [10].

Network-based models are being used as well, because high-throughput technology and bioinformatics methods have matured [10]. They model molecular interactions using a biological systems network analysis. This includes networks of interactions between drugs with their targets, drugs with each other and drugs with diseases. It also includes looking at transcriptional and signaling networks as well as protein-protein interactions. This network-based approach can characterize drug targets and identify potentially new targets. Finally, recent advances in ways to

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mine texts have enabled better use of information available from previous publications for repositioning drugs [10].

3.1.2 Select a therapeutic target or phenotype

Once a disease has been selected, therapeutic targets or anticipated effects on phenotypes are chosen [1]. At first, most prescription drugs were developed based on their effects on their effects on the phenotype, such as whether or not pathogenic Bacteria lived or died when exposed to penicillin [16]. Cell-based assays are used to see if a characteristic associated with the disease phenotype is affected. This is called phenotypic screening, forward pharmacology, classical pharmacology or forward chemical biology. The therapeutic target and biochemical mechanisms of action may remain unknown – even after the drug is approved. Often this is the case with rare diseases that have not been studied very much. Phenotypic screening is a systems-based approach that looks at entire, intact cells or organisms and not just isolated components present in them. NMEs discovered in such screens may have different mechanisms of action. Retrospective analyses have found that the targets and mechanisms of action of many older drugs (especially those approved in the 1970s) were unknown. This includes aspirin, or acetylsalicylic acid. It took over 100 years to discover its molecular target (cyclooxygenase, or Cox) and mechanism of action (anti-inflammatory) [16]. Modern uses of phenotypic screening will be discussed further in section 3.1.16

On the other hand, many prescription drugs have been developed by looking at their effects on a single therapeutic molecular target [16]. This is often referred to as reverse pharmacology or reverse chemical biology. Therapeutic targets were usually discovered using animal models of diseases or by doing biochemical analyses [16]. In the classic, reductionist paradigm, a drug was thought to bind to a single therapeutic target. If it bound to another target, it was expected to cause side effects – some of them very adverse. Table 1 lists several classes of single molecular targets and gives an example of a drug that targets them. A much longer list of such drugs that were FDA-approved from 1999-2013 and their molecular targets has been published [17]. In the modern systems approach, an effective drug will often bind to several therapeutic targets which interact with even more biopolymers (especially proteins) not just to exert pleiotropic (many) therapeutic effects, but also to minimize side effects.

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Table 1. Molecular targets, drugs that target them and diseases treated.

Molecular target	Drug targeting it	Disease/Condition	References
Acetylcholine esterase	Donepexil (Aricept®)	Alzheimer's disease	[1, 18]
HMG-CoA reductase[a]	Statins, like simvastatin®	Heart disease	[1, 19]
Bcr-Abl; cAbl kinase inhibitor	Imatinib (Gleevec®)	Cancer (CML[b])	[1, 20]
Angiotensin-converting enzyme (ACE)	Enalapril (Vasotec®)	Hypertension	[1, 21]
HIV[c] reverse transcriptase	Zidovudine (AZT®)	AIDs	[1, 22]
VEGF[d]	Bevacizumab (Avastin®)	Cancers (colon, lung, renal, glioblastoma)	[1, 23]
H^+/K^+ ATPase (proton pump)	Omeprazole (Prilosec®)	Acid reflux	[1, 24]
Dipeptidylpeptidase 4	Sitagliptin, (Januvia®)	Type-2 diabetes	[1, 25]
β-adrenergic receptor	Propranolol and other β-blockers	Hypertension, cardiac arrhythmias and heart attacks	[1, 26]

a – HMG-CoA is hydroxymethylglutaryl-CoA, the rate-limiting step in cholesterol biosynthesis
b – CML is chronic myelogenous leukemia
c – HIV is the human immunodeficiency virus
d – VEGF is the vascular endothelial growth factor A

After deciding which disease to study, the next step is to identify the target in reverse pharmacology [1]. At first glance, this may seem easy. For example, malaria is caused by parasites that are protozoans in the genus *Plasmodium* that are carried by mosquitoes. AIDS is caused by the human immunodeficiency virus (HIV). Sepsis (blood poisoning) is caused by a pathogenic bacterium such as *Staphylococcus aureus*. Black Death (bubonic plague) is caused by *Yersinia pestis*. So, frequently the target organism can be identified in infectious diseases. Moreover, molecular targets within a virus or infectious organism can be identified. For

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example, HIV needs enzymes to make copies of itself and then mature to be able to leave the host white blood cell and infect other cells. That is, HIV needs a reverse transcriptase to convert its RNA into DNA that the host cell can use. HIV also needs a protease to form mature, infectious viruses. The HIV protease catalyzes the hydrolysis of a large precursor protein into the proteins needed for HIV to mature and infect other white blood cells. So, two classes of drugs in HIV therapy are reverse transcriptase inhibitors and protease inhibitors. Sometimes, however the molecular target is unknown. For example, artemisinin treats malaria, even though its molecular mechanism of action was unknown when it was approved. Aspirin (acetylsalicylic acid) treated headaches and fevers for over a century before its molecular target and mechanism of action were described [1].

In contrast, many approved drugs were discovered by phenotypic screening and were later found to affect several unintended targets, causing harmful side effects in many cases [1]. Improved drugs that only bind to the therapeutic target were developed and approved. Many of these drugs bind to an enzyme, receptor protein, ion pump, ion channel, microtubules, RNA or DNA. Some of these targets are highly-connected hubs in the cellular networks. When these hubs are changed by mutation or any other way, it affects the many nodes to which it is connected. This can lead to metabolic imbalance and disease. Networks are more vulnerable to attacks on the hubs than the less connected nodes. Similarly, cells contain many proteins and other biopolymers, only a minor fraction of which are highly connected. There is plenty of redundancy in the cellular network, so that harmful effects caused by eliminating many less-connected nodes can be mitigated by other surviving nodes. When genes coding for such nodes are eliminated (knocked out) in mice, there may be no visible effect. However, when a gene coding for a hub, like *RAS* or *P53* is knocked out, the result can be incompatible with live birth. So, drugs can treat and sometimes cure diseases by targeting the malfunctioning hubs. However, such hubs, like the oncoproteins ras and p53, interact with so many cellular processes that targeting them almost always causes serious adverse side effects in organs that were not the intended target. As network theory and bioinformatics improve, more appropriate, less obvious targets are being found. Researchers are identifying nodes which are dynamic and can change their level of connectivity to other nodes, depending on the needs of the cell [1].

To understand this better, a systems-based nomenclature can be used [1]. That is, most nodes in cellular networks are problem solvers. For example, the enzymes in glycolysis catalyze reactions that are needed to

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produce energy from glucose. They resolve the problem of a lack of energy in a cell. Hubs, though, serve as problem distributors. They distribute signals to many other nodes, in response to changes in the intra- and extracellular environments. Both problem solvers and distributors can be rather predictable. Another type of node is less predictable and is called a creative element. These nodes are very dynamic and monitor almost the entire network by continuously changing the structures of the proteins to which they are bound either directly or indirectly. Creative elements may have few links at any given time, but can increase their connectivities when needed. Also, such nodes (like p53 and protein chaperones) have internal structures that are flexible, so they can bind different ligands or substrates at different times and in different environments. Creative elements function like bridges, joining diverse groups of interconnected nodes, called modules. Some of them occupy unique positions or have a monopoly on a certain function in the network, so they can become bottlenecks. These can be promising drug targets. Targeting such nodes can influence many more nodes and have important effects on cellular functions and diseases [1, 27]. Therapies that affect many targets have been successful already in treating AIDS, many types of cancer and other diseases [1]. Several other drugs that are undergoing clinical trials affect many targets. Each target may have only a small influence on the disease process by itself, but can have a major effect when combined with other targets to produce a synergistic effect [1].

Individual enzymes have been some of the most effective therapeutic targets for prescription drugs and some dietary supplements that were developed using reductionist thinking. Many biochemists and other scientists learned more about the causes of many diseases that were formerly studied using phenotypic screening. As hypothesis-driven research became popular, specific enzymes were targeted. For example, the Framingham Heart Study found that having a high concentration of cholesterol in the blood was a risk factor for cardiovascular diseases. So, the biochemical pathway for synthesizing cholesterol was studied. The rate limiting reaction (slowest step) in this pathway was found to be catalyzed by HMG-CoA reductase, also known as 3-hydroxy-3-methylglutaryl-coenzyme A. The next step was to find chemicals that could inhibit this enzyme. A Japanese scientist (Akiro Endo) knew that some fungi might produce natural inhibitors of HMG-CoA, since this enzyme is needed to make mevanolate, an important precursor of components of cell walls in many infectious organisms. In the early 1970s, this led to the isolation and characterization of mevastatin, which is produced by *Penicillium citrinum* [28]. This was followed up by the isolation of another HMG-CoA

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inhibitor from the mold *Monascus ruber* and later the soil fungus *Aspergillus terreus*. It was originally called monacolin K or mevinolin, but it is now known as lovastatin. Clinical trials were performed in the 1980s. Lovastatin (Mevacor®) was granted approval by the FDA on September 1, 1987 [28]. Subsequently, many other statins have been approved by the FDA and other governments' regulatory agencies. However, it should be noted that there is a natural substance, red yeast rice (*Monascus pupureus*), that has been used for centuries as an herbal medication in China and contains a mixture of statins [29]. Unlike statins that are sold as prescription drugs, the doses of statins that are in red yeast rice dietary supplements are not well controlled. The amount of lovastatin and/or other statins must be written on the label of the bottle that contains the red yeast rice. However, the actual amounts found by chemical analyses can vary considerably and some samples were contaminated [30]. This is an excellent example of an important difference between prescription drugs and dietary supplements.

One thing that some prescription drugs and dietary supplements have in common is the way that their inhibition of enzymes is analyzed. Of course, many prescription drugs and active ingredients in dietary supplements don't function by inhibiting an enzyme. However, for those that do, their effects on enzyme activity are measured at different concentrations of substrate (S) and inhibitor (I) at concentrations that are much higher (μM concentrations) than that of the enzyme (nM concentrations) used in the analysis. That is, enzymes (E) catalyze reactions involving one or more substrates to produce one or more products. For example, there is a biochemical reaction that links glycolysis with lipid metabolism. Dihydroxyacetone phosphate (an intermediate in glycolysis) reacts with a coenzyme, NADH (nicotinamide adenine dinucleotide, reduced form), to produce glycerol-3-phosphate (from which phospholipids are made). In addition, NAD^+ (nicotinamide adenine dinucleotide, oxidized form) and H^+ are produced, as shown in reaction (1). This reaction is catalyzed by the enzyme glycerol 3-phosphate dehydrogenase. It can catalyze the reaction in either direction, depending on the needs of the cell. The structures of dihydroxyacetone phosphate, glycerol-3-phosphate, NAD^+ and NADH are shown in Figures 1-4.

Reaction (1): Glycerol 3-phosphate + NAD^+ ⇌ Dihydroxyacetone phosphate (DHAP) + NADH + H^+

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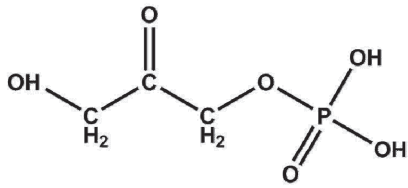

Figure 1. 2D structure of dihydroxyacetone phosphate (DHAP), an intermediate in glycolysis. At a physiological pH of about 7.2, the phosphate would be ionized.

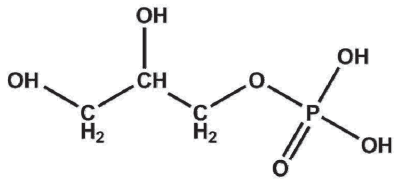

Figure 2. 2D structure of structure of glycerol-3-phosphate, from which phospholipids are made.

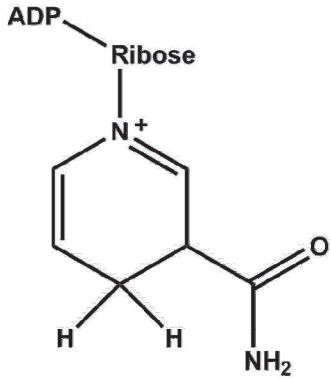

Figure 3. 2D structure of structure of NADH (nicotinamide adenine dinucleotide, reduced form), where ADP stands for adenosine diphosphate. NADH absorbs UV light at 340 nm. NADH is a coenzyme for many biochemical reactions that are catalyzed by the class of enzymes called dehydrogenases.

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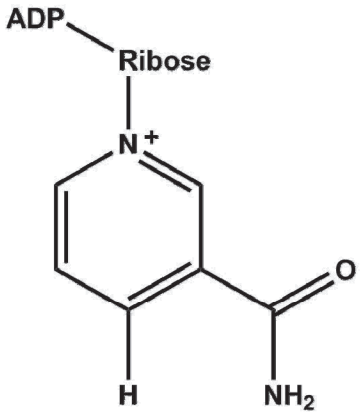

Figure 4. 2D structure of structure of NAD^+ (nicotinamide adenine dinucleotide, oxidized form), where ADP stands for adenosine diphosphate.

NAD^+ does not absorb UV light at 340 nm, but NADH does. NAD^+ is a coenzyme for many biochemical reactions that are catalyzed by the class of enzymes called dehydrogenases. So, the kinetics of reaction (1) can be studied by measuring the increase of UV absorbance at 340 nm after adding the enzyme to a mixture of a fixed, saturating concentration of NAD^+ and different concentrations of glycerol-3-phosphate. The rate or velocity of increase in the absorbance at 340 nm can be used to calculate the speed or velocity at which NADH is produced. The relationship between the velocity of the reaction (v) and the concentration of the substrate, S, is given by equation 1.

$$\text{Equation 1: } v = \frac{[S]V_{max}}{Km + [S]}$$

This is called the Michaelis-Menten equation [1, 31]. V_{max} is the maximum velocity of the enzyme-catalyzed reaction. It occurs when the concentration of substrate [S] is saturating, and K_m is the defined as the Michaelis-Menten constant of the substrate. This equation can be rewritten by taking the reciprocal of each side to obtain the equation (2):

$$\text{Equation 2: } \frac{1}{v} = \frac{1}{V_{max}} * \frac{K_m}{[S]V_{max}}$$

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A graph of v vs [S] describes a hyperbole, but a plot of 1/v vs 1/[S] describes a straight line. The y-intercept is $1/V_{max}$ and the slope is K_m/V_{max}. Note that K_m is not an equilibrium dissociation constant, like K_D in a complex formed between an agonist (or antagonist) and a protein receptor. Instead, K_m is a mixture of kinetic constants. Unless one knows the kinetic mechanism of the enzyme-catalyzed reaction, K_m can be difficult to interpret [1, 31]. So, a graph of 1/v vs 1/[S] is called a double reciprocal plot and looks something like the simulated graph in Figure 5. It is also called a Lineweaver-Burke plot.

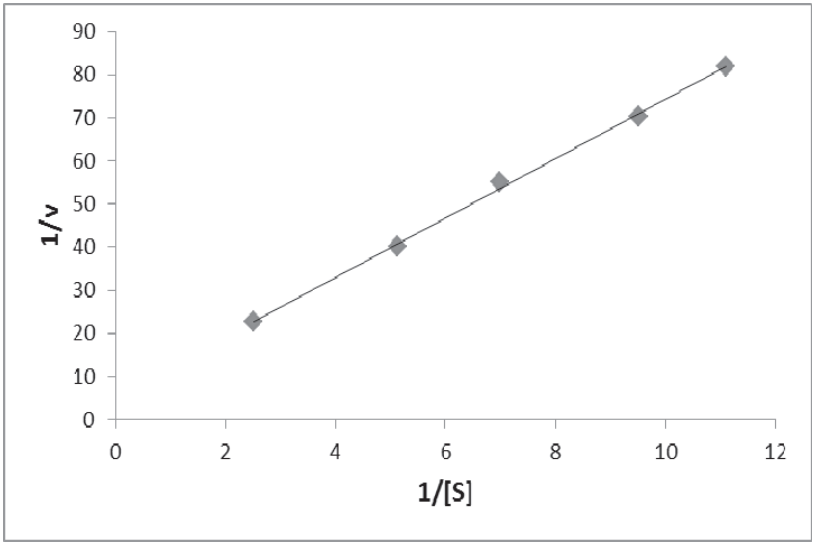

Figure 5. Double reciprocal plot of 1/v vs 1/S for a simulated enzyme-catalyzed reaction with no inhibitor present, with simulated experimental error (not a perfect straight line) [1, 31].

After measuring the reaction velocities at different concentrations of glycerol-3-phosphate and determining its K_m, a similar experiment can be done to study the reverse reaction. That is, the enzyme is added to varying concentrations of DHAP and a fixed, saturating concentration of NADH. This time, the decrease in absorbance at 340 nm is measured. From this, the K_m for DHAP can be determined. Then, if one were to find a compound that can inhibit this reaction, the type of inhibition can be determined. This is done by adding the inhibitor to increasing

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concentrations of the substrate (glycerol 3-phosphate or DHAP) and a constant concentration of coenzyme (NAD^+ or NADH). The reaction is started by adding the same amount of enzyme to each reaction mixture. If the inhibitor binds to the active site of the enzyme (where the substrate binds), it causes a competitive inhibition. That is, inhibition can be prevented if the concentration of the substrate is high enough. This can be shown by repeating the experiment by adding several other concentrations of inhibitor to obtain a series of double reciprocal plots that have the same y-intercept. A simulated example of this is shown in Figure 6. The data can be used to calculate the concentration of inhibitor needed to inhibit the reaction by 50%. It is abbreviated as IC_{50} [1, 31].

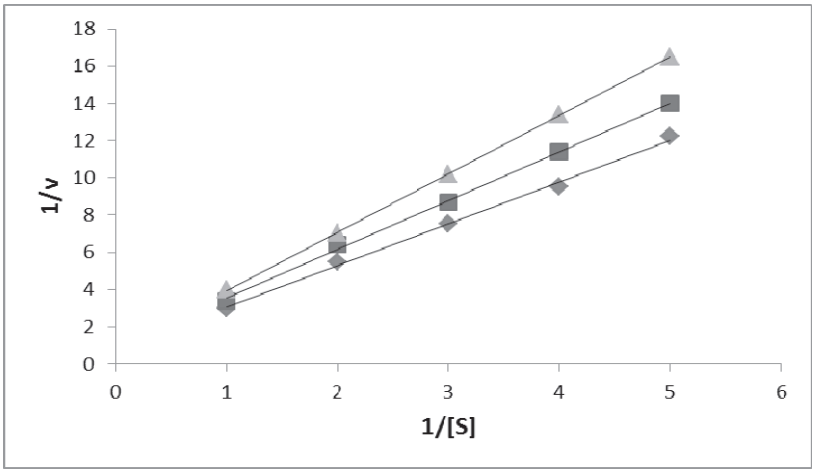

Figure 6. Double reciprocal plot of 1/v vs 1/S for a simulated enzyme-catalyzed reaction with different concentrations of inhibitor present, with simulated experimental error (not a perfect straight line). This type of result is obtained if the substrate binds to the enzyme's active site, causing a competitive inhibition [1, 31].

Some inhibitors of some enzyme reactions don't bind to the enzyme's active site [1, 31]. Non-competitive inhibition occurs when the inhibitor binds equally well to the free enzyme and the enzyme with substrate bound to it. In that case, the double reciprocal plots don't intersect at the same point on the y-axis. Many inhibitors of the enzyme called aldol reductase are non-competitive with respect to glucose. On the other hand, uncompetitive inhibition occurs when the substrate must bind to the enzyme before the inhibitor can bind to it. In this case, the double reciprocal plots are parallel to each other [1, 31].

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All of these enzyme kinetic experiments are valid only if the inhibition constant is much greater than the concentration of enzyme [1, 31]. If it is not, then the inhibitor binds very tightly. This type of binding occurs sometimes during drug discovery. For example, the test compound ZD5522 inhibits aldose reductase with an IC_{50} of 25 nM. That is, about 25 nM ZD5522 caused a 50% reduction in aldolase activity. Tight binding is suspected when the IC_{50} is very low [32].

Lineweaver-Burke plots have been used to study enzyme kinetics and inhibition since 1934 [1]. For quite some time, it was felt that there was only one conformation (or 3-dimensional structure) that an enzyme could have and still be biochemically active. This may be true for some simple enzymes, but it does not apply to larger proteins – especially those that have more than one subunit. For example, hemoglobin has four subunits that interact with each other. Moreover, the binding of oxygen (O_2) to hemoglobin is a cooperative process. As hemoglobin binds the first molecule of O_2, its affinity for more O_2 molecules increases. The plot of v vs [S] did not produce a hyperbole. Instead, it was sigmoid in shape. The reason for this was first explained by the eminent scientists Jean-Pierre Changeux, Jacques Monod and Jeffries Wyman. They showed that many proteins (enzymes and receptors) can exist in different states that are interconvertible. There are both relaxed and tense states (or structures) that are in equilibrium with each other. Molecules that affect the enzyme activity can bind to one state or another and shift the equilibrium. Some compounds can stabilize the active conformation of a protein, while inhibitors stabilize the less active or inactive conformations. Some inhibitors can bind at sites located some distance from the substrate binding site, called allosteric sites [1].

So, Jacques Monod and François Jacob used the term allosteric in 1961 to characterize the experiments of their student, Jean-Pierre Changeaux, on end product inhibition of *L*-threonine deaminase [1, 33]. The product of the reaction is *L*-isoleucine. It was proposed that it could inhibit the enzyme by binding to a different part of the enzyme. This made sense, since the structure of isoluecine is quite different than that of *L* –threonine [1, 32]. In 1965 the concept of allostery was modified in a journal article written by Monod, Wyman and Changeaux [33]. This MWC model was based on two important, but over-simplistic concepts [1, 33]. First, all allosteric proteins were thought to be oligomers consisting of identical monomers arranged symmetrically. Second, the allosteric effect is caused by binding of a ligand (like O_2) that leads to a change in the protein (hemoglobin) structure by rotating the subunits around an axis, thus maintaining the symmetry of the oligomer. In the original model, each

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subunit was thought to be able to exist in just two different states. Supposedly, they all had to be in the same state (R or T for hemoglobin) on any one protein molecule. An allosteric constant was even defined as being the ratio between the amount of protein in the T and R states, where the R (relaxed) state has more affinity for O_2 than the T (tense) state. This model explained how small changes in the concentration of O_2 can cause large changes in the allosteric constant. It explained the positive cooperativity, or synergism of oxygen binding. They also defined a partition function, Ξ, shown in equation 3 [1, 33]:

Equation 3: $\Xi = L(1+cK\lambda)^2 + (1+cK\lambda)^2$

L is the equilibrium constant between the free T and R protein structures (L = [T]/[R]). Next, K is the equilibrium binding constant for a subunit in the R state and c is the reduction factor for binding to the T state [1, 33]. Moreover, different interacting conformations of the four subunits of hemoglobin (quaternary structures) can bind O_2 in both the T and R forms. A change in equilibrium between the two conformers occurs when the ligand (O_2) binds to produce an allosteric effect. Similarly, the equilibrium between tense and relaxed states of an enzyme can change when a substrate binds to it. This model had many applications, from the original case of hemoglobin to membrane receptors and others [1, 33, 34].

However, the WNF theory has been modified further [1]. First, we now know that monomeric proteins can also have allosteric effects. Second, many proteins are intrinsically disordered. This enables them bind to many different ligands or substrates. Such proteins often are hubs in the protein interaction network and have special importance. Many oligomeric proteins are not symmetric. Third, many oligomeric allosteric proteins do not change their quaternary structure when they bind a ligand or substrate. Fourth, the WNF theory can't explain negative cooperativity, in which the binding of the first molecule of a substrate or ligand decreases the activity of the enzyme or receptor instead of increasing it. Instead, negative cooperativity was explained in a theory that was first described by Linus Pauling [35], and developed further by Daniel Koshland [36]. It is now called the KNF or Pauling-KNF model, after Koshland, Némethy and Filmer, who developed the sequential model [33]. It explained negative cooperativity by using a sequential model, with induced fit. That is, the binding of one molecule of an inhibitor to a subunit causes a conformation change in the next subunit. This decreases the affinity of the enzyme for the substrate (or ligand, for receptors). The partition function for this model is given by equation 4 [33]:

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Equation 4: $\Xi = 1 + 2K\lambda + \alpha K^2\lambda^2$

Here, the constant α is an interaction constant. It is >1 for positive cooperativity and <1 for negative cooperativity [33].

We now know that most proteins can exist in many different conformations [1]. This was not understood at first when X-ray crystallography was the only available method for determining the structures of protein crystals. That is because proteins have just one conformation in the solid phase. In contrast, when proteins are present in aqueous solutions in the cytosol of a cell and in fluid membranes they are flexible and can move [1, 33]. This was supported by the fact that many water-soluble enzymes could be denatured and rendered insoluble by heating them in aqueous solution. In the active state, an enzyme was thought to be folded into a single structure. Once denatured, it unfolded into a random coil. However, it was also noticed that there were many enzymes that were difficult or impossible to crystallize, so that they could not be analyzed by X-ray diffraction. As stronger magnets were made, NMR became capable of determining the structures of some of these proteins. In contrast to X-ray crystallography, NMR was able to analyze proteins that were dissolved in aqueous buffers, in which they are more mobile than they were as solid crystals. NMR showed that most proteins can exist in many interconvertible conformations in living cells. It is now known that the structures of active enzymes and protein receptors are flexible and this is vital to their proper physiological functions. So, proteins (and other biopolymers) have several structures that were determined by NMR, and they can be found in protein databases. Moreover, their motions can be quantified by molecular dynamics simulations [37, 38]. So, structures in protein databanks [39] that are based on X-ray crystallography show only one structure, but those based on NMR show several structures.

Still, X-ray structures have been and continue to be very helpful [1, 40]. As described by Perutz [40], hemoglobin has two quaternary structures in the solid, crystalline phase: T (deoxygenated) and R (oxygenated) for the tetramer and two tertiary structures (r and t) for each monomer. From the X-ray diffraction patterns of a series of crystal structures of unliganded T and ligand-bound R tetramers, he was able to show that there is important coupling between the tertiary structures of the individual monomers and the quaternary structure. Changes in tertiary structure precede changes in quaternary structure [1, 33, 40]. It should be noted that the primary structure of a protein is the amino acid sequence. The secondary structures are alpha helices, beta-pleated sheets and random

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coils. The tertiary structure is the three-dimensional (3D) structure of the monomer. The quaternary structure is the structure of the interacting subunits of a multimeric protein (tetramer in the case of hemoglobin).

Another former assumption has been invalidated by more recent data. It was once assumed that substrates and ligands could bind only to the lowest energy conformation of an enzyme or protein receptor. This was assumed to be the same conformation that was observed in X-ray crystallography of the crystallized enzyme or receptor. In the solied state (as crystals), proteins have one conformation and little movement. This led many to believe that there was only one conformation of an enzyme or receptor that could bind substrates or ligands. This was challenged by NMR data on dihydrofolate reductase (DHF reductase) [1, 33, 41], in which the substrate binds to a higher energy conformer of DHF reductase that has a higher affinity for it than the lower energy conformation. Unlike X-ray crystallography (which requires solid crystals), NMR looks at proteins (or any substance) that have been dissolved in a solvent. Proteins and other biochemicals dissolve in water (usually with the pH adjusted with a buffer). In the solid state, a protein's movement is severely restricted. So, it can only be in one conformation. Different conformations can be formed by mixing a protein with a substrate, substrate analogue or inhibitor. However, they can only be seen one at a time when they are in the solid state. In contrast, when a protein (or other biopolymer) is dissolved in water, it is quite flexible. It can move easily and change from one conformation to another. Moreover, we now know that proteins can exist in many structures that are interconvertible, not only when they are dissolved in the aqueous cytosol, but also when they are embedded in lipid membranes. So, instead of a single protein structure, there is an ensemble of structures.

In another twist, it has been found that both positive and negative cooperativity can occur in a single enzyme. One example is the class of proteins called chaperones. They are needed to help other proteins fold into their proper, active conformation after being translated from mRNA. They need the energy obtained from hydrolyzing ATP to make the necessary conformation changes on the unfolded protein substrate. Perhaps the best studied chaperone is the bacterial GroEL. It is shaped like a double ring [1, 33, 42]. It undergoes a structural change in each of the rings when they interact with a denatured (improperly folded) protein substrate. There is positive cooperativity when ATP binds cooperatively with one ring and negative cooperativity between the rings. So, the MWC model explains intra-ring cooperativity and the Pauling-KNF model explains the inter-ring negative cooperativity [1, 33].

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Another example of negative and positive cooperativity is the interaction between three proteins that can alter metabolism in cells infected by the adenoviral protein E1A [1, 43]. This protein uses its amino terminal region together with two other regions to bind several regulatory proteins cooperatively to subvert signaling pathways in infected cells [1, 43]. Two of them are the general transcriptional coactivator CREB (cAMP response element binding protein) and cAMP binding protein (CBP), its paralogue p300, and the retinoblastoma protein (pRb; also called RB1). The interactions between E1A, CBP and pRb can occur through either positive or negative cooperativity, depending on which interaction sites are available on E1A. So, the accessibility of the ternary and binary E1A complexes can be fine-tuned. This enables context-specific tuning of downstream signaling systems. This suggests that the conformations of intrinsically disordered proteins (IDPs) can be changed slightly to optimize their interactions with other proteins. The relative amounts of different protein conformations can be changed to optimize cellular properties and affect disease processes. It has been estimated that over 30% of the human proteome consists of IDPs. Most of them are involved in signal transduction. So, a single, well-defined structure is neither necessary nor sufficient for transmitting or transducing signals. Moreover, many proteins do not have only one structure that responds to a molecular signal. Instead, IDPs have an ensemble or collection of structures with sometimes very different binding sites for their substrates, ligands and/or inhibitors [1, 43].

Allostery can also occur in monomeric proteins, such as the bacterial chemotaxis protein Y, or CheY, since it can undergo several conformational changes, which are in equilibrium [1, 34, 44, 45]. The mechanism of CheY activation can be partly explained by both the MWC and Pauling-KNF models [34]. That is, the rotation of a critical tyrosine residue occurs prior to the displacement of another critical threonine, but the two processes are thermodynamically coupled, as predicted by the MWC model. However, the displacement of a beta sheet and an alpha helix is energetically favored only after the tyrosine has rotated. This is predicted by the Pauling-KNF model [34].

Negative cooperativity can also happen in homodimers, such as the catabolite activator protein, or CAP [1, 34]. It activates transcription by opening up the DNA sequence that is targeted, making it available to RNA polymerase. The N-terminus of CAP has a ligand binding domain that is also responsible for dimerization. Two molecules of cAMP bind CAP with negative cooperativity and function as allosteric effectors by increasing the CAP's affinity for the target DNA sequence. There are also intermediate

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conformers that can be populated. Researchers found that the negative cooperativity in the CAP protein can be explained better by changes in the protein dynamics than in the average structure upon binding the substrate [1, 34, 46]. This concept of a population shift is now widely accepted [34]. It emphasizes that some of the enzymes or receptors are in their active conformations. These are the dominant conformations after an allosteric transition. The dynamic nature of proteins, RNA and DNA is emphasized. Moreover, it is now known that ligands or substrates can bind almost anywhere and exert their effects throughout the proteins to which they bind. It has been suggested that all dynamic proteins are allosteric – even if there is no conformational change [1, 46]. There is also the idea that a change in the average structure of a protein is not needed for allostery, but rather a change in the distribution of the average structures [1, 34, 47]. These changes affect the subsequent binding affinity of substrates, ligands or inhibitors at distant sites [1, 34, 47].

So, proteins in buffered, aqueous solutions and in cells (be it the cytosol or a membrane) exist in an ensemble of conformations that can be characterized by an energy landscape [1, 48]. There are several valleys (energy minima) on the graph of the landscape that represent conformational sub-states. This landscape is dynamic. The population of different sub-states changes during an allosteric event that perturbs the structure of the protein, causing energetic strains. These changes propagate throughout the protein and shift the equilibrium distribution of sub-states. This causes changes in the shapes and properties of binding sites for ligands and substrates. New drugs are being discovered by combining evaluations of equilibrium fluctuation with genomic screens [1, 48].

Allosteric effects can be classified [1, 49]. This helps researchers better understand allosteric mechanisms, predict signaling at a molecular level and design new drugs. For example, G-protein coupled receptors (GPCR) are proteins that have seven alpha helices. Their typical conformational change was classified as conformational change, negative cooperativity and enthalpy driven. That is, ligands bind to an extracellular pocket on a GPCR. This causes a conformational change through the transmembrane helices that converts the GPCR from a resting to an activated state. The transmitted signal causes a change in the structure of the intracellular portion of the receptor. So, with GPCRs, a binding site is created for a target, which is a G-coupled protein. The trimeric G-protein in its inactive GDP-Gαβγ state catalyzes the exchange of GTP on the exchange protein for GDP on the G-protein. This causes the enthalpy-driven dissociation of the now active Gα and Gβγ and thus, negative cooperativity [1, 49]. Note that GDP and GTP stand for guanosine di- and triphosphate, respectively.

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For example, the epidermal growth factor (EGF) and its receptor, HER2 (human epidermal growth factor receptor 2), are allosteric proteins that undergo conformational change with positive cooperativity, enthalpy dominated, and the functional site is not at the substrate site [1, 49]. That is, binding of the EGF causes a conformational change in its receptor. This exposes a binding site that leads to the association of extracellular domains. This conformational change propagates through the cell membrane and induces intracellular dimerization of the two cytoplasmic kinase domains, thus causing positive cooperativity. Cytokine receptors also fit into this category [1, 49].

Notch receptors, CD4 receptors for the HIV-1 glycoprotein gp160 and the cAMP-activated exchange protein (EPAC, exchange protein activated by cAMP) are conformational change, positive cooperativity, enthalpy driven proteins [1, 49]. Notch receptors regulate cell fate. They are activated by a transmembrane protein, which enters the cell nucleus and affects DNA transcription. The HIV-1 gp160 protein is hydrolyzed to produce gp120 and gp41. The gp120 binds to CD4, causing a conformational change that results in exposing a binding site for the co-receptor CCR5 (C-C chemokine receptor type 5), causing positive cooperativity. The interaction between gp120 and its co-receptor causes a large conformational change in gp41 that relocates it from the gp120-gp41 interior to the host white blood cell membrane, enabling HIV to enter the cell. The EPAC is a receptor for the second messenger, cAMP. A salt bridge cluster called the ionic latch exists between the catalytic core and the cAMP binding domain (CDB). When cAMP binds to EPAC, it causes a conformation change in the CBD, shifting the equilibrium toward the open state. The salt bridges are weakened, facilitating movement of an alpha helix and enabling binding of Rap1 and Rap2 GTPases [1, 49]. Note that a GTPase is an enzyme that catalyzes the hydrolysis of GTP.

On the other hand, there are some "no conformational change, negative cooperativity, entropy driven allosteric proteins" [1, 49]. The catabolite activator protein (CAP) is an example. It controls the expression of over 100 genes. Each subunit of CAP has an N-terminal cAMP binding site and a C-terminal DNA binding domain. Two molecules of cAMP bind with negative cooperativity. However, binding of the first cAMP does not lead to a conformational change, so it is controlled by entropy [1, 49].

The ternary complex between the transcription factor CSL, Notch intracellular (NotchIC) and the coactivator called Mastermind is an example phosphorylation creating a binding site, with conformational change, negative cooperativity and is enthalpy driven [1, 49]. Ligand

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binding to the phosphorylated site causes NotchIC and mastermind to dissociate and become degraded or hydrolyzed [1, 49].

So, allostery applies to both protein receptors and enzymes. Moreover, recent research has focused on compounds that do not bind to the active site of target enzymes or ligand binding sites of receptors, but instead bind to allosteric sites [1, 50]. Active sites are often quite similar in isozymes that exist in different tissues and in different enzymes with similar functions. So, drugs that target the active site of one target enzyme often bind other proteins or isozymes and cause harmful side effects. This is less likely with allosteric drugs, because they bind on the protein surface that is far from the active site. As a result, they can be more specific and usually have fewer side effects. Since allosteric drugs don't bind to the active site, they don't act as off and on switches for enzyme or receptor activities. Instead, they can modulate those activities at relatively low doses and have therapeutic effects without harmful side effects. Examples include modulators of phosphodiesterase 4 activity that are used to treat neurodegenerative diseases. One excellent example is valium. More recent examples include maraviroc (Selzentry) and cinacalcet (Sensipar®) for treating AIDS and hyperparathyroidism, respectively. So, many current research projects are dedicated exclusively to the development of allosteric drugs [1, 49, 50]. This concept is being expanded to allo-network drug effects, in which drugs bind to an allosteric site on one protein and have many effects on proteins to which the target protein is linked. Combination therapies and multi-target drugs are examples of allo-network drugs. They cause several effects, often at places that are relatively far from the malfunctioning protein in the cellular network [1, 50].

Even though a molecular target was used in developing many drugs, this did not mean that their mechanisms of action were understood very well. Some were even misunderstood. As mentioned previously, statins are a good example of this [1, 51]. They were approved based on their supposed ability to prevent arteries from becoming clogged up with excess cholesterol. One of them, Lovastatin, was first discovered as a natural product in a fermentation broth of the fungus *Aspergillus terreus* [52]. Supposedly, statins can keep the arteries and veins from becoming clogged up with too much cholesterol. Since statins were such a huge commercial success, other pharmaceutical companies developed drugs that also lowered the concentration of cholesterol in the blood [53]. However, it was discovered that many of them did not decrease mortality, while statins did. The presence of elevated concentrations of cholesterol in the blood is a risk factor for cardiovascular disease by itself. However, it was not a significant risk for mortality. We now know that heart failure occurs in

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several steps, starting with smoldering inflammation, followed by the expression of cellular adhesion molecules in the endothelial cells [52, 54]. Then, leukocytes attach to the arterial wall. The vascular cell adhesion molecule-1 (VCAM-1) binds to monocytes and T lymphocytes, which are found in early atherosclerotic plaques. Oxidized lipids, nuclear factor-κB (NF-κB) and tumor necrosis factor-alpha (TNF-α) can also stimulate the expression of VCAM-1. When blood flow is disturbed, lesions start to form. Monocytes start to adhere to the arterial endothelium and enter the vessel walls, causing a fatty streak to start developing. The monocytes mature into macrophages, which will become foam cells. Next, T-lymphocytes of the adaptive immune system enter the arterial cell walls and cause more inflammation. The next stage is the formation of plaques, which can begin when a person is still in their early twenties. Finally, the plaques can rupture, leading to thrombosis or blood clots [52, 54]. So, statins save lives by having many (pleiotropic) effects and not just by reducing cholesterol [55-62].

Statins can not only lower the concentration of serum cholesterol, but also that of plasma low density lipoproteins (LDL, also known as LDL-cholesterol or LDL-C) and triglycerides (also known as triacylglycerides) [59-62]. These are risk factors for cardiovascular diseases and stroke. However, not all statins are equally effective [59]. That is, doses of 10 to 40 mg of rosuvastatin (Crestor®) lowered LDL-C more than atorvastatin (Lipitor®) or simvastatin (Zocor®). All the statins tested also lowered triglycerides, with rosuvastatin being the most effective [59]. It is also important to note that statins only lowered triglycerides in people who are already hypertriglyceridemic [60]. This is important because statins can be used to treat other illnesses in people with healthy (normal) concentrations of triglycerides. It is important that statins not cause hypotriglyceridemia in such patients. Moreover, almost all scientific articles and books that describe lipid dystrophy and other forms of imbalance in metabolism incorrectly use the term level instead of concentration. That is, the level of cholesterol (or any other substance in the blood or other tissues) is equal to the concentration times the total volume. Since the total blood volume can be low in people suffering from dehydration, it is important to know the difference. It is the concentration that is important in affecting health and toxicity – not the level. This fact is widely appreciated by the medical community, but it has not changed how researchers write about it or how peer reviewers evaluate such articles and books.

When statins inhibit HMG-CoA reductase, they inhibit the biosynthesis of not just cholesterol, but also other isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) [55].

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FPP and GGPP are substrates in the post-translational modification of many proteins. This includes the γ subunit of heterotrimeric G-proteins; heme-a; nuclear lamins; and the small guanosine triphosphate (GTP)-binding protein Ras; as well as the Ras-like proteins, such as Rho, Rab, Rac, Ral, or Rap [55]. Statins also inhibit the biosynthesis of isopentanyl adenosine, dolichols and the polyisoprenoid side chains of ubiquinone (also known as coenzyme Q_{10} and CoQ_{10}) [56]. These isoprenoids are needed to activate several intracellular signaling proteins (such as Ras, Rho, Rab, Rac, Ral snd Rap). This helps cause the pleiotropic anti-inflammatory, antioxidant, antiproliferative and immunomodulatory effects of statins. Statins also stabilize plaques, normalize sympathetic outflow, and prevent platelet aggregation [56].

Statins are not the only drugs that act by inhibiting enzymes [1]. Other enzyme-inhibiting drugs have many uses. Some are used to prevent heart attacks. Others are pain relievers, antibiotics, chemotherapeutic drugs to help cure cancer, anti-malarial drugs, sleeping pills, medicines for high blood pressure, and drugs to treat diabetes, asthma, HIV, seasonal flu, fungus infections, parasitic worm infections and osteoporosis. Enzymes that are therapeutic targets include acetylcholine esterase, AChE; L-amino acid decarboxylase; cyclooxygenase, or COX; angiotensin-converting enzyme, or ACE; dipeptidyl peptidase-4 (DPP-4); carbonic anhydrase; enzymes that catalyzes a key reaction in the synthesis of bacterial cell walls; bacterial DNA-gyrase; topoisomerase; penicillinase; bacterial tetrahydrofolate reductase; a reductase that catalyzes the synthesis of mycolic acids in *Mycobacterium tuberculosis*; renin; type 5 phosphodiesterase (PDE-5); farnesyl pyrophosphate synthase; inosine-5'-monophosphate dehydrogenase (IMPDH); calcineurin; HIV reverse transcriptase; HIV protease; hepatitis C virus (HCV) RNA polymerase; fungal enzymes; pyruvate-ferridoxin oxidoreductase; dihydrofolate reductase (DHFR); Na^+/K^+ ATPases; aromatase; topoisomerase; BCR-ABL tyrosine kinase; the proteasome; histone deacetylase; thymidylate synthase; viral neuraminidase, viral thymidine kinase; L-amino acid decarboxylase; xanthine oxidase; and dipeptidyl peptidase-4 (DPP-4). Some chemotherapeutic drugs for treating some types of cancer inhibit aromatase enzymes that catalyze the conversion of androgens, such as testosterone into estrogens, such as estradiol [1]. More recently, Rucaparib (Rubraca®) was approved by the FDA to treat BRCA-positive ovarian cancer [63]. It inhibits poly (ADP-ribose) polymerase (PARP) [63].

On the other hand, some drugs act by modulating instead of completely inhibiting enzyme activity [1]. There are allosteric drugs that target phosphodiesterase 4 (PDE4), but do not completely inhibit its enzymatic

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activity. There are even drugs that such as aminoglycosides that work by inhibiting the RNA-enzyme complex that comprises the small ribosomal subunit (30S) that is needed by Bacteria to make peptides and proteins, using an mRNA template and amino-acyl-tRNA [1].

There are also some proteins that are first activated by ligands and subsequently catalyze reactions [1]. So, they are both receptors and enzymes. Examples include receptor tyrosine kinases, which catalyze the addition of a phosphate to a tyrosine residue in a protein. Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) are tyrosine kinases. There are also serine/threonine kinases (STK) that catalyze the phosphorylation of serine and/or threonine residues in a protein. One of the most important STK is the mitogen-activated protein kinase (MAPK). It is part of a signaling cascade. That is, extracellular signals (ligands) bind to many types of kinases and initiate a cascade of signals that amplifies and transmits the message, stimulating growth and development. Drugs which inhibit specific kinases are being developed to treat several diseases, and some are currently in clinical use. This includes Gleevec® (imatinib) and Iressa (gefitinib). Imatinib is used to treat certain types of cancer. It is currently marketed by Novartis as Gleevec® (USA) or Glivec (Europe and Australia), and as its mesylate salt, imatinib mesilate. It has been reported recently that Gleevec may also help stroke victims when given in combination with a blood-clot dissolving drug, tissue plasminogen activator. Other approved anticancer signaling agents include Bortezomib, Dasatinib, Erlotinib, Everolimus, Lapatinib, Nilotinib, Sorafenib, Sunitinib, and Temsirolimus [1].

One very important prescription drug, metformin (Glucophage), is the first line treatment for type-2 diabetes [51]. It reduces the amount of glucose that is released from the liver and increases the transport of glucose into muscles, increasing insulin sensitivity and decreasing glucose absorption from the gastrointestinal tract. Metformin is also available in combination with the dipeptidyl peptidase (DPP-4) inhibitor, sitagliptin as Janumet®. Even though its mechanism of action was unknown when it was being developed, we now know that metformin increases the activity of one enzyme (adenosine monophosphate activated protein kinase, AMP-APK) in the liver and decreases the activity of another, protein kinase C [51].

On the other hand, some drugs act by binding to protein receptors that act as ion channels, pumps or transporters [1]. If a drug binds to a receptor and causes the same physiological response as the natural ligand, it is an agonist. If a drug binds to a receptor and prevents or blocks the physiological response by the natural ligand, it is an antagonist. To

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quantify the interaction between drugs, antagonists and receptors, the binding of ligands or physiological response at different concentrations of ligand and antagonist is measured. One plots the percent binding or percent response versus the log of the agonist concentration and looks for the log of the agonist concentration that elicits 50% of the response without an antagonist. The negative log is defined as pD_2. The binding of a compound, A, with a receptor, R can be written as an equilibrium binding, as shown in reaction 1 [1].

Reaction 1: $A + R \rightleftharpoons A\text{-}R$

On the other hand, the concentration (or chemical activity) of a compound that is free (not bound to the receptor) is represented as the letter F, and the amount of compound that is bound is often represented as the letter B [1]. So the equilibrium is often written as shown in equation 5.

Equation 5: $F + R \rightleftharpoons B$

The equilibrium dissociation constant, K_D, is given by equation 6:

Equation 6: $K_D = \dfrac{F[R]}{B}$

Let B_{max} be the maximum concentration of A-R, or bound ligand. Then equation 6 becomes equation 7:

Equation 7: $B = \dfrac{F * B_{max}}{K_D + F}$

There are many hormones that act by binding to receptors [1]. This includes the GPCRs. For example, the muscarinic acetylcholine receptors (M1 and M2) bind acetylcholine, and adrenoreceptors (β_1, β_2, α_1 and α_2) bind adrenaline. There are also GCPRs for angiotensin II, histamine, leukotrienes, opioids (μ and κ), prostanoids, somatostatin, serotonin ($5HT_1$, $5HT_2$, $5HT_4$), gonadotrophin-releasing factor and dopamine (D_2, D_3 and D_4). There are many therapeutic drugs for these receptors. They include tiotropium (for treating the lung disease COPD that is caused by smoking), atenolol (for hypertension), losartan (for hypertension), loratadine (for allergies), pranlukast (for asthma), morphine (an analgesic), epoprostenol (for primary pulonic hypertension), sumatriptan (for

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migraines), clozapine (for schizophrenia), goserelin (for cancer) and ropinirole (for Parkinson's disease) [1].

Receptor tyrosine kinases (RTKs) comprise another important class of membrane-bound receptors [1]. RTK class I is the epidermal growth factor receptor (EGFR) family. RTK class II is the insulin receptor family. RTK class III is the PDGFR family. All these receptors bind the ligands after which they are named. Their signals are transduced and amplified by downstream signaling pathways, often called signaling cascades. That is, a ligand such as the epidermal growth factor (EGF) binds to its receptor (EGFR), which catalyzes the phosphorylation of some of its own tyrosine residues, which activates downstream signaling cascades [1].

Opioid receptors have also been good therapeutic targets. Morphine, codeine, heroin and fentanyl bind to opioid receptors [1]. Fentanyl is 100 times as potent as morphine. It is used to treat severe and persistent pain. It is used in surgical procedures with intravenous or epidural administration. Analogues of this drug were made for other applications. One of them, called remifentanil is the shortest acting opioid (5-10 min) for use in humans [1].

Some membrane-bound protein receptors act as ligand-gated ion channels. Barbiturates work by binding to the gamma amino butyric acid-A receptor ($GABA_A$), which is a ligand-gated chloride ion channel that binds the inhibitory neurotransmitter, GABA [1]. Barbituates, such as sodium thiopental (Sodium Pentothal™) are potent $GABA_A$ agonists. Another barbiturate, Phenobarbital, also binds to GABA receptors and is used to prevent most types of epileptic seizures. Another pain killer (analgesic) that binds to a receptor is gabapentin (Neurontin®). It binds to a subunit of a multi-protein complex that regulates a specific voltage-gated calcium ion channel [1, 64]. Also, gabapentin binds to amino acid transporters in the brain, so that it can cross the blood-brain barrier and exert its analgesic effect [1, 64].

Diazepam (Valium®) binds to $GABA_A$ receptors, causing a conformation change that enhances the receptor's binding to GABA [1]. Zolpidem (Ambien®) is a sedative and hypnotic agent that binds to one of the subunits (α subunit) of the $GABA_A$ receptor in the brain, inducing sleep [1].

The 5-hydroxytryptamine (5-HT) receptors are a group of GPCRs that have 5-HT (more commonly known as serotonin) as their natural ligand [1]. The prescription drug sumatripan (Imitrex®) binds to 5-HT_{1D} and 5-HT_{1B} receptors and is an agonist. It is used to treat severe migraine headaches. Another drug, buspirone (BuSpar) binds to postsynaptic 5-HT_{1A} receptors and is used to treat generalized anxiety disorder [1]. A new

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generation of more effective anti-psychotic drugs binds to 5-HT_{2A} receptors, and others bind to metabotropic glutamate receptors (mGluR) [1, 65].

Adrenergic receptors at the junction between the postganglionic neurons and the innervated tissue in the sympathetic nervous system use norepinephrine (also known as noradrenaline) as their ligand [1]. Adrenaline and noradrenaline bind to two different classes of adrenergic receptors, α and β. Tamsulin (Flomax®) is an antagonist of α_{1a}-adrenergic receptors in the prostate. It relaxes the arteries and veins in the prostate so that fluids can pass more easily. Flomax® is used to treat benign prostate hyperplasia (BPH), caused by an enlarged prostate. On the other hand, the α_{2A} receptor mediates adrenergic suppression of insulin secretion. Many bronchodiators are β_2-adrenergic receptor agonists. For example, Salmeterol (Serevent®) is a long-acting bronchodilator that is used to treat severe, persistent asthma. Regular use of salmeterol and corticosteroids reduces the frequency and severity of asthma attacks. Side effects can include increased heart rate and insomnia. On the other hand, short acting β_2-adrenergic agonists provide faster relief. The combination of salmeterol and the corticosteroid fluticasone (Flovent®), known as Advair® is now used by most patients. Fluticasone propionate (the propionate salt of fluticasone) is a synthetic analogue of cortisol. An intensive search for drugs that did not have the same problems with side effects as cortisol led to the development of fluticasone [1].

The melatonin, glutamate, leukotriene and histamine receptors are also therapeutic drug targets [1]. Ramelteon (Rozerem®) is a melatonin receptor agonist that is used as a sleep aid. Memantine (Namenda®) is an antagonist of the N-methyl-D-aspartate (NMDA) subset of glutamate receptors that is used to treat Alzheimer's disease. Montelukast sodium (Singulair®) is a synthetic antagonist of a cysteinyl leukotriene receptor on the surface of airways in the lungs. This receptor binds cysteinyl leukotrienes, which are produced in response to irritation. Upon binding to the receptor, they cause bronchial and smooth muscles to contract and they produce too much mucus. Leukotrienes are pro-inflammatory and are involved in asthmatic and allergic reactions. Thus, antagonists like Montelukast sodium (Singulair®) prevent this. Loratadine (Claritin®) is an H1 receptor antagonist. It is used to treat seasonal allergies, rashes and food allergies. Some older antihistamines caused drowsiness, but Claritin does not. It has been the most widely used antihistamine since its introduction in 1981. Since 2002 it has been available without a prescription and is available as a generic drug. Ranitidine (Zantac®) is an H2 receptor antagonist. There are H2 receptors on the surface of parietal

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cells in the stomach that release acid. So, Ranitidine (Zantac®) is used to treat peptic ulcers caused by stomach acid [1]. Pitolisant (international nonproprietary name) or tiprolasant (in the USA) is an inverse agonist of the H3 receptor that received orphan drug status in the USA and EU for treating narcolepsy [1, 66]. It was recently approved for use in France for treating narcolepsy with or without cataplexy in adults [67]. It went through a successful clinical trial for treating cataplexy in adults [68]. There is also an investigational drug, Toreforant, that is a highly selective H4 receptor antagonist with a K_i of 8.4 nM [69]. It was shown to be safe in a Phase I clinical trial [69].

There are also many prescription drugs and dietary supplements that target nuclear receptors (NRs) [1]. Such receptors are especially important in reproductive medicine. They are transcription factors that affect metabolism. They bind ligands in the cytosol, cell membrane or cell nucleus. Those that bind ligands in the cytosol or cell membrane migrate to the cell nucleus, where they bind to specific parts of nuclear DNA to regulate gene transcription – often of adjacent genes. Ligands include lipophilic steroids, thyroid hormones, vitamins A and D, lipids, lipophilic environmental toxins and retinoids. They control many biological activities. For example, estrogen is involved in the development of the reproductive organs and central nervous system and in maintaining bone autopoiesis. NRs make good targets for therapeutic drugs [1].

The first oral contraceptive was called Enovid. It was approved by the FDA in 1960 [1]. Currently used contraceptives are taken at lower doses than Enovid. The estrogen component in them is ethinyl estradiol. It is combined with any of several available progestins, or synthetic proestrogens. For men, there is the hormone testosterone. It is approved for the treatment of testosterone deficiency as a male form of hormone replacement therapy. A synthetic form of testosterone called methyltestosterone is prescribed for some women (in combination with estrogen) as the female form of hormone replacement therapy. However, in 2006 it was reported that this can cause a higher risk of breast cancer, so this is not prescribed as much these days [1].

A synthetic steroid called Mifepristone (Mifeprex®) used to terminate pregnancies up to the 49th day of gestation [1]. It blocks the action of progesterone. It is used in some countries as a morning after pill to prevent pregnancy when taken within 12 hours of unprotected intercourse. It is also used in combination with Gameprost to terminate pregnancies between weeks 13 and 24. It can also be used to induce labor in cases of fetal death. For many years, it was known as RU-486. It was approved for use in Europe long before it was approved in the USA [1].

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Drugs are also available to induce labor by stimulating uterine contractions [1]. Oxytocin is a natural peptide hormone that does this. It binds to GPCRs, which initiate contraction. Also, prostaglandins can induce labor. Prostaglandin E_2 (PGE_2) (dinoprostone) is approved for the induction of labor. Misoprostol is a synthetic PGE_1 derivative that can also induce labor, but it is used in the USA mainly for preventing gastric ulcers that can be caused by non-steroid anti-inflammatory drugs (NSAIDs) [1].

Table 2 shows some examples of these and other nuclear receptor-binding drugs, their receptors and indicated uses [1].

Table 2. Lipophilic Nuclear Receptor-Binding Drugs, Receptors and Indications [1]

Drug	**Receptor**	**Indication**
Tamoxifen, Nolvadex®	Estrogen	Breast cancer
Raloxifene, Evista®	Estrogen	Osteoporosis
Conjugated estrogens, Premarin®	Estrogen and progesterone	Hormone replacement therapy
Norgestimate/ethinyl estradiol, Tri-Cyclen®	Estrogen and progesterone	Contraception
Bicalutamide, Casodex®	Androgen	Prostate cancer
Spironolactone, Aldactone®	Mineralocorticoid	Congestive heart failure
Fluticasone, Flonase®	Glucocorticoid	Asthma/hay fever
Calcipotriene, Dovonex®	Vitamin D	Psoriasis
Levothyroxine, Synthroid®	Thyroid hormone	Hypothyroidism
Isotretinoin, Accutane®	Retinoic acid	Acne
Gemfibrozil, Lopid®	PPARα	Cardiovascular disease
Rosiglitazone, Avandia®	PPARγ	Type-2 diabetes

PPAR is Peroxisome proliferator-activated receptor

Receptors are also important in the immune network [1]. For example, the sphingosine-1-phosphate receptor (S1P) is the target for the drug Fingolimod (Gilenya®). It is used as an immunosuppressant and is the first oral medication approved for treating relapsing multiple sclerosis. Sphingosine is a natural lipid that participates in cell signaling [1].

Receptors are very important targets for helping make vaccine effective [1]. For the vaccines to work, they need an adjuvant that provides

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an extra stimulus to the immune network [1]. They are made of things like bacterial cell wall components, liposomes, alum, and small pieces of DNA [1, 70]. These substances bind to receptors that are called toll-like receptors [71]. They get their name from the toll receptor that was first described in fruit flies [1]. A German-speaking scientist used the word toll, which means wild (or Wow!) to describe the first report on these receptors. Other receptors with similar structures, but different functions have been found in many organisms [1]. These toll-like receptors (TLRs) link the two categories of the vertebrate immune system, the innate and acquired immune systems [1, 71]. TLRs initiate the inflammatory response in immune cells. TLRs initiate signaling that activates the key transcription factor, nuclear factor kappa beta, NF-κB. This leads to the production of inflammatory cytokines [1, 71].

It is also possible to target proteins that are neither enzymes nor receptors. For example, the cytokine IL-17A is a target for the recently approved first in its class drug, secukinumab (Cosentyx®) [72-74]. It is used to treat severe plaque psoriasis, psoriatic arthritis and ankylosing spondylitis. It's a monoclonal antibody that binds specifically to the IL-17A cytokine and prevents it from initiating its pro-inflammatory signaling cascade. Even though many patients had been successfully treated with inhibitors of TNF, many of them suffered from significant harmful side effects, while others were not effectively treated by it. The IL-17 cytokine is produced by T_H17 immune cells, which cause inflammation and activate matrix destruction in cartilage and bone which leads to chronic inflammation. Secukinumab is also undergoing clinical trials to treat or cure other autoimmune diseases, including psoriasis, rheumatoid arthritis, Crohn's disease, dry eye, multiple sclerosis and asthma [73].

Some prescription drugs target or use DNA [1]. One of the most important is adriamycin (also known as doxorubicin). It is an anthracycline that was extracted from the fungus *Streptomyces peucetius* at Farmitalia, in Milan, Italy. It was discovered near the Adriatic Sea – thus the name Adriamycin. Daunorubicin is a very similar compound with one less oxygen. It is used mostly to treat myeloid leukemia, the most common form of leukemia in adults. Adriamycin (doxorubicin) is used to treat some forms of leukemia and Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lungs, ovaries, thyroid, soft tissue sarcom and multiple myeloma. Both adriamycin and daunorubicin act by being inserted (or intercalated) between base pairs in DNA, preventing its replication and its transcription into mRNA in rapidly dividing cancer cells. Adriamycin and doxorubicin also form a ternary complex between

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DNA and topoisomerase, an enzyme that catalyzes the unwinding of DNA [1]. Similar anthracyclines and topoisomerase inhibitors that are approved drugs include epirubicin, idarubicin, valrubicin, etoposide, irinotecan, mitoxantrone, teniposide and topotecan [1, 75]. Adriamycin is often given in combination therapy with other anticancer drugs, such as cyclophosphamide, taxotere, bleomycin, vinblastine, dacarbazine, vincristine, prednisone and 5-fluorouracil [1]. However, these drugs can kill any cell that is rapidly growing and dividing, so they are not specific for cancer cells. Adriamycin and daunorubicin can also cause cardiotoxicity. This stimulated the development of a new formulation that uses liposomes to encapsulate the adriamycin. It has lower cardiotoxicity. Also, many tumors can become resistant to adriamycin and other unrelated drugs, because cancer cells often start producing high levels of nonselective efflux pumps that belong to the ATP-binding cassette class, or ABC transporters [1].

Mitomycin is another drug that targets DNA [1]. It is used in combination with other drugs to treat cervical, stomach, breast, bladder, head, neck and lung cancer. Mitomycin is activated in liver cells and adds an alkyl group to bases in DNA. This kills the rapidly dividing cancer cells. Another alkylating agent is Ifosfamide, which is used to treat testicular cancer, breast cancer, lymphoma, testicular cancer, cervical cancer, bone cancer, soft tissue sarcoma, osteogenic sarcoma and ovarian cancer [1].

Carboplatin (Paraplatin®) is another chemotherapeutic agent that targets DNA [1]. It is given intravenously. It migrates to the cancer cells, where it is hydrolyzed to produce a platinum cation. The positively charged platinum ion binds to the nitrogen atoms of adenine and guanine, distorting the DNA double helix and preventing it from being copied. Carboplatin has been used to treat metastatic cancers of the ovary, testicles and bladder. The combination of bleomycin, etoposide and platins can often cure bladder cancer if it is found in the early stage. Similar approved drugs for treating cancer include carboplatin and oxaliplatin [1].

Bleomycin, actinomycin D, bis-chloroethylnitrosourea (BCNU), bendamustine, busulfan, CCNU (Lomustine), chlorambucil, cyclophosphamide, dacarbazine, hexamethylmelamine, ifosfamide, melphalan, methoxsalen, mitomycin C, nitrogen mustard, pipobroman, procarbazine, quinacrine, streptozotocin, temozolomide, thioTEPA, triethylenemelamine and uracil mustard also target DNA [1]. They act by damaging the DNA so it can't be copied or transcribed [1].

There are also antimetabolites and nucleosides that are approved anticancer agents [1]. Examples include 5-azacytidine, 5-fluorouracil, 6-mercaptopurine, allopurinol, calcium leucovorin, capecitabine, cladribine,

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clofarabine, cytarabine, decitabine, floxuridine, fludarabine, gemcitabine, hydroxyurea, methotrexate, nelarabine, pemetrexed, pentostatin and thioguanine [1, 72]. Some, like 5-fluorouracil can inhibit enzymes that are needed to synthesize DNA in rapidly growing cancer cells and others like 5-azacytidine can be incorporated into DNA and RNA [1]. This happens at relatively high doses. At lower doses, it can act as a DNA demethylase, catalyzing the removal of methyl groups, which tends to kill cancer cells [1].

Instead of targeting DNA, it is possible to do gene therapy [1]. When a patient has a mutated gene that codes for a defective protein, it may be possible to deliver the correct (wild type) gene to the patient. For example, children born with a defective gene that should code for the enzyme adenosine deaminase develop severe combined immunodeficiency (SCID) [1]. In 2016, the European Medicines Agency approved a gene therapy called Strimvelis to treat patients with SCID [76].

Research is also being done on using naked DNA in anticancer vaccines [77]. It has been shown that immunization with naked plasmid DNA that codes for tumor-associated antigens or tumor-specific antigens stimulates both cellular and humoral immune responses. Fortunately, vaccination with naked DNA does not induce autoimmune diseases in healthy animals. Moreover, DNA vaccines based on plasmid vectors can be produced and tested rapidly and economically. However, such vaccines often induce weak immune responses. So, several ways of improving the immune response are being investigated. This includes optimizing the antigen, using adjuvants and delivery systems like electroporation, co-expression of cytokines and co-stimulatory molecules in the same vector, as well as different vaccination protocols [77, 78].

In addition, pieces of bacterial DNA can be used to prevent diseases [1, 79]. Bacterial DNA contains CpG motifs that can stimulate the innate immune system by binding to TLR 9. This activates both the innate and the acquired (or adaptive) immune systems. In an effort to develop vaccines for neonates, the Bill and Melinda Gates Foundation, the Krembil Foundation and the Canadian Institutes of Health are trying to use CpG oligonucleotides as adjuvants in vaccines for diseases such as whooping cough (pertussis). Our human immune cells recognize CpG nucleotides as foreign, since human (and other eukaryotic) CpG dinucleotides are usually modified by the attachment of a methyl group. The bacterial CpG oligonulceotides selectively stimulate the T_H1-type immune response, which is needed for the vaccine to work. That is, there are two classes of helper T cells, T_H1 and T_H2. The T_H1 form is more active in neonates, so it

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is important to stimulate the T_H1 form, which is done by oligonucleotides containing several CpG dinucleotides [1, 79].

Viral DNA is being evaluated as a vaccine to prevent infection by the Zika virus [80]. DNA vaccines deliver DNA that codes for a viral antigen that is produced in the nuclei of host cells, which biosynthesize the antigen. The antigen then triggers an immune response that eliminates the virus. Fortunately, the DNA is more easily modified and engineered than live attenuated viruses that are used in some other vaccines. DNA is also comparatively easy to manufacture and purify. Finally, DNA (unlike many proteins and live attenuated viruses) is thermally stable, so it doesn't have to be stored or shipped refrigerated [80].

Different types of RNA can also be good therapeutic drug targets [81-87]. RNA therapeutics can be used to control the transcription of DNA [81]. Therapeutic RNAs can increase, decrease or stop the biosynthesis of specific proteins. They are being studied as possible ways to treat many human diseases including myocardial infarctions or heart attacks. Antisense oligonucleotides are designed to bind to an mRNA target to inactivate its translation into a protein. Ribozymes can recognize and catalyze the destruction (by hydrolysis) of an mRNA target. Aptamers bind can bind to a variety of targets and inhibit their activities. One example is Pegaptanib, which was approved for neovascular age-related macular degeneration. It is an aptamer that targets VEGF. It was discovered by the systematic evolution of ligands by exponential enrichment (SELEX) [81]. One starts with an ensemble of random RNA or DNA oligonucleotides, usually 20–40 nucleotides long [88]. Fixed regions containing binding sequences for reverse transcriptase (RT) and polymerase chain reaction (PCR) primers, as well as a promoter sequence for T7 RNA polymerase, and perhaps restriction endonuclease sites for cloning are attached to the randomized oligomers. Those aptamers that bind a target are selected, amplified by RT-PCR (reverse transcriptase PCR) and then reselected using a higher stringency assay. The process is repeated as many times as needed to achieve the desired affinity and specificity. The selected aptamer sequences then can be synthesized using solid-phase phosphoramidite chemistry after cloning and sequencing them [88].

Synthetic mRNA can also be used as a potential new drug class for mRNA-based cancer immunotherapies and infectious disease vaccines [83]. Synthetic mRNA can be designed to express proteins transiently by ensuring that its structure resembles the natural mRNA. In addition, mRNA can be used to replace or supplement proteins, make pluripotent stem cells and make mRNA-encoded designer nucleases [83].

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Modified mRNA has been used to induce vascular regeneration after myocardial infarction [84]. This is a cell-free approach for doing regenerative therapeutics. It was shown that intramyocardial injection of a synthetic modified RNA (modRNA) encoding human vascular endothelial growth factor-A (VEGF-A) expanded and differentiated endogenous heart progenitors in a mouse myocardial infarction model. The VEGF-A modRNA strongly improved heart function and increased the long-term survival of patients who received it. The results suggested that modRNA can be a versatile approach for expressing paracrine factors that control cell fate and enhance long-term organ repair [84].

Chemically modified nucleotides can also affect the translation of mRNAs [85]. So, chemical modified mRNAs can significantly increase the expression of proteins that they encode. Even though mRNAs that code for important functional proteins have been studied for various therapies, their instability and immunogenicity limits their use in humans. So, a recent study showed that mRNA that was chemically modified can increase their translation or expression into proteins. The modified mRNAs had *N1*-methylpseudouridine, 5-methoxyuridine and pseudouridine in them. So, these are promising modified nucleotides that may lead to the production of clinically relevant modified mRNAs [85].

Another type of RNA, microRNAs (miRNAs), may also become useful in cancer therapy because some of them can block angiogenesis [86]. They can modulate the expression levels of specific proteins based on sequence complementarity with their target mRNA. As a result, they play a key role in both health and disease. Angiogenesis is the process of forming new blood vessels from preexisting ones. This is particularly important cancer metastasis. That is, microRNAs are crucial regulators of signaling pathways in many types of cells, including endothelial and perivascular cells. Angiogenesis is activated when preexisting vessels become permeabilized in response to stimulating factors such as VEGF, PLGF (placental growth factor), and ANG-1 (angiopoietin-1). Also, extracellular matrix metalloproteinases (MMPs) catalyze the degradation of the basement membrane and extracellular matrix (ECM). The underlying endothelial cells (ECs) are attracted by the angiogenic stimulus produced by the tumor cells and the microenvironment. They migrate into the perivascular space. The pericyte coating becomes inadequate in the tumor vasculature. This leads to the formation of fenestrations and/or transcellular holes. These incomplete basal membranes lead to the formation of vessels with irregular diameters and structural abnormalities. At the same time, tumor blood vessel walls are formed by both endothelial and tumor cells. Angiogenesis is an essential step in cancer development

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and metastasis. It allows cancer cells to enter into the circulation and migrate to other organs. Once a tumor metastasizes, the patient's prognosis is bad. So, new antiangiogenic drugs are being developed to treat and hopefully cure many types of cancer [86].

At the same time, anti miRNAs are being developed to help treat Alzheimer's disease (AD) [87]. About 2650 mature human miRNAs have been found using existing technologies. However, only a few of them (40-50) are abundant in the human CNS. About one-quarter to one-fifth of them are significantly up-regulated in the brains of people who have AD. So, chemically modified miRNAs are being developed to target pathologically up-regulated miRNAs in the degenerating CNS. Many use locked nucleic acids (LNAs) to significantly increase the half-life and stability of the miRNA. So, LNAs have been used to synthesize the first miRNA-targeted drug Miravirsen®, an inhibitor of the liver-specific miRNA-122 that the hepatitis C virus needs to replicate itself [87].

So, many prescription drugs were developed and are being developed based on their abilities to target enzymes, receptors, DNA or different types of RNA. However, modern medicinal chemistry is now using the systems-based approach of network medicine [89]. Network-based approaches are being used to identify and prioritize drug targets. The system-wide effects of potential therapeutic targets are modeled to help identify new network drug targets. These network drug targets are not obvious from the traditional magic-bullet type analysis that tried to find the single most important cause of a given disease. By looking for drug targets that are important nodes in a network, additional hits can be found that were not obvious using a reductionist approach. Also, targeting edges that link nodes may make some of these hits even more specific. Drug target networks expose the system-wide target landscape and, when combined with other network methods, help reposition drugs for new applications. At the same time, some drugs can be designed to affect several targets by integrating the effects of the drugs on a systems level. It is also important to look for hidden connections. That is, allo-network drugs affect therapeutic drug targets that were not obvious at first glance. Such drugs can specifically modulate the targets without causing the many side-effects that can be result by direct targeting. Finally, by treating the whole cellular network (or a segment of it) as the therapeutic target, one obtains a conceptual epiphany of network description and analysis in drug design [89].

There are two strategies that can be used in network-based drug targeting: the central hit and the network influence strategies [89]. The central hit strategy is useful in finding new drug target candidates for

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curing infectious diseases and cancer. The goal is to damage the network integrity of the infectious agent or of the malignant cell in a selective manner. One tries to find hubs and central nodes (load-points) of various networks. They are also excellent targets in finding cures for infectious diseases. A detailed knowledge of the structural differences of host/parasite or healthy/malignant networks can help with this. In addition, choke points in metabolic networks are potentially excellent therapeutic targets. Also, directed nodes at the top of the hierarchy in the metabolic network of an infectious organism should be attacked by the central hit strategy. At the same time, anticancer drugs can be identified by analyzing metabolic networks that are specific for each type of human cancer. The network influence strategy is most useful in identifying new drug candidates for treating or curing complex diseases that are caused by several dysfunctional genes and/or proteins. The goal is to shift the malfunctioning network back to its normal, healthy state. An understanding of network dynamics in both healthy and diseased states is required for this approach. It is also useful to know as much as possible about the existing drug targets of the disease that is being investigated [89].

The arachidonic acid (AA) metabolic network contains several enzymes that are therapeutic targets, including 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) [90]. These enzymes are upstream from other enzymes and signaling molecules in the AA metabolic network. AA is an omega-6 (ω-6) polyunsaturated fatty acid with 20 carbons and four C=C bonds (20:4). Its structure is shown in Figure 7. The AA metabolic network produces inflammatory mediators, including leukotrienes (LTs) and prostaglandins (PGs) [1]. PGs were first discovered in the prostate gland. They all have 20 carbons, so they are eicosanoids, as are leukotrienes. PGs have a five-carbon ring and are derived from arachidonic acid. COX catalyzes their synthesis. They are found in almost all tissues and organs. They contribute to various diseases such as asthma, rheumatoid arthritis, atherosclerosis, Alzheimer's disease, and cancer [1].

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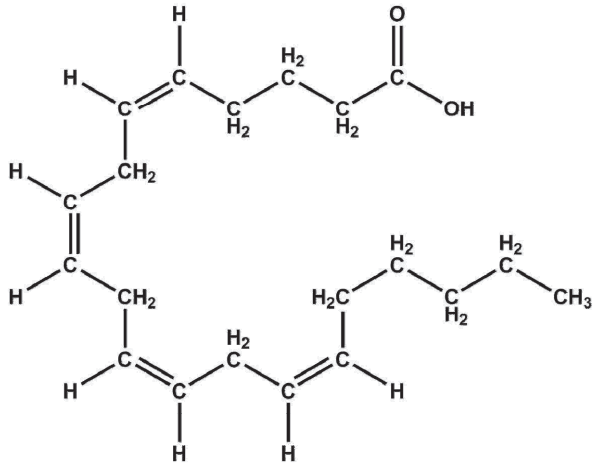

Figure 7. 2D structure of arachidonic acid, (5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenoic acid, 20:4(ω-6).

LTs are also 20-carbon derivatives of arachidonic acid, but their synthesis is catalyzed by 5-LOX [1]. As the name implies, leukotrienes have three carbon-carbon double bonds (C=C) and are made by leukocytes (white blood cells). They help regulate blood flow. One of them, leukotriene 4, attracts white blood cells to the site of an infection and increases the permeability of blood vessels. LTs are signaling molecules that can affect either the cells that produce them (autocrine signaling) or neighboring cells (paracrine signaling) to help regulate immune responses. LTs are also produced along with histamines and PGs, which are mediators of inflammation. One of the LTs (leukotriene D_4) triggers the contraction of smooth muscles lining the bronchioles in the lungs. When LTs are over-produced, it can lead to asthma. LT antagonists are used to treat it. On the other hand, PGs cause pain and inflammation, while inhibiting blood platelet aggregation and the formation of unnecessary blood clots. They are biosynthesized in reactions catalyzed by cyclooxygenase-1 and 2 (COX-1 and COX-2). The active ingredient in aspirin (acetylsalicylic acid) irreversibly inhibits both COX-1 and COX-2 by covalently attaching an acetyl group to a serine residue in the active site of each enzyme. This prevents the formation of pro-inflammatory PGs and helps to prevent heart attacks by inhibiting platelet aggregation [1].

When blood platelets aggregate, they can cause blood clots, which are the primary cause of heart attacks and stroke [1]. So, a low dose of aspirin (81 mg) taken once a day can help prevent heart attacks and stroke.

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Aspirin is given as first aid in case of a heart attack. Also, 81 mg is enough to change the activity of COX-2 in endothelial cells, so that it catalyzes a reaction that leads to the synthesis of the class of anti-inflammatory eicosanoids, called lipoxins in monocytes and leukocytes. This is catalyzed by 5-lipoxygenase (5-LOX), which also catalyzes reactions that initiate the synthesis of leukotrienes. Leukotrienes and lipoxins are potent mediators of the inflammatory response. Lipoxins counteract the effects of pro-inflammatory eicosanoids. Lipoxins are antiinflammatory for neutrophils and eosinophils. This helps them clear inflamed tissue. Also, lipoxins promote the restoration of an injured airway epithelium by indirectly blocking the release of the proinflammatory cytokines IL-6 and IL-8 and by inhibiting neutrophil transmigration. Aspirin helps resolve inflammation by inducing the synthesis of resolvins and protectins. Resolvins have anti-inflammatory actions that lead to the resolution of the inflammatory cycle. They are synthesized either from eicosapentaenoic acid (EPA) or from docosahexaenoic (DHA), which are omega-3 polyunsaturated fatty acids [1].

So, aspirin is a non-steroid anti-inflammatory drug (NSAID) [1]. Since it can lead to severe and sometimes fatal internal bleeding, other NSAIDs were developed that selectively and reversibly inhibit just COX-1 Examples include ibuprofen, naproxen (Alleve®), Vioxx® and Celebrex®. They provide similar pain relief as aspirin, but with greatly reduced risk of fatal or debilitating peptic ulcers [1].

Uncontrolled, smoldering inflammation can lead to diabetes, cardiovascular disease and many types of cancer [90]. They are seldom treated effectively by a traditional single-target drug. Instead, safer and more effective anti-inflammatory drugs are being developed by analyzing biological networks, including the AA network. The goal is to find drug candidates that target two or more proteins in the network. For example, licofelone (ML3000), an inhibitor of COX and two other enzymes, is undergoing phase III clinical trials. Similarly, the dual-target inhibitors, azelastine, tepoxaline, and licofelone are undergoing clinical trials [90].

In addition, the AA metabolic network and multitarget optimal interventions are being used to help understand traditional Chinese medicine (TCM) [90]. TCM uses mixtures of several medicinal plants that contain many active ingredients. So, molecular docking was combined with an analysis of the AA metabolic network to help understand the anti-inflammatory functions of herbs used in TCM. Several herbs that are related to “heat clearing” or “syndrome-relieving” were found to be involved with the AA metabolic network. Therapeutic targets were identified [90].

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So, using systems thinking to analyze metabolic networks and their dynamics is proving to be important in new drug development.

3.1.3 Find a lead compound

Once a disease and therapeutic targets have been identified, the next step is to find chemicals that either bind to the therapeutic target or have the desired effect on the disease phenotype. These are called hits. Next, the most promising of these hits is identified as a lead compound [1]. Systems thinking often looks at nature to find compounds that make good lead compounds. In traditional medicine, a long oral tradition has identified many plants, herbs and extracts from plants that have medicinal properties. Many of them have been analyzed to try to find the active ingredients. Using modern science and medicine, the chemicals present in these organisms can be separated by chromatography and identified by spectroscopic techniques, such as NMR and mass spectrometry. Also, modern medicinal chemists have libraries containing millions of compounds that have been synthesized and tested on at least one molecular target of a disease. As similar new targets emerge for other diseases, these compounds can be tested as well [1].

Once the basic biology and chemistry of a disease are understood, simple tests can be done [1]. This often begins with an *in vitro* screen of extracts of botanicals and is followed by a secondary function screen. Next, the metabolism and pharmacokinetics of the most promising compounds are evaluated. This should lead to a description of a pharmacodynamic model, followed by a disease model, and finally development of the best new drug candidate. In the past, reductionist thinking led researchers to do a primary screen to measure drug binding to a single therapeutic target. These days, systems-based thinking is used to determine the effects of the test compounds on cells and native tissues. The metabolism and pharmacokinetics of substances that pass the primary and secondary screens are determined. In the past, a pharmacodynamic assay was done using animals (usually rats and mice). The goal was to demonstrate that the drug will reach the target organ and determine the LD_{50}. So, animal models were used as a pre-clinical proof of concept and a preliminary test for toxicity [1]. However, these days, cell-based assays and tissues on a chip (especially liver on a chip) are used.

Once the basic research has been done, it is time for applied research [1]. That is, once we learn about how drug candidates function in glass test tubes or Petri dishes (*in vitro*) containing cells or single proteins, it is important to learn how they function in live organisms (*in vivo*) [1]. It has

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been estimated that an organization can use biochemical and cell-based *in vitro* assays to rapidly screen up to 100 000 compounds per day in high throughput screening [1, 91]. Animal models can test 100 to 100 000 compounds per year. Standard rodent toxicology tests can be done on 10 to 100 compounds per year. Tests on humans can only be done for only one to three compounds per year [1, 91].

High throughput screening (HTS) can be used to evaluate rapidly over a million compounds in the lab and on the computer (*in silico*) [1]. Sometimes lead compounds can be found through fragment-based drug discovery. First, small (molecular weight <300) compounds are identified that bind weakly to a biological target, such as a membrane-bound receptor or an enzyme. The parts of these compounds (pharmacophores) that bind to the target protein are identified. Once these important fragments are identified, more compounds can be synthesized that combine the different fragments to make a more potent NME. It is usually much easier to make NMEs by joining fragments than to synthesize much larger NMEs that are resemble large, lead compounds that are identified by more classical methods [1].

Isothermal titration calorimetry (ITC), NMR and X-ray crystallography can be used in an integrated approach to screen libraries of small compounds and discover new leads [1, 92]. That is, when a molecule binds to a target protein it causes a change in the temperature at which the protein unfolds, or 'melts' (ΔT_m). This can be quantified by ITC. The part of the test compound that binds to the target protein in an aqueous, pH-buffered solution can be identified by NMR. That is because when ^1H nuclei of the binding fragment are within 5 Å of the ^1H nuclei in the binding site of the therapeutic target, magnetic energy is transferred from the target protein to the test compound. The resulting NMR spectrum can show this binding by changes that occur in the chemical shifts (locations of the peaks in the spectrum). To verify that the NMR data are correct, scientists often use X-ray crystallography to show the exact structure of the complex that is formed between the test compound and its therapeutic target. So, ITC, NMR and X-ray crystallography were used to screen 1250 compounds to see which fragments bound to an enzyme made by the bacterium that causes tuberculosis (*Mycobacterium tuberculosis*). This interdisciplinary approach identified three distinct binding sites for fragments and enabled a successful structure-based development of potent inhibitors [1, 92].

So, an NME can be tested for its ability to bind to a single therapeutic target [1]. In the past, the molecular target was usually removed from its normal cellular environment. Test compounds that affect a purified

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molecular target were identified as hits. The hits were evaluated further in cell based assays, to try to find a lead compound. However, when the molecular target is in an environment that is closer to its natural (physiological) state, the assay is more relevant. That way, the effects on not just the single target, but on closely linked proteins can be determined [1].

So, cell-based HTS platforms have been developed that use microfluidics to allow small quantities of test compounds to be mixed with cells and enable metabolic reactions to occur [93]. HTS platforms that use microfluidics have been used to study membrane-bound proteins that are popular and are used to identify productive therapeutic targets. They have also been used to make organs on a chip that are more relevant than animal models for evaluating the safety and efficacy of NMEs. Microfluids can be manipulated in a perfusion, droplet and microarray mode. The perfusion mode is used to introduce reagents and samples and transfer fluids in microchannels, while combining and mixing reactants. It can be used to study a variety of cells. It can also be combined with mass spectrometry (MS) to identify and characterize metabolites and biomarkers for diseases. There is also a microfluidic platform for monitoring apoptosis in HTS. To make the system more physiologically relevant, 3D cell cultures can be perfused by making an array of micropillars in a microfluidic channel. They have been used to study carcinoma cell lines, hepatocytes and bone marrow mesenchymal stem cells. When separate compartments are used, it enables automated studies and the ability to monitor several parameters at a time [93].

There is also a droplet mode that uses water in oil microemulsions in which the reagents are in much smaller, nanoliter (nL) volumes [93]. The oil that separates from the aqueous phase in the droplets prevents cross-contamination between reagents in neighboring droplets. This reduces the non-specific binding of reagents with the surface of the channels. It also enables a nearly 500-fold increase in the rate of screening. The droplet mode can also be used to simulate tumors as 3D aggregates. This mode has also been used to do HTS of potential, new antibiotics. In many of these studies, droplets are created and handled in channels or in tubing. However, digital microfluidics (DMF) is another option. They manipulate nL to μL volumes of droplets on the surfaces of silicon chips through arrays of electrodes. DMF was used to study apoptosis when a drug was administered at several concentrations. Much lower amounts of reagents were needed, while the sensitivity and dynamic range of the analysis were improved. That is, detection limits were lower and a wider range of concentrations of test compounds were assayed [93].

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The third mode is based on microarrays [93]. It was used to make a miniaturized 3D cell culture array called the DataChip. Human cells were encapsulated and cultured to evaluate the potential toxicities of test compounds. It integrated 1080 different arrays of cells on a single chip and reduced the consumption of reagents by nearly 2000-fold. Hanging drops have also been used to make 3D cellular spheroids. They were used to monitor the self-organization of cells. There is also a sequential operation droplet array (SODA) that is used to deliver two or more drugs to cells. This way, possible synergistic effects can be seen. In these and other microfluidic methods, drug discovery is being accelerated by automation [93].

Previously, cells were almost always grown in culture in monolayers, which is not very natural [1]. Recently, though, researchers have developed a synthetic extracellular matrix so that cells can be grown in 3D cultures [1, 94]. This may help produce results that are more biologically relevant and help scientists learn how cells work and respond to test compounds.

This is especially important when developing anticancer drugs. HTS assays that only test the effects of molecules on cancer cells grown in a monolayer don't take into account the effects of the tumor microenvironment. So, a multilayered culture was developed that contained primary human fibroblasts, mesothelial cells and extracellular matrix in 384- and 1536-well microplates [95]. It reproduced the metastatic microenvironment of human ovarian cancer cells. Its usefulness was validated by testing known inhibitors of three different ovarian cancer cell lines. Its success means that complex 3-D cell cultures which recapitulate the metastatic environment of tumors can be adapted to a HTS format [95].

Cancer cells are not the only type of cells that contain organized networks of metabolic and signal transduction cascades that use proteins [1]. All cells use such networks. So, NMEs can affect cells by altering one or more steps in metabolism and/or signal transduction. Cells contain many proteins that are bound to the outer plasma cell membrane and to membranes on internal organelles. Signals from outside the cell act as primary messengers. Hormones, neurotransmitters, cytokines, adipokines and other molecules are examples of primary messengers. Cytokines are small proteins that are secreted by specific cells of the immune system and carry signals between cells. Many protein hormones circulate in nanomolar (10^{-9} M) concentrations that usually vary by less than one order of magnitude. In contrast, some cytokines (such as IL-6) circulate in picomolar (10^{-12} M) concentrations in a healthy state. However, their

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concentrations can increase up to 1000-fold during trauma or infection. Cytokines are different from hormones in that they are much more widespread throughout the body. Almost all nucleated human cells produce the cytokines IL-1, IL-6 and TNF-α. Also, cytokines are produced by adipocytes (fat cells) and are called adipokines. Examples include IL-6, TNF-α, adiponectin, apelin, retinol binding protein 4 (RBP4), leptin and resistin [1]. So, it is important to study the effects of test compounds on whole cells that contain intact membranes and organelles.

For example, immature oligodendrocyte progenitor cells were exposed to 100 000 different test compounds in separate culture wells [1, 96]. After six days, the cell cultures were assayed for their ability to produce myelin basic protein, which is made by myelin-forming cells. Next, the most promising compounds were further tested for their ability to form intact myelin sheaths. One of them, benztropine, produced much more myelin around axons than untreated cells. Benztropine blocked the activity of muscarinic cholinergic receptors. It was further tested in two mouse models of demyelinating disease that causes the same type of extensive demyelinated lesions and axonal damage that occurs in multiple sclerosis. "Benztropine substantially mitigated the disease and promoted remyelination in these mice" [1, 96].

So, HTS can be done on as many as 10 000 to 100 000 compounds per day using modern technology [1]. Pharmacology and toxicity screening can be done with many different cell lines, including 60 different cancer cell lines (the NCI 60). Also, genotoxicity tests such as the Ames test for mutagenicity, the micronucleus test and a test for clastogens (which cause chromosome damage) can be done. Moreover, *in vivo* tests can look at different organs for histological changes. Toxicogenomics can measure the effects of toxins on DNA and the amounts of mRNA transcribed from it. Proteomics can be used to determine the effect of a drug on the expression of proteins and their interactions with other proteins. NMR and LC-MS can be used to analyze physiological samples (such as blood and urine) to see which metabolites are produced. Some of the biological materials that have been used for *in vitro* cell-based testing are listed in Table 3.

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Table 3. Biological Materials for *in vitro* Cell-based Testing [1].

	Primary cells	**Cell Lines**
Definition	Directly derived from donor animal or human.	Originally from animal or humans, but can proliferate indefinitely in culture. Often derived from cancer cells
Disadvantages	Often short-lived, need tissue donors, differences between individuals	Loss of tissue-specific functions.
Advantages	Approximates *in vivo* systems. Best if done on single cells.	Long-term evaluation possible, don't need tissue donor.
Examples	Hepatocytes Proximal tubule cells Hematopoietic progenitor cells, Neutrophils Keratinocytes, skeletal myocytes, Tissue slices	HepG2, FAO MDCK, LLPKC1, Vero (kidney) HL60, TK6, L5178Y (hematopoietic) V79, CHO (fibroblasts)

Computational models are used to understand the structure and function of coupled nonlinear biological systems in health and disease [1, 97-99]. These models can be based on statistical learning, which describes relationships between ions, molecules and networks. The Virtual Physiological Human and Physiome Projects are creating such models. Statistical modeling is based on the fact that not all combinations of individual molecular states are equally probable in functioning organisms. Some configurations are far more likely to be observed than others. So, the favored states in health and disease are often markedly different and distinguishable. When analyzing genomic data, high dimensional patterns of biomarkers can be recognized. Data produced from gene microarrays and other high-throughput technologies are used to identify such network-based signatures. They help to detect diseases early and improve patients' prognosis. Multiscale mechanistic models are being developed to understand better the lungs, cardiac electromechanics and microcirculatory, as well as the function of the renal, gastrointestinal, and neuroendocrine immune systems in health and disease [1, 97].

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High throughput screening (HTS) can be combined with systems thinking and network medicine to find lead compounds and NMEs [100]. Many parameters can be monitored in HTS. Examples include extracellular pH, dissolved O_2, intracellular ions (especially Ca^{2+}), small biological compounds (such as cAMP, cGMP, and inositol triphosphate, or IP_3), proteins, mRNA, total RNA, the expression of genes, epigenetics, enzyme activities, receptor binding, protein-protein interactions, cells (growth and/or cytotoxicity) and phenotypes. NMEs can also alter the transport of critical molecules, such as the neurotransmitter serotonin. Other NMEs can alter the membrane electropotential (voltage) by binding to ion channels. Many NMEs will also alter cellular metabolism. Lead compounds and NMEs can alter several of these parameters and generate huge amounts of data. So, mathematics and systems analysis are essential for mining the data and understanding it [100].

Automated procedures are used for HTS by pharmaceutical companies and others [100-103]. Automated compound management and retrieval systems are used with 384- and 1536-well plates, along with an acoustic compound dispense ultrasound technology to dispense 2.5 nL droplets. In addition, an extensive informatics infrastructure is essential. *In vivo* tests are used to look at different organs for histological changes. Proteomics are used to determine the effect of a drug on the amounts and types of proteins that are produced in a test. At the same time, NMR and LC-MS can be used to analyze urine to see which metabolites are produced [100-103].

Through automation, millions of compounds can be screened in a month or less, producing many hits [100, 101]. The hits that are obtained are prioritized, optimized and annotated. Next, structure-activity relationships (SARs) are evaluated to obtain druggable profiles, in which each compound has been characterized by many *in vitro* and *in vivo* tests. The goal is to optimize leads faster and better by testing more diversified compounds. This will provide better 'tool molecules' for validating therapeutic targets and for doing successful *in vivo* studies that demonstrate efficacy. Throughout the screening, informatics and data mining tools are used to build an expertise-centric, technology-integrated and efficient lead optimization process [100-102].

Once one or more lead compounds have been identified, HTS can be used to measure their solubilities, cell permeabilities, stabilities and ADMEs (absorption, distribution, metabolism and elimination), as well as their abilities to bind to select proteins and inhibit enzymes involved in drug metabolism (cytochrome P450s, or CYPs) [100-102]. The HTS process also produces a focused high-throughput chemical library. One

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goal is to produce technologies that can be leveraged to provide more complete data packages. They will influence decisions on which NMEs to synthesize in large enough quantities to do initial clinical trials. Another goal is to make better decisions about which NMEs to select for *in vivo* testing in disease models. Moreover, many research activities focus on mechanistic studies, along with target identification and validation [100-101].

However, it is essential that HTS provides high quality data [100]. This is consistent with the 'Six Sigma' concept of TQM [93, 104]. Quality is assured, in part, by using a fully integrated automation system to prepare and track compounds [100-102]. This also helps minimize environmental insults by reducing compound consumption 100-fold. So, NMEs and lead compounds have been evaluated using common assays for binding to GPCRs, protein kinases, nuclear hormone receptors, proteases and ion channels. Structure-liability relationships (SLRs) and QSARs are done using HTS. Several advances have allowed researchers to prepare panels of disease targets. This includes enhanced cellular reagent support capabilities, improved non-tip dispensing of liquids and multi-mode plate readers. There is an immunology panel that has several immune markers and immune cell lines. There are also multi-mode functional assays within a disease-relevant cellular context and multi-parameter endpoints for various *in vitro* assays. For example, over 40 assays for kinase activities have been done in parallel using 14 cell-based assay panels that test for cytotoxicity. New informatics tools are also continuously improving the efficiency of lead optimization [100-102].

It is also important to monitor the expression of genes in HTS [100-103]. So, one can use arrays of 96, 384 or 1536 wells in microplates containing polymerase chain reaction (PCR) components to analyze the expression of genes. Historically, 96 well μL (or microfilter) plates were used to quantify DNA and/or antibodies from viruses and pathogenic bacteria. These days, specific genes and even whole cells can also be put into the wells [100-103]. The amount of viral DNA can be determined using quantitative PCR (qPCR), which was its original application [100, 105]. Over 100 catalogues of genes that code for proteins in a variety of signal transduction pathways are available commercially. Conveniently, users can put the genes of their choice into custom-made arrays. In addition, real time, reverse transcriptase (RT^2) PCR arrays can quantify the translation of mRNA into proteins that are important in signaling pathways. Fluorescent probes are used to monitor the production of DNA and/or RNA, as well as the binding of proteins to them. Sometimes several

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fluorescent dyes are used to monitor many processes simultaneously, in what is called multiplex technology [100-102].

One PCR method uses the 5′-3′ exonuclease activity of the heat-stable DNA polymerase Taq (from the thermophilic Bacteria *Thermus Aquaticus*) [100, 106]. Taq catalyzes the degradation of a non-extendable fluorescent DNA probe following hybridization and extension in the PCR [62]. Probes containing specific DNA sequences are labeled with a fluorescence probe (such as fluorescein) and quencher (such as rhodamine). They are bound to the 5′ and 3′ ends of the probe. The fluorescence of the reporter is quenched by fluorescence resonance energy transfer (FRET) to the quencher when they are in close proximity to each other. The probe is inserted between the forward and reverse primers on the target sequence, where it is hydrolyzed. In the absence of the PCR product from a target sequence, the probe does not bind and is not hydrolyzed. As a result, the fluorescence remains quenched on the reporter. A different fluorescent probe can be used for each gene-specific probe. Reactions are multiplexed so many genes can be quantified in a single reaction [101, 107].

Another option is to use two fluorescent oligonucleotide probes (one at the 3' and the other at the 5' end) [100-103]. The 3' end is phosphorylated to prevent extension of the DNA chain. After the DNA target is amplified by the PCR, DNA primers hybridize to the target. This brings the fluorescent probes close together. So, FRET-based fluorescence emission occurs when the donor fluorophore is irradiated by a light emitting diode (LED). FRET is fluorescence resonance energy transfer. The probe does not have to be hydrolyzed. One can also use a fluorogenic hairpin primer. The 5' and 3' ends interact and quench the fluorescence. If a target DNA sequence is present, it will cause unwinding of the hairpin probe, separating the ends and stopping the quenching, so fluorescence emission occurs [100, 106].

Another option is to use an intercalating dye, SYBR Green [100-103]. When it binds to double-stranded DNA, a strong fluorescent signal is emitted. These dyes cost less than DNA probes. Since they are not specific for any base sequence, they can be used to study any reaction. Non-fluorescent dyes have also been developed to quench the fluorescence. Fortunately, they don't fluoresce on their own, so there is less noise. They are useful in high throughput multiplexed analyses because they decrease the number of wavelengths needed to monitor each reaction and they decrease the spectral overlap of fluorophores. There are also compounds that bind to the minor groove in DNA. They are attached to probes that increase the temperature needed to separate the two strands of DNA. In

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this way, mismatched sequences can be distinguished. This increases the specificity, so SNPs can be detected [100-103].

Also, short and highly specific probes can be used [100, 106]. A 96 well plate often contains 84 genes that are specific for a certain signaling pathway or disease. The plate also contains at least five housekeeping genes and two negative controls. The housekeeping genes are present in all cells and should be detected with equal efficiency. So, they are excellent positive controls. Kinetic imaging plate readers can identify allosteric ligands that bind to many protein targets simultaneously, while only partially inhibiting them. The goal is for a new drug to have far fewer adverse side effects than drugs that are currently being used that target the single active site of an enzyme and inhibit it completely [100, 106].

Mass spectrometry (MS) is another powerful tool for detecting lipids, proteins, RNA and DNA, as well as complexes formed between them and NMEs [100-103]. The amount of mRNA in a sample is usually quantified by first converting it to DNA using a reverse transcriptase, followed by analysis of the DNA. Matrix assisted laser desorption and ionization with time of flight mass spectrometry (MALDI-TOF/MS) can separate and detect proteins and other biopolymers with molecular weights up to $1.5x10^6$ Daltons. For example, the lipid profiles of red blood cells and post-mortem brains of people who had schizophrenia or bipolar disorders were determined by LC-MS. That is, lipids were separated using ultraperformance liquid chromatography (UPLC) and detected by TOF/MS [100, 107]. Quantitative analysis of unique proteins produced by cancer cells (proteomics) have been used to discover cancer biomarkers [108]. The extent of methylation of DNA that codes for miRNA (miR-200 family and miR-205) was determined in tissue samples, biopsies and cell lines of people with invasive bladder cancer by MALDI-TOF/MS [109]. Instead of using common crystallized mixtures of matrix and analyte, microdroplets of a free liquid beam were used as the matrix in the laser target for liquid beam ionization/desorption (LILBID) coupled to MS [110]. DNA duplexes and complexes between DNA, RNA and proteins with masses up 1.6 million to can be measured using LILBID-MS [100, 110].

However, multi-cellular assays only detect the average responses of those cells. More reliable and useful information can be obtained by looking at only one cell at a time. This can be done by using high content screening, or HCS, with automated image processing of images from fluorescence microscopes [100, 111]. Even cell lines that have been cloned can develop subpopulations that respond differently to chemical probes or other stimuli. These subpopulations can have very different properties than

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the rest of the cells being assayed. Some very important signaling proteins can have different subcellular locations in different cells. So, single cells were observed using quantitative, colocalization-based microscopy to determine the subcellular locations of the oncoprotein Ras [100, 112]. Also, the proteins in cells being evaluated by NMEs are in their native configurations, in which they are often bound to other proteins or membrane lipids. So, they will have different properties than when they are studied in isolation in dilute solution, using reductionist thinking. With HCS, many phenotypic features can be measured that are relevant to the desired therapeutic and non-toxic properties of NMEs. Since it is slower than high throughput screening (HTS), HCS is seldom used for the first stage of screening compounds. Instead, it is used to prioritize hits and develop better leads in screens of smaller, specific libraries of compounds and is especially useful in early toxicity screening assays [100, 112].

Another tool to emerge recently in HTS and HCS is activity-based proteomics, or ABP [100]. In contrast to classical proteomics that identifies all the proteins produced in a system, ABP just focuses on a single class of proteins, such as proteases [100, 113]. The ABP method uses small molecules that contain a 'warhead' that binds covalently to the active site of an enzyme, along with a linker region and a 'tag' that can be used to manipulate and measure modified targets. The components of the probe are made using solid phase synthesis. So, large libraries of probes can be produced. These probes have been used to find new proteases and improved our understanding of their activities and roles in diseases such as cancer, malaria and other tropical diseases [100, 113].

Computer-aided drug discovery (CADD) is also proving useful in HTS [100, 114], just like computer-aided design (CAD) and computer-aided manufacturing (CAM) in other industries. CADD can be used for virtual screening of more NMEs faster than *in vitro* or *in vivo* methods. There is even a 'computer mouse' that can simulate the immune system of a non-obese diabetic mouse. It is far easier to manipulate than real mice. CADD can use the three-dimensional structures available on protein databases or homology modeling to predict structures from amino acid sequences. CADD also designs NMEs *de novo* by connecting molecular fragments to produce a new molecule with the desired properties [100, 114].

It is important to use combinatorial search techniques to reduce the number of NME structures that should be considered [100]. Computers are needed for simulated annealing [115], genetic algorithms [116], molecular dynamics [117], evolutionary-based [118] and particle swarm algorithms [119] for *de novo* drug design. Databases containing structures of small molecules can be used as an alternative to *de novo* drug design. The ability

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of NMEs to dock (or bind) to active sites on enzymes or ligand binding sites of receptors can be tested. Molecular dynamics (MD) simulations that include tightly-bound water molecules on proteins can provide better accuracy than simpler docking programs. The molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) [120] method and linear interaction energy method (LIE) [121] are examples of MD approaches. The MM/PBSA method approximates binding affinities by combining molecular mechanics (MM) and continuum solvent approaches. The quantum mechanics/molecular mechanics (QM/MM) hybrid method is another way to measure binding affinity. QM/MM combines the strength of QM (accuracy) and MM (efficiency) to study biological systems [100, 122].

MM-PBSA and MM-GBSA are useful methods that are based on molecular docking and virtual screening [100, 123]. They use a single structure of the minimum energy configuration of a NME and implicit solvation models to approximate scoring functions. These are used to estimate binding energies, which can be correlated with experimental values. The accuracy of this approach was compared to other methods. The results showed that MM-PBSA and MM-GBSA more accurately ranked various inhibitors of dihydrofolate reductase (DHFR) from *Plasmodium falciparum*. They also compared the correlations that were obtained using the average structure of DHFR obtained by molecular dynamics snapshots. The MM-PBSA and MM-GBSA approaches are rapid because they use just one structure. This makes HTS feasible. MM-PBSA and MM-GBSA combine molecular mechanics and continuum solvent models to estimate the binding affinities of ligands to their cognate receptors. MM-PBSA and MM-GBSA are much faster than calculations of free energy perturbation (FEP) and thermodynamic integration (TI) [100, 123].

QSARs ae also used to optimize leads [100]. In 3-D QSARs, physicochemical and structural parameters of molecules are used as descriptors to express biological properties. Comparative molecular field analysis (CoMFA) uses both interactive graphics and statistical methods to correlate the shapes of ligands with their biological activities [124]. A quantum mechanics-based QSAR, or QMQSAR, called AMPAC uses the AM1 Hamiltonian to calculate the lowest energy conformation of molecules and generate over 400 molecular descriptors [125]. The data from the AMPAC calculations can be input into another program called CODESSA [126], which uses the descriptors to develop the QMQSAR.

Protein-protein interactions can be targeted using computational drug design [100, 127]. Drugs can target interactions between monomers in

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oligomeric proteins, between different proteins or between antibodies and antigens. Two databases that contain small molecule protein-protein inhibitors are the TIMBAL database [128] at the University of Cambridge (http://www-cryst.bioc.cam.ac.uk/databases/timbal) and the 2P2I database [129] at CNRS in France (http://2p2idb.cnrs-mrs.fr/). Two drugs that target protein-protein interactions that are clinically available are tirofiban and maraviroc [100]. They target integrins that attach the cellular skeleton to the extracellular matrix. They are used to treat cardiovascular disease (tirofiban) and to block HIV viral entry (maraviroc) by preventing the HIV gp120 from interacting with the CCR5 receptor [100].

One complication is that interfaces between interacting proteins are broad and flat [100, 127]. So, scoring functions that were designed for small ligands that bind to tight, deep pockets may be inapplicable for predicting the binding of ligands to proteins that interact with other proteins. However, there are usually 'hot spots' that contribute most of the free energy of binding. They make good targets for fragment-based drug design. The fragment based ligand design approach has been particularly successful when designing NMEs that block interactions between interleukin-2 (IL-2) and its receptor (IL-2Rα). For example, Ro26-4550, disrupts this interaction. Another nanomolar inhibitor of this interaction is Sp4206. There is also an NME, ABT-263, which disrupts interactions between B cell lymphoma dimers of Bck2 and Bcl XL. Through lead optimization, it was modified to make an improved NME, Navitoclax, that is undergoing clinical trials for hematologic and solid tumors as well as chronic lymphocytic leukemia. There are also benzodiazepinediones. They are anticancer drugs that block the interaction between HDM2 (human protein double minute 2) and the oncoprotein p53, thus blocking its ability to activate the transcription of other genes that lead to cancer. There are many other examples of drugs in development that target protein-protein interactions and databases that categorize and classify these interactions [100, 127].

There are also computational methods that are used to identify specific druggable hot spots in protein-protein interfaces [100, 127]. Other software packages that can identify hot spots include FTMAP (http://ftmap.bu.edu), the CastP server (http://sts-fw.bioengr.uic.edu/castp/index.php), FTFLEX (http://ftflex.bu.edu), Qsitefinder (http://www.modelling.leeds.ac.uk/qsitefinder/), PocketQuery (http://pocketquery.csb.pitt.edu), ANCHOR (http://structure.pitt.edu/anchor) and FastContact 2.0 (http://structure.pitt.edu/servers/fastcontact/). There are also software packages that predict protein-protein binding rate constants that can be found at http://pipe.sc.fsu.edu, GridDock at http://pocketome.org/, and

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another program for predicting and disrupting protein-protein interactions in bacterial outer membrane and eukaryotic mitochondria beta barrel membrane proteins at www.uic.edu/~jliang [100, 127].

Fragment-based drug design has been useful in other applications [100, 130]. The process starts start with initial building blocks that have a desired property, such as a specific biochemical activity. These building blocks can be made larger, connected directly or connected by a linker. Binding can be measured by NMR. It was used to do a QSAR and identify inhibitors for FK506-binding protein (FKBP), factor Xa, matrix metalloproteinases and B-cell lymphoma 2 family proteins, more popularly known as Bcl-2, an apoptosis factor. This fragment-based drug design process is repeated until NMEs with improved properties are obtained. Sometimes, the term *de novo* design is used when smaller fragments are used, but the terms *de novo* and fragment-based drug design are frequently interchanged. Regardless of the term used, this process has been used to design inhibitors of the estrogen receptor, thrombin, factor Xa, cathepsin D, tRNA-guanine transglycosylase, dipeptidyl peptidase, Aurora A kinase, HIV protease and others. One advantage of fragment-based drug design is that a relatively small number of fragments can be mixed, matched and combined to cover most of the possible properties present in much larger sets of drug-like molecules. Another advantage is a much higher hit rate [100, 130].

Interactions between proteins can change in cancer cells. So, a cancer-perturbed protein-protein interaction database has also been constructed [100, 131]. It uses a nonlinear stochastic modeling to describe individual and cooperative protein interactions. By examining the topology of this database, 17 hubs in the protein-protein interaction network of apoptosis were identified that could be good druggable targets, including the Bcl-2 protein family members [100, 131].

Network medicine and HTS have also been used to develop new antibiotics [100, 132]. The changing concentrations (flux) of metabolites in *E. coli* were analyzed so essential metabolic reactions could be identified. Common antibiotic targets in *E. coli* and *S. aureus* were found by identifying metabolic reactions in their metabolic networks that shared tissue-specific functions or were essential to both Bacteria. Dozens of potential inhibitors of some of the enzymes that catalyze metabolic reactions were found by virtual screening. Experiments showed that a subset of these NMEs inhibited both enzyme activities and the viability of the Bacteria. However, some of the inhibited enzymes were found to be essential only for *S. aureus*. This includes most enzymes of the type-II bacterial fatty-acid biosynthesis (FAS II) pathways. The malonyl-CoA-

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acyl carrier protein transacylase (MCAT, FabD) was of special interest. It catalyzes the first committed step in the fatty-acid biosynthesis pathway. There is also a ZINC lead library that contains about 10^6 small molecules that were prefiltered for drug-like properties. The most promising NMEs were docked to either the crystal structures of *E. coli* FabD or to a FabD homology model that was derived for *S. aureus* strain N315. This blueprint is applicable to any organism whose DNA has been sequenced and for which there is a high-quality metabolic map available. So, this could be used to develop anti-infective therapies that are specific for a given strain of Bacteria [132].

HTS can also be used in personalized medicine when cells come from the individual patient. So, the potential cardiotoxicities of NMEs that inhibit protein tyrosine kinases (PTKs) were evaluated by HTS that used human induced pluripotent stem cells to produce cardiomyocytes, endothelial cells and cardiac fibroblasts. That way, the effects of several FDA-approved PTK inhibitors on different types of heart cells were determined. One unexpected discovery of the study was that cardiotoxicity can be alleviated by activating insulin and insulin-like growth factor signaling [133].

Stem cells may also become useful in regenerating tissues [134]. For example, limbal cells have been approved for treating ocular burns. Phase I clinical trials are being conducted to treat spinal cord injury, heart failure and macular degeneration with human plutipotent stem cells (hPSCs). For these to work and for HTS screening of drugs that act on iPSCs to be successful, they must have an effective supporting substrate. This includes materials called Matrigel™ and one of its components, the protein called laminin. More recently, substrates that have epitopes that can bind to cells have appeared. One of them is called Synthemax™. It can support the self-renewal of human embryonic stem cells (hESCs). In order to discover more substrates, a HTS screening method has been developed. Instead of testing libraries of drugs, libraries of synthetic polymers are being tested. Certain peptide ligands, parts of molecules (moieties) and appropriate physical properties (elastic moduli and topography) have been identified that can influence the behavior and fate of stem cells [134].

So, HTS is an important part of new finding lead compounds during drug development.

3.1.3.1 Protein-protein interactions (PPIs)

Systems-based thinking is being used to find lead compounds that affect the whole network and not just one protein. Proteins interact and

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affect each other. As mentioned previously, prescription drugs can exert their effects through PPIs [100, 135-137]. Moreover, protein-peptide interactions between antibodies and antigenic peptide fragments have been well-known for many decades in immunology [100]. The immune system performs an essential function in autopoiesis and life. It helps us define ourselves on a molecular level. That is, the immune system (or network) differentiates between self and non-self. This is consistent with the first rule of communication – know your audience. One of the main functions of the immune system is to ensure that our bodies can recognize and respond properly to its audience – the human cells within us. Our human eukaryotic cells have specific proteins (human leukocyte antigens, or HLAs) on their surface. They are antigens that define self. Class I HLAs display peptide fragments of intracellular proteins on their cell surface. As a result, they will be identified as self and not be destroyed by T-lymphocytes. In contrast, Class II HLAs present extracellular protein antigens to helper T (T_H) cells, activating them and starting an immune response, when necessary. In addition, our immune system must distinguish between peptide antigens that come from food and antigens that come from a piece of a parasite, virus or bacterium. It must also be able to distinguish peptide antigens that come from commensal Bacteria that are essential to life and good health from peptide antigens derived from pathological Bacteria. So, protein-peptide interactions are essential for two pillars of systems thinking – communication and feedback [100].

In addition, there is a PPI interaction network that is a crucial part of all organisms [138]. It is crucial for the proper processing of inter- and intracellular signals and decision making, such as cell proliferation or death [135]. The same type of control theory that is used in the design of electric circuits, manufacturing processes, communication systems, aircraft, spacecraft, and robots was used to do a controllability analysis of a PPI network. The components of this network exhibit feedback and feed forward control. The PPI network consisted of 6339 proteins and 34 813 interactions. The analysis found that proteins could be classified as dispensable, neutral or indispensable. This was based on how they affected the number of driver nodes in the network when they were removed. The indispensable proteins were found to be the primary targets of disease-causing mutations, human viruses and therapeutic drugs. This suggested that changing the control property of a network can be critical for the transition between healthy and disease states. In addition, data from 1547 cancer patients indicated that 56 genes that are frequently amplified or deleted in nine different types of cancer are indispensable. Interestingly, 46 of them had not been previously associated with any cancer. So,

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controllability analysis can very useful in identifying genes that can help cause diseases and may become potential therapeutic drug targets [135].

The PPI network is also a core element of a disease-specific PPI network that associates proteins with one or more diseases [138]. This disease-specific PPI network can be used to calculate a heuristic energy score to predict the possible amount of synergy that might happen when two or more drugs are used to affect PPIs. This heuristic energy score is based on the topology of the connection and centrality of the drug targets. The topology score of two drugs is high when they target proteins that are close to each other in the PPI network and have high network centralities. Another type of evaluation, the agent score, was used with the topology score in an algorithm called NIMS (network target-based identification of multicomponent synergy) to evaluate drug synergy. The agent score measures the similarity of the phenotypes produced by the two drugs. This was determined by the descriptors in the OMIM (On-line Mendelian Inheritance in Man) database. It was calculated by a text mining method. Also, a predictive synergy score was calculated by multiplying the topology score by the agent score. Researcers used this method to recognize some synergistic ingredients in traditional Chinese medicines that are anti-angiogenic and may prevent cancer metastasis. Others used a disease-specific PPI network to calculate a Topological Score of Drug Synergy (TSDS). It was used to evaluate the effects of drug combinations that have several therapeutic targets. The TSDS was based on the topological features of the target. This included the reachability of the node as well as the global and synergistic effects. It identified new therapeutic targets that may lead to successful synergistic therapies for type-2 diabetes. So, computational methods based on the PPI network can shed light on the relationships between complex diseases and potential treatments with new or already available prescription drugs [138].

At the same time, scientists and physicians realize that proteins are packed together tightly in cell membranes, the cell nucleus and cytosolic multi-protein complexes. Last century, it was very difficult to study the effects of drug candidates on PPIs. Instead, it was much easier to study the kinetics of the inhibition of enzyme activity on a single purified enzyme that was present in a rather dilute solution (nM concentration). Such experiments did produce many life-saving prescription drugs. However, many drug candidates that bound to the active site of an enzyme or ligand binding site on a receptor had unacceptable side effects. This was often due to the fact that isozymes located in tissues not targeted by the drug candidate were also affected. Moreover, the structures of active sites of many enzymes with very different functions had similar shapes – often

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resembling deep pockets. So, enzymes were often inhibited that were never the intended therapeutic target – causing severe adverse side effects. In contrast, allosteric sites and places where PPIs occur often have unique structures. So, drugs that bind to such sites may be much less likely to bind to other proteins. This would result in far fewer adverse side effects.

For these reasons, medicinal chemists are now developing new drugs that don't bind to an enzyme's active site or the ligand binding site of a protein receptor. Instead, they bind at allosteric sites and affect an enzyme's or receptor's interactions with other proteins, RNA and/or DNA. So, allostery occurs not only with individual proteins, but also large groups of proteins that interact with each other as well as with RNA and/or DNA [100, 139]. That is, a drug that binds to an allosteric site on one protein can affect the structures of allosteric sites on proteins that interact with the target protein. Such effects can spread across the cell membrane, through the cytoplasm and into organelles. Diseases can occur through allosteric changes that shift the populations of interacting proteins from their active to inactive states, or vice-versa [100, 139].

Moreover, the interactions between proteins can be dynamic and transient [136]. Proteins are flexible. The structures of their interfaces can change when allosteric ligands for receptors or enzyme inhibitors bind to them. The binding can expose transient parts or cavities in the protein that attain different structures on the surface of the bound form. Such structures are often not apparent from the X-ray or NMR structures that are in protein data banks. However, tools for molecular simulation can complement or replace X-ray and NMR methods. Such analyses have shown that there are some general trends in PPIs. Unlike the active sites of enzymes and the ligand binding sites of receptors, the sites of PPIs usually do not contain relatively deep cavities, but are almost flat. As a result, many of the atoms in transient PPIs are on the solvent-accessible surfaces of the interacting proteins and would be accessible to many new drug candidates. The interfaces between interacting proteins can be divided into a core and a rim region. The rim contains amino acid residues that are not buried within the interior of either protein. The core region does contain buried atoms and about 55% of all the amino acid residues at the interface. Moreover, there are hotspots that contribute more to the total binding energy than other parts of the interacting proteins. They are less flexible and tend to occur in clusters that are usually found on both of the interacting proteins. They also are often in contact with each other and form a network of interactions called the hot region. Amino acid residues in hotspots are usually located around the center of the interface and are protected from bulk water by relatively hydrophobic amino acid residues

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that form an O-ring. However, it should be noted that the term hotspot can also mean those parts of any therapeutic target that bind tightly to low molecular weight drugs [136].

These hotspots can be excellent therapeutic targets for fragment-based drug design [100]. They have been especially useful in designing inhibitors of the interactions between interleukin-2 (IL-2) and its receptor (IL-2Rα). A new molecular entity (NME), Ro26-4550, disrupts this interaction. Another nanomolar inhibitor of this interaction is Sp4206. There is also a NME, ABT-263, which disrupts interactions between B cell lymphoma dimers of Bck2 and Bcl XL. Through lead optimization, it was developed into ABT-263. There is also Navitoclax, a new Bcl-2/Bcl-xL inhibitor. It is currently in Phase I clinical trials for hematologic and solid tumors and Phase II for chronic lymphocytic leukemia. Also, HDM2 (human protein double minute 2) is a therapeutic target for treating cancer. It blocks interactions between the p53 protein and HDM2, which binds to p53 and blocks its transcriptional activity. Other drugs, including Sp304, disrupt the interactions between the human papilloma virus (HPV), E2 viral transcription factor and the viral helicase E1. There are many other examples of drugs in development that target protein-protein interactions and databases that categorize and classify protein-protein interactions [100, 127, 140].

To help develop new drugs that target PPIs, a structural database called 2P2Idb v2.0 has been created [141]. It is dedicated to finding ways to modulate PPIs. It is a compilation of structural information on inhibitors of PPIs and their targets. The 3D structures of proteins are taken from the RCSB Protein Data Bank (PDB). The 2P2Idb contains data on all the structures that are available for both the protein–protein and protein–inhibitor complexes [141]. This includes 26 PPIs and 242 small molecule inhibitors that have been found at the interface between proteins that interact with each other directly. There is also a protein-protein interface analysis tool called 2P2I Inspector. It can calculate physical and chemical descriptors of protein-protein and protein-ligand interfaces from 3D structures. A complex that is being analyzed can be visualized using a Jmol applet. There is also a 2P2I scoring function that predicts the potential druggability of protein-protein interfaces using six different interface descriptors. Next, there is a 2P2I hunter that is a filtering tool that can be used to design focused chemical libraries dedicated to the inhibition of protein-protein complexes [141].

In addition, potential disease genes and drug targets are being identified using a controllability analysis of the human protein interaction network [135, 142]. Control theory and analysis involve the design and

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analysis of the stability of dynamic systems that receive information and produce measurable outputs. Control and regulation are essential when studying biological systems, which sense and process both external and internal signals. They use a network of interacting molecules, including proteins. The dynamic regulation of this molecular network can move the system to various functional states – such as initiating cell proliferation or inducing apoptosis. So, specific input signals can direct networks from an initial state to a specific functional state. The ways that biological systems are controlled affects the evolution of molecular interaction networks. Note that the term *state* has a control context and the *state space* of a control system is the space containing all values that the state variables can attain. For a protein-protein interaction (PPI) network, the *state variables* are the specific protein concentrations and the state space is the total number of proteins in the PPI network [135].

A dynamic system is controllable if it can be pushed from any initial state to any desired final state in finite time, if given suitable inputs [135]. The components of such networks exhibit properties of control systems. This includes proportional action, feedback control, and feed-forward control. However, there are important challenges that can complicate the systematic controllability analysis of biological networks. They include our limited understanding of large-scale biologically relevant networks and efficient tools to analyze their controllability. In order to do a careful controllability analysis, two resources were used. One was a directed human PPI network and the other was an analytical framework that could characterize the structural controllability of directed weighted networks. The directed human PPI network gives a global snapshot of the flow of information in cell signaling. For each weighted and directed network associated with linear time-invariant dynamics, the analytical framework identified a minimum set of driver nodes, whose control was sufficient to fully control the dynamics of the whole network. So, the protein nodes in the network were classified as indispensable, neutral or dispensable, based on the change of the minimum number of driver nodes needed to control the PPI network when a specific protein node is absent. In addition, the roles of different types of nodes in the etiology of human diseases were determined. Using known examples of disease causing mutations, virus targets and drug targets, indispensable nodes were identified that are essential in mediating the transition between healthy and disease states. The results showed the potential application of network controllability analysis for identifying genes that can help cause diseases. In the network, there were nodes that were connected with edges. To test the robustness of the nodes, the effects of rewiring (or reconnecting) nodes and changing the

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direction of their edges were evaluated. When indispensable nodes were rewired or their edges flipped, some were affected (Type-I), while others were not (Type-2). Type-1 nodes tended to be hubs (well-connected nodes), while Type-2 nodes could not be distinguished from other nodes based on the network properties that were analyzed. However, both types were enriched in genes involved in cancer and other diseases. Data on 1547 patients in The Cancer Genome Atlas (TCGA) and cBioPortal for Cancer Genomics were analyzed. They had nine different types of cancer. The investigators found that 56 Type-II genes were among the top 1% of the most frequently deleted or amplified genes. Of these, 17 regulated cell proliferation. Eleven of them induced proliferation, eight stopped proliferation and two of them were able to do both [135].

That study produced a DirectedPPI database that enables researchers to navigate the directed human PPI network with predicted controllability [135]. Users can enter a gene or upload several genes. The tools in the database accept genes and their properties. This includes mutation frequency, *P* values from GWAS and changes in the extent to which the genes are expressed. Indispensable, neutral and dispensable genes can be identified based on the node shape and color. So, this database can be used to identify indispensable nodes and their interconnections. The analysis of the human PPI network identified 36% of the nodes as driver nodes, which is similar to the percentage (30%) found in metabolic networks. Indispensable nodes were found to be well-conserved and highly regulated at the level of translation and post-translational modifications. They are also important in directing the transition from healthy to disease states. This can help future investigators understand the basic science behind the diseases they are studying, as well as identify potential therapeutic targets. It should be noted that mutations that help to cause diseases, as well as pathogenic viruses and therapeutic drugs that target fragile, indispensable nodes control the number of driver nodes, instead of the driver nodes themselves. So, controllability may be crucial in the transition from a healthy to a disease state. Furthermore, controllability analysis can complement the analysis of the network properties that have been used previously to identify genes that can help cause diseases. So, as the quality and completeness of interactome maps increases and the dynamics of network analysis are added to the studies, our understanding of biological networks and their effects on diseases will continue to improve [135].

In addition, computer calculations are being used to design and discover low molecular weight (LMW) molecules that are most likely to affect PPIs [136, 143]. This has dispelled the misconception that it would be difficult to find such LMW compounds. That misconception was based

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on the idea that the interfaces between interacting proteins were almost always flat and large, with no cavities that were large enough to accommodate LMW molecules. Moreover, there were no endogenous LMWs that were known to affect PPIs and act as leads. So, it was not possible to modify their structures to improve on them. However, our understanding of the structures of the regions where proteins interact with each other has improved. It was already known that the parts of the proteins that form transient PPIs are almost always buried in a surface area of about 900 $Å^2$ (about 500 $Å^2$ provided by each partner) with about 12 amino acid residues on each partner being involved. Many of the atoms in the transient PPI interfaces are accessible to the water solvent. The interfaces were divided into a core region and a rim region. The rim surrounds the core. The rim region contains amino acid residues that are not fully buried. The core contains about 55% of all the residues at the PPI interface. There is also a support zone that has a similar amino acid composition as the interior of most proteins. There are also hotspots that are responsible for most of the binding energy (about 2 kcal/mol). When they are removed by site-directed mutagenesis the strength of the PPI decreases significantly. Hot spots tend to be found in clusters and are on both partners of a PPI. They form a hot region. They can be identified by site directed mutagenesis (which can be time-consuming and labor-intensive) or by computer calculations. The two can also be combined to predict an overall contact area. Hotspots usually contain some of the most highly conserved amino acid residues and are located around the center of the interface. They are protected from bulk water solvent by amino acid residues that form a hydrophobic structure that resembles an O-ring. The surface area that contains hotspots is only about 600 $Å^2$. This is small enough to be able to bind LMW compounds [136, 143].

The desire to find LMW compounds is based on the hypothesis that it is more likely to find a good lead compound if one focuses on compounds with a molecular weight ≤500 Da (or ≤500 g/mol) [1, 144]. This is part of a guideline that is often called Lipinski's rule of five for obtaining a drug-like compound: molecular mass ≤500 Da; calculated LogP (cLogP) ≤5; number of hydrogen-bond donors ≤5; and number of hydrogen-bond acceptors ≤10 [144]. This is called the rule of five "because the cutoffs for each of the four parameters were all close to five or a multiple of five" [144]. This rule became quite popular because compounds that meet all four criteria in the rule of five will have physical and chemical properties that give them acceptable aqueous solubility and intestinal permeability [145]. These two properties are especially important if a drug is going to be prepared as an oral dose, which is the most convenient dosage form for

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patients. However, the rule of five does not "apply to natural products or any molecule that is recognized by an active transport system" [146]. That is due, in part, to the chameleon-like nature of many natural products [147, 148]. That is, they can change their shape and polarity when the amount of hydrogen bonding changes as the natural product goes from an aqueous, hydrophilic environment to a hydrophobic or lipophilic environment in a cell membrane [147, 148]. Moreover, many natural products exert their health effects by preventing or disrupting PPIs. For example, many phenolic compounds which are in a variety of colorful fruits and vegetables bind to the protein called the nuclear erythroid-2 like factor-2 (Nrf2) transcription factor [149]. These phenolic compounds bind to Nrf2 and break its bonds with an inhibitor protein called Keap1 (Kelch-like enoyl-CoA hydratase-associated protein 1) [149, 150]. That is, when cells have low oxidative stress, Nrf2 is bound to Keap1, which is anchored to actin in the cytoskeleton in the cytosol [149, 151]. This complex makes the Nrf2 protein accessible to reaction with ubiquitin [149, 152]. Ubiquination causes many transcription factors (including Nrf2) to be broken down (hydrolyzed) in subcellular organelles called proteasomes when DNA transcription should not be activated. However, this breakdown of Nrf2 can be prevented by breaking the bonds between it and Keap1 [149-152]. That is, the Keap1 protein contains several cysteine residues with sulfhydryl groups that can react with RONS and electrophiles, thus breaking the bonds between Keap1 and Nrf2. Once the bonds are broken, Nrf2 translocates to the cell nucleus, where it can bind to regulatory regions of DNA that turn on the transcription of genes coding for antioxidant elements [149-152]. As a result, many colorful fruits and vegetables can prevent smoldering inflammation and diseases caused by it by disrupting the PPI between Nrf2 and Keap1 [1, 149]. Inflammation, dietary antioxidants and phenolic compounds will be discussed further in Chapter 6.

So, studying PPIs can lead to potentially useful prescription drugs that may treat or even help cure many deadly diseases. One example of this is the development of the first in its class drug, Venetoclax [63]. It inhibits an anti-apoptotic protein Bcl-2 that was named after B-cell lymphoma 2 because it was the second protein discovered that is affected by translocations between chromosomes 14 and 18 in follicular lymphomas [153]. As a result, Venetoclax is approved for treating chronic lymphocytic leukemia (CLL) [63]. The Bcl-2 protein interacts with other proteins to help control the intrinsic pathway of apoptosis (Type I programmed cell death) [63, 100]. So, Venetoclax is the first approved prescription drug that acts by disrupting PPIs [153].

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The intrinsic pathway of apoptosis begins within the cell, while the extrinsic pathway of apoptosis is initiated by signals that come from outside the cell [51]. In both pathways, caspases (cysteine proteases) catalyze reactions that dismantle dead and dying cells. When this apoptosis is disrupted, cancer can emerge. Abnormal cells should die, but they don't. The mitochondria, which regulate apoptosis in healthy cells, become defective. Not only do they not initiate apoptosis, but their metabolism changes. Despite the ready availability of O_2, glycolysis occurs as if it was in an anaerobic environment and the final product is lactate, not pyruvate. This is called the Warburg effect, after the scientist who discovered it [51].

The intrinsic pathway can be initiated by DNA damage, growth factor deprivation and oxidative stress [154]. The *BCL-2* gene is just one member of the *BCL-2* gene family. All members of the Bcl-2 protein family contain up to four Bcl-2 homology (BH) regions in their protein structures. Some members of this family are anti-apoptotic (such as Bcl-2 and Bcl-X_L, Bcl-W, myeloid cell leukemia 1 or MCL1, and A1, also known as Bcl-2A1). They antagonize the BH3-only proteins and they inhibit the essential apoptosis effectors Bcl-2 antagonist killer 1 (BAK) and Bcl-2-associated X protein (BAX). Two other BH3-only proteins, Bcl-2-interacting mediator of cell death (BIM) and BH3-interacting domain death agonist (BID) suppress anti-apoptotic proteins located at the mitochondria and endoplasmic reticulum. A combination of NMR-based screening, fragment analysis and structure-based drug design produced BH3 mimetics. They disrupt the PPIs between anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family. This led to the development of Venetoclax, which binds to the Bcl-2 protein with nanoMolar affinity and induces apoptosis that depends on BAX and BAK. More recently, Venetoclax was shown to be safe and effective in treating relapsed or refractory CLL [154, 155]. Cancer cells often undergo mutations after being treated with tyrosine kinase inhibitors [51]. As a result, they survive and become dependent on one or just a few oncogenes to maintain the malignant phenotype [51]. Two of them are PUMA and BIM. PUMA is a member of the Bcl2 family of proteins [156]. It is activated when the PI3K-Akt signaling pathway is inhibited in both HER2-amplified breast cancers and lung cancers that have a mutation in the gene coding for EGFR. BIM is also a Bcl2 family member that plays a key role in gefitinib and erlotinib-induced apoptosis in lung cancers that have a mutation in the gene coding for EGFR and in lapatinib-induced apoptosis in HER2-amplified breast cancers. That is, there are some types

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of cancers that depend on specific driver mutations for survival, in a phenomenon called oncogene addiction [156].

It is also interesting to note that oncogenes can be amplified not just by parts of DNA that are in chromosomes, but also in circular extrachromosomal DNA (ecDNA) [157]. So, a research group has developed a software package called ECdetect to find and analyze ecDNA. They found ecDNA in almost half of human cancers. The frequency at which they occurred varied with the types of tumors, but they were almost never found in normal, healthy cells. Driver oncogenes were amplified most frequently in ecDNA, thus increasing their transcription into oncoproteins. They also performed some mathematical modeling. It predicted that ecDNA amplification would increase the number of copies of oncogenes and the intratumoral heterogeneity more effectively than chromosomal amplification. These predictions were validated by quantitative analyses of cancer samples. The results strongly suggest that ecDNA can accelerate the evolution of cancer cells [157].

In contrast, apoptosis is undesirable if it causes immune cells to die rather than stay alive and so they can kill cancer cells. For cells in the immune system to properly control the balance between co-stimulatory and co-inhibitory signals, checkpoint inhibitors are used [158]. Two of them are especially important, the cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell-death protein 1 (PD1). They inhibit the activation of cytotoxic T-cells. They play essential roles in maintaining self-tolerance and in regulating the amplitude and duration of T-cell responses. Three checkpoint inhibitors are approved for treating malignant melanoma: ipilimumab (Yervoy®), nivolumab (Opdivo®) and pembrolizumab (Keytruda®). Pembrolizumab was the first anti-PD1 therapy to be approved by the FDA. It was approved relatively fast for treating unresectable malignant melanoma – just three years after the first clinical trials [158]. More recently, it was approved for treating any cancer that has the PD-1 biomarker [159]. Previously, the FDA only approved cancer treatments that were based on the organ in which the cancer originated. This is the first anticancer drug that has been approved for treating any unresectable or metastatic solid tumor [159]. Nivolumab has been approved for treating non-small cell lung cancer [160]. The PD1 protein also interacts with a protein ligand called PDL-1 [63]. The prescription drug atezolizumab (Tecentriq®) targets it and is approved for treating CLL [63]. In addition, a combination of anti-PD1 and CTL4A antibodies is effective in treating several cancers [161]. So, there are some drugs that have been approved that use the body's own immune system to treat cancers as they affect PPIs.

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3.1.3.2 Systems view of allostery and allo-network drugs

As systems-based thinking continues to spread, drugs are being developed that affect multiple targets, many of which are hidden at first glance. That is, an NME might bind to one protein and exert a minor effect on it that subsequently spreads throughout the PPI network. That is, cells contain an ensemble of interconvertible conformations of enzymes and receptors [1, 48]. Proteins are dynamic, robust and adaptable [3, 100]. As a result, global changes in protein structure can emerge from local, dynamic changes. Proteins can undergo structural changes without losing their function, making them robust. They are also adaptable in that new biological activities can emerge [3, 100]. This flexibility is essential for their proper physiological functions [100, 162, 163]. For example, many important proteins (such as p53) that are hubs in the cellular network can bind many different substrates or ligands to cause a variety of physiological effects. These proteins have flexible portions that can be converted into several different, less flexible structures after they bind their substrates or ligands. These proteins are almost impossible to crystallize when no ligand is bound to them. Instead, NMR can be used to look at them in a buffered, aqueous solution. The spectra obtained can be analyzed to produce several structures that exist at the same time in solution. So, for the structures of proteins in a protein data bank based on NMR will show several structures, while data based on X-ray crystallography will show just one [162].

This is consistent with systems thinking, in which active enzymes, receptors and other native proteins are known to be flexible and interact with each other synergistically [162]. That is, proteins in solution have an ensemble of different conformations. Their populations are tunable throughout a range of physiological conditions, including ligand or substrate binding and protein-protein interactions. There are several distinct pathways through which the effects of the binding of an allosteric ligand propagate through the protein structure to exert its physiological effects. Moreover, when an allo-network drug binds to one protein, it can cause conformational or dynamic changes in adjacent proteins and proteins to which they are linked. This propagation of conformational changes can affect one or more signal transduction pathways that are seldom obvious. NMR is an excellent tool for finding the pathways through which the effects of binding of an allosteric ligand propagate [162].

So, a new view of allostery has emerged and is described in detail by others [162, 164-174]. An excellent example of this is p53, which is a

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tumor suppressor and a hub in the cellular and metabolic networks [51, 162, 163]. Structural changes caused by mutations can be robust, adaptive or even lethal [162, 163]. The tetrameric domain of p53 is especially flexible, enabling this crucial protein to bind to many different ligands and cause a variety of physiological effects. When looking for new drugs that bind to proteins like p53, it is important to evaluate the extent of independent motions in amino acyl residues by doing a number of clusters (NOC) analysis. It evaluates of the amount of movements in independent amino acyl groups. When NOC increases after mutations, the p53 structure becomes more flexible, which causes a loss of function. When NOC decreases, the p53 structure becomes less flexible and whole protein is more stable. Also, protein structures are studied by network analysis. The amino acyl residues are the nodes, while the distances between them are the links in the graphs of the network. Topological amino acid networks have also been called contact networks or graphs, protein structure networks and residue interaction graphs (RIGs). So, new drugs are being developed based on their effects on these networks when they bind to their therapeutic targets [162, 163].

So, there is now a unified view of how allostery works from three different standpoints: thermodynamics, the free energy landscape of population shifts and structure [162, 163]. They all have the same allosteric descriptors. The free energy landscape shows the Gibbs free energies of different conformations of proteins. They can convert from one conformation to another, due to their flexible structures. The difference in Gibbs free energy (ΔG) between the active and inactive states or conformations can be calculated. Allosteric ligands can change the conformation of proteins even when the natural ligand is bound to them and change the ΔG. However, unlike orthosteric ligands, the amount of allosteric activation or inhibition depends on the extent to which ligand binding stabilizes the active conformation and not its equilibrium binding constant to the allosteric site [162, 163].

This has also led to new ways of studying enzyme kinetics. As described in section 3.1.2**,** enzyme inhibition has been evaluated in the past by double reciprocal Lineweaver-Burke plots (1/v vs 1/S) of enzyme activities (v) in the presence of increasing concentrations of substrates (S) and inhibitors [1]. Inhibitors that bind to the active site of an enzyme and prevent the binding of one of the enzyme's substrates were called competitive inhibitors. They produced a series of plots that intersected at about the same point on the y-axis (1/v). In such cases, enzyme inhibition can be prevented at high concentrations of substrate (S) [1]. This led to the development of derivatives of 2-phenylaminopyrimidine like the anticancer

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drug imatinib. They were classified originally as competitive inhibitors of kinases with respect to the substrate ATP [20, 175, 176]. However, NMR spectra of imatinib bound to the oncogenic Bcr-Abl (breakpoint cluster and Abelson) protein tyrosine kinase showed that imatinib binds to not just the part of the active site to which ATP binds, but also to an allosteric site, making it a type II allosteric inhibitor [177, 178].

Allosteric regulation of proteins can also happen when ions, lipids, other proteins, DNA or RNA bind to them, as well as by post-translational modifications, point mutations and reactions with small molecules [162, 165]. When the allosteric state is perturbed, new protein conformations are not created. Instead, the relative distribution of conformations is altered [162, 165]. There are different pathways through which the effects of binding of an allosteric ligand extend through the protein structure to exert its physiological effects [162, 166]. This allosteric propagation causes different sites within a protein to communicate with each other and affects signaling pathways, which lead to cellular responses. Cellular communication occurs through both direct contacts between proteins and indirectly through long-range interactions. The communication exists throughout the cellular network and the entire organism. That is, cellular networks are made from interconnected signaling pathways that undergo extensive cross-talk. Network dynamics are changes in the interactions and connectivities of these pathways [162, 166].

As a result, allostery exists in single proteins and large groups of proteins that interact with each other, as well as with RNA and DNA [162, 179]. Allosteric effects can spread across the cell membrane and enter the cytoplasm and organelles. Diseases can occur through allosteric changes that shift the populations of proteins to and from states with different activities. Also, individual atoms within an allosteric protein can be either drivers or anchors that create attractive (pulling) or repulsive (pushing) forces between interacting biopolymers. The strength of the allosteric effect is not based so much on the structure of the allosteric site as on the extent to which it shifts the populations of active and inactive states. Coupling between allosteric and active sites identifies those amino acyl residues that trigger these changes in populations [162, 179].

At the same time, allosteric drugs can be anchors or drivers [139, 162]. Anchors bind to an allosteric pocket in a protein without changing the pocket's structure. Anchors provide a foundation upon which drivers exert a push or pull to or from the active state. However, the atoms in a drug or ligand that are anchors when binding to one protein can become drivers when binding to a different protein. Moreover, similar drugs that bind to

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the same allosteric site can cause very different physiological effects [139, 162].

So, allosteric sites and allosteric drugs are being identified [162, 167, 179]. For example, there is an allosteric database that had 1473 targets, 71538 modulators, 75462 interactions, 1930 sites, 56 pathways, 261 networks and 3350 allosteric diseases as of August 4, 2015 [162, 167, 168]. It is updated annually and is freely available [162, 168].

To help understand the allosteric effect, proteins can be modeled as amino acid residue interaction networks [180]. That is, proteins can be thought of as a complex network of interacting residues. These amino acid residues interact both covalently and non-covalently (through hydrogen bonds, ionic interactions, van der Waals forces and hydrophobic interactions) to form the complex 3D structures of the protein in which they exist. Proteins can perform many different functions with great specificity. Their specificity depends on their structure, which depends on the interactions between their amino acid residues. So, it is important to analyze proteins collectively through these interactions. In addition, proteins can be understood better by analyzing the interactions of their amino acid residues and by looking for holistic or emerging properties. They can be modeled as sub-networks of residues that have similar physical properties. Also, simulations of molecular dynamics can be connected to the protein contact network to help understand better the dynamic properties that are caused by the binding of a ligand or substrate. At the same time, biological networks can rearrange their interactions to produce important effects. This allows them to adapt to stress and biochemical signals to perform many different biological functions. An understanding of network theory helps. This has been reviewed [180, 181].

Proteins function holistically [182]. That is, local perturbations resonate through the entire structure of the protein. Allostery is a consequence and emerging property of this general characteristic of proteins. Perturbations don't necessarily involve a conformational change. Instead, perturbations travel across a protein's structure as a type of energy signal. The atomic interactions within the amino acid network provide the structural support for this signaling highway. Network descriptors that describe aspects of network signaling efficiency explain allostery in terms of signal transmission. A complex network paradigm provides a new perspective in medicinal chemistry and biochemistry. That is, allo-network drugs are being developed based on allosteric effects. This is a promising application of the contact network formalism [182].

So, there is much research being done to find NMEs that do not bind to the active site of target enzymes, but instead bind to allosteric sites [1].

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Active sites are quite similar in isozymes that exist in different tissues and in different enzymes with similar functions. That is, the active site of the form (or isozyme) of lactate dehydrogenase that exists in muscles is quite similar to the isozyme that exists in the liver. Similarly, many different enzymes have the same structural motifs (like the pleckstrin homology domain) to which ligands or substrates bind. So, drugs that target the active site of a target enzyme often bind other proteins or isozymes in different parts of the body, thus causing harmful side effects. This is unlikely to happen with allosteric drugs, because they bind on the protein surface that is some distance from the active site. Thus, they can be more specific, and almost always have fewer side effects. Examples include drugs that bind the pleckstrin homology (PH) domain of proteins in the PI3K-Akt pathway. They are selective, potent inhibitors of individual Akt isozymes. Since allosteric drugs don't bind to the active site, they don't function as off and on switches. Instead, they can modulate protein and biological activities at relatively low doses. They have a therapeutic effect with little or no adverse side effects. For example, modulators of phosphodiesterase 4 (PDE4) activity are useful in treating neurodegenerative diseases. A classic example is valium. More recent examples include maraviroc (Selzentry) and cinacalcet (Sensipar) for treating AIDS and hyperparathyroidism, respectively. Increasing numbers of new drug pipelines are being dedicated exclusively to the development of allosteric drugs. This concept is also being expanded to include allo-network drug effects. That is, binding of a drug to an allosteric site on one protein can have multiple effects on proteins to which the target protein is linked. Many combination therapies and multi-target drugs act this way. They cause multiple effects, often at places distant from the malfunctioning protein in the cellular network [1, 50]. For example, the plexstrin homology (PH) domain occurs in many receptors and enzymes that are involved in the phosphoinositide-3 kinase (PI3K)-Akt signaling pathway. Drugs have already been developed that bind to the active site of PI3K. Phosphoinositide analogues inhibit Akt, but may also bind other proteins containing the PH domain and cause undesirable side effects. On the other hand, some PH domain-dependent inhibitors are selective and potent inhibitors of individual Akt isozymes. In addition, allosteric drugs do not block the active site, so they don't compete with the natural substrate (ATP in this example). As a result, they do not act as an on/off switch for a receptor, but instead allow intermediate levels of activity [1, 50].

Modulating activity can be beneficial. For example, allosteric drugs that target PDE4 do not completely inhibit its enzymatic activity [1, 50]. They don't cause emesis (vomiting), a dose-limiting side effect of existing

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active site PDE4 inhibitors. These advantages occur in other allosteric drugs, from valium and the benzodiazepines to the more recently approved drugs maraviroc (Selzentry) and cinacalcet (Sensipar). So, many research projects are dedicated exclusively to the development of allosteric drugs. Recent examples of allosteric drugs include RDEA119/BAY 869766, an allosteric inhibitor of mitogen-activated protein/extracellular signal-regulated kinase (MEK), pyrvinum, a potent allosteric inhibitor of Wnt signaling, efavirenz, an allosteric reverse transcriptase inhibitor and SCF-I2, an allosteric inhibitor of (yeast) F-box protein Cdc4. Wnt is named after the wingless gene (Wg) in *D. melanogaster* (fruit flies) that is important in the formation of the body axis during embryonic development. Allosteric drugs are especially useful when they target proteins (nodes) in the metabolic network that have been identified by new mathematical tools and algorithms that identify nodes which are dynamic. They interact with other nodes at different times and/or different conditions. Such hubs (called date hubs) preferentially connect functional modules to each other, whereas party hubs preferentially act inside functional modules. Some of them have been identified in yeast by observing how its interactome responds to perturbations. They mediated the transmission of perturbations between signaling modules and are important in network cooperation [1, 183]. So, multitarget drugs that partially inhibit several nodes may be needed to modify many cellular functions, with little specificity for a particular disease process.

3.1.3.3 Using NMR to develop allo-network drugs

NMR is becoming an important tool for developing new allosteric and allo-network drugs [184]. When an organic compound or biochemical (like a protein) is placed in a strong magnetic field, the ^{1}H, ^{15}N and ^{13}C nuclei in it become magnetized and are able to absorb radiofrequency (rf) energy. The exact frequencies that are absorbed are called chemical shifts. They depend on the chemical environment of the nuclei. The more rf energy that is absorbed, the larger the signals (or peaks) that occur in the spectra. So, rf transmitters can be set at the range of frequencies that ^{1}H, ^{15}N and ^{13}C nuclei absorb. In addition, clever sequences of rf pulses can be used to transfer magnetization from one ^{1}H to other ^{1}H, ^{14}N and/or ^{13}C nuclei that are located next to them or are close to them. So, a variety of one and two-dimensional NMR spectra can be used to compare the ensembles of structures that exist in the free protein and ligand-bound protein (or inhibitor-bound protein). The results are used to study the allosteric properties of proteins. Almost all proteins may be capable of

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being regulated allosterically. Allosteric changes in proteins redistribute the populations within an ensemble of structures. Allostery can occur without a conformational change. Information is transmitted from the allosteric site to the substrate site through communication pathways within the protein. In this network view, a protein is represented as a graph where nodes are amino acid residues and edges are defined by the relationship between them. Communication from one site to another may occur along the edges of the graph. A single X-ray structure, several NMR structures or an ensemble of structures obtained from molecular dynamics (MD) simulations can be used to define various networks within proteins. Networks of protein-protein interactions have also been analyzed. They showed that hot spots located at their interfaces tend to occur in clusters and are highly centralized. Moreover, cells depend on properly functioning cellular networks such as protein-protein interactions, signal transduction, gene transcription and metabolic networks. Evolution has optimized cells to respond rapidly to changing environments by using modular functional organization and large multimolecular assemblies [184].

NMR has been very useful not only in characterizing the solution structures of proteins that are potential therapeutic targets, but also the changes in their structures that occur when substrates or ligands bind to enzymes or protein receptors, respectively [184, 185]. Moreover, structures based on NMR spectra show hydrogens and tightly bound water molecules, while those based on X-ray crystallography do not. So, a methyl group ($-CH_3$) in NMR-based structural models appears as a large sphere (due to C) with three overlapping smaller spheres (the three hydrogens), while X-ray structures show methyl groups as a single sphere. The detailed structures of proteins in solution have enabled NMR to be useful at every stage of drug development [186]. It has provided many structures of target proteins with and without attached ligands that are freely available from data banks [185]. There are also NMR screening assays for evaluating ligand binding [184].

Many parts of NMR spectra change upon the binding of small molecules to proteins [184, 186]. One especially useful technique is to test the ability of a small molecule to bind to a ^{15}N-labeled protein. Changes in the chemical shifts (rf frequencies absorbed) of the amide nitrogens upon binding to potential ligands (organic compounds, nucleic acids and peptides) are used to identify the binding sites. One can also identify small molecules that bind weakly to various sites on the target protein. The parts of the molecules that bind (pharmacophores) to sites on the target protein are identified. The parts of two or more molecules that bind to nearby locations on the protein can be linked by organic synthesis to produce new

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ligands that bind tighter and more specifically to the protein than do the individual molecules [184, 187]. This is called structure activity relationships through NMR or SAR through NMR [184, 188, 189].

In a method called saturation transfer difference NMR (STD-NMR), the magnetization of the target protein is transferred to bound ligands [184, 188]. Magnetization is saturated by irradiating the mixture of protein plus ligand with only those radio frequencies (rf) that the nuclei in the protein will absorb and not those of the ligand. The NMR spectrum of the rf-saturated protein is subtracted from that of the spectrum obtained without rf saturation. The bound ligands transfer magnetization to unbound ligands that are in free in solution, producing a spectrum that only contains signals due to bound ligands. The spectrum is obtained using a 50 to 100-fold molar excess of ligand and a low (μM) concentration of protein. The STD method can be combined with other NMR techniques to produce a variety of STD-NMR spectra, such as STD-TOCSY or STD-HSQC [184, 188].

In another method called waterLOGSY, the bulk magnetization of the water solvent is partially transferred through the ligand-protein complex to free ligand [184, 190]. The signals of compounds that don't bind to the protein appear as negative peaks and are weaker than those of bound molecules. On the other hand, genuine ligands often bind through tightly-bound water molecules in the target protein. Magnetization can be transferred through such water molecules and through acidic, ionizable hydrogens (protons) in carboxyl, phenol, amino, imidazole, guanidinium and amide groups. As a result, waterLOGSY is a relatively sensitive method for screening compounds to see if they bind to target proteins. However, it is difficult to identify ligands that bind tightly to proteins using waterLOGSY. Instead, reporter screening can be used. In this method, the ligand that binds stronger (lower inhibition constant or K_i) is used to displace the test compound [184, 190].

It is also possible to detect ligand binding by observing changes in chemical shifts of proteins labeled with ^{15}N and/or ^{13}C [184, 190]. This requires more protein (about 50 μM) than other methods, but can be used on larger proteins (with molecular weights up to about 35 kDa). Even larger proteins (up to 100 kDa) can be analyzed as perdeuterated (completely deuterated) proteins and specifically labeled proteins using transverse relaxation optimized spectroscopy (TROSY). When ligands bind to the target protein, the ^{1}H, ^{15}N and ^{13}C resonances of the protein change. This enables the spectroscopist to find binding sites and affinities. When fast methods such as FAST-HMQC are used with autosamplers and low volume cryogenic probes, small molecule libraries containing many compounds can be screened in just a few days. For example, NMR-based

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methods have been applied successfully to develop new drugs that target protein kinases, phosphatases and the Ras superfamily of small guanosine triphosphatases (GTPases) that are important in cancer and other diseases [184, 190]. This is especially important for Ras GTPases, because *RAS* is one of the most commonly activated oncogenes in human cancers, but the Ras protein and its signaling pathway have been difficult to effectively target without using ligands that bind to similar enzymes and cause harmful side effects [1, 184]. So, it is interesting to note that one group screened 3285 molecular fragments by NMR and identified 266 ligands. The binding sites were mapped by ^{15}N-HSQC using the GDP-bound GTPase domain. This enables the further identification of 25 compounds that interacted with an inducible pocket normally occupied by tyrosine 71, which also forms hydrogen bonds with the SOS protein that binds to Ras. Another group screened 11 000 fragments for their ability to bind to K-Ras that had GDP bound to it. They found 140 compounds that did bind. Most of them were close to the SOS binding site. Two of them were covalently linked to make a ligand with much higher affinity [184, 190].

Also, an NMR-based fragment screening study was done to identify allosteric inhibitors of the Bcr-Abl oncoprotein that causes chronic myelogenous leukemia, or CML [177, 184, 191]. It is a protein that is coded for by a gene formed by a reciprocal translocation and fusion of the *ABL1* gene on chromosome 9 to the *BCR* gene on chromosome 22. This "Philadelphia chromosome" contains the gene that codes for the fusion protein, in which the Abl kinase is constitutively activated, causing uncontrolled growth, lack of differentiation and reduced apoptosis of hematopoietic cells. The normal, c-Abl protein is closely regulated by an autoinhibitory mechanism in which myristate binds to an allosteric site. Myristate is the form (carboxylate anion) of the saturated fatty acid, myristic acid, 14:0, that exists at the nearly neutral pH in cells. The fusion protein has no covalently bound myristate, so it is not inhibited by this mechanism [184, 192]. So, NMEs are being studied for their abilities to bind to the allosteric site to which myristate binds [184, 192]. In addition, a fragment library was screened by NMR for the ability of fragments to inhibit Pak1, a p21-activated kinase (Paks are a family of serine/threonine protein kinases). Some of the compounds in the library were found to be allosteric inhibitors. They are being used to study how Pak1 helps to maintain tumors. In other studies, a fragment library was screened by NMR against farnesyl pyrophosphate synthase (FPPS), the enzyme that is targeted by bisphosphonates that are used to treat osteoporosis. Some of the chemicals that were tested inhibited FPPS by binding to a previously unknown allosteric site. They were not bisphosphonates, but they still

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bound to FPPS at nanomolar concentrations. An NMR assay identified those chemicals that bind to bone mineral and may potentially help to treat osteoporosis [184, 192].

So, NMR is being used to study ligand binding as well as protein-protein interactions [184]. NMR can also be used to identify allosteric binding sites and to map allosteric networks, especially in multidomain signaling proteins and intrinsically disordered proteins [184, 193-196]. Chemical shifts of some key ^{1}H, ^{13}C and ^{15}N nuclei are different in the various conformations of a protein [184, 196]. These differences can be used to study the structural dynamics of proteins. If two different conformations exchange magnetization relatively quickly compared to the time required to produce the chemical shifts, the observed chemical shifts will be weighted linear averages of the chemical shifts of the nuclei in the two conformations. When ligands bind to a protein or mutations occur that cause changes in the structure and/or location of amino acids, the conformational equilibrium and chemical shifts can change, both locally and at locations that are relatively far from the perturbation. Amino acid residues with nuclei that have co-varying changes in chemical shifts are probably involved in the same conformational change and are expected to be in the same amino acid network [184, 196].

These changes can be detected using chemical shift covariance analysis (CHESCA) [184, 196]. It can detect allosteric networks [184, 194, 196]. When combined with ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) analysis, CHESCA can identify allosteric cycles in proteins, such as protein kinase A (PKA) [184, 194]. Allosteric regulation is extremely important in signaling proteins that have several structural domains [184, 195]. These proteins often contain flexible inter-domain linkers in which crucial functional sites are located. CHESCA provides a set of pairwise, linear inter-amino acid correlations that are displayed as a matrix. The CHESCA correlation matrix shows not only the correlations that exist inside a structural domain, but also correlations in linkers, such as those that occur in PKA. CHESCA has shown that flexible linkers can function not only as “covalent threads” between different domains in proteins (like PKA), but also can be active elements of allosteric signaling [184, 195].

PKA and other protein kinases often have critical roles in cellular processes and pathology [184, 197]. Unfortunately, previous 2D and 3D NMR methods have not been able to describe their structures in solution. However, improvements in transverse relaxation-optimized spectroscopy (TROSY) are enabling researchers to acquire high quality NMR spectra of these relatively large protein kinases. TROSY spectra identify strong associations between protein motion and kinase activity. The dynamic

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changes that occur at ligand binding sites and other regulatory domains can propagate to conserved core regions that mediate the catalytic functions of these kinases. TROSY spectra also show that protein kinases have dynamic structures. They are regulated by inter-domain interactions, ligand binding, and covalent modifications that change the motion and conformational equilibrium of the kinases. These can be correlated with physiological function. So, TROSY NMR is providing a unique way to learn about how protein dynamics can affect protein kinase regulation and catalysis, with important implications for discovering new drugs [184, 197].

PKA structure and function have also been studied using a statistical analysis of the chemical shifts [184, 197]. Coordinated chemical shifts behavior (CONCISE) observes protein conformational equilibria by following the linear trajectories of NMR chemical shifts. CONCISE quantifies the population shifts that are associated with adding increasing amounts of ligand and estimates the degree of collectiveness of the amino acid residues' response to binding ligands. This gives a good view of the structural transitions. The combination of CONCISE NMR spectra with thermocalorimetric and kinetic data can illuminate a protein's approximate conformational energy landscape. This method was tested with the catalytic subunit of PKA. When complemented with chemical shift covariance analysis (CHESCA), this new method provided both a collective response and residue-specific correlations caused when ligands bind to proteins [184, 197].

CHESCA is also used together with site-directed mutagenesis in which alanine (Ala) is changed to glycine (Gly) [184, 198]. This adds a methyl group to each of the Gly residues that are mutated. This can change the conformational space that is accessible by the part of the protein backbone that is close to the mutation. Also, longer-range structural dynamic changes can occur that cause changes in chemical shifts. By studying the wild type and mutated α subunit of tryptophan synthase, amino acid networks were shown to be not static, but change in different functional states of the enzyme. Instead, the switching of allosteric pathways is probably caused by changes in conformational dynamics that were triggered by ligand binding [184, 198].

$^{1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectra can be used to identify key amino acids in allosteric and ligand binding sites [184, 191, 199, 200]. ^{15}N chemical shifts are strongly affected by the identity and conformation of the preceding amino acid in the protein [184, 196]. The NMR chemical shifts of amino acids that change when a ligand binds to a protein form a network that can be characterized [184, 192, 201,

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202]. The allosteric networks can be identified by CHESCA [184, 201, 202]. This combination of HSQC and CHESCA was used to identify allosteric binding sites and study the binding of NMEs to them on potentially therapeutic drug targets [184, 201].

In $^{1}H-^{15}N$ HSQC, a specific sequence of rf pulses is used to cause the magnetization of proton (^{1}H) nuclei to be transferred to nitrogen (^{15}N) nuclei [184, 198]. These spectra can be acquired rapidly, enabling many compounds to be screened for their ability to bind to target proteins [184, 189]. Every amino acid residue except proline forms an NH bond that is part of the amide groups that covalently link amino acyls. So, the amide NH bonds can be detected by $^{1}H-^{15}N$ HSQC. The rf of the ^{15}N (ω ^{15}N) is plotted on the y-axis against the rf of the ^{1}H (ω ^{1}H) on the x-axis. However, the differences in rf that various ^{1}H and ^{15}N nuclei absorb in NMR are so small that it is much more convenient to express them as parts per million (ppm) than megaHerz (mHz) [1, 184]. That is, when using an NMR that has a magnet with a field strength of 11.75 Teslas (11.75 T), ^{1}H nuclei will absorb rfs at or near 500 MHz. The amide H in one amide bond might absorb rf at about 500.036 MHz (7.2 ppm). Many others might absorb rf at about 500.040 MHz (8.0 ppm) and some might absorb rf as high as 500.047 (9.4 ppm). It would be very inconvenient to label the x-axis in a $^{1}H-^{15}N$ HSQC spectrum in units of MHz ranging from 500.036 to 500.047 MHz, so ppm is used instead. At the same time, amide nitrogens (^{15}N) will absorb rf at or near 50.782 MHz. Again, the differences are so small that their chemical shifts (rf absorption frequencies) are expressed in units of ppm. When NMR spectra are acquired using magnets with higher field strength, the sensitivity and resolution of $^{1}H-^{15}N$ HSQC and other NMR spectra increase. However, even when $^{1}H-^{15}N$ HSQC spectra are acquired using NMR spectrometers with different magnetic field strengths, the results are directly comparable if the axes are labelled in units of ppm. So, NMR spectrometers with different sized magnets can be used to characterize allosteric binding sites and protein-protein interactions. As one protein interacts with a second protein or a piece of DNA (as in transcription factors), the interactions between the second protein and other proteins (or other genes) can change. So, allosteric signals can be transmitted from one protein to other proteins, or to DNA and/or RNA that are far from the original protein. So, a relatively new term has been coined for allosteric drugs that affect biopolymers that are not in direct contact with protein that binds the drug – they are allo-network drugs [1, 184].

The HSQC method was recently improved and renamed FAST-HSQC [184, 203, 203]. In a technique called ASAP-HSQC (HSQC as soon as possible), spectra of unlabeled small molecules can be obtained in less

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than 30 sec [280, 297]. The digital resolution is enhanced by keeping all unused ^{1}H magnetizations along the *z* axis during acquisition. There is also a modest gain in sensitivity [184, 203]. This and other new NMR methods for analyzing small molecules have been reviewed [184, 204].

NMR data has been used to help build an allosteric database that can be used to identify possible therapeutic targets for allo-network drugs [1, 184, 205]. NMR data are being combined with network-based analyses to play an important role in developing new drugs [184, 206]. For example, a network analysis recently showed that targets of drugs often are good spreaders of perturbations in the human interactome. It also found that proteins involved in colorectal cancer were good spreaders of perturbations and had a high centrality. In contrast, proteins related to type-2 diabetes had an average spreading efficiency and average centrality in the human interactome. This is because rapidly proliferating cancer cells are attacked most effectively at their central, hub proteins, while differentiated cells like those involved in type-2 diabetes are best attacked at proteins that are neighbors of hub proteins [184, 206].

Still, the interactions of drugs with therapeutic proteins *in vitro* can be quite different than with proteins in intact cells [184, 207]. That is, high throughput screening of drugs acting on tissues or combinations of more than one cell measures the effects that are due to an average of the responses in all of the cells. This can easily lead to false conclusions. So, the single value decomposition (SVD) method was recently described for characterizing protein-protein interactions in a single cell. It is a mathematical technique for identifying the principal components of a matrix that contribute the most to the variance in the elements of the matrix. It was used to evaluate data from structural interaction-NMR (STINT-NMR) spectra acquired from two proteins that are involved in the proteasomal degradation of *Mycobacterium tuberculosis*. The SVD analysis distinguished concentration-dependent interactions from concentration-independent interactions and identified the amino acids that were involved in the interactions [184, 207].

3.1.3.4 Pharmacokinetics

One of the biggest problems in developing new drugs and getting their regulatory approval in the past is the misleading information that is often obtained from doing pharmaco- and toxicokinetic studies on animals. That is, many developmental drugs were found to have very low toxicities in test animals, but ended up exhibiting serious toxicities in a small, but important fraction of the human subjects in clinical trials. At the same

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time, many developmental drugs that failed the toxicity tests in animals may have had little or no toxicity in humans. This is because of important biological and medical differences between animals and humans. Still, almost all the prescription drugs that have already been approved have passed the toxicity tests in animals. Subsequently, the pharmacokinetic parameters obtained in human studies were used to help establish the appropriate doses given in clinical trials.

So, pharmacokinetics determines how much of the test compound reaches the target organ or organs and how much is eliminated at different times after giving different doses, sometimes in various dosage forms [51]. It also evaluates efficacy. Drugs can have multiple effects on the proteome, transcriptome, epigenome, metabolome and interactome of cells, tissues and organisms. Toxicokinetics are similar to pharmacokinetics, except toxicity is measured, instead of efficacy. That is, when the dose of a test compound is too high, it becomes toxic. So, toxicokinetics are much like pharmacokinetics, but at higher doses. Important parameters in pharacokinetics include the maximum drug concentration seen in the blood or target organ, C_{max}, the time it takes to reach maximum concentration, t_{max}, the area under the curve, AUC, bioavailability, clearance, volume of distribution and the half-life for clearance, $t_{1/2}$. There are physiological based pharmacokinetic models (PBPK) that match a test compound's properties to its physiological properties. The absorption, distribution, metabolism and elimination (ADME) of the test compound are measured. Drug metabolism occurs mostly in the liver and intestine. Phase I metabolism adds functional groups (-OH, -SH, -NH_2, -COOH), while phase II involves biotransformation. Phase II enzymes add larger molecules and groups [51].

3.1.3.5 Systems technology, Chemical Screening Based on Adverse Outcome Pathways (AOP)

Even though there are two phases of drug metabolism in both test animals and humans, there are often important differences in the toxicities of test compounds. Still, for many decades, animal testing seemed to be the best option. Unfortunately, the best that previous technologies allowed was to determine the ADME in test animals without understanding the mechanisms by which toxicity occurred [208]. Instead, the effects of an NME on mortality, reproduction, growth and development were determined by *in vivo* tests for toxicity. The traditional, reductionist way of doing toxicology was time-consuming, expensive, low-throughput, and provided little information about the mechanisms of toxicity. Many people

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also considered it immoral. This is because of animal suffering and that animal testing almost always ended by killing any animals that survived the kinetic analyses [208]. So, in 2007, the US National Research Council (NRC) published the report Toxicity Testing in the 21st Century (TT21C): A Vision and a Strategy [209]. It defined toxicity pathways as, "response pathways that result in an adverse health effect following sufficient perturbation" [209]. It recommended changing the way that toxicity testing is done. No longer should high-dose studies in laboratory animals be performed. Instead, *in vitro* methods should be used that can evaluate changes in normal cellular signaling pathways. Such tests could use cells or tissues that are relevant to humans. The term "toxicity pathway" was coined. It means determining whether a normal signaling process is significantly perturbed, and if the result is an adverse outcome to the cells or tissues. Recently, this idea of basing toxicity studies on changes in mechanistic pathways has been named the adverse outcome pathway (AOP). It starts by identifying the chemical reactions that occur during the initial event(s). This should lead to identifying further key events and finishes with a specific adverse outcome that leads to an adverse outcome that is relevant to risk assessment and regulatory decision-making. The concept of AOP improves on the former concept of "toxicity pathways", which focuses on the initial events (such as absorption and distribution) and key measurable endpoints (such as illness or death), but not on the adverse outcome. AOP links the first event with the following progression of a defined series of key events. They should be measurable changes in the biological state and be necessary for the adverse outcome to occur. Thus, AOP includes the whole process from the first event to observing the adverse consequences [209].

This approach of identifying the mechanisms of toxicity has been called systems toxicology [210]. It uses computational tools to model chemically induced pathophysiology, as is done in systems biology. It combines some of the tools used in classical toxicology (such as quantifying drugs and their metabolites by LC-MS) with a quantitative analysis of large networks of molecular and functional changes that occur in many levels of biological organization. The systems-wide molecular changes that are caused by administering a test compound are quantified. The upstream events that cause an observed toxicity can include measuring interactions between receptors and ligands, as well as DNA binding and cellular responses such as activating the transcription of genes, protein production and altered signaling pathways. The results are integrated with the succession of causative downstream molecular events that link exposure to the test compound with the observed toxicities. Then,

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computational models are built that try to describe these processes in as accurate of a quantitative way as possible. The resulting integrated analyses helps researchers and medicinal chemists find out how key biological pathways are changed by exposure to the test compound. This, in turn leads to predictive computational models of toxicological processes and improves the accuracy of risk assessment [210].

So, a conceptual framework was developed to examine exposure and ADME properties and see how they relate to a molecular initiating event, or MIE [211]. The concept was evaluated by using a previously established AOP for the inhibition of acetylcholinesterase (AChE). They evaluated 30 chemicals that were found to inhibit AChE in the ToxCast™ assay. They incorporated exposure and ADME properties into the conceptual workflow. They eliminated ten low-priority chemicals that may otherwise have undergone additional, costly analyses. Their procedure can increase one's confidence in interpreting *in vitro* results by identifying possible false negatives [211].

This concept of making safety decisions using toxicity pathways and not *in vivo* testing on rodents was evaluated for the natural compound quercetin [212], which is found in capers (salt-brined flower buds of the *Capparis spinosa* plant), all types of onions, dark red or blue fruits (such as blueberries, apples, cherries and raw black plums) and many vegetables (such as red leaf lettuce, raw green chili peppers, kale, spinach and asparagus) [213]. Even though quercetin is a healthy antioxidant that can activate the Nrf2/ARE antioxidant system [214, 215], it can be toxic if taken at too high of a concentration in some cancer patients because this antioxidant system is over-activated in multi-drug resistant cancers. Moreover, all substances are toxic if given at too high of a dose [1, 216]. That is, the dose is the poison [170]. Using systems thinking, a multidisciplinary team of chemists, biologists, modelers and risk assessors collaborated to see if previously published data on the *in vitro* dose-response of quercetin could accurately predict its safety [210]. The bioinformatics methods used databases such as the Comparative Toxicogenomics Database, KEGG, MetaCore and International Pharmaceutical Abstracts (IPA), in combination with -omics data to identify toxicity pathways. This produced a list of prioritized pathways, but provided very little understanding of their dynamics. So, computational systems biology pathway (CSBP) models that provide a better understanding of the experimental data were also used. It was also important to realize that biological systems are inherently nonlinear, so nonlinear dose-response curves are to be expected, even though they have been assumed to be linear and have been extrapolated to zero dose in the

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past (when reductionist thinking was used). Moreover, biological systems pathways are populated with many different recurring motifs. They protect systems from excessive changes due to perturbations while moving systems from one state to another by using negative feedback [210, 217]. When homeostasis is maintained, cells respond to perturbations by making slight adjustments that result in normal function also being maintained [210]. This region of the dose-response curve can be used to establish the no observed adverse effect level (NOAEL) of the compound being evaluated. At higher doses the adaptive responses become over-saturated, leading to adverse effects. That is, there is a tipping point between adaptive and adverse effects. So, it is important to determine this tipping point when assessing safety. So, an attempt was made to construct a TT21C-based risk assessment in a human cell line derived from a biopsy of a fibrosarcoma from a 35-year old male Caucasian. It looked at pathway biomarkers as well as markers of cell-cycle, apoptosis and micronuclei formation, plus gene transcription. This cell line expresses the p53 signaling pathway in a normal, healthy way and has been used to study signaling through this hub in cellular metabolism. Dose-response curves containing 18 points were produced to determine the doses that caused significant perturbation. NOELs and benchmark doses were compared to the output from biokinetic modeling. The results were encouraging. They provided a step towards the practical implementation of TT21C [210]

At the same time, much progress has been made in producing *in vitro* platforms for evaluating liver toxicity [218]. That is because the liver is susceptible to damage by chemicals, drugs and their metabolites. Moreover, hepatotoxicity is a major cause of drugs being withdrawn from the market. This includes troglitazone, nefadazone and trovafloxacin, as well as diclofenac and acetaminophen, which are still being sold but pose a significant risk of hepatotoxicity. The human toxicity of troglitazone, nefadazone and trovafloxacin was not predicted by animal testing (which showed little or no toxicity). To become toxic in humans, they need specific human metabolism. They also have a latency period between their initial administration and the onset of toxicity. Even though animal testing was popular in the past, *in vitro* methods have emerged. This includes using cell cultures of primary hepatocytes alone or in combination with other cells in two- and 3D formats. Liver cells grown in monolayers (two-dimensional, or 2D) don't live long, so only acute and short-term studies are possible. In contrast, when an appropriate matrix is inserted between hepatocytes, they can survive up to seven weeks. Matrices include Type 1 collagen, Matrigel™ and pro-electrolyte layers. Systems thinking was used to establish that hepatotoxicity is not due to just pathological lesions

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in the liver parenchyma, but also interactions between hepatocytes and the resident liver immune, stellate, Kupffer and endothelial cells. So, 3D formats are preferred. Moreover, animal models don't mimic the interactions between human cells and human toxicity very well [218].

So, macroscale and liver on a chip systems have been developed and commercialized recently [218]. There is a macroscale transwell system that cultures hepatocytes and non-parenchynal cells that are in a nearly physiological ratio. There is also a 3D microtissue spheroid culture that uses a gravity-enforced cellular assembly that enables the formation of cellular contacts. It produced more accurate TC_{50} values than more conventional 2D cell cultures. TC_{50} is the concentration that causes toxicity in 50% of the cells. There is also a co-culture of discrete micropatterned islands of hepatocytes that are stabilized with stromal cells called Hepatotec. It is functional up to six weeks. There are also liver on a chip platforms that are made using lithography, which is a type of 3D printing. They are very similar to *in vivo* structures. So, they have a 3D microarchitecture and have a ratio of cells to nutrients that is close to that which exists in the liver. They are dynamic microsystems that enable a precise control of the flow of growth media and the removal of waste products. There is also a microfluidic liver sinusoid model that uses lithography to create an artificial barrier that is similar to the porous liver sinusoid. It eliminates the need for endothelial cells by constructing a structural barrier (with posts) that shields hepatocytes from media stress and simultaneously allows nutrients to be exchanged. There is also a multi well plate platform that uses hepatocytes with non-parenchymal cells and flow of growth media that is induced by a pneumatic controlled underlay. It creates an environment that is similar to the liver in that it allows fluids to flow, while providing an oxygen gradient and shear stress. There is also a microfluidic microscale cell culture analog (μCCA) that allows several tissues to interact in a physiologically-based pharmacokinetic model. Next, there are many assays and biomarkers that can assess the physiological mechanisms of toxicity in multi-component liver platforms that include human hepatocytes. There are also platforms for determining genomics (or toxicogenomics), proteomics and metabolomics by analyzing tissues, blood and/or urine. Toxicogenomics studies have shown that specific gene-expression profiles and mechanisms of toxicity are linked. Some of this research is being done at the US National Center for Toxicogenomics (NCT) at the National Institute of Environmental Health Sciences (NIEHS). In addition, the EU Framework Project published the results obtained by a consortium of 15 pharmaceutical companies, two small companies and three universities. They reported the transcriptomics,

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proteomics and metabolomics resulting from 16 compounds that were administered in two-week rat studies. They found that the transcriptomics (total RNA) provided enough data to show the mechanism of toxicity while proteomics and metabolomics supported the results from transcriptomics [218].

So, the FDA is compiling a Liver Toxicity Knowledge Base (LTKB) that contains diverse data [218]. It includes mechanisms of drug-induced liver injury, drug metabolism, histopathology, therapeutic use, targets, side effects that are associated with individual drugs. The FDA is using systems thinking to integrate the data with assessments of drug-induced liver injury and prediction of such injuries. Similarly, the National Library of Medicine (NLM) and the National Institute of Diabetes and Digestive and Kidney Diseases have set up a Liver toxicity website (Livertox.nih.gov, 2013). The LiverTox website provides up-to-date, comprehensive and unbiased information about drug-induced liver injury that is caused by prescription and nonprescription drugs, as well as herbal remedies and dietary supplements. It is provided as a mixture of text and data with many references. The US Environmental Protection Agency (EPA) also has resources for environmental toxins. This includes the Aggregated Computational Toxicology Resource (ACToR) and the Toxin and Toxin-Target Database (T3DB) [218-220].

When developing drugs to treat cardiovascular diseases, it is also important to see if they cause any types of cardiotoxicity. Cardiovascular diseases (CVDs) include coronary heart diseases (such as myocardial infarctions or heart attacks and hypertensive heart disease), atherosclerosis, hypertension, and other vascular cellular dysfunctions that can lead to coronary occlusion and thrombosis [221]. CVDs are the leading cause of death in the USA [221, 222]. In the past, there were many failures in clinical trials for CVDs, due to unexpected adverse side effects on the heart [221]. Moreover, some of the previously approved drugs had to be removed from the market because of such side effects. The high failure rates were due, in part, to relatively ineffective toxicity tests on animals or in 2D cell cultures. So, microfluidic cardiovascular organ chips are being developed for improved toxicity testing on drugs that are being developed to treat CVDs. In addition, a variety of biomaterials can be mixed with different types of cells (such as iPSCs and rat neonatal ventricular cells) to develop 3D structures that model cardiovascular diseases. They are being used to test the efficacy of NMEs. Some researchers are also investigating the possible use of transplanting embryonic stem cells, cardiac progenitor cells, skeletal myoblasts and iPSCs to repair heart tissue. To assist in this effort, a microfluidic device

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was made by co-culturing skeletal myoblasts and cardiomyocytes in separate chambers and connecting them with permeable microvalves. It showed that skeletal myoblasts can repair cardiomyocytes that have been damaged by hypoxia through intercellular interactions. Another group used hydrogels and microfluidic systems to mimic valvular tissue and disease. A thin, porous membrane separated valvular endothelial cells (VECs) and valvular interstitial cells (VICs). The VICs resembled fibroblasts. They were encapsulated in gelatin methacryloyl (GelMA) hydrogels. The VECs were seeded on fibronectin-coated polydimethylsiloxane (PDMS) microchannels. Together, the VICs and VECs facilitated the adhesion of cells inside the microfluidic channels. Growth medium was continuously perfused to mimic shear stress caused by blood flow in myocardial tissue. As a result, a system was produced that can test the safety and efficacy of NMEs, lead compounds and newly developed drugs [221].

Still others are engineering cardiac muscle tissue and making heart on a chip devices that include even more of the cells that are in native heart tissue [221, 223-226]. They also include electrical stimulation and cardiomyocyte anisotropy [221]. This is important because native cardiomyocytes need to be electrically stimulated by automated pacemaker cells that are in the sinoatrial node. These electrical signals travel through the left and right atria and go to the atrioventricular node. Then, the signals reach the right and left ventricles. This causes the cardiomyocytes to become anisotropic (aligned) and depolarized. This, in turn, produces regular, synchronized contraction of cardiac muscle [221]. So, anisotropically organized cardiomyocytes were put on a heart on a chip using a muscular thin film platform [221, 225]. Self-organized cardiomyocytes were electrically stimulating using platinum electrodes to mimic the native (*in vivo*) production of electrical signals by pacing cells in the heart. The electrical stimulation increased the alignment of the cells as well as the differentiation, and functionality of the engineered cardiac tissue [220, 224, 225].

In addition, microfluidic platforms are being used to mimic human vasculature and study vascular networks and angiogenesis [220, 227-229]. They create 3D vascular beds using a variety of hydrogels. The mechanical properties and extracellular matrix can be fine-tuned to target specific regions of interest in the vascular system. They can mimic the microenvironment of vascular tissue, which is making the discovery of new drugs to treat CVDs easier and more productive [220]. One important example is the use of bioprinting to make 3D microarchitectures that mimic different vascular regions of the human body [220, 230, 231]. These devices are being used to test NMEs in a fast, controllable

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inexpensive way on a large scale [220]. However, these approaches are not personalized.

So, it is important to note that advances are also being made in developing 3D models from iPSCs that are unique (or personalized) for every patient [232]. The iPSCs were derived from human perinatal foreskin fibroblasts and human adult dermal fibroblasts. Digital 3D bioprinting was used to produce a triculture model from human induced pluripotent stem cells (hiPSCs), human umbilical vein endothelial cells (HUVEC) and adipose-derived stem cells in a microscale hexagonal structure. It had good morphological organization, higher expression levels of genes that are specific to the liver, increased secretion of metabolic products and enhanced induction of cytochrome P450 enzymes that are essential in proper liver metabolism. This model will enable personalized drug development, disease modeling and prediction of toxicity [232].

In addition to labs on a chip for the liver and heart, similar devices have been made for the lungs, blood-brain barrier, intestines (gut on a chip) and cancer tissues [233]. 3D printing and its predecessor, rapid prototyping, often use UV stereolithography to make plastic models one layer at a time on a structure that is supported by polymers that must be removed after printing the model [233, 234]. One pre-polymer that is frequently used in UV stereolithography is Cyracure® UVR-6105 (Ultraviolet resistant 6105) [234, 235]. Its major component is 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate [234]. It was originally developed as a replacement for another widely-used epoxy pre-polymer, EPON™ 828. Its primary component is the diglycidyl ether of bisphenol A (DGEBA), also known as bisphenol A diglycidyl ether (BADGE). Like most chemicals that have phenyl rings, it degrades (or is bleached) by extended exposure to UV light. So, to make cured epoxies that aren't degraded by lengthy exposure to the sun, Cyracure® UVR-6105 was developed (the UVR stands for UV resistant). Since the supports needed to make stereolithographic models had to be removed by hand, most people also appreciated that Cyracure® UVR-6105 was not as mutagenic as BADGE [234]. The structures of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and BADGE are shown in Figures 8 and 9.

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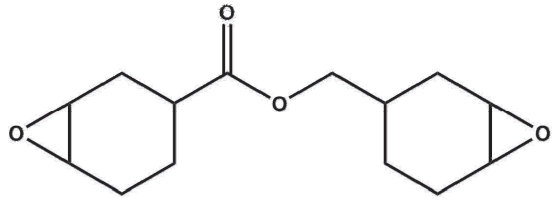

Figure 8. 2D structure of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate. It is the major component of Cyracure® UVR-6105.

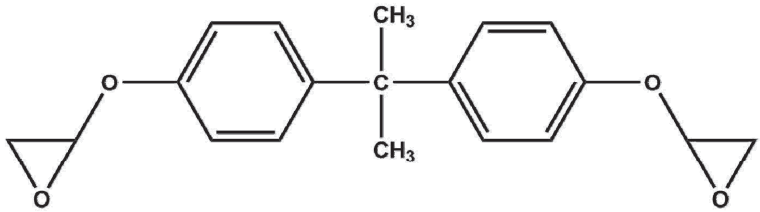

Figure 9. 2D structure of bisphenol A diglycidyl ether (BADGE), the major component of EPON^{TM} 828. In three dimensions, the phenyl groups are at a 90^{o} angle to each other. That is, they are orthogonal and not coplanar.

However, the bioavailability of BADGE is much lower than that of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its hydrolysis products [234]. So, a worker should not use his or her bare hands and fingers to remove the supports from a 3D-printed model made from Cyracure® UVR-6105. That is because if the worker were to touch his or her mouth or eat food before thoroughly washing his or her hands, some of the uncured (unreacted) 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate can enter the blood and reach stomach and liver, where it can be hydrolyzed by in a reaction catalyzed by either acid (H^{+}) in gastric fluid or an esterase in liver to produce the metabolites 3,4-epoxycyclohexylmethanol (ECHM) and 3,4-epoxycyclohexanoic acid (ECHHA), whose structures are shown in Figures 10 and 11. ECHM has is more genotoxic that either BADGE or 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate [234]. The genotoxicity of ECHHA has not been tested.

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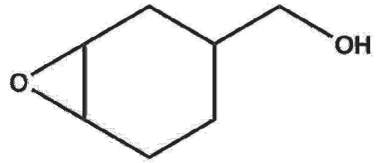

Figure 10. 2D structure of 3,4-epoxycyclohexylmethanol (ECHM), a metabolite of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate.

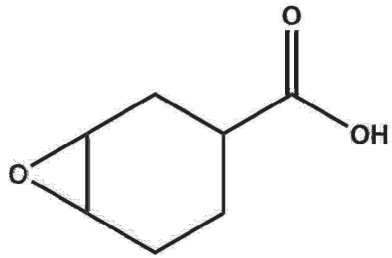

Figure 11. 2D structure of 3,4-epoxycyclohexanoic acid (ECHHA), a metabolite of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate.

Both ECHM and ECHHA are more soluble in water and the aqueous environment of cells than either BADGE or Cyracure® UVR-6105. ECHM and ECHHA also contain –OH groups that can react in the acidic environment of gastric fluid with the epoxy (or oxirane) group in uncured 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate. So, a very interesting thing occurs when you compare what happens when BADGE or Cyracure® UVR-6105 are mixed with simulated gastric fluid (water at pH 1.6 made by adding HCl). At first, both BADGE and Cyracure® UVR-6105 don't mix with the aqueous solution, but form a separate oily layer. For about the first 15 minutes, only the surface of the oily globules of BADGE or 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate are exposed to the acidified water. So, only a very small portion of either epoxy is hydrolyzed. However, after about 15 min of stirring, the mixture of Cyracure® UVR-6105 in acidified water at pH 1.6 will start to turn cloudy, while the mixture containing BADGE will not. That is because the ester groups in enough molecules of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate have been hydrolyzed to produce hydrophilic products. This leads to the formation of micelles and/or microemulsions that can suspend the unreacted 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate. In contrast, the hydrolysis products of

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BADGE are not very soluble in water, so neither micelles nor microemulsions can be formed. In contrast, once the unreacted oily globules containing molecules of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate from UVR6105 are broken down into micelles, many more molecules of 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate will become exposed to the acidic, aqueous medium. They can be hydrolyzed to make more ECHM and ECHHA, so more micelles are formed and more 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate molecules are exposed. That is, the micellar structure makes more copies of itself and becomes an autopoietic system. However, unlike autopoiesis in living systems, this form of autopoiesis is more nearly associated with death caused by genotoxicity, which can lead to cancer. Interestingly, the autopoietic system made from 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its breakdown products evolves rapidly. That is, the H^+ in the acidic environment catalyzes reactions between epoxy groups and –OH groups to produce molecules such as those shown in Figures 12-15.

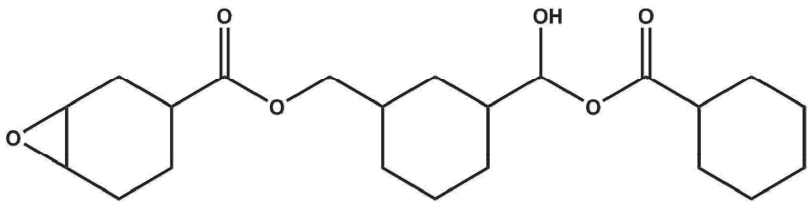

Figure 12. 2D structure of one product of the acid-catalyzed reaction between 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its hydrolysis products.

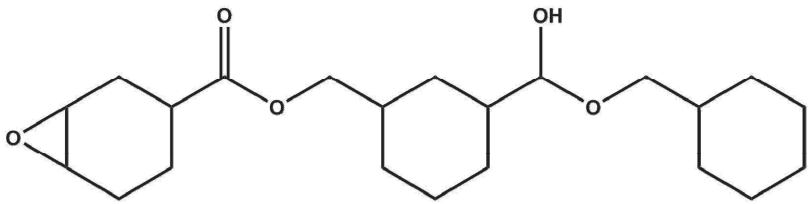

Figure 13. 2D structure of another product of the acid-catalyzed reaction between 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its hydrolysis products.

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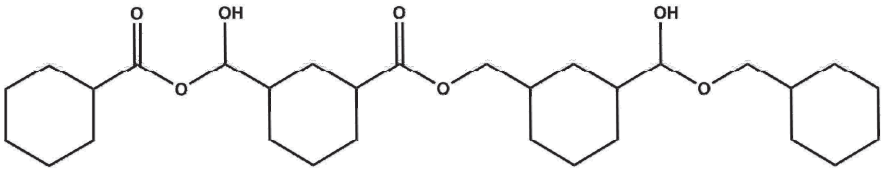

Figure 14. 2D structure of a third product of the acid-catalyzed reaction between 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its hydrolysis products.

Then, after a few more minutes, the many different products of the reactions between 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate and its hydrolysis products are further hydrolyzed until all that is left are 3,4-dihydroxycyclohexylmethanol and 3,4-dihydroxycyclohexanoic acid. Their structures are shown in Figures 15 and 16. They are both quite water soluble. So, after 30 minutes of stirring, the mixture turns clear and no more micelles are formed. This does not happen with BADGE or epoxy-containing silicones that are used in dental applications [236].

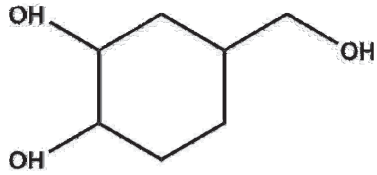

Figure 15. 2D structure of 3,4-dihydroxycyclohexylmethanol - one of the final hydrolysis products of UVR 6105.

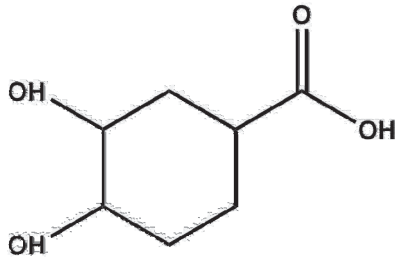

Figure 16. 2D structure of 3,4-dihydroxycyclohexanoic acid - one of the final hydrolysis products of UVR 6105.

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Since many toxicologists and oncologists know that epoxies are carcinogenic, the term oxirane is used instead of epoxy in the common (or trivial) names of these silicones. They are called siloranes. The structures of two of them, bis-3,4-epoxycyclohexylethyl-phenyl-methylsilane (Ph-Sil) and 3,4-epoxyclohexylethyl-cyclopolymethylsiloxane (Tet-Sil) are shown in Figures 17-18.

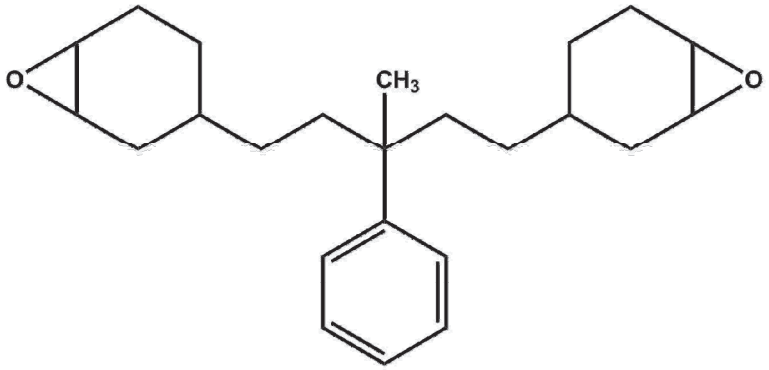

Figure 17. 2D structure of the silorane bis-3,4-epoxycyclohexylethyl-phenyl-methylsilane (Ph-Sil).

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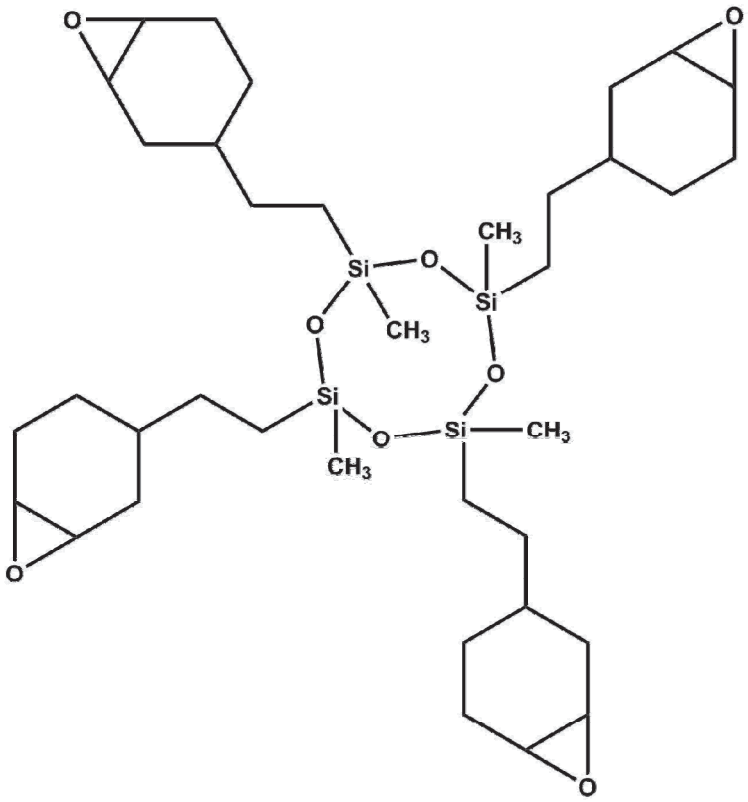

Figure 18. 2D structure of the silorane 3,4-epoxyclohexylethyl-cyclopolymethylsiloxane (Tet-Sil).

So, it is possible that siloranes might be a better choice than Cyracure® UVR-6105 for preparing 3D printed structures for bone engineering or any other application. That is, even though most of the Cyracure® UVR-6105 is polymerized into an insoluble polymer, no polymerizations done in industry are complete. There is always some unreacted monomer remaining. In the case of Cyracure® UVR-6105, the unreacted monomer can be hydrolyzed by esterases and H^+ in gastric fluid to produce mutagenic compounds that are more soluble and bioavailable than the unreacted monomer.

Another safer alternative for 3D printing is *N*-isopropylacrylamide [237]. It can be polymerized grafted with hyaluronan and blended with methacrylated hyaluronan to make high-resolution scaffolds to make a

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bioink to make stratified tissues like articular cartilage. Since hyaluronan is a natural substance that is safely consumed in many dietary supplements (usually in combination with chondroitin sulfate) and even used in clinical applications, it is a logical choice for a bioink. This idea is supported by the fact that this bioink had no direct toxicity on cells that were cultured on its surface [237]. So, many advances have been made in biological and toxicological applications of 3D printing.

3.1.4 Improve the lead compound

The toxicity of the first lead compound that is discovered is often unacceptable. Moreover, some lead compounds have low solubility in aqueous solutions, so they can't be given as an oral dose. Other lead compounds either don't reach the target organ sufficiently or reach other unintended organs where they can cause harmful side effects. So, it is usually necessary to improve the lead compound.

For example, salicylic acid was found to be the active ingredient in willow bark, which was used for centuries to treat pain and inflammation [1]. However, salicylic acid irritates the stomach and can cause severe internal bleeding as a potentially fatal side effect. So, willow bark is not used to treat pain or inflammation any more. Instead, aspirin is used. That is, the German scientist Felix Hofmann discovered in 1897 that he could obtain an equally effective pain reliever by converting the COOH in salicylic acid to an acetyl ester. The result was acetylsalicylic acid, or aspirin. There are over 40 000 tons of it made each year [238].

In modern medicinal chemistry and new drug development, lead compounds are often improved by rational drug design [1]. Their structures can be systematically changed to make new compounds that are more effective and have fewer side effects. For example, cocaine is a highly addictive, illegal drug with deadly side effects - even though it is a good local anesthetic. It was also present in many 'patent remedies' that existed before the creation of the FDA and other governments' regulatory agencies. To make a safer anesthetic, the structure was modified to produce procaine, the oldest man-made anesthetic. The structure of procaine resembles that of cocaine (Figures 19 and 20). They have similar parts, called pharmacophores.

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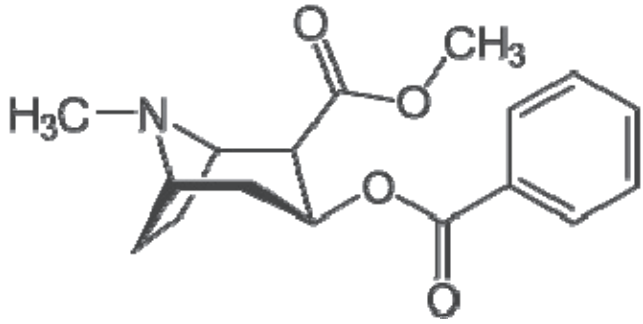

Figure 19. 2D structure of cocaine.

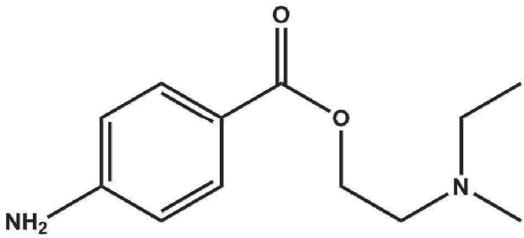

Figure 20. 2D structure of procaine.

Subsequently, the ester linkage of procaine was replaced with an amide linkage to make procainamide hydrochloride, which is a cardiac depressant, local anesthetic and antiarrhythmic [1]. Another powerful drug with serious adverse side effects is morphine. It binds to opioid receptors in the brain to produce its analgesic effects. Its structure was modified in a relatively healthy way to produce codeine, but in a deadly way to produce heroin. Codeine was further modified to make dextromethorphan, the major ingredient in most cough medicines. Now, medicinal chemists can look for specific pharmacophores or fragments in libraries of compounds. Vemurafinib was the first FDA-approved drug that was developed by fragment-based drug discovery. That is, small fragments of compounds are identified that bind weakly to a biological target, such as a protein receptor. Next, more compounds are made that combine the different fragments. Vemurafinib inhibits the proto-oncogene B-Raf, a protein kinase that plays an important role in regulating the MAPK signaling pathway [1].

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Compounds can also be evaluated *in silico* in computer calculations. 3D structures are drawn, so that physical and chemical properties can be calculated [1]. Then, statistical correlations are made. In addition, structures of target proteins that were determined by X-ray crystallography and/or NMR can be downloaded from databanks. As a result, compounds that were drawn can be docked *in silico* to the target proteins and interaction energies calculated. Quantum mechanical calculations produce hundreds of molecular descriptors. The descriptors that are the most important in determining the biological, chemical and/or physical properties of the compounds are identified. The molecular and quantum mechanical descriptors can be correlated to the experimentally measured properties. This is the basis for determining quantitative structure-activity relationships, or QSARs, also known as structure-activity relationship, or SAR. Finally, databases of biochemical and medicinal interactions can be evaluated using chemoinformatic theory and network theory to find new therapeutic targets. Different types of QSARs and their applications have been reviewed previously [1].

Some of the chemoinformatic tools that are available for empirical predictive modeling include Daylight [239], OpenEye Scientific Software [240], ChemAxon [241], The Chemistry Development Kit102 [242], RDkit [243], Dragon descriptors103 [244] and Batch Modules for ACD/Percepta [245]. They combine chemistry, computer science and information science with topology and chemical graph theory, as well as information retrieval and data mining of the chemical space. Some of the statistical tools used include the R Project for Statistical Computing [246], Bioclipse59 [247] and JMP statistical discovery software [248]. There are linear and generalized linear models, nonlinear regression models, time series analysis, classical parametric and nonparametric tests, clustering and smoothing. Pipelining tools include Pipeline Pilot [249] and Knime104 [250]. They help automate the process of accessing, analyzing and reporting a variety of scientific data [1].

The US FDA and EPA also have freeware that can be downloaded. The FDA's National Center for Toxicological Research (NCTR) has an endocrine disruptor knowledge base (EDKB), which contains QSAR models for predicting the binding affinity of test compounds to estrogen and androgen nuclear receptors. The FDA also applied a computer automated structure evaluation program to their databases to develop a battery of QSARs for predicting carcinogenicity and genotoxicity, along with reproductive and developmental toxicities. It was called MC4PC (http://multicase.com/) [251].

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3.1.5 Drug delivery

After the best compound has been chosen, it must be formulated in a suitable delivery system [1]. Systems thinking is essential. That is, the initial research that shows that a drug can bind to an isolated therapeutic target *in vitro* using reductionist thinking is of little use when considering the complications that exist in the entire system. That is, the drug must be stable in the bloodstream if given intravenously. It should also reach just the target organ or tumor and not other organs where it can exert adverse side effects. Finally, it must be able to enter the target organ, tumor and cancer cells. So, drugs must be formulated in a way that makes them stable and ensures that the drug (or prodrug) reaches the target organ, pathogenic virus or microorganism. When the drug enters unintended parts of the body, it can cause adverse side effects. For example, many chemotherapeutic anticancer drugs that have been used for decades also kill all the fastest growing cells in the body. This includes not just cancer cells, but hair and other cells. There can be other side effects that are severe. One often hopes that the drug kills the cancer before it kills the patient [1].

Even if a drug or vaccine reaches its molecular target, sometimes substances are added to the dose vehicle so the drug can have its maximum effect [1]. One example is preparing better adjuvants for immunizations. Adjuvants are substances that are added to vaccines to boost the specific immune response to the antigens in them, but cause minimal toxicity and no long-lasting side effects on their own. That is, the 'drug' in a vaccine is an antigen (or combination of antigens), often made from pieces of viruses or Bacteria. The antigens must reach the target cells in the immune system, but that is not enough. The immune cells must be activated, and this requires adding adjuvants, such as aluminum compounds, paraffin oil and bacterial products to the delivery systems, or dose vehicles. So, researchers are trying to find ways to get drugs to the target cell and organ, and not to the rest of the body. They want to make the drugs or vaccines more effective and activate them only at the target organ, to minimize or eliminate adverse side effects [1].

One way to do this is to enclose a drug in a liposome (a small sphere of lipids that enclosed a smaller aqueous solution) and put something on its surface so it will bind to the target cell [1]. For example, the drug Doxil® is used in anticancer therapy [1, 252]. It contains adriamycin (also known as doxorubicin), which for decades has the first line (preferred) therapy for more cancers (leukemias and lymphomas, as well as breast, uterine, ovarian and lung cancers) than any other chemotherapeutic and has saved millions of lives [252]. Unfortunately, its side effects include cumulative dose-dependent cardiotoxicity, or heart attacks, especially in children.

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Severe myelosuppression, nausea and vomiting also occur frequently. These adverse side effects happened more often when the naked drug was given. However, when enclosed inside of liposomes the size of nanometers (nm), doxorubicin can be passively targeted to cancer cells. So, Doxil® was developed. Doxorubicin is encapsulated in a liposome that contains an ordered lipid bilayer made up of relatively high melting point (53°C) phosphatidylcholine plus cholesterol to which poly(ethylene glycol), or PEG is attached. This stabilizes the liposome, helping the drug to stay in the bloodstream much longer. These are sometimes called stealth liposomes. The PEG helps the liposomes evade macrophages, which usually attack any foreign object in the blood, including old-fashioned liposomes that don't have PEG. Doxil® has enhanced permeability and retention, in what has been called the EPR effect. This means the drug is accumulated selectively in tumors that have many porous capillaries that are permeable to particles (like liposomes) that have a diameter <100 nm. In addition, tumors have poor lymphatic drainage, so nm-sized liposomes are retained longer. At the same time, the liposomes don't accumulate in healthy tissues, so there is much less cardiotoxicity. The nano sterically stabilized liposomes (nSSLs) help the drug reach the target cells and organs, and makes it more effective and less toxic. To help the drug get into cancer cells, ammonium sulfate, or $(NH_4)_2SO_4$, is dissolved in the water that is inside the liposomes. So, there is much more NH_4^+ inside the liposome than inside the cancer cells. This NH^{4+} gradient exerts a force that drives the doxorubicin into the cancer cells, increasing its effectiveness [252].

Note that even though PEG is made up mostly of ether linkages that are usually thought to be hydrophobic, it is quite soluble in both water and in hydrophobic solvents like chloroform. So, PEGylated liposomes and micelles will be soluble both in the aqueous bloodstream, and in hydrophobic cell membranes. The structure of PEG is shown in Figure 21. Note that molecular weight of the PEG depends on how many repeat units, $-CH_2-CH_2-O-$, are present. So, PEG100 (with a molecular weight of about 100) has two repeat units, while PEG1000 has an average of 30 repeat units. That is, 1000 is the average molecular weight, so PEG1000 has some PEGs with 29 repeat units, too.

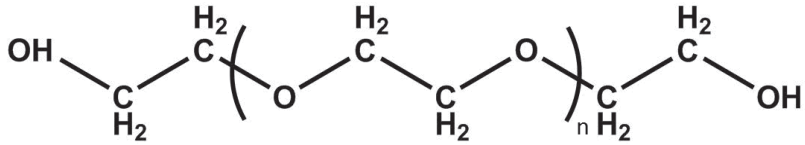

Figure 21. 2D structure of polyethylene glycol ($C_{2n}H_{4n+2}O_{n+1}$), where the number of repeat units, n, varies with the molecular weight of the PEG.

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Another version of liposomal doxorubicin, called Myocet® is used together with cyclophosphamide to treat metastatic breast cancer [1]. Other drugs have been encapsulated in liposomes, including AmBiosome®, which contains an antibiotic (amphotericin B). It is used to cure fungal infections in immunocompromised patients; Depocyt®, which contains the anticancer drug, cytarabine; DaunoXome®, which contains the anticancer drug, daunorubicin; Epaxal® (a hepatitis A vaccine); Inflexal® (an influenza vaccine); Depodur®, which contains morphine; and Visudyne®, which contains verteporfin (for macular degeneration, pathologic myopia, and ocular histoplasmosis). There is also Estrasorb®, which is a micellar form of the estradiol. It is a topical emulsion for treating vasomotor symptoms associated with menopause [1].

Nanoparticles that are 1-100 nm in size are quite consistent with systems thinking, since they have inherently nonlinear effects in complex adaptive systems [253]. Some of them have important diagnostic or therapeutic uses. Due to their small size and large ratio of surface area to volume, they exhibit nonlinear biological, chemical, thermal, optical and electromagnetic properties. Their large surface area gives them a high capacity to adsorb other substances and have better bioavailability than larger particles. Moreover, they can cross cell membranes that are impermeable to larger dosage forms. This includes the blood-brain barrier. They can also exert their therapeutic effects at lower doses and for longer periods of time than larger dosage forms. One important new area in which nanoparticles are proving useful is the combination of therapy and diagnostics, or theranostics. There are magnetic nanoparticles that can release their active agent only after they enter their intended specific cancer cell target and are activated. They are used for imaging and/or treatment. This is possible because of their nonlinear magnetic properties. In addition, nanoparticles can enter cancer cells rather easily because of the increased vascular leakiness of tumors resulting in passive and/or active uptake processes from ligands on the surfaces of the nanoparticles. When nanoparticles are used for targeted drug delivery, there is a large reduction in toxicity, due mostly to the much lower doses of drugs that are required to achieve the desired therapeutic and/or diagnostic effects. That is, some nanoparticles can initiate hormesis at doses that are far below that of the no-observed-adverse-effect level (NOAEL) of the bulk drug. So, hormesis is often thought of as “a dynamic adaptive response or biological plasticity of a complex living system at the level of the whole organism to intermittent mild stressors of various categories” [253].

Some researchers are using systems thinking as they borrow from nature to make nanoparticles that can persist in the bloodstream much

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longer than stealth liposomes based on PEG [254]. That is, the protein CD47 that is on the cell membranes of red blood cells helps them circulate in the body for 100-120 days. They signal their cognate receptor on phagocytes (white blood cells that ingest, or phagocytose foreign particles) to keep them from destroying the red blood cells. So, nanoparticles were created using a synthetic peptide that mimicked CD47. They increased the circulation of these nanoparticles in the blood [254]. This concept was then used to make modified lentiviruses (120 nm in size) that overexpress the CD47 protein [255]. They may prove useful in anticancer therapy, including treating deadly glioblastomas [255].

Another anticancer agent, paclitaxel, can be prepared in nanoparticles made from the common protein, albumin [1, 256]. It is called Abraxane®. It takes advantage of albumin's ability to enter tissues and target cancer cells with a higher effective dose of paclitazel. Nanoparticles made from liposomes and many other materials can deliver therapeutic or diagnostic agents by either encapsulating or physically attaching them directly to the nanoparticles. Liposomes made from PEG are called stealth liposomes, because the PEG keeps the encapsulated drug from being detected by the mononuclear phagocyte system (MPS), formerly known as the reticuloendothelial system (RES). The MPS contains phagocytes, which are specialized cells that line the liver, spleen, bone marrow, and lymphatic tissue. The MPS recognizes xenobiotics and foreign molecules and removes them from the circulation. There is also a second generation of nanovectors. They have a bio-recognition molecule attached to their surface. This enables them to target specific markers that are over-expressed on cancer cells. A third generation is being developed that can not only avoid the RES, but also target membrane receptors and deliver the therapeutic agent only to those cells that have the desired therapeutic target. They are designed to perform automatically in a pre-programmable sequential manner. These nanoparticles head towards the therapeutic target, pass through one or more biological barriers and finally add a degree of selective binding to the target [1, 256].

There are also drugs and siRNAs that are inserted into polymeric micelles [1, 257-262]. These include NK911® and NK105®, which contain doxorubicin and paclitaxel, respectively, in micelles made of poly(ethyleneglycol)-copoly(L-aspartic acid) and Genexol® PM, which has been approved for treating breast cancer [1, 257, 258]. It contains paclitaxel in micelles made of poly(ethyleneglycol)-copoly(D,L-lactic acid) [1, 257]. Micelles are colloidal particles with diameters of 5-100 nm [258]. They are made from amphiphilic molecules that have hydrophilic portions on the outside (exposed to the aqueous phase) and hydrophobic

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parts on the inside [263]. That is, amphiphilic molecules have both hydrophilic and hydrophobic parts [263]. Polymeric, amphiphilic micelles have been made for targeted chemotherapy using platinum-based anticancer drugs [259]. The plasma membranes on the surface of cancer cells often have a unique pattern of glycosylation. Many of them overexpress epitopes that contain covalently attached sialic acid, mostly as *N*-acetylneuraminic acid. The prognosis of many types of solid tumors has been correlated with higher levels of sialic acid. So, researchers have found that phenylboronic acid (PBA) has a specific affinity to sialic acids. PBA is relatively nontoxic, nonimmunogenic and inexpensive. So, it can be covalently attached to the PEG terminal region of block copolymers made with poly(L-glutamic acid) and used to make micelles that contained the anticancer drug (*trans-l*-1,2-diaminocyclohexane)platinum(II), also known as DACHPt. This micelle-encapsulated drug is called NC-4016. It is undergoing clinical trials [259].

However, liposomes have some disadvantages, such as their high cost and the fact that not all the liposomes get to the target cancer cells, but many go to the liver and spleen [1]. Instead, polymers are being developed that can target cancer cells more selectively. For example, the polymer made from *N*-(2-hydroxypropyl)methacrylamide targets the deadliest cancer cells of all, the metastatic cells. Cancer usually becomes deadly after some of the original cancer cells break off, enter the bloodstream and move to a different organ (like the brain) in the process called metastasis. New blood vessels must be produced for metastatic cancer cells, so nutrients can be delivered to them. So, polymers are being developed in which a therapeutic drug can be attached to them by a covalent bond that can only be broken when the polymer-drug system enters a cancer cell [1].

An especially useful type of polymer that offers much promise is called a dendrimer [1]. Dendrimers have many side groups that look like the thin appendages or arms. They enable scientists to attach many types of drugs. Although the dendrimers are much larger than monomers, they are still small compared to cells. They can be made as small as 2 to 10 nm. They are being evaluated as carriers of doxorubicin and other chemotherapeutic drugs. They are also being tested as carriers of DNA for use in gene therapy. This is important because other dosage forms don't deliver genes as specifically as they should, causing toxicity. It is anticipated that dendrimers, or other techniques based on nanometer-sized therapy will be less toxic [1].

Molecular modeling can be used to design efficient dendritic nano-carriers for DNA and siRNA [1, 264]. The multivalent character of dendrites and dendrons enables many binding sites to be created between

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the positive charges on dendrons and negatively charged DNA or siRNA. Poly(amidoamine) (PAMAM) dendrimers are the best known dendritic molecules. Five critical design parameters have been identified. They are size, shape, surface chemistry, flexibility (or rigidity) and architecture. Dendron and dendrite structures have been modeled by all-atom force fields as well as semi-empirical and *ab initio* methods [1, 264].

In addition to dendrimers, nanodots and nanobots (very small robots) are being developed that are as small as 1 – 100 nm [1]. They are part of the next generation of drug delivery systems, drugs, regenerative therapeutics and imaging systems that are stimulating a medical revolution that is environmentally friendly [1, 265-268]. That is, starting materials that are the size of atoms can be made that automatically self-assemble and form structures with amazing properties [1]. These can also be used as programmable self-replicating manufacturing systems that could start a new Industrial Revolution [1]. They have two central elements: a universal computer and a universal constructor [1, 265]. The universal computer contains a program that controls the universal constructor [1, 265]. The computer is not an electronic device, but a molecular sensor or probe that can determine if and when the constructor should be activated. The universal constructor, in turn, manufactures both another universal computer and another universal constructor. Once construction is finished, the program contained in the original universal computer is copied to the new universal computer. At the same time, nano-structures are being developed that can identify a cancer cell and kill it. The goal is to create nanostructures that act like small molecular computers, and have several molecular building blocks and a supply of a therapeutic drug (like adriamycin). They are being designed to circulate freely throughout the body and periodically determine whether the binding sites on cancer cells are or are not occupied. The extent to which the binding sites are occupied will determine how much of the drug should be delivered. The nano-device could include many binding sites and would monitor the levels of many appropriate proteins on the surface of cancer cells. The computer will determine if the profile of these proteins (mostly receptors) fits a pre-programmed cancerous profile and if so, would release the drugs that kill the cancer cells [1, 265].

Nanotechnology can also make materials for regenerative medicine [1, 265, 267]. Research is being done to assist in head and neck surgery, make artificial limbs and organs, regenerate nerves, heal burns and wounds, and make artificial skin with nanoelectronic sensors. Diagnostic tools are also being developed. Nanodots have been made that have a small piece of a protein (a peptide) attached. The peptide recognizes glycoproteins on the

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surfaces of cancer cells and produces colored or fluorescent signals. There are also microelectronic mechanical systems (MEMS). They are laboratories on a chip that can analyze samples from a patient. They can analyze the concentrations of nutrients, drugs and toxins. Some MEMS are being developed to analyze a person's genome so that drugs can be tailor-made for each individual. For example, there are cytochrome P450 (CYP450) enzymes in our liver, GIT and blood-brain barrier. They metabolize drugs and toxins and play important roles in the breakdown and synthesis of some hormones. However, there are many different forms of the genes that code for the various isozymes (types) of CYP450. The goal is for physicians to be able to analyze their patients' genomes so they can determine how fast each patient will metabolize each different drug. This way, the optimal dose and delivery schedule can be established. One important application is to reduce or eliminate infant deaths due to taking infant cold medicine. For example, some infants are unable to metabolize the active ingredient, dextromethorphan, and have died from an overdose, while millions of other infants can take the medicine without any danger and are relieved of the cold symptoms [1, 265, 267].

One of the most exciting applications of very small particles is the use of natural single-domain nanobodies [269-276]. They are obtained from the blood sera of one-humped camels (*Camelus dromedarius*) and two-humped camels (*Camelus bactrianus*), as well as llamas (*Lama glama* and *Lama guanicoe*) and vicugnas (*Vicugna vicugna* and *Vicugna pacos*) [269]. These animals are in the Camelidae family. Unlike humans and other mammals, these species of Camelidae produce not just conventional heterotetrameric antibodies, but also uniquely functional heavy (H)-chain antibodies (HCAbs), also called nanobodies (Nbs). The H chain of these homodimeric antibodies consists of one antigen-binding domain, the VHH, and two constant domains. HCAbs don't have light (L) chains that are in heterotetrameric antibodies. These smaller HCAbs are relatively easy to prepare and (unlike heterotetrameric antibodies and enzymes) are quite stable. So, they don't have to be stored refrigerated. They can be stored for months at 4^{o}C and even longer at -20 ^{o}C. In addition, the crystal structures of several HCAbs (Nbs) have been determined by X-ray crystallography. The knowledge obtained has encouraged their engineering and design for use as research tools in biotechnology and medicine. The NHH domain has a prolate structure, similar to that of a rugby ball. It forms a convex paratope surface. This makes it ideal for inserting into cavities on the surface of an antigen. This type of interaction increases the total interaction surface of the paratope. However, some therapeutic targets can switch their conformations rapidly. So, Nbs can

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trap and stabilize the desired conformation of the target, to produce an optimal effect. This type of interaction increases the actual interaction surface of the paratope. In addition, Nbs can be combined with larger proteins in which the Nb provides the specificity and targets the antigen, while the other protein exerts a therapeutic effect. Also, Nbs can be chaperones that stabilize membrane-bound proteins that are highly susceptible to degradation, unfolding, and/or aggregation. Nbs are also ideal for radiolabeling and imaging, because they are so small that they are well below the renal cutoff and are not removed by the kidneys [269].

So, Nbs may be useful in studying, diagnosing, visualizing and/or inhibiting cancer-specific proteins and processes [270, 274]. They also have much therapeutic potential as protein receptor antagonists and for delivering drugs or drug-loaded nanoparticles to tumors [270, 274]. Nbs are also being developed for inhaled therapies for treating lung diseases [270]. They are being used to make modular drugs with superior pharmacological profiles. That is, monomeric single Nb modules are being used to make multivalent and/or multi-specific constructs. A combination of Nbs may bind to the same target or different targets when they are prepared by genetic fusion of the monomers through short peptide linkers. Bi- or multivalent constructs have two or more Nbs with the same specificity. They are linked together with a flexible linker sequence that results in Nbs that bind much tighter to the therapeutic target, thus increasing the potency while minimizing adverse side effects. Examples include a bivalent anti-TNFα Nb which is 500 times more potent than the individual monovalent molecules. There is also an Nb (ALX-0171) that was made into a trimer that can bind to the human Human respiratory syncytial virus (RSV) fusion (F) protein, which naturally exists as a multimer. This increased its potency by more than three orders of magnitude (1000-fold). So, it inhibits an early step in the replication cycle of RSV. Two Nbs that bind different and non-overlapping epitopes can be combined into a biparatopic format. They can be used to make drug candidates with superior pharmacological properties. One example is a biparatropic Nb that targets the chemokine receptor CXCR2 [272].

Throughout the process of developing better drug delivery systems, pharmaceutical companies and others are using systems thinking to listen to the voice of the patient [277]. That is, drugs must be designed so that patients will want to take them. “The best way to do all this is to have the patient’s voice incorporated into the drug development process as early as possible” [277]. This is supported by the FDA, which issued a guidance for industry on the Size, Shape, and Other Physical Attributes of Generic Tablets and Capsules [278]. It provided data and insight on the correlation

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between the difficulty in swallowing certain dosage forms and patients' willingness to take them [278]. Usually, the best option is to make orally disintegrating tablets (ODTs) [247]. They are effective in many therapies and are essential for patients who have trouble swallowing. Extended-release formulations are also quite useful. They deliver a steady dose that produces a nearly constant concentration of the drug in the blood serum. This prevents spikes in concentration. Advanced techniques like OptiShell^{TM} technology enable the delivery of controlled release matrix formulations in a soft capsule. Extended-release formulations enable less frequent dosing. This leads to better patient adherence and is especially convenient for drugs that have low bioavailabilities when given in other dosage forms [247].

3.1.6 Manufacturing and cGMP

3.1.6.1 cGMP for prescription drugs and medical devices

After the new drug has been optimized and the best dosage form determined, the next step is to file an Investigational New Drug (IND) application with the FDA or another country's regulatory agency. It must be manufactured in compliance with the FDA's cGMP (current Good Manufacturing Practice) or other countries' regulatory policies [1]. That is, the World Health Organization (WHO), China, Singapore and the European Union have their own versions of cGMP (WHO-GMP, GMP in China, Pharmaceutical Inspection Co-operation Scheme (PIC/S) in Singapore and EU-GMP) [279]. However, these regulations do have their differences – especially when dealing with herbal remedies [279].

The rules and regulations for the version of cGMP that is enforced by the FDA are in title 21 of the Code of the Federal Register (21 CFR 210 and CFR 211) [280]. It specifies that the building used for manufacturing the drug or NME and all equipment inside must be clean and hygienic. It also specifies that all employees must be properly trained and that the training be documented. Moreover, all steps in the manufacturing process and the analysis of the drug or IND must be documented in Standard Operating Procedures, or SOPs. Quality must be built into the drugs and medical devices being manufactured. Each step of the manufacturing process must have good quality control. The possibility of contamination must be minimized. This is done by using high quality raw materials that must be analyzed and by ensuring that the employees follow good, safe and hygienic practices. Also, there must be a quality control (QC) organization and quality management system (QMS) that has a set of

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policies, methods, documentation and support from all levels of management. There must be detailed documentation of every lot and batch of drug that is produced. This includes master manufacturing and batch records, in which it is certified that each step in the manufacturing process was done in accordance with the SOPs [280].

Batch records must show which equipment was used, along with the details of the reaction conditions used to make the final drug product, including all reactants, catalysts and solvents, along with the reaction times and temperatures [1, 280]. Often, drugs must be purified by removing unreacted reagents and unwanted byproducts of reactions sued to synthesize them. All of this must be part of the batch record, too. Drugs are almost always synthesized and purified in the presence of organic or aqueous solvents. Batch records must also show how the solvents were removed – usually in a clean vacuum drying oven. The batch records also need to show that the dried drug was analyzed and found to contain little or no residual water. Logs are kept of who used each piece of equipment in the manufacture and analysis and when they were used. Batch records must be kept for at least one year after the expiration date of the batch. Also, master production and control records must be kept for each drug product. They must indicate the size of each batch that was made, and include the name, strength of the drug product, list and weights of all ingredients, and the dosage form. There must be statements of the theoretical weight or measure at appropriate phases of processing and the theoretical yield, including the maximum and minimum percentages of theoretical yield. That is, basic principles of chemistry, called stoichiometry, can be used to predict how much drug should be produced if all the reactants are converted to products. This is called the theoretical yield, or the amount obtained if the yield of product were 100%. Very little, if anything, that is done by humans is 100% perfect. So, when developing the method for synthesizing the active pharmaceutical ingredient (API), manufacturers will weigh the reactants and the API and use the data to calculate the actual yield. After repeating the synthesis a few times, they can establish the maximum and minimum percentages of theoretical yield. So, all drugs that are manufactured for pre-clinical and clinical studies, as well as for sales to the public (after they are approved) must be analyzed to ensure their identity and purity. The Appendix describes some of the different analytical methods that are used. Also, the stability (or shelf life) of a drug must be determined. It is important to measure the stability for at least six months. This requires analytical methods for measuring the concentration of the drug and the possible

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appearance of breakdown products. There are many more details and nuances of cGMP that have been described in detail [1, 280].

The QC organization reviews all data and performs internal quality audits [280]. They maintain extensive files of data on the production and analysis of all lots and batches of drugs that are made. They ensure that all the equipment (flasks, ovens, etc) used to manufacture a new drug have been thoroughly cleaned to remove the previous drug that was made in that equipment. Logs are kept of who used each piece of equipment in the manufacture and analysis of the drug and when it was used. Also, appropriately labeled reserve samples must be retained. Enough API shall be retained so that there is "at least twice the quantity necessary for all tests required to determine whether the active ingredient meets its established specifications, except for sterility and pyrogen testing" [280].

In addition, almost all drug manufacturing organizations emphasize continuous improvement, even though this is not specifically required in cGMP regulations [281]. This requires systems thinking and its core values of teamwork, communication and the involvement of all employees. By using statistical process control methods, the amount of variation in the production process can be decreased. In addition, many companies write their own quality manual that summarizes their QMS in a process that was borrowed from the International Standards Organization-900 (ISO-9000). It contains a written acknowledgement of the company's quality assurance policies and its commitment to following cGMP regulations. There is also a revision history and a page that contains approval signatures by management [281].

There is a quality assurance tool that is important in ensuring that contamination is prevented [281]. It is called Hazard Analysis and Critical Control Points (HACCP, pronounced "hassip"). It is used not just by the FDA, but also by the U.S. Department of Agriculture to help regulate food production and other governments' regulatory agencies. The seven principles of HACCP are: analyze hazards, identify critical control points (CCPs), establish procedures to monitor CCPs, provide for corrective actions that should be taken when a critical limit is not met and establish ways to verify that the manufacturing system is functioning as intended and effective record keeping. Even though HACCP is not required by cGMP, many manufacturers of prescription drugs, dietary supplements and medical devices incorporate it into their QMS. Moreover, the WHO has provided guidance on the use of HACCP in the manufacture of pharmaceuticals [281].

The FDA's cGMP became part of the law of the land when the Food, Drug and Cosmetic Act (FD&C Act) was passed in 1938 [1, 281, 282],

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after many people had died from Elixir Sulfanilamide, which was used at the time to treat *Streptococcal* infections. Sulfanilamide had been effective in a tablet or powder form, but the elixir was a liquid. The sulfonamide was dissolved in diethylene glycol, which we now know is toxic. It is now used as antifreeze. The company (S.E. Massengill) that made Elixir Sulfanamide did not test the toxicity of its Elixir Sulfanilamide. At the time (1937), the food and drugs law did not require that safety studies be done on new drug formulations. What S.E. Massengill did was wrong, but it was legal. On October 11, 1937, the American Medical Association (AMA) received reports from physicians in Tulsa, Oklahoma that an unfamiliar sulfanilamide compound was responsible for many deaths. S.E. Massengill already knew that the the liquid dosage form of sulfanilamide was poisonous. They sent telegrams to more than 1000 salesmen, druggists, and doctors. However, the telegrams merely requested the return of the product and failed to indicate the urgency of the situation or say that this formulation of the drug was lethal. The FDA Commissioner, Walter Campbell, had been pushing for better federal regulation of drugs. He pointed out how the inadequacy of the law had contributed to the disaster. This led to Congress passing the 1938 Food, Drug and Cosmetic Act. The phrase "current good manufacturing practice" appeared in section 501(B). This phrase, and its abbreviation, cGMP, has been used ever since. It applies to manufacturing foods, pharmaceutical products and medical devices. A slightly modified version is required when manufacturing dietary supplements. So, the goal of cGMP is to establish the "basic standards of operation at the manufacturing level, to ensure that finished products meet established specifications for identity, purity, strength, composition and limits on contaminants" [1, 280, 281].

The FDA and other governments' regulatory agencies do regular inspections and issue untitled letters and warning letters when they find violations in the manufacture of medical devices and drugs [283]. Untitled letters simply request a written response from the manufacturing organization within a reasonable time. Warning letters, though, require a written response. They are issued when the FDA finds violations that have regulatory significance. Warning letters indicate the violative activities and require a written response within 15 days. These letters also tell the violative organization that if they fail to take prompt, corrective actions, serious consequences might follow. This can include seizing the violative products, issuing an injunction or even prosecution in court. Warning letters can be especially problematic for foreign manufacturers, since the FDA can withhold approval of requests for export certificates, or approval of pending new drug applications made by the manufacturer or

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pharmaceutical company. From January 1, 2007, to December 31, 2014, a total of 997 warning letters were issued by the FDA regarding violations in cGMP. Of these, 685 were issued related to medical devices, 223 were about finished pharmaceuticals, 18 were about biological products and 14 dealt with the processing of pharmaceuticals. To avoid receiving such letters, manufacturers should follow the principals of TQM. They should communicate regularly and at all stages of drug development with the FDA or other governments' regulatory agencies. It is especially important to pay attention to changes in the FDA's policies and regulations. For example, the FDA enacted a new enforcement strategy in 2009 that manufacturers should provide a "clear, complete and adequate response" to the FDA Form 483 [283].

The FDA Form 483 is issued to the management of a manufacturing firm when an inspection has shown that the firm may have violated the FD&C Act and related acts [284]. "The FDA Form 483 does not constitute a final Agency determination of whether any condition is in violation of the FD&C Act or any of its relevant regulations. The FDA Form 483 is considered, along with a written report called an Establishment Inspection Report, all evidence or documentation collected on-site, and any responses made by the company. The Agency considers all of this information and then determines what further action, if any, is appropriate to protect public health" [284].

Even though all these regulations may seem burdensome to some people, they are much better than the unregulated system that existed before the FDA was created. Still, there is frequently a shortage of badly-needed pharmaceuticals, including anticancer medicines [285]. This is due, in part, to a lack of flexibility and robustness in many parts of the pharmaceutical sector. To address this shortage, the vision for the FDA's Pharmaceutical Quality for the 21st Century is to "promote a maximally efficient, agile, flexible pharmaceutical sector that reliably produces high-quality drugs without extensive regulatory oversight" [285, 286]. This has led to the implementation of quality-by-design (QbD) and increased use of process analytical technology (PAT) to design, analyze and control drug manufacturing [285]. This is in contrast to batch processes, in which raw materials are put into the manufacturing system at the beginning and final product is removed at the end. Instead, raw materials and final products can be added to the system or removed from it at any time during a continuous process. Still, continuous manufacturing can make batches – even though a batch is defined as "a specific quantity of drug or other material that is intended to have uniform character and quality within specified limits and is produced according to a single manufacturing order

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during the same cycle of manufacture" [285, 286]. So, in continuous manufacturing or continuous processing, a "lot" is synonymous to a "batch". That is, the regulatory definition of "batch" is based on the amount of material and not the way it is manufactured. Moreover, continuous processes can rapidly increase the amount of drugs that are produced when a shortage seems imminent. At the same time, continuous manufacturing is consistent with the FDA's QbD paradigm. Robust, continuous manufacturing uses a good understanding of the product and manufacturing processes. This enables manufacturers to "adopt advanced manufacturing controls to produce uniformly high-quality products with reduced waste resulting from the generation of out-of-specification material" [285].

Certainly, reducing waste is good for the environment. So, QbD can be thought of as a green technology and an essential part of TQM. Using systems thinking, pharmaceutical companies realize that their prescription drugs and medical devices won't work if the societies in which they are introduced are destroyed due to Global Climate Change. So, it is important to note that scientists from Boehringer Ingelheim, Pfizer, Novartis, Genentech, Eli Lilly, Bristol-Myers Squibb, Merck, Amgen and two universities collaborated in writing an article that describes green and sustainable drug manufacturing [287]. They analyzed 46 drug manufacturing processes from nine major pharmaceutical companies. From this analysis, they developed a new metric called the Green Aspiration Level (GAL) to help establish badly needed consistency in establishing smart, green goals for manufacturing. It assesses the amount of waste produced. This complements Life Cycle Analysis (LCA), which looks at the safety of a process, as well as energy used and the environmental impact. GAL is used to calculate the Relative Process Greenness (RPG) and to generate a Green Scorecard. Finally, systems thinking was used to work with the International Consortium for Innovation & Quality in Pharmaceutical Development and the American Chemical Society's (ACS) Green Chemistry Institute Pharmaceutical Roundtable (ACS GCI PR) to communicate their results and the GAL methodology. That is, "History teaches us that most breakthrough innovations are … [integrated] … through networks" [287, 288].

GMPs are also being used to control the quality of radiophamaceuticals [243] and biologics [290, 291]. Additional regulations exist to ensure the safety of workers who produce and use radioactive pharmaceuticals [289]. This includes optimizing the air pressure in clean rooms used to make them and wearing personalized dosimeters. So, the European Association

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has published "Guidelines on Current Good Radiopharmacy Practices (cGRPP) in the preparation of radiopharmaceuticals" [289, 290].

Some cGMP guidelines are also being developed for large-scale manufacturing of [290]. Glybera was the first drug based on recombinant adeno-associated viruses (rAAVs) that was approved in Europe for treating a deficiency of lipoprotein lipase by gene therapy. However, its manufacturer (UniQure) is not going to ask European authorities to renew its marketing authorization, due to a lack of demand. The safety and efficacy of rAAV vectors has been shown in over 70 clinical trials. However, many of the methods used to make the rAAVs are not amenable to being scaled up. To produce enough rAAVs for use in larger clinical trials and eventual sales, cell suspension is preferred. The safety of the rAAVs is assessed by analyzing them for impurities and contamination. The final rAAV products are characterized by determining the viral genome titer, infectious titer (potency), the identity of the viral capsid and genome, as well as the therapeutic gene identity. The stability of the rAAV is also measured at predetermined times and at the end of a clinical trial. Still, the QC methods need to be standardized to enable an unbiased comparison of investigational new drugs (INDs) for treating a variety of diseases [290].

Cell-based medicines are also being developed, along with appropriate cGMP guidelines [291]. Computerized monitoring systems are being used to control the temperature, relative humidity and CO_2 concentration when making cells for regenerative medicine. This helps to reduce costs, improve productivity and ensure the conformity of the specifications for making these cells. These computerized systems are used to train workers, analyze risks, establish security, store data, apply electronic signatures, provide an audit trail, ensure the accuracy of data processing, document and audit suppliers, as well as manage incidents and control changes that are made. Since there is always a potential for risks, the computer monitoring system also performs Quality Risk Management (QRM) and provides a system for Corrective Actions and Preventive Actions (CAPA) [291].

Quality must also be assured in regenerative medicine [292]. For example, a new type of therapy uses regenerated cells in disease microenvironments. Regenerative medicine is especially important useful in patients who have degenerative and chronic diseases that are difficult to cure completely. Patients who have a myocardial infarction (an acute occlusion of the coronary circulation that causes widespread cell death in the heart) usually recover from the initial treatment. That is, mortality has dropped to about 5% in such patients after being treated in a hospital.

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However, many of them suffer from post-ischemic remodeling that can degrade into heart failure. So, average survival after five years is only about 30%. So, it is hoped that regenerative medicine can restore the health of such patients by stimulating and reactivating repair and regeneration mechanisms that are specific to the target organ (such as the heart). Stem cells that can become heart cells or promote endogenous healing mechanisms are being developed to protect, repair and regenerate damaged hearts. There are also cell-based regenerative products for treating cartilage defects and corneal damage, as well as for immunotherapy. As scientists, physicians and engineers start designing and implementing first-in-human and larger clinical studies, they must consider the problems inherent in manufacturing. This includes scaling up the production of cells, as well as documenting the supply chain and installing QC systems that are cGMP-compliant. Even though engineers can make a product right, it is also important that the right product is being built [292].

Regenerative medicine presents some important challenges to making a process cGMP-compliant that don't exist when making prescription drugs [292]. That is, if a manufacturer starts with the same qualified lots of raw materials and use validated SOPs, he or she can produce a drug whose properties are acceptable and predictable. As a result, the quality of the final product depends on the quality of the starting materials. This depends on a "mathematically formalized, measurable understanding of the system" [292]. However, when cells are being made for regenerative medicine, the same cells are the raw material and the final product. Regenerative cells are chosen for use based on the expression of certain protein markers on the surface of their cell membranes. They are characterized by these specific antigens. This has been the reference point for QC, but it is of limited value. Even if cells have the appropriate antigenic proteins on their surface, they can have very different regenerative potential. If patient-specific heterogeneity is not considered, the starting materials (cells) may be poorly qualified. On top of that, there is no mathematical framework at the present time that can relate measured parameters of cells with their regenerative performance. There is also uncertainty due to variables such as cell culture conditions and the often unpredictable interaction with the patient's microenvironment. So, organ chips are now being developed for improved QC and cGMP compliance [292].

Organ chips are engineered to mimic and recapitulate important aspects of physiology [292]. They can be used to assess the phenotype of the cells that are manufactured by measuring the expression of genes, as well as the structure, metabolism and function of the cells that are seeded

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into microenvironments that recapitulate healthy or diseased conditions. When cells are taken from the patient, they can be used to specifically model inherited pathologies cost effectively and on personalized genetic backgrounds. Moreover, organ chips are easier to describe mathematically and provide a unique platform for computational modeling. That is, a computational model of muscle mechanics and mechanotransduction was used to design a minimal *in vitro* model of the infarct border zone. Next, microenvironments were made that mimicked the elasticity and composition of the extracellular matrix of healthy and fibrotic myocardium. Finally, cell pairs composed of primary and stem cell-derived cardiomyocytes were engineered on these substrates. They were used to recapitulate important *in vitro* observations made in preclinical and clinical studies. As a result, organ chips were made that regenerated cell products with the desired phenotypes in complex microenvironments. The result was cardiac cell therapy on a chip platform [292].

Since stem cells are being developed for many applications, a version of cGMP called Good Cell Culture Practice (GCCP) has been described by an international collaboration of scientists [293]. It is a proposed modification of the original GCCP. It was needed because increasingly diverse cell types and culture formats are being used in research, product development, testing and the manufacture of biotechnology products and cell-based medicines. It described the basic principles of best practice to enable data to be compared between different laboratories and experiments done at different times. This included recommendations for how to document and report cell culture conditions. It was intended as guidance to facilitate the production of reliable data from cell culture systems. It did not conflict with pre-existing regulatory requirements. It expanded on the main principles of the original GCCP: authenticate cell lines and establish appropriate documentation and reporting practices. However, the original GCCP began before the advent of human pluripotent stem cells (hPSCs) and organs on a chip. So, the newly proposed version, GCCP 2.0, provides guidance on using hPSCs and organs on a chip. This includes establishing and maintaining a sufficient understanding of these systems and relevant factors that can affect them. This is especially important for the dynamic hPSCs that can change their phenotypes by differentiating into different types of cells. It is important to maintain these cells in their undifferentiated state if they are to be used properly. So, the conditions of the cell culture must be properly controlled and documented. They must also be handled, maintained and preserved properly. Microbial, viral and cellular cross-contamination must be avoided. The methods used to reprogram and differentiate the hPSCs must be controlled, too. As in

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cGMP, GCCP 2.0 specifies that the quality of all the materials and methods must be assured. The identity, karyotype and type of HLAs (human leukocyte antigens) should be verified. There are many other details of GCCP 2.0 that have been described [293].

At the same time, societies around world are concerned about the environment and want sustainable drug manufacturing [287].

3.1.6.2 cGMP for dietary supplements

The cGMP regulations for dietary supplements are almost the same as those for prescription drugs [294, 295]. However, they do not require manufacturers to analyze all the raw materials used in their production. Even though many American manufacturers do analyze all the raw materials that they obtain in the USA, it can be very difficult to analyze or even completely control raw materials that come from other countries. Moreover, the final specifications for the products are made by the manufacturer, since there are no widely-available compendia or standard specifications for dietary supplements. This is in contrast to prescription drugs, for which the US Pharmacopeia (USP) has many standard methods. At the same time, manufacturers of dietary supplements can choose any testing method they want to characterize its raw materials or products. Very few manufacturers of dietary supplements have the more expensive, but very useful analytical instruments (like NMR and LC-MS) that pharmaceutical companies, universities and research institutes have. Also, manufacturers of dietary supplements don't have to tell their customers what is in the supplements. They can simply say that they are a proprietary mixture of extracts of fruits, vegetables, herbs and/or spices. They also don't have to do stability studies, even though they are expected to provide the shelf life, based on data that might be available on similar food products. They do still have to create standard processes, train qualified people, follow SOPs, maintain clean, hygienic facilities, have a QMS system for QC, maintain detailed records that follow the principles of ALCOA (Attributable, Legible, Contemporaneous, Original and Accurate). They must also define the parameters for qualifying suppliers of active ingredients and enforce vendor compliance [294].

3.1.7 Clinical trials

Potentially new prescription drugs, medical devices and even many dietary supplements are first investigated in a pre-clinical setting [1]. This is done before the drug is tested in human clinical trials. The proof of

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principle is established for NMEs and formerly, animal testing was done. Moreover, suitable formulations are identified and sufficient amounts of the NME for future clinical trials are prepared. Next, an internal review board (IRB) reviews all the data so protocols can be prepared. Then, documents are filed for putting the IND (Investigational New Drug) into a phase I clinical trial. The rules and regulations for filing an IND are in Title 21 of the Code of the Federal Register (21 CFR 312) [250]. It is a best to document all studies as they are done, instead of waiting until the last minute [1]. It is also a good idea to schedule a pre-IND meeting with the FDA and/or other countries' regulatory agencies so any potential problems can be identified and fixed. Once this is done, the FDA will either approve the IND within 30 days of receiving it or ask for more information [1].

Subsequently, some dietary supplements are tested in clinical trials that seldom follow the same strict regulatory requirements that apply to prescription drugs and medical devices. Still, many dietary supplements are advertised as being clinically tested or even clinically 'proven' to be effective. This is in contrast with potentially new prescription drugs [1]. They are not just manufactured in compliance with cGMP, but also are tested in several phases of clinical trials that follow Good Clinical Practice [297].

In most phase I clinical trials, the absorption, distribution, metabolism and elimination (ADME) of the IND are determined in healthy subjects, as are the safety and tolerability [1]. First, a single dose is given, followed by dose escalation and repeat dosing on 20-80 subjects. The goals are to define the drug metabolism and pharmacological activity, identify the appropriate dose range for Phase II and longer term toxicology testing, as well as establish protocols and provide documents for Phase II trials. In phase IIa, the ADME is tested in patients who have the disease that is going to be treated, to establish the tolerability and efficacy. These are small studies (<100 patients) that aim to identify side effects and prove the concept or mechanism of action and look for any possible toxicities or adverse side effects. Also, protocols are established and documents prepared for Phase III trials. Before they can be done, though, phase IIb trials are conducted on 100-400 patients to demonstrate efficacy, identify the best commercial formulation and prepare more documents. The goal is to select the dosage and regimen for phase IIIa trials. The phase IIIa trials are done on even more subjects. The goals are to do definitive studies of efficacy and document any side effects. Next, phase IIIb trials are done to support any additional claims that will be put on the label for the drug and to support the launching of the new drug, is it is approved. Once the FDA

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and/or other governments' regulatory agencies approve the drug, then the drug is launched. Finally, phase IV post-market surveillance is done. The efficacy and safety are monitored. Additional studies can be done to support marketing. Pharmaceutical companies are required to document all reports of adverse side effects, but they also frequently monitor the effect of their new drugs on the life spans of the patients who take them and the possible effects they may have on other diseases. Many more details of the IND and clinical trials have been described elsewhere [1].

These procedures are quite different than what is done for most dietary supplements. Instead of studying the efficacy of the supplement in double-blind clinical trials, personal testimonials are usually given. This practice pre-dates the FDA or any other country's regulatory agency. That is, in the 19th century many remedies were sold to anyone who was foolish enough to buy them. An important part of the sales pitch was to have someone give their personal testimonial about how it made them feel much better. Since many of these remedies contained opium and/or cocaine, it would be easy to find someone who could give a very favorable testimonial. Fortunately, very few dietary supplements still have opium or cocaine in them, but some do. Still, the people who make and market dietary supplements know that a personal testimonial from a trusted friend or family member can be very convincing. Since only the safety and not the efficacy of dietary supplements needs to be shown, this is perfectly legal.

3.1.8 Many drugs that were developed and approved had a single therapeutic target

One of the best examples of a class of drugs that was originally thought to have a single therapeutic target is statins [1, 55, 56, 298-300]. They inhibit the rate limiting step of cholesterol biosynthesis, catalyzed by 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase [1, 55, 56]. In the process, they also inhibit the biosynthesis of essential isoprenoid intermediates. This includes farnesyl pyrophosphate, geranylgeranyl pyrophosphate, isopentanyl adenosine, dolichols and polyisoprenoid side chains of ubiquinone, heme A, and nuclear lamins. These compounds activate several signaling proteins. Examples include Ras and Ras-like proteins like Rho, Rab, Rac, Ral, or Rap. They all have essential roles in many cellular processes. When the concentrations of these proteins in circulating blood decrease, these signaling proteins are not activated. This leads to antiinflammatory, antioxidant effects, antiproliferative and immunomodulatory effects. They also increase the stability of plaques, normalize sympathetic outflow and prevent platelet aggregation [1, 55,

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56]. In addition, farnesyl pyrophosphate and geranylgeranyl pyrophosphate are needed to attach an isoprenyl group to the key G-protein Rac1 [1, 298, 299]. If Rac1 is not isoprenylated, it can't trigger the formation of lamellipodia that are needed for angiogenesis. Statins were found to be inhibitors of lymphangiogenesis by doing high content screening of the database called the Library of Pharmacologically Active Compounds, or LOPAC® [1, 298]. They used individual human lymphatic endothelial cells along with automated image acquisition and analysis to look for a specific phenotype – the formation of lymphatic vessel sprouts [1, 299]. Lymphangiogenesis is important because the lymph nodes are the first sites of metastasis in most epithelial cancers and malignant melanomas [1]. Moreover, malignant tumors cause lymphatic vessels to grow [1].

Even more effects of statins were discovered by doing proteomic and secretome analyses of statins [300]. They looked at the blood plasma, urinary, skeletal muscle, brain, plasma membrane lipid rafts and cancer cell proteomes, as well as the secretome of atherosclerotic plaques and vascular cells. They showed that statins may be useful in treating thrombosis in patients with antiphospholipid syndrome. They can also reduce the risk of stroke in patients at high risk of CVD and in patients with noncardioembolic stroke or transient ischemic attack. They may also be useful as chemopreventive agents and in combination therapy with current anticancer drugs. Finally, there are many publicly available resources that one can use in proteomic applications for new drug development [300].

In general, proteomics and other –omics methods are useful in network pharmacology [300, 301]. It emphasizes the "paradigm shift from a one target–one drug to a network targeted, multi-target drug approach" [300, 301]. This concept has been elaborated further [302]. That is, network pharmacology has led to "drug repurposing, polypharmacology, chemogenomics, phenotypic screening and high-throughput *in vivo* testing of mixture-based libraries in an integrated manner" [302]. So, instead of trying to find a single lead compound that binds to one target, systems thinkers are looking for 'master key' compounds that interact favorably with several therapeutic targets. This also means that methods that were previously used to develop structure-activity relationships (SAR) for single biological and/or biochemical endpoints are being modified so they can model structure-multiple activity relationships [302].

Systems thinkers are also interested in finding cures for many orphan diseases that affect only a few people. So, there is a database that identifies prescription drugs that have already been approved by the FDA, but may be useful in treating these diseases [303].

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3.1.9 Combinations of drugs are also used and are being developed

Certain combinations of drugs are being used successfully while new ones are being developed. For example, a mixture, or cocktail of antiretroviral drugs is given to HIV-positive patients in what is often called highly active antiretroviral therapy, or HAART [51]. This includes drugs that inhibit the entry of HIV into host cells (entry inhibitors), as well as nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors and maturation inhibitors. Without HAART treatment, the progression from HIV infection to AIDS used to have a median time of nine to ten years, while HAART treatment can extend that by many decades. Also, elephantitis is treated with albendazole, in combination with either ivermectin or diethylcarbamazine. Standard chemotherapy for lung cancer usually combines two or more drugs. Commonly used combinations include paclitaxel plus carboplatin, cisplatin plus vinorelbine tartrate, cisplatin plus etoposide (VP-16), and carboplatin plus VP-16. A combination of fluticasone furoate, an inhaled corticosteroid and vilanterol, a long-acting β2-adrenergic receptor agonist is used to treat chronic obstructive pulmonary disease (COPD). Duavee® is used to treat the vasomotor symptoms of menopause. It is a mixture of conjugated estrogens and bazedoxifene, which is a selective estrogen receptor modulator (SERM) designed to mimic the beneficial qualities of estrogens, while blocking estrogen in tissues where it can be harmful. Corlanor® (ivabradine) is a combination of two antihypertensive drugs (valsartan and sacubitril) that is used to lower blood pressure. A combination of 1.2% clindamycin phosphate and 3.0% benzoyl peroxide is used to treat acne. Obinutuzumab (Gazyvaro®) is used with chlorambucil to treat adult patients with previously untreated chronic lymphocytic leukemia (CLL) who have comorbidities making them unsuitable for a more popular type of chemotherapy (full-dose fludarabine). It is a glycoengineered humanized monoclonal antibody that binds to the CD20 cell surface protein and prevents B-cell cancers. A combination of parenteral ascorbate (vitamin C) and the conventional chemotherapeutic agents carboplatin and paclitaxel can synergistically reduce toxicity associated with chemotherapy in patients with ovarian cancer. Etanercept, infliximab and adalimumab are more effective in treating rheumatoid arthritis when used in combination with methotrexate [51].

Drugs and natural products that are not antibiotics can be used as antibiotic resistance breakers (ARBs) [304]. They can directly inhibit

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bacterial growth, lower the minimum inhibitory concentration of the antibiotic and/or modulate the host's defense by altering inflammation and autophagy. For example, the antifungal agent Ciclopirox inhibits the biosynthesis of surface coat lipopolysaccharide (LPS). It makes Gram-negative Bacteria susceptible to antibiotics that are usually effective only for Gram-positive Bacteria. There is also Loperamide, which is used to decrease the frequency of diarrhea. It is an agonist for the μ-opioid receptor. It has no innate antibacterial activity but acts synergistically with many antibiotics, including tetracyclines, cephalosporins, novobiocin, and polymyxin B. When administered intravenously, Loperamide does not show opiate-like effects, as it cannot cross the blood-brain barrier due to efflux by P-glycoprotein. There are also some important natural products that are ARBs. This includes epigallocatechin-3-gallate (EGCG), a polyphenol found in green tea. It acts synergistically with antibiotics against both Gram-positive and Gram-negative Bacteria. Also, curcumin, a constituent of turmeric, has direct antibiotic activity against many Gram-negative and Gram-positive species of Bacteria. It also exhibits synergism with antibiotics of broad range against methicillin-resistant *Staphylococcus aureus* (MRSA). The alkaloid berberine, which is found in goldenseal (*Hydrastis canadensis*), also has ARB properties. It is very effective against streptococcal, staphylococcal, and enterococcal species, including multidrug resistant strains of *Mycobacterium tuberculosis* and *S. aureus*. It is also effective against *E. coli*, *Giardia lamblia*, *Vibrio cholerae* and *Klebsiella* species. Berberine inhibits the sortase enzyme in Gram-positive Bacteria and cell division protein FtsZ in the Gram-negative *E. coli*. Also, berberine improves the defense response of the host by suppressing pro-inflammatory responses. Moreover, Bacteria develop little resistance to berberine [304]. However, EGCG, berberine and curcumin activate the Nrf2/ARE antioxidant system [305, 306], which is over-activated in some forms of multidrug-resistant cancer and cardiovascular diseases [307-309]. So, people who are being treated for cancer or who either had or are susceptible to cardiovascular diseases might want to refrain from taking dietary supplements that contain pure EGCG, curcumin or berberine in them.

Another approach is to deliver drugs to their specific target through antibody-drug conjugates and fusion proteins, in which one part (such as the antibody) binds specifically to the target and the other part is released and has the desired therapeutic effect [1, 310]. An example of this is the drug called trastuzumab emtansine (Kadcyla®), also known as T-DM1 and ado-trastuzumab emantsine. It's a combination of trastuzumab (the "T" in T-DM1), better known by the brand name Herceptin®, and a very

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powerful anticancer drug called emtansine (the "DM1" part). It was developed jointly by Genentech (the maker of trastuzumab) and Immunogen, which makes emtansine. Trastuzumab and DM1 are covalently linked with a stable thioether linker. This limits the exposure of normal tissue to DM1. Herceptin binds to the human epidermal growth factor receptor 2, or HER2, a specific receptor on breast cancer cells. The linker remains intact in the cytosol. The antibody-drug conjugate (ADC) binds to HER2 receptors on the surface of breast tumor cells and is internalized [1, 305]. The monoclonal antibody, trastuzumab, is broken down (hydrolyzed) in reactions catalyzed by proteases. As a result, emtansine is released into the cytosol to bind to microtubules, leading to cell cycle arrest and apoptosis [1, 310, 311].

So, emtansine only reaches the cancer cells in the breast and produces none of the severe side effects caused by Herceptin alone [1, 312]. T-DM1 is effective in patients who have HER2-positive metastatic breast cancer (MBC). The *HER2* gene is amplified in about 20-25% of all primary breast cancers [1, 313]. This type of tumor is aggressive, so patients have poor prognoses. So, T-DM1 offers hope to patients with Stage 3 or Stage 4 HER2-positive MBC that is resistant to previous treatment methods [1, 313]. It has been approved by the FDA and other countries' regulatory agencies.

So, there is an increased interest in developing more ADCs [1, 312]. Another example of an ADC is brentuximab vedotin (SGN-35). It is used to treat Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL). Brentuximab vedotin has a chimeric IgG1 monoclonal antibody (mAb) that targets the cell surface protein, CD30. It is conjugated to the antimicrotubule drug monomethyl auristatin E (MMAE) through a peptide linker. After the being taken up by cells that have CD30, the linker is hydrolyzed in reactions catalyzed by lysosomal proteases. MMAE is released into the cytosol where it binds to microtubules and arrests the cell cycle, leading to apoptosis. Glembatumumab vedotin is another ADC. It is undergoing clinical trials. It is a fully human IgG2 mAb that is specific for the melanoma glycoprotein NMB. It is conjugated to MMAE and is used to treat metastatic breast cancer and melanoma. There is also Lorvotuzumab mertansine, a humanized IgG1 mAb that is specific for CD56. It is conjugated to the maytansine derivative DM1 for treating small cell lung cancer, Merkel cell carcinoma, ovarian cancer and multiple myeloma. Another example is Inotuzumab ozogamicin, a humanized mAb specific for CD22 that is bound to calicheamicin. It is used to treat diffuse large B-cell lymphoma, indolent non-Hodgkin's lymphoma. Finally, there is Mylotarg® (gentuzumab ozogamicin). It was the first ADC to get FDA

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approval. It received accelerated approved in 2000 for acute myeloid leukemia, but was withdrawn in 2010 after a clinical trial showed the drug increased patient death and added no benefit over conventional cancer therapies [1, 312]. However, additional phase II clinical trials were encouraging, so the FDA has agred to review a re-submission of a Biological License Application for it.

There are also recombinant immunotoxins, in which a fusion protein is made that contains a bacterial toxin and a fragment which targets the specific cancer cells needing treatment [1, 314]. For example, anti-Tac(Fv)-PE38 or LMB-2, contains a toxin fragment of the endotoxin produced by *Pseudomonas* and an mAb against the cell surface protein, CD25 that is on cancer cells [1, 314]. Radionuclides can also be conjugated to antibodies. One example is ^{131}I-tositumomab (Bexxar®) and ^{90}Y-ibritumomab tiuxetan (Zevalin®), which target the CD20 protein on cancer cells [1, 315]. It is used to treat non-Hodgkin's lymphoma. There is also the antibody-drug conjugate brentuximab vedotin (SGN-35), which is now FDA-approved [1, 315]. There is also a hu14.18-IL2 fusion protein which consists of IL-2 covalently bound to a humanized mAb that binds to the GD2 disialoganglioside that is expressed on neuroblastoma cells [1, 316].

Small inhibitory RNAs (siRNAs) are also being developed for targeted drug delivery [1, 317]. Fusion proteins consisting of single-chain fragmented antibodies and positively charged peptides can deliver siRNAs into specific target cells. For example, Polo-like kinase 1 (PLK1) siRNAs that were fused with a Her2-ScFv-protamine peptide fusion protein (F5-P) suppressed the growth of HER2+ breast cancer cell lines and primary human cancers in breast cancer models. PLK1-siRNAs inhibited gene expression, reduced proliferation and induced apoptosis of HER2+ breast cancer cell lines and primary human cancer cells *in vitro* without triggering a potentially toxic response by interferons [1, 317]. "Intravenously injected F5-P/PLK1-siRNA complexes concentrated in orthotopic HER2+ breast cancer xenografts and persisted for at least 72 hours, leading to suppressed expression of the PLK1 gene and programmed cell death (apoptosis) of the tumor cells" [317].

More recently, antibody directed enzyme prodrug therapy (ADEPT) has been used to produce cytotoxic drugs from nontoxic prodrugs only in the cancer cells that are targeted [318]. Cancer cells produce tumor associated antigens that can be targeted by antibodies. Many different antibodies have been used to deliver therapeutic drugs selectively to tumors. For example, the FDA and other governments' regulatory agencies have approved two antibody-drug conjugates: Adcetris®, for the

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treatment of some lymphomas and Kadcyla® for certain breast cancers. Adcetris® contains a monoclonal antibody (brentuximab) that targets the CD30 antigen that is on Hodgkin lymphomas. Each monoclonal antibody molecule is covalently bound to three to five molecules of the antimitotic agent monomethyl auristin E through apeptide-based linker that can be hydrolyzed once Adcetris® reaches the targeted tumor cells. ADEPT is an improvement on these. It delivers a prodrug that has much lower toxicity than the active drug. The prodrug is converted to an active form in an enzyme-catalyzed reaction once it reaches the tumor cells that it is targeting. Even though several enzymes have been evaluated, the only one to be used in clinical trials in an ADEPT application is carboxypeptidase G2 (CPG2). It is a bacterial enzyme that is quite specific for the prodrug, and has no known human analogue. It catalyzes the hydrolysis of folates, including methotrexate. ADEPT is also being used to make vascular disrupting agents, DNA repair modulators and immunomodulatory drugs that may prove useful in a combined therapy for cancer. Even though this may lead to an effective treatment for solid cancer, more work needs to be done in an interdisciplinary and iterative process [318].

However, many anticancer drugs target signaling pathways that occur earlier than those that are in the mitochondria and merge subsequently onto the intrinsic senescence pathway [304]. Cellular senescence is a state of irreversible arrest of cell growth that is an important factor in aging and cancer. However, drugs that target the upstream signaling pathways can lose their efficacy due to the emergence of drug resistance. This can be avoided by using drugs that directly target the mitochondria. Cancer cells have elevated concentrations of lactate due to high metabolic activity and the activation of the membrane Na^+/H^+-antiporter that pumps out H^+ and makes the extracellular environment more acidic. This lowers the cellular uptake of many anticancer drugs. So, proton pump inhibitors (PPIs) can sensitize multidrug-resistant cancer cells to anticancer drugs. Due to the high energy demand of cancer cells, drugs that cause depletion of ATP may sensitize resistant cells. So, adding 2-deoxyglucose, metformin and lonidamine to conventional anticancer drugs lowers the concentration of ATP in multidrug resistant cancer cells and sensitizes them to the therapy [304].

To help develop more synergistic drug combinations, a method called MASCOT has been developed to identify them from curated signaling networks [319]. It leverages machine learning-based target prioritization in a given disease-associated network with the Loewe additivity theory from systems pharmacology, which assesses the non-additive effects obtained from administering a combination of drugs. Researchers examined two

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disease-related signaling networks to demonstrate the superiority of MASCOT compared to existing approaches. MASCOT identified known drug combinations that are biologically relevant. In addition, the generic and adaptable nature of MASCOT allows other disease-specific restraints to be included in the analysis. This includes various –omics data as well as information about drugs and diseases. MASCOT excludes drug combinations that are too similar to monotherapies, while avoiding activators of pro-disease targets [319].

So, there is much research and development that is developing better ways to deliver drugs to their specific target. To increase efficacy, lower the dose and prevent serious side effects.

3.1.10 Some targets are now known to have formerly unexpected biological roles

Systems thinking has led to the realization that some therapeutic targets have formerly unexpected biological roles. For example, the renin-angiotensin system (RAS) is the therapeutic target for inhibitors of the angiotensin converting enzyme (ACE) [1, 320]. Note that this should not be confused with the *RAS* oncogene or the ras oncoprotein. Angiotensin II (Ang II) is a hormone that helps regulate blood pressure. It is produced by the hydrolysis of the precursor protein angiotensinogen in a reaction catalyzed by ACE. Ang II has been known to exert its effects through AngII type 1 and type 2 receptors (AT1R and AT2R, respectively). These receptors are mutually antagonistic in the control of blood pressure, water, and electrolyte homeostasis. They were originally thought to be controlled by the brain and kidneys. There are several ACE inhibitors that have been approved by the FDA and other countries' regulatory agencies. This includes captopril, which was developed by modifying a lead compound that is a venomous peptide made by the lancehead viper. However, we now know that RAS plays an important role in cancer (as does the ras oncoprotein). Many parts of the RAS can be produced by resident or infiltrating cells, such as mast cells. They are produced when needed during an infection or injury. Moreover, we also know that there is an alternative pathway for angiotensin. In it, a newly identified enzyme, ACE2, liberates angiotensin 1-7 (Ang1-7), which binds to the Mas receptor. It opposes many of the actions of the AngII-activated receptors. In addition, we now know that RAS (but not the ras oncoprotein) also plays an important role in wound healing and drives malignant processes in tumors through the AT1R receptor. So, AT1R blockers and ACE inhibitors may be useful in combination anticancer therapy. However, this

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was not obvious at first. Some meta analyses showed that AT1R blockers (ARBs) were associated with a small increased risk of new cancer diagnosis. This led to headlines like ‘‘ARBs Cause Cancer!’’ and ‘‘ARBs Increase the Risk of Cancer!’’ even though the authors advised caution when interpreting the results of their analyses. Subsequent studies showed that there was no significant risk of worsening cancer incidence or progression and no increase in death, while some researchers reported finding a reduced risk of cancer with ARB use. When systems thinking was used, it became apparent that all these seemingly contradictory reports could be true. That is, the findings depended on the circumstances and timing of the observations. That is, long-term (5+ years) use of ARBs and ACEIs progressively reduces the risk of cancer by reducing oxidative stress and genetic damage. However, for the first few years, ARBs and ACEIs might slightly increase the risk, especially when a combination of ARBs and ACE inhibitors (ACEIs) is used by tobacco smokers who already had precancerous nodules. This is because ARBs and ACEIs can push the RAS towards ACE2, Ang1-7, and Mas activation that promote the growth of cancer cells. Also, ARBs and ACE inhibitors can help cancer patients survive by blocking chronic inflammation and tissue remodeling processes in the tumor microenvironment. Finally, ARBs and ACE inhibitors have many healthy effects for people who have advanced stages of cancer. They increase survival, improve performance status, reduce reoccurrence and reduce toxicity when used together with radiotherapy or chemotherapy [320].

3.1.11 Some drugs may be useful in treating other diseases

Metformin (Glucophage®) is one of the most important drugs that was originally approved to treat one disease (type-2 diabetes, T2D) but may be useful in treating others, and possibly even slowing down aging [321-327]. It is the drug that is most frequently prescribed for lowering blood glucose in the first-line treatment of patients who have T2D, which affects one in nine adults in the USA [327]. It can also help reduce the occurrence of the top five comorbidities of T2D: cardiovascular diseases, cancer, depression, dementia and frailty. However, the extent to which metformin does this depends on the phenotype of the patient [327]. Metformin is also quite useful in preventing T2D in people who are pre-diabetic [323]. It not only reduces the concentration of glucose in the blood, but also offers protection against cardiovascular diseases [321]. Metformin also decreases the concentration of insulin, decreases IGF-1 signaling and inhibits mTOR (mammalian target of rapamycin) [323]. It reduces the incidence of cancer

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and mortality and helps people retain proper cognitive function. It does this by inhibiting the mitochondrial complex 1 in the electron transport chain, thus reducing the endogenous production of reactive oxygen species (ROS) [323]. At the same time, it targets histone acetylation in cancer-prone epithelial cells [325]. It may also affect aging by activating AMP-activated protein kinase (AMPK) and reducing DNA damage. So, metformin favorably influences metabolic and cellular processes that are closely linked to the development of age-related problems, such as inflammation, autophagy, and cellular senescence [323]. Since it is so inexpensive and easy to obtain, metformin could be especially useful in countries where many people don't have much money [321].

Perhaps the most exciting potential use of metformin (Glucophage®) is in extending the lifespan of not just people who have diabetes, but also in slowing down the aging process [321-327]. It does this by affecting several biochemical pathways that are important in how prediabetics age [322]. This includes increasing the concentrations of the following proteins while activating genes that code for them: *N*-glycans (non-invasive surrogate markers of aging), SIRT1, p53 and p66Shc (an adaptor protein in the member of the SHC, or sarcoma homology family that functions as a redox enzyme that is linked to apoptosis). Metformin also increases the activity of SIRT1 and AMPK, while increasing the accessibility of the SIRT1 promoter, the length of telomeres and the accessibility of the SIRT1 promoter on chromatin [322]. It also decreases IGF-1 signaling, inhibits mTOR and the mitochondrial complex 1 in the electron transport chain, while reducing the production of ROS and reducing DNA damage [323]. Metformin also favorably influences metabolic and cellular processes that are linked to the development of age-related conditions, such as inflammation, autophagy, and cellular senescence [323].

Metformin (850 mg twice daily) was administered to subjects in a Diabetes Prevention Program (DPP) [323]. It also reduced the incidence of T2D by 31% compared to placebo after three years and prevented diabetes, as defined by HbA1C concentrations. Moreover, metformin improved risk factors for cardiovascular diseases and subclinical atherosclerosis (coronary artery calcium) in male participants [323].

Partly due to these important health benefits of metformin, some scientists are rejecting the idea that we die of old age [326]. Instead, we die of cumulative failures that occur within our cells and tissues. These failures are not inevitable breakdowns, are reversible conditions of aging. One of the most important of these is AMPK. Its concentration is relatively high when we are young, but decreases with age. Recent studies

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have suggested that increasing the activity of AMPK can prevent and possibly reverse the life-shortening effects of aging [326]. Some scientists are beginning to refer to AMPK as a suppressor of aging itself [326, 328]. It has been called a "metabolic master switch" [326, 329]. It monitors the energy status of every human eukaryotic cell within us and triggers responses that maintain it within a healthy range [326]. That is, too little available energy starves the cell, while too much energy can produce too many ROS and disrupt cellular components. As AMPK decreases when we age, we become less energetic and more obese, while becoming increasingly vulnerable to cancer and diseases associated with impaired DNA and protein function. As a person accumulates abdominal fat, this leads to reduced insulin sensitivity, system-wide smoldering inflammation and metabolic syndrome. This, in turn, can lead to many forms of cancer, as well as cardiovascular, neurodegenerative and autoimmune diseases (including T2D). The modern Western lifestyle with its overabundance of nutrients and low level of physical activity exacerbates this. When a person's caloric intake is too high and/or physical activity too low, AMPK activation decreases. As a result, cells decrease their energy-releasing ATP-generating activities and shift to energy-storing processes that generate new fat deposits and make excess new glucose. Moreover, energy inefficiency eventually leads to the dysfunctions that are often described as inevitable diseases (or symptoms) of aging. So, restoring the activity of AMPK in the elderly may not only increase longevity, but also help to fight the symptoms of aging [326]. This hypothesis was supported by a clinical study in which subjects with T2D either received metformin (which activates AMPK) or placebo [328]. They were compared to subjects who did not have T2D. It was found that the subjects who were diabetic and received metformin lived a median of 15% longer than did matched controls without diabetes [328]. So, there is a clinical trial underway, called Metformin in Longevity Study (MILES) [330] and another that is being planned called Targeting Aging with Metformin, or TAME, that will look at the effects of metformin on longevity [324].

There are many other possible applications for drugs that are already approved by the FDA and other governments' regulatory agencies. So, there is a database called SWEETLEAD that is being used to help in computer-aided drug discovery [331]. It includes approved drugs, as well as regulated chemicals, and herbal isolates [331]. In addition, current trends in computational drug repositioning have been reviewed [332]. It lists several sources of chemical, genetic, pharmacogenetic and clinical data and describes computational methods, such as machine learning, network analysis and text mining. It also describes validation methods. It

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also gives some examples of drugs that are being studied for other uses (drug repositioning). This includes not just metformin, but also auranofin for the treatment of gastrointestinal stromal tumors, irinotecan for the treatment of breast cancer, the anthelmintic drug mebendazole for the treatment for colon cancer and trifluoperazine as a potential anti-CSC (cancer stem cell) agent [332]. Finally, the popular active ingredient in many cough medicines, dextromethorphan (Robitussin®) is being evaluated for treating neurological and neuropsychiatric disorders [333]. This includes depression, stroke, traumatic brain injury, seizure, pain, methotrexate neurotoxicity, Parkinson's disease and autism [333].

So, there is much work being done on to find new applications for drugs that have already been approved and have already been shown to be safe in millions of people.

3.1.12 The Reemergence of Phenotypic Screening

In systems thinking and TQM, we use all the resources at our disposal to solve problems and develop new drugs. That is, we not only look for NMEs that affect therapeutic targets, but also NMEs that cause a desired phenotype, such as the death of an infectious agent or cancer cells. As a result, there has been a reemergence of phenotypic screening in new drug development [334-355]. This is not an evil conspiracy but an open collaboration between pharmaceutical companies that used to work in isolation [338]. That is, scientists from Genetech, Pfizer, Eli Lilly, Novartis and Roche gave an industry perspective and described the challenges of phenotypic drug discovery. This idea was supported by conferences supported by the New York Academy of Sciences and the Keystone Symposia on Molecular and Cellular Biology. In addition, a PDD special interest group has been established under the auspices of the Society for Laboratory Automation and Screening. The review article describes ways to translate new scientific discoveries into safe and effective new drugs. It also mentions advanced cellular models including tumour spheroids, tissue-on-a-chip, structured co-cultures and multicellular organoids. The goal is to find NMEs that act through previously unidentified mechanisms to produce first in a class prescription drugs. Still, phenotypic drug discovery and targeted drug discovery should be thought of as complementary and not competing methods [338]. So, we are shifting from reductionism to the realization that human health and diseases are complex entities that cannot be understood effectively by treating each part in isolation [354]. In this new paradigm, phenotypic screening has reemerged for finding NMEs and providing insights into the

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underlying cellular processes. This is leading to new opportunities for drug discovery [354]. This is consistent with systems thinking, since the screening looks at the phenotype of the entire organism and not just one isolated therapeutic target. That is, phenotypic screening does not start with any hypotheses about how the new drug might work. In contrast, targeted approaches do start with a hypothesis, such as the idea that inhibiting HMG-CoA reductase will reduce the concentration of cholesterol in the blood, which will prevent heart attacks. Still, both approaches have identified many small molecules that became new, safe and effective prescription drugs [1, 51]. That is, many traditional medicines or remedies were discovered by looking at the effects of various foods, herbs and spices on the phenotype of the organism. This includes their effects on many symptoms of diseases and their abilities to kill infectious organisms. However, traditional medicine and phenotypic screening alone were not as effective as one might have hoped. Many traditional remedies have been shown to be unsafe and ineffective. This led many scientists to believe that it was more important to find the single root cause of a disease and target it. As discussed previously in this chapter, many new drugs that were developed based on their effects on a single therapeutic target were approved by the FDA and other governments' regulatory agencies. One group looked at 113 first-in-class drugs that were approved by the FDA from 1999 to 2013 [335]. They found that the majority (78) of them were discovered through target-based approaches (45 small-molecule drugs and 33 biologics). In addition, of 33 drugs that were identified without knowing the therapeutic target or targets, 25 were found through a chemocentric approach in which compounds with known pharmacology served as the starting point. Only eight were developed through phenotypic screening [334]. However, medicinal chemists and other researchers are realizing that when a desired effect is seen in a phenotypic screen, computational models of the disease can be used to learn how the drug works [332]. That is, advances in proteomics and the computational analysis of biological pathways have made it possible to identify the therapeutic targets of phenotypic hits. Once the possible targets have been identified, medicinal chemists can return to phenotypic screening to test the new predictions made with the computational model. This iterative and integrated approach is called quantitative systems pharmacology (QSP). It originated in about 2010, when the U.S. National Institutes of Health (NIH) sponsored a workshop to integrate the distinct fields of systems biology and pharmacology [333]. Since proteomics and other –omics are being used with computational

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models phenotypic screening can be thought of as a new discipline rather than as a return to an ancient or classical approach [335].

Modern phenotypic screening aims to identify the mechanism of action of NMEs and identify the effective target or targets responsible for these phenotypes [353]. Both are important in optimizing lead compounds. One group statistically aggregated signals from the biological signatures of several compounds to find other compounds that could produce a desired phenotype in the cell. This enables the expansion of targeted hits and the identification of new therapeutic targets. They found some new nanomolar inhibitors of cellular division that reproduce the phenotype and the mode of action of reference natural products. They also found blockers of the NKCC1 (Na^+-K^+-Cl^-) cotransporter for autism spectrum disorders. Their results were confirmed in both cellular and biochemical assays. These examples provided new insights into the information content and biological significance of such biological signatures from HTS, and their applicability to discovering other new drugs. Their results showed that open access bioactivity data can be used for prospective drug discovery. This includes those cases in which the effective target or mode of action of a particular NME is not known [353].

Phenotypic screening is often done by looking for morphological or other changes in cells grown in culture [336-340]. Such tests should be simply, speedy, sensitive and specific as well as distinctive [340]. For example, a 3D cell culture chip can be used for high-throughput phenotypic screening of a human neural progenitor cell line (NPC) encapsulated within Matrigel™ [336]. The toxicities of 24 compounds were evaluated using both undifferentiated and differentiating NPCs. Five compounds had significant differences in their IC_{50} values between undifferentiated and differentiating NPCs. This platform can also be used in phenotypic screening to study the toxic effects of NMEs on human stem cells [336]. High-content, image-based screens are also being used to identify chemicals that induce cellular responses similar to those of known drugs but through different targets [337]. To do this properly, suitable imaging biomarkers must be used. So, a method was developed to systematically identify optimal reporter cell lines for annotating compound libraries (ORACLs), whose phenotypic profiles most accurately classify a training set of known drugs. They produced a library of fluorescently tagged reporter cell lines, and let analytical criteria identify those that best classified chemicals into multiple, diverse drug classes [337].

Phenotypic screening with primary neurons has been used to find drugs that can help treat neurodegeneration and reduced growth of axons that occurs in CNS injury and neurodegenerative diseases [343].

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Phenotypic screening has also been useful in finding natural products that can cure deadly diseases [340, 341, 351]. The Nobel Prize in Physiology or Medicine was awarded in 2015 to Satoshi Ōmura and William Cecil for their discovery of ivermectin and artemisinin that are used to cure infections caused by roundworms and malaria, respectively. Other phenotypic screens are being used to find better antifungal drugs and anticancer agents [340, 341]. Even though most of the therapeutic antibodies that have already been approved were developed using targeted screening, phenotypic screening is becoming more popular and useful [341]. It is being used to develop therapeutic antibodies against cancer and infectious diseases. Alemtuzumab is the first FDA-approved therapeutic antibody that was developed through phenotypic screening. Its clinical efficacy was demonstrated long before its mechanism of action on the CD52 antigen was elucidated [341].

Angiogenesis (the process of growing new blood vessels) is an important phenotype that medicinal chemists and others are studying and evaluating for therapeutic applications [345-350]. It is important not just in cancer metastasis, but also in other diseases that depend on angiogenesis [345]. This includes autoimmune diseases, atherosclerosis and age-related macular degeneration. So, it is important to use systems thinking and realize that angiogenesis is an organizing principle in biology. By doing so, researchers have obtained important insights into the molecular mechanisms behind many previously unknown (or hidden) phenomena. That is, if a molecular mechanism is discovered for one disease, it would be much easier to show that it is important for another disease if it is known ahead of time that the two diseases are linked to each other. For example, bevacizumab is approved to treat colorectal cancer and non-small-cell lung cancer and has been used to treat macular degeneration in an off-label application. Also, the anticancer drugs erlotinib (Tarceva®), cetuximab (Erbitux®) and vandetanib (Caprelsa®) were originally developed as inhibitors of the tyrosine kinase EGFR. So, they are anti-oncoprotein signal transduction inhibitors. However, they were subsequently found to inhibit angiogenesis as well. They do this by blocking the VEGF receptor. Cetuximab also inhibits the secretion of some pro-angiogenic proteins, including VEGF, basic fibroblast growth factor and transforming growth factor-α [345]. There are also natural products that can inhibit angiogenesis. This includes phenolic compounds from the mushroom sclerotium of *Pleurotus tuber-regium* [348].

At the same time, systems-based thinking leads us to realize that angiogenesis must be properly controlled. Not only is too much angiogenesis unhealthy, but so also is too little angiogenesis [350]. So, it is

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also important to realize that angiogenesis is regulated by a complex interaction of biophysical and biochemical cues from the extracellular matrix (ECM), angiogenic factors and several types of cells. As a result, better ways to design pro-angiogenic drugs are also being described. Such drugs and devices should be biomimetic, incorporate angiogenic factors and mimic cooperative interactions between growth factors and the ECM. They could be useful in regenerative medicine to generate new blood vessels so blood flow can be restored in ischemic tissues and to improve tissue regeneration. It has been estimated that more than 300 million people in Western nations might benefit from pro-angiogenic therapies [350].

For phenotypic screening to work properly, the genetics, epigenetics, extracellular miRNAs and signaling networks must be understood as well as possible [354, 355]. For example, a biochemical pathway-based meta-analysis was used to identify 43 pathways that are associated with T2D in European populations [353]. They analyzed several genome-wide association studies (GWAS) that detected SNPs that are associated with complex traits. They used their general procedure called Summary-based Adaptive Rank Truncated Product (sARTP) to do meta analyses of genes and biochemical pathways. They also developed an R package and a web-based tool for sARTP for analyzing pathways with thousands of genes and tens of thousands of SNPs [354]. Another group developed Pascal (Pathway scoring algorithm) to calculate gene and pathway scores from SNP-phenotype association summary statistics [355].

So, phenotypic screening is becoming more important in new drug development, since we can now interpret the results of various –omics and GWAS studies better and calculate the relevant biochemical pathways.

3.1.13 Other new approaches to new drug discovery

In addition to phenotypic screening, other novel approaches are emerging. Systems thinking rejects dogma and realizes that today's solutions can come from yesterday's problems. In business, it is said that it is important to turn your deficits into assets. In the TQM process of new drug development, viruses that have caused deadly epidemics in the past are now being modified to make them oncolytic, or able to destroy cancer cells. In addition, whole new classes of drugs are emerging. This includes modified RNA (modRNA), pre-mRNA, mRNA and gene therapy. New uses are being found in anticancer therapy for a safe drug that has been used for decades (fenofibrate) in combination with a safe imaging agent (2-deoxy-D-glucose). Also, an oncoprotein (gankyrin) that interacts with

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many other proteins is emerging as a promising target for anticancer therapy. Finally, network dynamics in the tumor microenvironment as well as interactions between blood platelets and tumor cells are being targeted.

Cytotoxic and immunogenic mechanisms of recombinant oncolytic poliovirus

An aspect of systems-based thinking is that today's solutions often come from yesterday's problems. For example, the poliovirus (PV) once terrorized humanity by killing and paralyzing millions of people. Also, the rhinovirus is the most common infectious agent in humans and causes the common cold. Now scientists are modifying the poliovirus to target inflamed cancer cells in one of the deadliest forms of cancer – glioblastoma [356]. The modified poliovirus is a type of oncolytic virus, in that it preferentially infects and kills (lyses) cancer cells. It does this by infecting and lysing cancer cells while simultaneously activating pro-inflammatory, antiviral biochemical pathways. So, researchers prepared an oncolytic poliovirus by replacing the PV's internal ribosomal entry site (IRES) with that of the human rhinovirus 2 (HRV2). This destroys the toxicity and neurovirulence of the PV. The recombinant oncolytic virus is called PVSRIPO for **PV**(**S**abin)-**R**hinovirus **I**RES **P**V **O**pen reading frame. Note that an open reading frame is the part of a chromosome that contains a reading frame that begins with a start codon (such as AUG) and ends with a stop codon (such as UAA, UAG or UGA). The PVSRIPO oncolytic virus is in a phase I clinical trial for treating glioblastoma. It targets the CD155 protein that is on the surface of host human cells and is required for the virus to enter them. Since the same CD155 protein is also upregulated in solid tumors, it binds the PVSRIPO oncolytic virus selectively. The researchers are learning more about the mechanism, so the PVSRIPO oncolytic virus may become useful in treating and possibly curing other types of cancer [356]. The uses of this and other oncolytic viruses will be discussed further in section 3.3.

Effects of chemically modified mRNA on protein expression

Chemically modified mRNAs (modRNAs) are also being investigated as potential new drugs and therapies [357-360]. *In vitro* transcribed (IVT) mRNA can be used to deliver genetic information [360]. This type of synthetic mRNA can be engineered to express proteins transiently. IVT mRNA can be used two different ways. It can be transferred into the patient's cells *ex vivo* and subsequently infused back to the patient. This method is being investigated for genome engineering, genetic reprogramming, immunotherapies based on T cells and dendritic cells to treat cancer and

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infectious diseases, and protein replacement. Alternatively, the IVT mRNA can be delivered directly to the patient. This method is being used for applications in oncology and infectious diseases, allergies treatments and for other protein-replacement therapies. For both *ex vivo* transfection and direct vaccination, the endogenous enzymes in the transfected cells are used to translate the modRNA into the desired protein, which is the pharmacologically active product. Note that modRNA works in the opposite way that siRNA works, since siRNAs inhibit the expression of proteins. Also, IVT mRNA is engineered so its structure is very similar to that of naturally occurring mature and processed mRNA in the cytoplasm of eukaryotic cells. The modRNA is synthesized in a cell-free system by *in vitro* transcription from a DNA template. The template DNA is subsequently broken down by reactions catalyzed by DNA hydrolases (DNases) and the modRNA is subsequently purified [360].

The goal is to produce modRNAs that can code for any protein that is wanted or needed [357-360]. So, if a patient can't produce enough of an important protein or makes a defective version of the it, a chemically modRNA could fix the problem. This is thought to be potentially much better than gene therapy, in which the genetics of the patient is permanently altered – often with unwanted consequences. In addition, modRNAs can be used to make proteins that are not secreted by cells. Such proteins are difficult if not impossible to prepare using current techniques, such as bioengineered cells [357]. So, modRNAs that are chemically synthesized using N_1-methylpseudouridine, 5-methoxyuridine and pseudouridine have been made that code for firefly Luciferase and enhanced green fluorescent protein [361]. The modRNAs that contain these chemically modified nucleotides are resistant to degradation and have relatively low immunogenicity. Similar modRNAs may have many diverse biomedical applications in the near future [361]. They are already being used to make iPSCs by overexpressing appropriate protein factors in somatic cells [362].

Also, modRNA that coded for human vascular endothelial growth factor-A (VEGF-A) directed the fate of heart progenitor cells and induce vascular regeneration after myocardial infarction in a mouse model [363]. The function of the heart improved and the mice lived longer than the controls. The pulse-like expression of the modRNA was shown to be better than that of plasmid DNA in reducing the size of the infarct, as well as enhancing myocardial perfusion [363]. In addition, modRNAs are being clinically tested for their use in cancer immunotherapies and vaccines for infectious diseases [360, 362]. Other potential applications include making pluripotent stem cells and doing genome engineering using designer

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nucleases. The history and principles behind modRNA therapies have been reviewed [360, 362].

However, it is not always easy to find ways to get the modRNA into target cells by conventional means. Instead, nanotechnology is being used [364]. Cationic lipids have become the most used nanosystems for delivering *in vitro* transcribed mRNA to cells. They form lipoplexes spontaneously with the mRNA through electrostatic interactions. This protects the mRNA from RNA hydrolases in the blood, so the mRNA can reach the cells. In addition, the positive charges on the lipids promote their uptake into cells. These and other types of nanostructures are being evaluated and were described in a recent review [364].

pre-mRNA and mRNA-based therapeutics - developing a new class of drugs

Not only chemically modified mRNA, but also other oligonucleotides are being developed [365, 366]. For example, an antisense drug called Spinraza™ (synonyms: Nusinersen, IONIS-SMNRX, ISIS-SMNRX) was approved by the FDA on December 23, 2016 [365]. It is the first drug that has been approved for treating spinal muscular atrophy (SMA), an all too common disease that affects infants and children. SMA is caused by low concentrations of the Survival Motor Neuron (SMN) protein caused by deletions or mutations in the *Survival Motor Neuron 1* (*SMN1*) gene. SMA is a unique genetic disorder, in that there is a second copy of the *SMN* gene, *SMN2*. However, there is a translationally silent mutation of C to T (C6U in the mRNA) at the sixth position in exon 7. This exon is spiced inefficiently to produce a shorter, truncated protein, SMNΔ7 that is unstable and only partly functional. Still, *SMN2* can potentially produce the complete, full-length SMN2 protein if the mRNA that codes for it is spiced properly. This can be done using antisense oligonucleotides (ASOs). They are short and are designed to anneal to complementary base sequences in the target gene. By doing so, the ASO can block the binding of *trans*-acting proteins to the pre-mRNA. One such protein is Intronic Splicing Silencer N1 (ISS-N1). It inhibits the inclusion of *SMN2* exon 7. When the proper ASO is used, the ISS-N1 protein is sequestered and a full-length transcript of the SMN2 protein can occur. As a result, Spiranza™ has been approved for treating children who have already been diagnosed with SMA and have experienced damage in their motor neurons. This is being followed by a clinical trial (NCT02386553) in which Spiranza™ is being given to infants who have mutated *SMN1* genes, but who have not yet shown any symptoms of SMA [365].

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Other oligonucleotide therapies are being developed and are undergoing clinical trials to treat more nervous system disorders [366]. In addition, two more oligonucleotide drugs have been approved by the FDA. They are mipomersen (Kynamro) and eteplirsen (Exondys 51), for treating homozygous familial hypercholesterolemia and Duchenne muscular dystrophy, respectively. ModRNAs can be designed so that they modulate the spicing of selected pre-mRNAs. They bind to the part of the pre-mRNA that has mutated exons, causing them to be excluded from the final mRNA that the targeted cells use to make the desired protein. There are also some small-activating RNAs (saRNAs) that are synthetic and double-stranded. They can upregulate the expression of target genes. This technology is being developed by a company based in London, MiNA Therapeutics. They made a bioinformatics platform for designing saRNAs that activate the targeted genes (http://minatx.com/ 5/29/16). Their work has led to the first clinical study of a therapeutic saRNA, MTL-CEBPA. It is being tested for treating patients who have advanced hepatocellular carcinoma (ClinicalTrials.gov ID# NCT02716012). MTL-CEBPA is a dsRNA that is designed to activate the *CEBPA* gene, which codes for a protein transcription factor that is involved in the differentiation of certain blood cells. It is delivered in amphoteric iminolipid nanoparticles. The use of saRNAs, modRNAs and other types of modified oligonucleotides has been reviewed and their and their unusual pharmacokinetics described [366].

Gene therapy as a potential tool for treating neuroblastoma

Even though there has been much promising work done on modRNA, gene therapy is still being studied as a way of providing a functional protein to patients who lack it or who make a dysfunctional, mutated version of it [367]. One potential application is treating neuroblastoma, in which several oncogenes are not properly regulated. The goal of gene therapy is to restore the normal expression of these oncogenes. One main obstacle to successful gene therapy is a lack of effective delivery systems. To improve this situation, retroviral and adenoviral vectors are being evaluated for delivering the functional genes. Engineered retroviral vectors can integrate into the host genome and permanently transfer the gene of interest. They work together with the host's immune system. Some of these vectors can express chemokines that help the immune system eliminate tumors. For example, T-cells that have the surface proteins CD4+ and CD8+ increase the potency of this type of cancer immunotherapy. On the other hand, adenoviruses that have defective replication have lower toxicity than retroviruses. They are used as vectors

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for delivery vehicles and vaccines. This is different than oncolytic viruses that can replicate properly. They recognize specific tumor cells and kill them [367].

A combination of gene and cell therapy is being used to fight neuroblastoma [367]. Genetically engineered adenoviruses are being used with human mesenchymal stem cells (MSCs). The MSCs compensate for the inability of adenoviruses to target tumors. They are multipotent adult stem cells that can be isolated from several different tissues (such as dermis, adipose tissue and peripheral blood). They can not only differentiate into many different types of cells, but also engraft (incorporate in a firm or permanent way) tumors when delivered intravenously. When MSCs are delivered with engineered adenoviruses, the adenoviruses can reach the targeted tumor, where they are replicated [367].

Another novel approach: Combine 2-deoxy-D-glucose with fenofibrate

Another exciting recent development in anticancer therapy is the use of 2-deoxy-D-glucose (2-DG) with the FDA-approved drug, fenofibrate [368]. Fenofibrate (a PPARα agonist) has been used for over 40 years to lower the concentration of triacylglycerides and cholesterol in the blood. So, it is quite safe. Similarly, 2-DG has been used safely to make images of tumors and to monitor the uptake of glucose in them. Since tumor cells depend on increasing their glucose metabolism for their energy, 2-DG causes metabolic stress by inhibiting it. Also, 2-DG is an analogue of mannose, so it causes stress in the endoplasmic reticulum (ER) by interfering with *N*-linked glycosylation. Under normoxic conditions, a low concentration (< 2 mM) of 2-DG is nontoxic. However, tumor cells are under more stress than normal cells, due to their uncontrolled growth and proliferation, as well as the abnormal microenvironment of a tumor. So, 2-DG can be toxic to tumors. In addition, fenofibrate can inhibit the function of mitochondria in tumor cells that already suffer from ER stress. Moreover, 2-DG can induce mitochondrial stress, which increases the sensitivity of tumor cells to fenofibrate. Thus, 2-DG and fenofobrate work synergistically to kill many types of cancer cells, due to their universal vulnerability [368]. Interestingly, a more recent study showed that a combination of 2-DG and berberine (a natural compound found in goldenseal) can synergistically induce apoptosis in human lymphoblastoid TK6 cells [369]. They are effective when given together at doses that are so low that neither one by itself can induce apoptosis. So, this might be another exciting development in preventing, treating and possibly even curing many types of cancer [369].

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Small-molecule binder of the oncoprotein gankyrin

There is another recent advance in treating many types of cancer [370]. A small molecule called cjoc42 has been found to bind to an oncoprotein called gankyrin and prevent it from interacting with other proteins and exerting its pro-cancerous effects. Gankyrin has potent cell-cycle stimulatory and anti-apoptotic properties. It is overexpressed early in hepatocellular carcinoma. Its upregulation is associated with poor prognosis in esophageal squamous cell carcinoma, glioblastomas, colorectal, pancreatic and lung carcinomas. Existing treatment options for these cancers are limited, and no small-molecule inhibitors of gankyrin had been identified previously. The structure of cjoc42 is shown in Figure 22. Elevated concentrations of gankyrin lead to increased degradation of the p53 tumor suppressor. In addition, several proteins bind to and/or interact with gankyrin. So, much effort was put into finding small molecules that can inhibit these PPIs. These efforts were successful when cjoc42 was tested. It inhibits gankyrin activity in a dose dependent manner. It prevents the decrease in p53 concentration. So, the sensitivity of cells to DNA damage is maintained. Hopefully, cjoc42 and/or other small molecule inhibitors of gankyrin will eventually be used to cure many types of cancer [370].

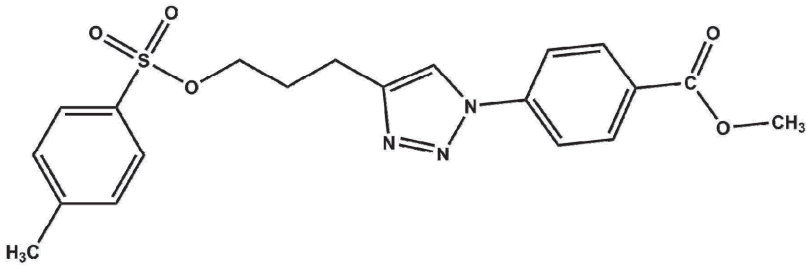

Figure 22. 2D structure of cjoc42.

Targeting network dynamics in the tumor microenvironment

Systems-based thinking is also being used to help understand the network dynamics in the tumor microenvironment [371]. We have learned that the evolutionary path from tumor initiation to metastasis can understood best by viewing cancer cells as part of an ecosystem. This evolutionary process involves genetic and epigenetic changes in somatic cells and clonal selection of tumor cells. The evolutionary fitness of clonal tumor cells is not determined in isolation, but by their interactions with other clones, the extracellular matrix, hormones, soluble factors and stromal tissues within the tumor microenvironment. Interactions between

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many different types of cells in the tumor microenvironment can provide growth advantages, resist anticancer treatments and allow tumors to remain dormant for a long time. There are now computational models of the tumor microenvironment based on extensive data and many molecular details. They have been built from emerging high-throughput technologies. These models can identify mechanisms of dysregulation and suggest specific drug targets and suggest useful follow up experiments [371].

Targeting interactions between blood platelets and tumor cells

Blood platelets are an important part of the tumor microenvironment. There is direct signaling between platelets and cancer cells [372]. The interactions between platlets and cancer cells prime cancer cells for subsequent metastasis. Tumor growth factor β (TGFβ) from platelets and direct platelet tumor cell contacts activate the TGFβ/Smad and NF-κB pathways in cancer cells synergistically. This triggers a transition to an invasive mesenchymal-like phenotype and enhanced metastasis. Lung cancer metastasis can be prevented by inhibiting NF-κB signaling in cancer cells or stopping the expression of TGFβ1 in platelets. So, we now know that blood platelets are not just physical shields, as once thought. Instead, they are a crucial source of TGFβ for cancer cells in the vasculature. They are necessary for tumor cell extravasation and metastasis. So, the metastatic potential of tumor cells continues to evolve outside of the primary tumor site, in response to interactions between the tumor and the host in the bloodstream and at the site of metastasis [372].

3.2 The difference between how prescription drugs, dietary supplements and ‘super-foods’ are developed and used

As described in section 3.1, prescription drugs are developed in a nonlinear, iterative process. There are several important steps in the process. They include select a disease, identify a target, verify the target, find a lead compound, quantify drug-target interactions, determine solubility, pharmacokinetics and toxicity, improve the lead compound, find the best way to deliver the drug to the patient, manufacture the drug, apply to the FDA to do investigational studies, do the clinical studies, apply to the FDA for drug approval and do post-registration studies [1]. In contrast, legal dietary supplements are developed and marketed in many different ways. Many of them do have specific therapeutic targets. For example, red yeast rice contains statins that target the HMG-CoA reductase enzyme that catalyzes the rate limiting step in the biosynthesis

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of cholesterol. Also, there are ACE inhibitors from natural sources like fish proteins and Hawthorn trees (*Crataegus* species). However, unlike prescription drugs, the concentrations of the active ingredients in dietary supplements are seldom known. Vitamins are important exceptions. The identity and dose of every vitamin are known and are indicated on the label. Similarly, the identity and dose of active ingredients in over-the-counter (OTC) medications are also known and indicated on the label. Some of them, such as Nexium®, or esomeprazole magnesium) were once prescription drugs. Nexium® is so safe and effective that it is now available without a prescription. Other examples include a nicotine patch and Nicorette® gum to help people stop smoking, Children's Advil and Children's Motrin (ibuprofen), Orudis KT and Actron (ketoprofen), and Aleve (naproxen sodium) for pain relief and fever reduction; Femstat 3 (butoconazole nitrate) for vaginal yeast infection; Pepcid AC (famotidine), Tagamet HB (cimetidine), Zantac 75 (ranitidine hydrochloride), Axid AR (nizatidine), and Prilosec OTC (omeprazole magnesium) for heartburn; Rogaine (minoxidil) for hair growth; and Claritin (loratadine), the first non-sedating antihistamine [373]. There are specific regulations that describe the process by which drugs can be switched from prescription to OTC drugs (Rx to OTC switch) [373].

There are also OTC drugs that were never prescription drugs. Perhaps the best example of this is aspirin. It was developed and brought to the market before the FDA and other countries' regulatory agencies were even formed. Even though physicians routinely recommend it for treating pain and fever in some patients, it can be very dangerous to others. For example, it should not be given to infants, children and adolescents because it has been linked to Reye's syndrome [374]. In addition, aspirin can cause gastrointestinal bleeding. More important, neither aspirin nor any other NSAIDs should be taken by people who have chronic kidney disease. Instead, they can take acetaminophen (Tylenol®). However, it can cause liver toxicity and even death if taken at too high of a dose (more than eight 500 mg doses per day) or when mixed with stronger pain medications.

It is unlikely that aspirin would receive approval from any government's regulatory agency if it were an IND, due to its adverse side effects. Still, millions of lives are saved by people who take a low dose of aspirin to prevent heart attacks. In fact, aspirin is part of the first aid for a heart attack. This raises an extremely important difference between prescription drugs, OTC drugs and dietary supplements. It is difficult, time-consuming and expensive to develop and get regulatory approval for prescription drugs. Even though many INDs may be shown to be safe and

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effective for most people, it only takes a few cases of adverse side effects to get the IND rejected. As a result, many INDs that could have helped millions of people never reach the market because they might hurt or kill a few hundred or a few thousand people. So, it can be very difficult or impossible for the millions to get the potentially beneficial IND. This is consistent with the Hippocratic Oath that physicians take. That is, "first do no harm". It also avoids a potentially very serious problem. It can be very difficult to determine if any single individual will benefit from an IND (or a prescription drug). For this reason, pharmaceutical companies are required by law to monitor and report all adverse side effects that occur in patients who take prescription drugs that are already approved.

This is in total contrast with OTC drugs and legal dietary supplements. Since large-scale phase III clinical trials are not done on these, it is possible that a few hundred out of the millions of people who take them could suffer from severe adverse side effects or even death without being noticed. This means that the millions of people who are helped by the OTC drugs and dietary supplements can still get them in this theoretical example. Besides, it is very difficult to establish in a court of law that an OTC drug or dietary supplement was the single cause of a health problem or even a person's death. It is just as difficult to establsih this with prescription drugs, but pharmaceutical companies err on the side of caution. They don't require proof before they stop trying to get an IND approved if only a small fraction of subjects in a clinical trial suffer adverse side effects. So, it's wong to claim that the FDA is in a conspiracy with physicians and pharmaceutical companies to keep people sick and prevent them from taking dietary supplements, so they will have to buy prescription drugs. The opposite is true. The FDA, physicians and pharmaceutical companies are in an open collaboration to prevent people from becoming sick and to treat them effectively with prescription drugs. Instead of a conspiracy to keep people from taking dietary supplements, there is an open collaboration to keep people from taking potentially harmful INDs and prescription drugs that are contraindicated due to a patient's specific state of health. No government organization or pharmaceutical company is tryng to keep people from having access to any legal dietary supplement that they might want to take. Similarly, physicians don't have the power to prevent people from taking dietary supplements. They can only warn them about the potential dangers or, when appropriate, recommend that they take some of them, like low dose aspirin, acetaminophen or vitamins.

This brings us to another extremely important difference between how people decide whether or not to take prescription drugs and dietary

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supplements. Quite often, the decision to take a prescription drug is made by a physician and not the patient. For example, if a person is brought to the emergency room of a hospital and is unconscious, a physician may prescribe a treatment without the patient even knowing about it. Even if the patient is conscious and simply in their physician's office, they can feel like they are being forced to take prescribed medicines. They might not blame the physician, but still feel that they have been victimized because it was not their decision. In contrast, the decision to take an OTC drug or most types of dietary supplements is made by the person who will take them and not a physician. This can make people feel empowered and in control of their own health. In addition, most people start taking dietary supplements because a trusted friend or family member recommended them. In total contrast to scientists and physicians, lay people and companies that sell dietary supplements use words like prove, as in "it has been proven to be effective in clinical trials". Scientists and physicians are taught to be skeptical and use language like I used in the Introduction to this book. That is, science can't prove anything. So, where a lay person might say that something is definitely true, a scientist or physician would say that there is a link between an IND and a disease or that evidence supports the hypothesis that it is safe and effective for those subjects that were in a clinical trial. Moreover, scientists and physicians know that bad, unhealthy things can happen to good, healthy people. They also realize that there is a medical equivalent to the Heisenberg uncertainty principle in chemistry that states that you can never know the exact position and momentum of an electron. The medical equivalent is that you can never know if any diet, drug or lifestyle will be effective on any one person. This is due to our current lack of knowledge and not an inherent property of people, as is the uncertainty principle for electrons. That is, using current technology, we can only predict that the diet, drug and/or lifestyle will improve the health and prevent diseases in most people. That is, even though medical research and clinical studies can show that a healthy diet and lifestyle as well as an IND or new drug will help most people in a large group, there is no guarantee that they will work for any individual person. In contrast, many people who market legal dietary supplements will claim that the supplement saved their lives and it will save yours – if you just try it. This is driven by huge market forces. If people are willing to spend billions of dollars on supplements that don't work, just think what they will spend on something that you can make them think does work.

There is another important difference between the ways that prescription drugs and dietary supplements are developed. Toxicologists are involved in the development of prescription drugs. They realize that all

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substances are potentially toxic if given at too high of a dose [1, 170]. That is, the dose is the poison [1, 170]. Unfortunately, many people who sell and/or consume dietary supplements think that if a little bit of a superfood or nutraceutical is good for you, then more of it is even better. For example, we all know that eating fruits and vegetables is healthy, as is drinking green tea. However, few people eat enough of them. Moreover, nobody can eat all of the thousands of fruits and vegetables that are available. So, some people think that a dietary supplement that contains a proprietary extract of countless fruits and vegetables would be just as good or even better [375]. Also, some people think that green tea extract or even the purified ingredient, EGCG, is healthier than just green tea. Similarly, many people take dietary supplements that contain other purified antioxidants, such as resveratrol, curcumin, pterostilbene, sulforaphene, quercetin, ellagic acid, genistein, luteolin and many others. Even though these and many other antioxidants can help prevent many types of cancer and other diseases, some of them may promote tumor metastasis, multidrug resistance and even cardiovascular diseases – especially those that stimulate the Nrf2/ARE antioxidant system [376-379]. However, there has not been enough research to determine conclusively which cancers metastasize more readily when any or all of these antioxidants are over-consumed. It is left up to each individual to decide whether or not to take such supplements. Still, it is always wise to let your primary care physician and oncologist know if you are thinking about taking these or any other dietary supplements or have actually started taking them. So, this raises three more key differences between prescription drugs and dietary supplements. Your primary care physician and oncologist will know about the drugs that they prescribe for you. They will be very confident that they work and they will be well aware of any contraindications and possible side effects. In contrast, they will only know which dietary supplements you are taking if you tell them. They will know little or nothing about the alleged effectiveness of most supplements (especially if they aren't effective). Note that some vitamins – especially folic acid and iron – are important exceptions. Physicians will often recommend them and be well aware of their health benefits – especially for pregnant women. Finally, very few physicians will know about the contraindications and frequently occurring adverse side effects of dietary supplements. In fact, very few people know about them – especially people who take them based on personal testimonials. So, it is quite possible that the same antioxidants that are quite healthy when consumed at low doses as part of a whole food (like pomegranates), might be quite dangerous when taken at high doses in a purified dietary supplement.

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This does not mean that all dietary supplements are dangerous – especially those that are legal. We simply don't have enough information about many of them to know and may never know for sure. This is especially true for dietary supplements for extending longevity that are made from natural ingredients. Pharmaceutical companies can't patent them, so very few dietary supplements have ever or will ever undergone clinical trials. This is especially true for any clinical trial for longevity. Such trials could take decades to conduct and cost billions of dollars. This means that people who take dietary supplements that supposedly extend one's lifespan or (more importantly), slow down the aging process and keep people healthy for much longer are taking a leap of faith. They may not work and may cause adverse side effects. Still, that is their choice. Moreover, most elderly people are more concerned about staying free from pain, full of energy and free from diseases as long as possible. It is not the total number of years that a person lives, but the number of healthy years that counts.

So, it is important to mention some dietary supplements that are based on sound science and may help accomplish this. Some of this science comes from learning about the pleiotropic effects of a prescription drug. As mentioned previously, the prescription drug metformin may accomplish these things due, in part, to its ability to stimulate AMPK [282]. That is, restoring the activity of AMPK in the elderly may not only increase longevity, but also help to fight the symptoms of aging [280]. That is because AMPK and mitochondrial function decrease with age, making us less energetic and more obese, while becoming more vulnerable to cancer and diseases associated with impaired DNA and protein function [280, 380]. In addition, the concentration of NAD^+ decreases with aging [380-382]. This can be prevented in mice by feeding them a precursor of NAD^+, nicotinamide mononucleotide, or NMN. It can restore the proper concentration of NAD^+ in mitochondria and other markers of mitochondrial function [380, 381]. So, clinical trials are being done so scientists can see if these effects also occur in humans [382]. A phase I trial of a supplement called NIAGEN™ was conducted to test the safety of two doses of 500 mg daily for six weeks in healthy, older subjects. It contains nicotinamide riboside (NR). It is also looking at physical and cognitive function, systemic inflammatory markers, and endothelial function. This is being followed by a phase II trial that will look at the effects of three different doses (100, 300 and 1000 mg) in 140 middle aged overweight healthy adults on urinary methylnicotinaide, as well as blood, urinary and muscle concentrations of NR metabolites, resting energy metabolism, branched chain amino acids and adverse side effects. At the

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same time, another supplement (BASIS^{TM}) contains both NR and pterostilbene, which activates the Nrf/ARE antioxidant system [383] and increases mitochondria function [384]. Pterostilbene is a dimethylated analogue of resveratrol. Their structures are shown in Figures 23 and 24. Its safety was tested in a phase I clinical trial by giving 120 older adults either the recommended dose (250 mg NR plus 50 mg pterostilbene), twice the recommended dose or a placebo every day for eight weeks. No serious adverse side effects were seen and the concentration of NAD^+ in the blood increased by 40-90% [382]. So, it is still not known if adding pterostilbene to NR makes the supplement better or worse. If these were INDs that were being evaluated as possible new prescription drugs, the effects on the Nrf2/ARE antioxidant system, multi-drug resistant cancer and cardiovascular diseases would probably be determined before any regulatory agency would make a decision about them. This would take many years. In the meantime, both supplements are readily available and are widely consumed.

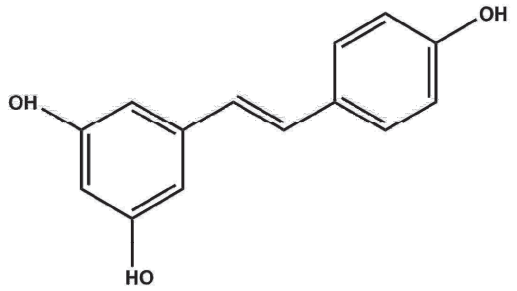

Figure 23. 2D structure of resveratrol, also known as *trans*-3,5,4'-trihydroxystilbene and 5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol (the IUPAC name), $C_{14}H_{12}O_3$.

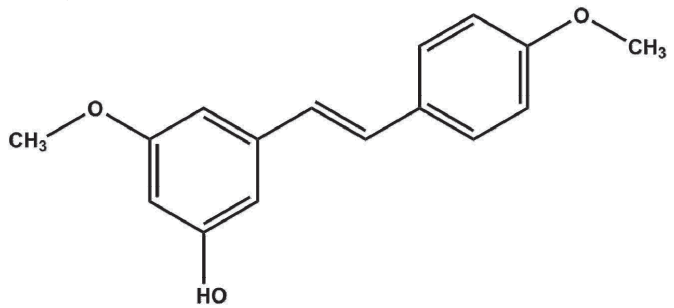

Figure 24. 2D structure of pterostilbene, also known as 3',5'-dimethoxy-4-stilbenol and 4-[(*E*)-2-(3,5-dimethoxyphenyl)ethenyl]phenol (the IUPAC name), $C_{16}H_{16}O_3$.

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At the same time, J. Craig Venter has started a new company, Human Longevity Inc™ (HLI) [385]. Its goal is "to give everyone access to the power of data-driven health intelligence. HLI combines state-of-the-art DNA sequencing and expert analysis with machine learning, to help change medicine to a more data-driven science. With a multidisciplinary team of research scientists, computing experts and physicians, we are empowering every part of the healthcare system to work more efficiently" [385]. They have already published the results of deep DNA sequencing of 10 545 human genomes [386]. A wholly owned subsidiary of HLI, called The Health Nucleus does extensive testing of clients [387]. They analyze their genome, gait and fecal microbiome. Clients get a prognosis of their present and future state of health. The data gives them control to modify their diet and behavior [387]. In addition, Google and Arthur D. Levinson started a company called Calico that aims to "harness advanced technologies to increase our understanding of the biology that controls lifespan. We will use that knowledge to devise interventions that enable people to lead longer and healthier lives" [388]. There are other companies that are using data-driven –omics data to improve wellness and/or longevity by providing personalized advice on nutrition [389]. They include Arivale [390], Day Two [391] and Google Baseline [392]. There are even some companies that do personalized analyses of microbiomes [389]. They include American Gut [393], Enterome [394], Second Genome [395], Seres Health [396] and Vedanta Bioscience [397]. So, this is another example of how industry is working with people in an open collaboration to improve health. None of these companies or organizations is in a conspiracy to keep people sick.

3.2.1 'Superfoods'

Dietary supplements are not the only thing that has captured the imagination of the public. So have 'superfoods', functional foods, herbal medicines and nutraceuticals [398-403]. Superfoods have been defined many different ways. For example, they have been called a subset of functional foods, which have "special constituents that possess advantageous physiological effects" [398, 399]. However, 'superfoods', functional foods, herbs and nutraceuticals have no legal, scientific or regulatory definition [398, 400, 401]. Existing definitions in dictionaries can illustrate the confusion. That is, The Oxford English Dictionary defines an herb as "a plant of which the stem does not become woody and persistent (as in a shrub or a tree) but remains more or less soft and

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succulent, and dies down to the ground (or entirely) after flowering; spec. applied to plants of which the leaves, or stem and leaves, are used for food or medicine, or in some way for their scent or flavor" [401]. Based on this definition, many of the products sold as 'herbal remedies' or 'herbal medicines' are not really herbs. This includes the Amazonian fruit called açaí, which is picked from three different species of trees (*Euterpe oleracea* Mart., *E. edulis* Mart. and *E. precatoria* Mart.). This should not be too surprising since even the term 'food' has many different definitions in different cultures. Some things that are considered to be food in one culture are thought to be sinful or even deadly poisons in others.

The term 'superfood' is not used as often in scientific communications as functional food [400]. 'Superfood' is used widely in marketing, on food packages and in the popular media. Its use was clarified in a recent Ph.D. thesis that used systems thinking to describe the history of 'superfoods'. That is, it used a 'biography-of-things' approach that used assemblage theory, actor network theory and circuits of culture theory. It was an interdisciplinary approach that included nutritional science, analysis of liberal trade agreements, traditional indigenous knowledge, food processing and transportation technologies. This approach led to an understanding of how "our cultural obsessions and anxieties feed into practices of science, and the sciences reciprocally nourish our cultural fixations". Even though this can make it easy to dismiss 'superfoods' as a marketing tool designed to entice unknowing consumers, it is important to recognize the real fears that consumers have [400].

Certainly, one of the main goals of this book is to recognize these fears and the fact that many people, through no fault of their own, don't trust science in general or pharmacy in particular. Hopefully, the information in this book – and especially in this section will provide useful information to scientists, physicians and people who work in the pharmaceutical industry so they can rebut false claims and misinformation that are all too common. This is especially important when describing 'superfoods' and contemporary 'superfood' cults [398].

That is, a type of cult has emerged from the marketing that tries to increase the sales of 'superfoods' [398]. This cult was built through Critical Discourse Analysis (CDA). "CDA describes the values, conventions and understandings that are made and reinforced by advertisements" [398]. These values, conventions and understandings connect with larger "social processes and structures which give rise to the production of a text, and of social structures and processes within which individuals or groups as social historical subjects create meanings in their interactions with texts" [398, 402]. In the process, CDA reveals "political,

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economic and sociocultural assumptions while also considering the power relationships that constitute and divide interested parties. The discursive framework of neoliberalism, gender bias and nutritionism are the most important structures that make up the discussions about superfoods" [398]. Neoliberalism "uses the language of commercialism, privatization and deregulation to encourage individualism, competition and consumption. Moreover, 'superfood' advertisements overwhelmingly target women. Nutritionism uses reductionist thinking to disconnect food from its production and eventual consumption, while minimizing the differences between whole and processed foods (such as "nutritious" candy bars and beverages)" [398]. As a result, 'superfoods' are advertised as having exceptional (or superior) nutritional benefits making them capable of preventing diseases. It uses gender bias to encourage girls and women to focus on improving their appearance or beauty [398], which is also a social construct, subject to the whims of fashion and with no scientific basis. The term 'superfood' can give many foods and beverages a 'healthy-halo' which influences consumer preferences [403].

This type of confusion has led some to call for stricter regulations and a food fraud policy to regulate false health claims of 'superfoods' like açaí [404]. Even though açaí has many potential health benefits [405], there is much confusion about it. For many years, it was marketed in the USA and other countries as a 'fat burner' that can help a person lose weight. In fact, açaí has been consumed for centuries by under-nourished natives of the Amazon rain forest as an excellent source of calories. It has also become popular among endurance athletes because it is a good source of calories, without having much sugar. Even though açaí berries may have very little fructose, glucose, sucrose or any other free sugar, they do contain carbohydrates as fatty acyl glycosides that are not soluble in water and are covalently bound to fats, which are not soluble in hexane or chloroform. That is, the concentration of total free sugars (also known as total soluble solids or TSS) in fruits and vegetables is determined by analyzing either an aqueous extract or a juice (which is also an aqueous solution). This would not detect fatty acyl glycosides. Also, the fat content of fruits, vegetables as well as other foods and biological samples is measured by extracting them with either hexane or chloroform – neither of which will solubilize or extract fatty acyl glycosides. They are extracted using methanol [406, 407]. In addition, the vast majority of articles written about the composition of açaí indicate that it contains fatty acids, when it actually contains triacylglycerides [408]. That is, unlike the short chain fatty acids (SCFAs) that were discussed in Chapter 2 of this book, the fatty acyls in triacylglycerides don't have a carboxylic acid functional group (-COOH)

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and are not acids. Generalized structures of triacylglycerides and a fatty acyl glycoside found in the 'superfood' called noni (*Morinda citrifolia*) [406] are shown in Figures 25 and 26.

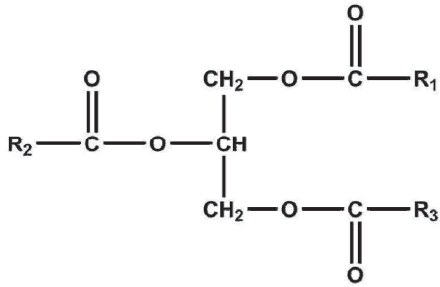

Figure 25. 2D generalized structure of a triacylglyceride, where the R_1, R_2 and R_3 groups are hydrocarbons, such as $–(CH_2)_{14}CH_3$ in tripalmitoylglyceride.

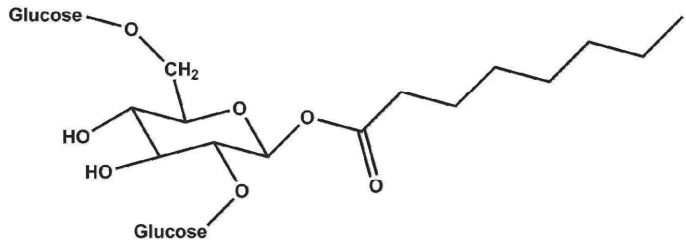

Figure 26. 2D structure of a fatty acyl glycoside found in noni (*Morinda citrifolia*). Its IUPAC name is 2,6-di-*O*-(*α*-D-glucopyranosyl)-1-*O*-octanoyl-*α*-D-glucopyranose. It is also known as nonioside B.

Nutraceuticals are often considered to be even better than 'superfoods' [409]. Some scientists think that they can be especially useful in preventing diseases. In at least one journal article, nutraceuticals were considered to be extracts of plants or animals that are concentrated and administered almost as if they were prescription drugs. They defined nutraceuticals as "a food or part of a food that provides benefits health in addition to its nutritional content" [409]. Even though many nutraceuticals may have important health benefits for some people, it can be difficult or impossible to know who will benefit and who will suffer from adverse side effects. In addition, they seem to be based on reductionist thinking, in which a linear dose-response effect is expected – even at high doses. This ignores the very real possibility that some ingredients, like pterostilbene or

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resveratrol activate the Nrf2/ARE antioxidant response, which is over-activated in multidrug resistant cancer cells and some types of cardiovascular diseases [307-309].

More importantly, it is important to use systems thinking, in which the whole diet is important and not the inclusion of any specific ingredient or 'superfood'. For example, cinnamon (*Cinnamomum zeylanicum* and *C. cassia*) has many health benefits [410, 411]. However, when it is consumed as part of a sweet roll made from white flour, *trans* fats and large amounts of sucrose, its health benefits are outweighed by the harm done by over-consumption of these unhealthy major ingredients. Moreover, there are very few legal substances that can counter the harmful effects caused by consuming too many calories and simple carbohydrates by simply adding them to your diet. There might be some exceptions. Many spices (like Cayenne pepper) can make foods tastier to some people. At the same time, one of their main health benefits is that they tend to make a person eat slower and feel full faster (increased satiety), so they eat less. Similarly, whole fruits, vegetables, seeds and nuts contain dietary fiber that increases satiety. Finally, if all it takes for a food to be included in the list of 'superfoods'or functional foods is that they have healthy ingredients with advantageous physiological effects, then almost all foods could be classified this way. If one were to stretch this definition further, even unhealthy candies that are full of sucrose and artificial dyes could be said to be functional foods. They do make children (and many adults) feel happy, which is usually beneficial to one's health. However, systems-based thinking will help their parents realize that this happiness is only temporary and candy has many adverse health effects. So, this is another example of how reductionist thinking can be dangerous. It can lead some to believe that some foods are superior, no matter how they are prepared and regardless of what else one eats even though their total diet can be quite unhealthy and hazardous to their health. Still, some fruits and vegetables can be made healthier by increasing their levels of essential vitamins, antioxidants and phytochemicals. This can be done through proper post-harvest treatment and metabolic engineering [411].

At the same time, there are many foods and spices that can be an important part of a healthy, balanced diet. One of them is turmeric (*Curcuma longa*) which contains many anti-inflammatory phenolic compounds called cucurminoids. This includes curcumin, which is also available as a purified compound in dietary supplements. Its structure is shown in Figure 27. It is readily soluble in olive oil, other vegetable oils and in organic solvents that have low polarity. It has very low solubility in water. However, as a beta-diketone, it is readily hydrolyzed by water. So,

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if one adds curcumin to one's hot tea or soup, it will break down into compounds that don't provide the same health benefits that curcumin and turmeric have [412]. Curcumin can also act synergistically with the polyunsaturated fatty acid, DHA to help improve brain health and prevent anxiety disorders [413]. However, some sources of turmeric have been found to be contaminated with lead chromate [414], in which the chromium is in the +6 oxidation state, making it a known human carcinogen. Chromate (as either chromate or potassium dichromate) is yellow, as is curcumin. So, it can be very difficult to tell just by looking at it whether or not a sample of curcumin is contaminated with this deadly poison. However, chromate salts (including lead chromate) are readily soluble in water. So, you can do a quick test on any sample that you may have. You can put some of your turmeric root or powder into a glass or cup of water and see if the water stays clear or if it quickly turns yellow. If it turns yellow, it may be contaminated with lead chromate. Still, you don't want to confuse this with the yellow color that will appear if you add curcumin to boiling water or cook with it, since its reaction product is yellow and water soluble. It is probably better to just dissolve turmeric powder in a vegetable oil (such as olive oil) and add it to your food after it is cooked. On the other hand, the curcuminoids in whole turmetic root are inside plant cells that are surrounded by relatively impermeable cell walls. So, it takes much longer for water to enter and hydrolyze the curcumin that is in it. However, after cutting off a piece of turmeric the remaining part can turn a dark red due to polyphenol oxidade enzymes being activated. The products of this reaction are not water-soluble. Similarly, other fruits (like bananas) can develop dark spots when they are bruised or cut.

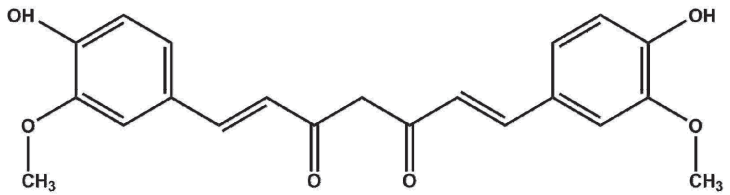

Figure 27. 2D structure of curcumin, $C_{21}H_{20}O_6$, molecular weight 368.39 g/mol. Its preferred IUPAC name is (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

However, there is another potentially serious limitation to consuming curcumin and turmeric. A recent study showed that a relatively low concentration of curcumin (10 μM) can increase the ability of free iron (as Fe^{2+}) to cause cancer [415]. As we will see in Chapter 2 of the second

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volume, unliganded or poorly liganded iron can undergo a reaction with naturally produced hydrogen peroxide to produce very harmful free radicals that can help cause not just cancer, but also cardiovascular and neurodegenerative diseases. The main sources of dietary iron are red meat and multivitamin supplements. Since these were not consumed in ancient India, Ayurvedic medicine recognized whole turmeric as a very healthy food or spice. So, turmeric may be much more effective in preventing cancer in vegetarians than in people who eat red meat.

3.2.2 Dietary supplements: from amphetamine to fish oil to vitamin D and folic acid

Contamination, adulteration and fraud are also big problems with illegal dietary supplements. Perhaps the most concerning was a weight loss supplement that was sold over the black market by calling a friend or a cell phone number provided on internet message boards [416]. It contained 100-120 mg of amphetamine per capsule. It was not available in stores or even on freely accessible websites. It was sold as capsules with no imprint on them and no list of ingredients on the label [416]. There are many other examples of mislabeled and/or adulterated dietary supplements.

The most common consumer complaints made to the FDA are due to adulterated dietary supplements. The FDA maintains a website on dietary supplements in which consumers, physicians and poison centers can obtain information and report an adverse event [417]. Adulterated supplements not only can cause illness and death, but also can cause consumers of such products to lose their jobs when they fail a urine test because they consumed a supplement that contained an illegal drug, even though its presence was not indicated on the label of the supplement [418]. Moreover, even though the FDA recalled many dietary supplements because they contained banned ingredients, many of them still contained those toxic ingredients in supplements that were purchased at least six months after they were recalled [419]. So, the FDA has issued a warning about weight loss dietary supplements [420].

In addition, the FDA and other countries' regulatory agencies have been analyzing dietary supplements for illegal adulterants [421]. These include narcotics (such as amphetamine, heroin, hydrocodone and other opioids), steroids, hormones (including flutamide, which is used for transgender hormone therapy), male sexual enhancement aids, alkaloids, a toxin in monkshood (*Aconitum variegatum*), a toxin in mushrooms, detergents, statins, muscle relaxants, antihistamines, stimulants, analgesics, antidepressants, diuretics, a cardiac glycoside (ouabain) that is used by

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indigenous people as an arrow poison, a laxative, nasal decongestants, anticonvulsants, estrogen mimitics (bisphenol A or BPA), antidiabetes drugs and a bronchodilator. A list of 242 such adulterants is shown in Table 4.

Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) can separate analytes from each other and measure the *m/z* of ions with sufficient accuracy to unequivocally determine the molecular formula of the parent compound. That is, some compounds may have the same masses to within ± 0.5 Dalton, such as cyclohexane (C_6H_{12}), cyclopentanone C_5H_8O and cyanopropylamine ($C_4H_8N_2$). However, their exact masses are 84.0939, 84.0575 and 84.0688, respectively. So, HRMS with a resolution of 140 000 at *m/z* of 200 and <1 ppm mass accuracy can distinguish between them. So, the molecular formulas and masses of the molecular adducts with one proton (H^+) attached (abbreviated as $[M+H]^+$) that are produced by positive ion LC-HRMS is included in Table 4. Actually, MS measures the mass to charge ratio, which is abbreviated as *m/z*. The *m/z* value of each of the molecular adducts is abbreviated as Q_1. Since all the $[M+H]^+$ ions listed have a charge of +1, the *m/z* is equal to the molecular weight plus one (due to the H). The data can be used to do a non-targeted analysis of dietary supplements for illegal and banned substances as well as bioactive ingredients that are not on the product's label. In the field of MS, a method is not usually considered to be valid if only the mass of one molecular adduct is used. An analyst must also look for other molecular adducts (such as $[M+Na]^+$ and $[M+NH_4]^+$) or fragments that are produced. This is explained in more detail in the Appendix, which is a primer on analytical chemistry, including mass spectrometry (MS). It also contains a Table that lists the *m/z* values of two other molecular adducts that are abbreviated as Q_2 and Q_3. Note that some laboratories might want to detect some of these adulterants as negative ions. This might include phenolic compounds like the ubiquitous environmental toxin BPA. Its molecular formula is $C_{15}H_{16}O_2$ and its molecular weight (or mass) is 228.286 Daltons (228.286 Da). Its 2D structure was shown in Figure 28. The molecular formula of 228.286 takes into consideration that 1% of carbons are the isotope ^{13}C and not ^{12}C. It also accounts for the natural abundance of ^{2}H and ^{17}O isotopes. Likewise, the molecular formulas of all the compounds shown in Figures throughout this book take into account the natural abundances of the isotopes of the atoms that make them up. However, HRMS can distinguish between $C_{15}H_{16}O_2$ in which all the carbons, hydrogens and oxygens have masses of 12.0000, 1.007825035 and 15.99491463 Da, respectively, and $C_{15}H_{16}O_2$ in which one of the carbons has a mass of 13.003355 Da. The molecular

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weight of the form of BPA that contains only the major isotopes is called the monoisotopic mass. So, mass spectroscopists are more interested in the monoisotopic mass than in the molecular weight (or molecular mass) that accounts for the natural abundance of isotopes, especially ^{13}C. The point for now is that BPA can be detected as either a positive ion – usually the H^+ adduct that is abbreviated as $[M+H]^+$ or as a negative ion in which the BPA has lost a hydrogen (abbreviated as $[M-H]^-$). So, the monoisotopic mass of BPA is 228.115036 Da. The monoisotopic mass of the $[M+H]^+$ ion is 229.12231 and the monoisotopic mass of the $[M-H]^-$ ion is 227.107211.

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Table 4. Analytes in illegal dietary supplements, with molecular formulas, Q_1 values and adducts [421].

Compound	Type	Formula	Q_1 (*m/z*)	Adduct
1,3-DMAA dimethylamylamine	Stimulant	$C_7H_{17}N$	116.14338	M+H
1,3-DMBA 1,3-bimethylbutylamine	Stimulant	$C_6H_{15}N$	102.12773	M+H
17-α-Ethynylestradiol	Hormone	$C_{20}H_{24}O_2$	297.18491	M+H
17-α-Methyltestosterone	Hormone	$C_{20}H_{30}O_2$	303.23186	M+H
17-β-Estradiol	Hormone	$C_{18}H_{24}O_2$	273.18491	M+H
19-Norandrostenedione	Steroid	$C_{18}H_{24}O_2$	273.18491	M+H
1-Androstenediol	Steroid	$C_{19}H_{30}O_2$	291.23186	M+H
1-Androstenedione	Steroid	$C_{19}H_{26}O_2$	287.20056	M+H
1-Testosterone	Hormone	$C_{19}H_{28}O_2$	289.21621	M+H
2,4-DNP, or 2,4-dinitrophenol	Weight loss	$C_6H_4N_2O_5$	185.0193	M+H
2-Hydroxypropylnortadalafil CIALIS®	Male sexual enhancement	$C_{24}H_{23}N_3O_5$	434.17105	M+H
2-Methoxyamphetamine	Stimulant	$C_{10}H_{15}NO$	166.12264	M+H
3-Methoxyamphetamine	Stimulant	$C_{10}H_{15}NO$	166.12264	M+H
4-Hydroxytestosterone	Stimulant	$C_{19}H_{28}O_3$	305.21112	M+H
4-*para*-Nonylphenol	Detergent	$C_{15}H_{24}O$	221.18999	M+H
4-tert-Octylphenol	Detergent	$C_{14}H_{22}O$	207.17434	M+H
5-HTP 5-Hydroxytryptophan	Aid for mental health[a]	$C_{11}H_{12}N_2O_3$	221.09207	M+H
7-Hydroxymitragynine	Alkaloid in Kratom	$C_{23}H_{30}N_2O_5$	415.22275	M+H
α-Amanitin	Toxin in mushrooms	$C_{39}H_{54}N_{10}O_{14}S$	919.36144	M+H
Acetaminophen	Analgesic	$C_8H_9NO_2$	152.07061	M+H
Acetildenafil	Male sexual enhancement	$C_{25}H_{34}N_6O_3$	467.27652	M+H
Acetyl vardenafil	Male sexual enhancement	$C_{25}H_{34}N_6O_3$	467.27652	M+H
Aconitine	Toxin from monkshood	$C_{34}H_{47}NO_{11}$	646.32219	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Aminopterin	Cancer chemotherapy	$C_{19}H_{20}N_8O_5$	441.16294	M+H
Aminotadalafil	Male sexual enhancement	$C_{21}H_{18}N_4O_4$	391.14008	M+H
Aminotadalafil condensation product	Male sexual enhancement	$C_{27}H_{21}N_4O_6$	498.15339	M+H
Amphetamine (R, -)	Stimulant	$C_9H_{13}N$	136.11208	M+H
Apomorphine	Opioid	$C_{17}H_{17}NO_2$	268.13321	M+H
Aspirin	Analgesic	$C_9H_8O_4$	203.03148	M+Na
Atorvastatin	Statin for dyslipidemia	$C_{33}H_{35}FN_2O_5$	559.26028	M+H
Atropine	Analgesic	$C_{17}H_{23}NO_3$	290.17507	M+H
Avanafil	Male sexual enhancement	$C_{23}H_{26}ClN_7O_3$	484.18584	M+H
β-alanine	Athletic performance enhancer[b]	$C_3H_7NO_2$	90.05496	M+H
Benfluorex	Weight loss	$C_{19}H_{20}F_3NO_2$	352.15189	M+H
Benzphetamine	Weight loss	$C_{17}H_{21}N$	240.17468	M+H
Berberine	Alkaloid	$C_{20}H_{18}NO_4$	337.13086	M+H
Bisphenol A	Estrogen mimetic	$C_{15}H_{16}O_2$	229.12231	M+H
Bolandiol	Steroid	$C_{18}H_{28}O_2$	277.21621	M+H
Bolasterone	Steroid	$C_{21}H_{32}O_2$	317.24751	M+H
Boldenone	Steroid	$C_{19}H_{26}O_2$	287.20056	M+H
Boldione	Steroid	$C_{19}H_{24}O_2$	285.18491	M+H
Brompheniramine	Antihistamine	$C_{16}H_{19}BrN_2$	319.08044	M+H
Brucine	Alkaloid	$C_{23}H_{26}N_2O_4$	395.19653	M+H
Bumetanide	Prescription diuretic	$C_{17}H_{20}N_2O_5S$	365.11657	M+H
Bupropion	Antidepressant	$C_{13}H_{18}ClNO$	240.11497	M+H
Caffeine	Stimulant	$C_8H_{10}N_4O_2$	195.08765	M+H
Calusterone	Steroid	$C_{21}H_{32}O_2$	317.24751	M+H
Captopril	Anti-hypertension	$C_9H_{15}NO_3S$	218.08454	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Cerivastatin	Statin dyslipidemia	$C_{26}H_{34}FNO_5$	460.24938	M+H
Cetilistat	Weight loss	$C_{25}H_{39}NO_3$	402.30027	M+H
Chloropretadalafil	Male sexual enhancement	$C_{22}H_{19}ClN_2O_5$	427.10553	M+H
Chlorpheniramine	Antihistamine	$C_{16}H_{19}ClN_2$	275.13095	M+H
Chlorzoxazone	Muscle relaxant	$C_7H_4ClNO_2$	170.00033	M+H
Cimetidine	Antihistamine	$C_{10}H_{16}N_6S$	253.12299	M+H
Citric Acid	Bind to metals to increase water solubility	$C_6H_8O_7$	193.03428	M+H
Clenbuterol	Weight loss	$C_{12}H_{18}Cl_2N_2O$	277.0869	M+H
Clenbuterol Hydrochloride	Weight loss	$C_{12}H_{18}Cl_2N_2O$	277.0869	M+H
Clostebol	Steroid	$C_{19}H_{27}ClO_2$	323.17723	M+H
Codeine	Analgesic	$C_{18}H_{21}NO_3$	300.15942	M+H
Colchicine	Alkaloid	$C_{22}H_{25}NO_6$	400.17546	M+H
Cortisone Acetate	Steroid	$C_{23}H_{30}O_6$	403.21152	M+H
Cyclopentynafil	Male sexual enhancement	$C_{26}H_{36}N_6O_4S$	529.25915	M+H
Cyproheptadine	Antihistamine	$C_{21}H_{21}N$	288.17468	M+H
Danazol	Steroid	$C_{22}H_{27}NO_2$	338.21146	M+H
Dapoxetine	Male sexual enhancement	$C_{21}H_{23}NO$	306.18524	M+H
Dehydrochlormethyltestosterone	Steroid	$C_{20}H_{27}ClO_2$	335.17723	M+H
Desmethylsildenafil	Male sexual enhancement	$C_{21}H_{28}N_6O_4S$	461.19655	M+H
Desoxymethyltestosterone	Steroid	$C_{20}H_{32}O$	289.25259	M+H
Desvenlafaxine	Antidepressant	$C_{16}H_{25}NO_2$	264.19581	M+H
Dexamethasone	Steroid	$C_{22}H_{29}FO_5$	393.20718	M+H
Dextroamphetamine Sulfate	Stimulant	$C_9H_{13}N$	136.11208	M+H
Dextromethorphan	Cough suppressant	$C_{18}H_{25}NO$	272.20089	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
DHEA Dehydroepiandrosterone	Steroid	$C_{19}H_{28}O_2$	289.21621	M+H
DHEA (minus H_2O)	Steroid	$C_{19}H_{26}O$	271.20564	M+H
Diclofenac	Analgesic	$C_{14}H_{11}Cl_2NO_2$	296.02396	M+H
Diclofenac Sodium Salt	Analgesic	$C_{14}H_{11}Cl_2NO_2$	296.02396	M+H
Diethylpropion	Weight loss	$C_{13}H_{19}NO$	206.15394	M+H
Digitoxigenin	Steroid	$C_{23}H_{34}O_4$	375.25299	M+H
Digitoxin	Steroid	$C_{41}H_{64}O_{13}$	765.44197	M+H
Digoxigenin	Steroid	$C_{23}H_{34}O_5$	391.2479	M+H
Digoxin	Steroid	$C_{41}H_{64}O_{14}$	781.43688	M+H
Dimethylsildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_4S$	489.22785	M+H
Diosgenin	Steroid	$C_{27}H_{42}O_3$	415.32067	M+H
Dithiodesmethylcarbodenafil	Male sexual enhancement	$C_{23}H_{30}N_6OS_2$	471.19953	M+H
Drostanolone	Steroid	$C_{20}H_{32}O_2$	305.24751	M+H
Emetine	Antiemetic	$C_{29}H_{40}N_2O_4$	481.30608	M+H
Ephedrine Hydrochloride	Stimulant	$C_{10}H_{15}NO$	166.12264	M+H
Epigallocatechin gallate (EGCG)	Antioxidant	$C_{22}H_{18}O_{11}$	459.09219	M+H
Eserine	Alkaloid	$C_{15}H_{21}N_3O_2$	276.17065	M+H
Estrone	Steroid	$C_{18}H_{22}O_2$	271.16926	M+H
Ethylestrenol	Steroid	$C_{20}H_{32}O$	289.25259	M+H
Fenfluramine	Weight loss	$C_{12}H_{16}F_3N$	232.13076	M+H
Finasteride	Steroid	$C_{23}H_{36}N_2O_2$	373.28495	M+H
Fluoxetine	Antidepressant	$C_{17}H_{18}F_3NO$	310.14133	M+H
Fluoxymesterone	Steroid	$C_{20}H_{29}FO_3$	337.21735	M+H
Flutamide	Transgender hormone therapy	$C_{11}H_{11}F_3N_2O_3$	277.07945	M+H
Fluvastatin	Statin dyslipidemia	$C_{24}H_{26}FNO_4$	412.19186	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Formebolone	Steroid	$C_{21}H_{28}O_4$	345.20604	M+H
Furazabol	Steroid	$C_{20}H_{30}N_2O_2$	331.238	M+H
Furosemide	Diuretic	$C_{12}H_{11}ClN_2O_5S$	331.015	M+H
Furosemide (-NH3)	Diuretic	$C_{12}H_8ClNO_5S$	313.98845	M+H
Gamma Aminobutyric Acid (GABA)	Neurotransmitter	$C_4H_9NO_2$	104.07061	M+H
Gendenafil	Male sexual enhancement	$C_{19}H_{22}N_4O_3$	355.17647	M+H
Genipin	Cardiovascular disease[a]	$C_{11}H_{14}O_5$	227.0914	M+H
Gestrinone	Steroid	$C_{21}H_{24}O_2$	309.18491	M+H
Glibenclamide	Antidiabetic drug	$C_{23}H_{28}ClN_3O_5S$	494.1511	M+H
Glutamine	Amino acid	$C_5H_{10}N_2O_3$	147.07642	M+H
Heroin	Opioid	$C_{21}H_{23}NO_5$	370.1649	M+H
Homosildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_4S$	489.22785	M+H
Hordenine	Alkaloid	$C_{10}H_{15}NO$	166.12264	M+H
Hydrastine	Alkaloid	$C_{21}H_{21}NO_6$	384.14416	M+H
Hydrochlorothiazide	Weight loss diuretic	$C_7H_8ClN_3O_4S_2$	297.97175	M+H
Hydrocodone	Opioid	$C_{18}H_{21}NO_3$	300.15942	M+H
Hydrocortisone	Steroid	$C_{21}H_{30}O_5$	363.2166	M+H
Hydroxyacetildenafil	Male sexual enhancement	$C_{25}H_{34}N_6O_4$	483.27143	M+H
Hydroxyhomosildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_5S$	505.22277	M+H
Hydroxythiohomosildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_4S_2$	521.19992	M+H
Hyoscyamine	Relieves muscle cramps	$C_{17}H_{23}NO_3$	290.17507	M+H
Ibuprofen	Analgesic	$C_{13}H_{18}O_2$	207.13796	M+H
Ibuprofen (Observed Mass)	Analgesic		393.20938	M+
Icariin	Male sexual enhancement	$C_{33}H_{40}O_{15}$	677.244	M+H
Isopiperazinonafil	Male sexual enhancement	$C_{25}H_{34}N_6O_4$	483.27143	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Ketoconazole	Treatment for fungal infections	$C_{26}H_{28}Cl_2N_4O_4$	531.15604	M+H
L-DOPA	Neurotransmitter	$C_9H_{11}NO_4$	198.07608	M+H
Leucine/isoLeucine	Amino acid	$C_6H_{13}NO_2$	132.10191	M+H
Levorphanol	Opioid	$C_{17}H_{23}NO$	258.18524	M+H
Lobeline	Alkaloid	$C_{22}H_{27}NO_2$	338.21146	M+H
Lodenafil Carbonate	Male sexual enhancement	$C_{47}H_{62}N_{12}O_{11}S_2$	1035.41752	M+H
Lorcaserin	Weight loss	$C_{11}H_{14}ClN$	196.08875	M+H
Lovastatin	Statin for dyslipidemia	$C_{24}H_{36}O_5$	405.26355	M+H
Lovastatin Hydroxy Acid	Statin for dyslipidemia	$C_{24}H_{38}O_6$	423.27412	M+H
Lovastatin Related Compound A	Statin for dyslipidemia	C24H38O5	407.2792	M+H
Mazindol	Stimulant	$C_{16}H_{13}ClN_2O$	285.07892	M+H
Megestrol (acetate)	Steroid	$C_{24}H_{32}O_4$	385.23734	M+H
Melatonin	Sleep aid	$C_{13}H_{16}N_2O_2$	233.12845	M+H
Meldonium	Stimulant	$C_6H_{15}N_2O_2$	148.12063	M+H
Mestanolone	Steroid	$C_{20}H_{32}O_2$	305.24751	M+H
Mesterolone	Steroid	$C_{20}H_{32}O_2$	305.24751	M+H
Metenolone	Steroid	$C_{20}H_{30}O_2$	303.23186	M+H
Metformin (Glucophage®)	For diabetes	$C_4H_{11}N_5$	130.10872	M+H
Methandienone	Steroid	$C_{20}H_{28}O_2$	301.21621	M+H
Methandriol	Steroid	$C_{20}H_{32}O_2$	305.24751	M+H
Methasterone	Steroid	$C_{21}H_{34}O_2$	319.26316	M+H
Methylephedrine, N-	Weight loss	$C_{11}H_{17}NO$	180.13829	M+H
Methyl Pseudoephedrine, N-	Weight loss	$C_{11}H_{17}NO$	180.13829	M+H
Methylsynephrine (oxilofrine)	Weight loss	$C_{10}H_{15}NO_2$	182.11756	M+H
Methyl-1-testosterone	Hormone	$C_{20}H_{30}O_2$	303.23186	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Methyldienolone	Steroid	$C_{19}H_{26}O_2$	287.20056	M+H
Methylnortestosterone	Steroid	$C_{19}H_{28}O_2$	289.21621	M+H
Methylphenidate (Ritalin)	Stimulant and treatment for ADHD	$C_{14}H_{19}NO_2$	234.14886	M+H
Methyltestosterone	Steroid	$C_{20}H_{30}O_2$	303.23186	M+H
Metribolone (methyltrienolone)	Steroid	$C_{19}H_{24}O_2$	285.18491	M+H
Mibolerone	Steroid	$C_{20}H_{30}O_2$	303.23186	M+H
Minoxidil (Rogaine®)	Anti-hypertension and baldness	$C_9H_{15}N_5O$	210.13494	M+H
Mitragynine	Alkaloid in Kratom	$C_{23}H_{30}N_2O_4$	399.22783	M+H
Modafinil	For narcolepsy	$C_{15}H_{15}NO_2S$	274.08963	M+H
MPEA (Methylphenylethylamine)	Weight loss	$C_9H_{13}N$	136.11208	M+H
Nandrolone	Steroid	$C_{18}H_{26}O_2$	275.20056	M+H
Naproxen	Analgesic	$C_{14}H_{14}O_3$	231.10157	M+H
n-Butylnortadalafil	Male sexual enhancement	$C_{25}H_{25}N_3O_4$	432.19178	M+H
N-desethyl vardenafil	Male sexual enhancement	$C_{21}H_{28}N_6O_4S$	461.19655	M+H
N-Desmethylsibutramine	Weight loss	$C_{16}H_{24}ClN$	266.167	M+H
N-Didesmethylsibutramine	Weight loss	$C_{15}H_{22}ClN$	252.15135	M+H
N-Formyl-N,N-didesmethylsibutramine	Weight loss	$C_{16}H_{22}ClNO$	280.14627	M+H
Nitrosoprodenafil	Male sexual enhancement	$C_{27}H_{35}N_9O_5S_2$	630.22753	M+H
N-Octylnortadalafil	Male sexual enhancement	$C_{29}H_{33}N_3O_4$	488.25438	M+H
Noopept (N-phenylacetyl-L-prolylglycine ethyl ester)	Nootropic	$C_{17}H_{22}N_2O_4$	319.16523	M+H
Noracetildenafil	Male sexual enhancement	$C_{24}H_{32}N_6O_3$	453.26087	M+H
Norbolethone	Steroid	$C_{21}H_{32}O_2$	317.24751	M+H
Norclostebol	Steroid	$C_{18}H_{25}ClO_2$	309.16158	M+H
Norephedrine	Stimulant	$C_9H_{13}NO$	152.10699	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Norethandrolone	Steroid	$C_{20}H_{30}O_2$	303.23186	M+H
Norneosildenafil	Male sexual enhancement	$C_{22}H_{29}N_5O_4S$	460.2013	M+H
Orlistat	Weight loss	$C_{29}H_{53}NO_5$	496.39965	M+H
Ouabain (arrow poison)	Cardiac glycoside	$C_{29}H_{44}O_{12}$	585.29055	M+H
Oxabolone	Steroid	$C_{18}H_{26}O_3$	291.19547	M+H
Oxandrolone	Steroid	$C_{19}H_{30}O_3$	307.22677	M+H
Oxycodone	Opioid	$C_{18}H_{21}NO_4$	316.15433	M+H
Oxymesterone	Steroid	$C_{20}H_{30}O_3$	319.22677	M+H
Oxymetazoline	Nasal decongestant	$C_{16}H_{24}N_2O$	261.19614	M+H
Oxymetholone	Steroid	$C_{21}H_{32}O_3$	333.24242	M+H
Paroxetine	SSRI[c] antidepressant	$C_{19}H_{20}FNO_3$	330.15	M+H
Pentazocine	Opioid	$C_{19}H_{27}NO$	286.21654	M+H
Phendimetrazine	Weight loss	$C_{12}H_{17}NO$	192.13829	M+H
Phenformin	Antidiabetic drug	$C_{10}H_{15}N_5$	206.14002	M+H
Phenibut	Antidepressant	$C_{10}H_{13}NO_2$	180.10191	M+H
Phenolphthalein	Laxative	$C_{20}H_{14}O_4$	319.09649	M+H
Phentermine Hydrochloride	Weight loss	$C_{10}H_{15}N$	150.12773	M+H
Phenylephrine	Nasal decongestant	$C_9H_{13}NO_2$	168.10191	M+H
Phenytoin	Anticonvulsant	$C_{15}H_{12}N_2O_2$	253.09715	M+H
Picrotin	Performance enhancer	$C_{15}H_{18}O_7$	311.11253	M+H
Picrotoxinin	Performance enhancer	$C_{15}H_{16}O_6$	293.10196	M+H
Piperazinonafil	Male sexual enhancement	$C_{25}H_{34}N_6O_4$	483.27143	M+H
Pravastatin	Statin for dyslipidemia	$C_{23}H_{36}O_7$	425.25338	M+H
Pravastatin-1,1,3,3-tetramethyl	Statin for dyslipidemia	$C_{23}H_{36}O_7$	425.25338	M+H
Pregnenolone	Steroid	$C_{21}H_{32}O_2$	317.24751	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Pregnenolone (minus H_2O)	Steroid	$C_{21}H_{30}O$	299.23694	M+H
Primidone	Anticonvulsant barbituate	$C_{12}H_{14}N_2O_2$	219.1128	M+H
Progesterone	Steroid	$C_{21}H_{30}O_2$	315.23186	M+H
Prostanozol	Steroid	$C_{25}H_{38}N_2O_2$	399.3006	M+H
Pseudoephedrine	Decongestant	$C_{10}H_{15}ON$	166.12264	M+H
Pseudovardenafil	Male sexual enhancement	$C_{22}H_{29}N_5O_4S$	460.2013	M+H
Quinbolone	Steroid	$C_{24}H_{32}O_2$	353.24751	M+H
Ricinine	Alkaloid	$C_8H_8N_2O_2$	165.06585	M+H
Rimonabant	Weight loss	$C_{22}H_{21}Cl_3N_4O$	463.08537	M+H
Rosuvastatin	Statin for dyslipidemia	$C_{22}H_{28}FN_3O_6S$	482.17556	M+H
Scopolamine	For motion sickness and nausea	$C_{17}H_{21}NO_4$	304.15433	M+H
Sertraline	Antidepressant SSRI[c]	$C_{17}H_{17}Cl_2N$	306.08108	M+H
Sibutramine	Weight loss	$C_{17}H_{26}ClN$	280.18265	M+H
Sildenafil	Male sexual enhancement	$C_{22}H_{30}N_6O_4S$	475.2122	M+H
Simvastatin	Statin for dyslipidemia	$C_{25}H_{38}O_5$	419.2792	M+H
Solanine	Alkaloid	$C_{45}H_{73}NO_{15}$	868.5053	M+H
Spironolactone	Diuretic	$C_{24}H_{32}O_4S$	417.20941	M+H
Spironolactone (minus C_2H_4OS)	Diuretic	$C_{22}H_{28}O_3$	341.21112	M+H
Stanozolol	Steroid	$C_{21}H_{32}N_2O$	329.25874	M+H
Stenbolone	Steroid	$C_{20}H_{30}O_2$	303.23186	M+H
Strychnine	Alkaloid	$C_{21}H_{22}N_2O_2$	335.1754	M+H
Sucralose	Sweetener	$C_{12}H_{19}Cl_3O_8$	397.02183	M+H
Sulfoaildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_3S_2$	505.20501	M+H
Sulfosildenafil	Male sexual enhancement	$C_{22}H_{30}N_6O_3S_2$	491.18936	M+H
Super-4-DHEA	Steroid	$C_{26}H_{42}O_2$	387.32576	M+H

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Compound	Type	Formula	Q_1 (*m/z*)	Adduct
Super-Ra-DHEA	Steroid	$C_{26}H_{42}O_3$	403.32067	M+H
Super-Rb-DHEA	Steroid	$C_{30}H_{50}O_3$	459.38327	M+H
Synephrine	Weight loss	$C_9H_{13}NO_2$	168.10191	M+H
Synephrine (Minus H_2O)	Weight loss	$C_9H_{11}NO$	150.09134	M+H
Tadalafil	Male sexual enhancement	$C_{22}H_{19}N_3O_4$	390.14483	M+H
Testosterone (Propionate)	Steroid	$C_{22}H_{32}O_3$	345.24242	M+H
Tetrahydrogestrinone	Steroid	$C_{21}H_{28}O_2$	313.21621	M+H
Theanine	For anxiety and high blood pressure	$C_7H_{14}N_2O_3$	175.10772	M+H
Theophylline	Bronchodilator	$C_7H_8N_4O_2$	181.072	M+H
Thiohomosildenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_3S_2$	505.20501	M+H
Thioquinapiperifil	Male sexual enhancement	$C_{24}H_{28}N_6OS$	449.21181	M+H
Topiramate	Anticonvulsant	$C_{12}H_{21}NO_8S$	340.10606	M+H
Trenbolone	Steroid	$C_{18}H_{22}O_2$	271.16926	M+H
Udenafil	Male sexual enhancement	$C_{25}H_{36}N_6O_4S$	517.25915	M+H
Valine	Amino acid	$C_5H_{11}NO_2$	118.08626	M+H
Vardenafil	Male sexual enhancement	$C_{23}H_{32}N_6O_4S$	489.22785	M+H
Vardenafil hydrolysis product	Male sexual enhancement	$C_{17}H_{20}N_4O_2$	313.1659	M+H
Xanthoanthrafil	Male sexual enhancement	$C_{19}H_{23}N_3O_6$	390.16596	M+H
Yohimbine	Alkaloid	$C_{21}H_{26}N_2O_3$	355.20162	M+H
Yohimbine *1*	Alkaloid	$C_{21}H_{26}N_2O_3$	355.20162	M+H

a Unproven use in traditional medicine

b Unproven use

c SSRI is a selective serotonin reuptake inhibitor

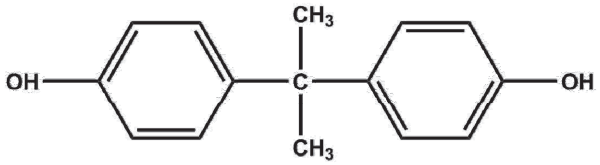

Figure 28. 2D structure of bisphenol A, also known as BPA. Its 3D structure would show that the two phenyl groups are perpendicular to each other and not coplanar, as they appear to be in the 2D structure.

It should also be noted that the analysis of potentially adulterated dietary supplements (or almost anything) by LC also includes determining the length of time that the analyte spends on the column that is used. This is called the retention time. Also note that an analyte is the compound being analyzed. So, verified standards of all the compounds listed in Table 4 (including BPA) were also analyzed by LC-HRMS and retention times recorded. That is, to verify that a particular analyte (and not one of its isomers) is present in a sample, not only the masses (or *m/z* values) must match that of the standard, but the retention times must also match. The retention times will depend on the type of column (usually an octadecyl silica or C_{18} column) and the mobile phase used (usually a mixture of either methanol and water or acetonitrile and water with formic acid added). The basics of LC and gas chromatography (GC), as well as MS and NMR are presented in the Appendix. Finally, for an analytical method to be properly validated, it is important to add a carefully measured amount of the analyte to the sample and see if the method can accurately detect and quantify it. This is often called spike recovery, since one spikes the sample and then measures the recovery of the analyte. For pharmacokinetic studies of prescription drugs that are not naturally present in the environment, an analyst will usually spike the analyte (the drug) at as low a concentration as possible and measure its recovery. If the percent recovery is acceptable (as defined by the organization's QC organization) the method is thought to be valid and a limit of quantitation can be indicated in the SOP for that method. It is important to be able to detect as little of the analyte as possible (such as μg/g or ppm), so the analyst can still quantify it a long time after the drug was administered. This helps to establish more accurately the rate that the drug is eliminated from the blood and target organs. However, when validating a method for analyzing dietary supplements, it is important NOT to spike at the lowest level possible – especially when analyzing for BPA, which will be present in any sample (including extremely pure water) at very low, but detectable

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concentrations. Instead, it is much better to spike at mg/g levels. That is, if there are only a few micrograms (μg) of almost any adulterant in a dietary supplement, it would have no physiological or regulatory significance, but a few mg could be quite dangerous.

In addition, there are some adulterants that may seem harmless to many people, but which can be deadly to others. For example, people with chronic kidney disease should not take non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin [422]. People who are already taking prescription statins to treat their lipodystrophy (such as elevated concentrations of cholesterol) should avoid dietary supplements like red yeast rice that also contain statins, to avoid an overdose. Some people can't tolerate statins due to severe muscle pain. Many people take dietary supplements because they want to avoid the potential side effects of prescription drugs. So, it is very important that the labels on bottles containing dietary supplements should indicate all the prescription drugs that are in them. It is also problematic when dietary supplements contain drugs that were formerly approved by the FDA, but have since been removed from the market, due to adverse side effects and toxicities.

Some dietary supplements are dangerous to a small portion of the population, even though they are quite healthy for most people. Fish oil is an example [51]. It is an excellent source of omega-3 fats, but some people are allergic to it. In addition, some people don't want to consume the mercury or dioxin that is in some fish and fish oil. For them (and many others), prescription fish oil is preferred. Unlike natural fish oil that contains triacylglycerides, prescription fish oil (Lovaza®) contains ethyl esters of fatty acids (but no free fatty acids). So, it is not naturally occurring and was able to be patented. The ethyl esters can be digested and are converted to free omega-3 fatty acids, which can be re-attached to glycerol in human cells as fatty acyls to make mono-, di- and triglycerides, which can be used in the body, as well as phospholipids that are especially important in the brain [51].

Even less obvious are the problems that might occur when taking seemingly harmless and potentially very healthy compounds. This includes an important antioxidant found in green tea, epigallocatechin-3-gallate (EGCG) [423]. It activates the Nrf2/ARE signaling system that destroys reactive oxygen species (ROS) that are unhealthy. Their presence at a modest concentration in green tea may be a major factor in its health benefits. That is, drinking green tea has been linked to a decrease in overall mortality due to cardiovascular diseases in both men and women and less mortality from cerebrovascular disease and respiratory disease in men. On the other hand, green tea consumption was not linked to a

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decrease in mortality due to cancer. However, many studies have described how green tea extract can prevent several types of cancer. This apparent discrepancy could be due to the fact that overactivation of the Nrf/ARE signaling system can lead to multidrug resistant cancer. That is, people who drink green tea may also consume green tea extract or pure EGCG. This was not controlled in previous clinical studies. When EGCG is present at a relatively low concentration in green tea, it might be quite healthy. When EGCG is consumed at much higher doses in a dietary supplement, it might become dangerous [423]. That is, people who have been diagnosed with cancer and are beginning chemotherapy might want to avoid dietary supplements containing EGCG to prevent their cancer from becoming resistant to the chemotherapeutic drugs. However, a cause and effect relationship has not yet been approved. It is also quite possible that the overactivation of the Nrf2/ARE signaling system does not cause cancers to become multidrug resistant, but only occurs after tumors become resistant to therapeutic drugs. So, future clinical trials may still be needed. For example, a clinical trial is being planned that will evaluate the effects of EGCG supplementation on multiple system atrophy (MSA), which is caused by aggregation of α-synuclein [424]. Since EGCG can inhibit this aggregation it may also be useful in treating Parkinson's disease that can also caused, in part, by the same aggregates of α-synuclein.

Another popular dietary supplement that activates the Nrf2/ARE signaling system is CoQ10 [425]. It is also known as ubiquinone because it is a quinone that is ubiquitous in many organisms and in the mitochondria in human eukaryotic cells [426]. Humans have the form of CoQ that has 10 repeat units, but other organisms can have between 6 and 10 isoprenyl repeat units, as shown in Figure 29 [1]. It is important for energy metabolism, as part of complex I (also known as NADH:ubiquinone oxidoreductase) in the respiratory chain in mitochondria and in many Bacteria [1, 427]. Complex I or NADH:ubiquinone oxidoreductase catalyzes the the transfer of two electrons from NADH to ubiquinone (Q) to form NAD^+ and ubiquinol, or QH_2. This is coupled to the translocation of four protons (H^+) across the bacterial or inner mitochondrial membrane [1], as shown by the reaction depicted in Figure 30. The structure of the reduced form of ubiquinone, called ubiquinol, or QH_2, is shown in Figure 31. It I also a popular dietary supplement and is advertised as being absorbed better that CoQ10.

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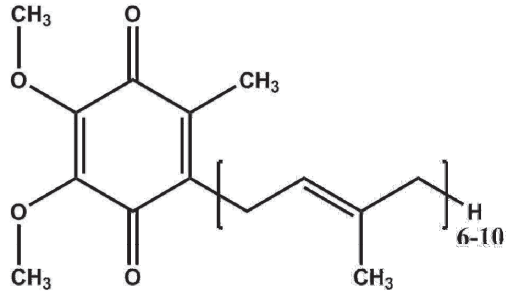

Figure 29. 2D structure of coenzyme Q. Human eukaryotic cells have 10 repeat isoprenyl units, so it is called CoQ10, and is also known as ubiquinone.

$$NADH + H^+ + Q + 4H^+_{in} \rightleftharpoons NAD^+ + QH_2 + 4H^+_{out}$$

Figure 30. Reversible reaction catalyzed by NADH:ubiquinone oxidoreductase.

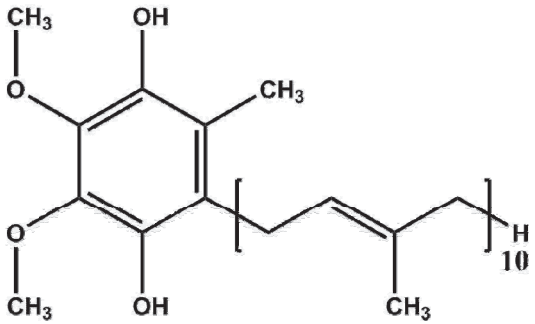

Figure 31. 2D structure of ubiquinol, or QH_2.

However, all our eukaryotic cells can produce CoQ10, including heart cells. If there were not enough of it, we could be vulnerable to heart attacks [1]. It is also an antioxidant that can reduce ROS and free radicals that can damage DNA [428]. It is taken by many people who take prescription statins to lower the concentration of cholesterol in their blood, since it has been thought to alleviate muscle pain [51, 426]. It is "the most popular therapy for statin myalgia among both physicians and patients despite limited and conflicting evidence of its efficacy" [429].

This makes it an excellent example to illustrate some important differences between how dietary supplements and prescription drugs are developed. Both are usually developed based on what seems at first to be a

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compelling idea. In this case, statins were shown to decrease the concentration of CoQ10 in blood plasma and lymphocytes [426]. So, it might seem reasonable to believe that taking CoQ10, or its reduced form, ubiquinol, could also increase its concentration in muscle cells and prevent muscle pain [427, 428]. There has even been a book written about CoQ10 that claims that CoQ10 supplements should be taken, since they can (supposedly) prevent and even cure cancer [430] - especially breast cancer [1, 431]. It has also been used to treat cardiovascular diseases, high blood pressure, diabetes, cancer and even infertility [1, 432]. It is widely thought that when people become very old, start taking statins or are suffering from heart disease, they may not be able to make enough of our own CoQ10 and require supplements [1]. In clinical studies, CoQ10 improved two markers of cardiac health in people suffering from cardiomyopathy [1, 433]. In a different study, CoQ10 helped the performance of world-class endurance athletes. It helped Finnish cross-country skiers [434]. It has been used to treat muscular dystrophy [435]. It helped cause a complete regression of tumors in breast cancer patients when given together with a conventional protocol of therapy. In one patient, the cancer had spread, but CoQ10 helped to completely eliminate the metastatic cancer [432].

A group of scientists and doctors, called the International Coenzyme Q10 Association even issued a position statement on the adverse effects related to the use of statins because they block the biosynthesis of CoQ10 [1, 436]. Moreover, patients who already have had congestive heart failure are thought to be especially susceptible to damage due to the decrease in CoQ10 caused by statins [1, 436]. The letter went on to say, “It is possible that the recently reported statin-related deaths are the tip of a side effect iceberg and the magnitude of the potential problem cannot be overstated. It is urgently incumbent upon the scientific community, the pharmaceutical industry and the United Sates Food and Drug Administration to be certain that we are not inadvertently creating a life-threatening deficiency of an essential co-factor in many millions of patients” [436]. There is also a document on the FDA website that reviews data on the effects of CoQ10 in humans and animals [437]. So, people taking prescription statins and/or red yeast rice often take CoQ10 supplements [1]. Also, CoQ10 is thought by some to be a critical addition to conventional treatments for patients with congestive heart failure [1]. In addition, a clinical trial showed that supplementation with 300 mg of CoQ10 per day can increase the activities of antioxidant enzymes while lowering the amount of inflammation in patients with coronary artery disease [438]. However, more recent randomized clinical trials of CoQ10 found that it had no effect on muscle pain in patients with statin myalgia [429, 439]. Moreover, NMR analysis

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of skeletal muscle oxidative metabolism in statin users found no significant change in mitochondrial function in subjects who received four weeks of statins plus CoQ10 [440]. So, despite the theory that CoQ10 deficiency plays a role in statin-associated myopathy [441], clinical trials have shown that taking it does not reduce muscle pain for most patients [439]. Still, many people are convinced that it is effective in them. Moreover, neither the FDA nor any other government's regulatory agency is trying to prevent anyone from having access to it. At the same time, ubiquinol has been granted orphan drug status by the European Medicines Agency (EMA) for treating "primary CoQ_{10} deficiency, a serious rare disease which produces muscular, nervous system and kidney disorders and can cause failures of other organs" [442]. As a result, if NMEs were being developed that had all the properties of CoQ10 and showed the same results in clinical trials, they most likely would not be approved by any government's regulatory agency. Still, millions of people take CoQ10 or Ubiquinol. This is despite it activating the Nrf2/ARE antioxidant system that is overactivated in multidrug resistant cancers. As mentioned previously, this does not mean that there is a cause and effect relationship. That is, linkage does not imply causation. When two things are linked, they can have a common cause or one may cause the other. Still, this would cause enough uncertainty to require any pharmaceutical company that is developing a NME with similar properties to do many more studies. So, prescription drugs go through a much more rigorous procedure to become approved by any government's regulatory agency. Dietary supplements, though, do not have to demonstrate efficacy – just safety. They do not require FDA approval.

Green tea extract and EGCG are two other examples of dietary supplements that are quite popular, despite them activating the Nrf2/ARE antioxidant system [423]. Many people think that they are safe and effective because green tea certainly is. Green tea made from the leaves of *Camellia sinensis* has been widely consumed for thousands of years and is generally recognized as a very healthy beverage [423, 443]. It is second only to fresh water in popularity. However, not everyone can tolerate the taste of green tea, so there are many dietary supplements that contain green tea extracts. Many of these have been tested and found to have important health benefits [444-449]. One of them, sinecatechins 15% or Veregen®, is an FDA-approved ointment for the treatment of external genital and perianal warts caused by the human papillomavirus [450]. The active ingredients are catechins that are extracted from green tea. They are immunomodulators that inhibit major viral functions. On the other hand,

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extracts of green tea can be toxic, especially when consumed along with energy drinks and other sources of caffeine [451-453].

Still, green tea has been recognized for centuries for its ability to prevent and treat many diseases, largely due to its antioxidant activity [423, 454, 455]. The antioxidants were thought to act by destroying reactive oxygen species (ROS) that are produced in normal metabolism [456]. ROS and accumulated oxidative damage were considered to be major sources of diseases caused by smoldering inflammation [51, 456]. However, the idea that dietary antioxidants act by reacting directly with ROS *in vivo* is largely discredited. Instead, they alter cell signaling and mitochondrial function [51, 456]. A recent article described how the many ingredients in green tea act synergistically to affect many enzymes in the drug-target-pathway-disease interaction network that contains six groups of target proteins [455]. However, most of the recent literature on the effects of green tea polyphenols on health has focused on Nrf2 [423, 448, 457-460].

Drinking green tea has been shown to be associated with a lower incidence of death due to cardiovascular disease in both men and women and mortality from cerebrovascular disease and respiratory disease in men [461, 462]. No association was found between green tea consumption and total cancer mortality [461]. However, to the best of my knowledge, the consumption of green tea extract or dietary supplements containing green tea polyphenols was not controlled. That is, the effects of green tea might be overwhelmed by the consumption of dietary supplements that provide much higher doses of epigallocatechin-3-gallate (EGCG) than from drinking green tea. Still, green tea has been used in many parts of the world for centuries as a health tonic [443]. Epidemiological data have indicated that green tea may prevent stroke and cardiovascular diseases [423, 443].

One of the major risk factors for cardiovascular diseases is obesity, or metabolic syndrome [51]. Green tea catechins, including EGCG may be able to help prevent metabolic syndrome from developing [423, 463]. Green tea catecins lower the concentration of LDL-cholesterol in blood [464]. Also, green tea extract caused significant weight loss, reduced waist circumference, and decreased the concentrations of total cholesterol and low density lipoproteins (LDL) in blood plasma without any adverse side effects in obese women. It was suggested that this was at least partly due to inhibition of ghrelin secretion, leading to increased adiponectin concentrations [423, 464]. Ghrelin and adiponectin are protein hormones produced by the hippocampus and adipose cells [51]. Ghrelin increases the amount of food intake and fat mass while activating the reward network

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(mesolimbic system) of the brain. Adiponectin is anti-inflammatory and anti-atherogenic. It stimulates the appetite and its concentration increases during starvation [51].

Also, obesity can damage the endoplasmic reticulum (ER) and lead to the unhealthy accumulation of misfolded proteins [423, 465]. This is a form of intracellular stress that occurs whenever the protein-folding capacity of the ER is overwhelmed. This can lead to reduced blood flow, cardiac hypertrophy and heart failure. ER transmembrane sensors are supposed to detect the accumulation of unfolded proteins. They should activate transcriptional and translational pathways that deal with unfolded and misfolded proteins. This is known as the unfolded protein response (UPR). If the UPR fails to control the concentration of unfolded and misfolded proteins in the ER, apoptotic signaling is induced. Furthermore, oxidative stress and subsequent cellular damage can damage the myocardium after ischemia/reperfusion and in cardiac hypertrophy secondary to pressure overload. To help prevent this oxidative stress, there are antioxidant defense systems that should ensure that the response to oxidants is adequate. Nrf2 is closely linked to the UPR sensor called pancreatic endoplasmic reticulum kinase. Interventions against ER stress that activate Nrf2 can reduce myocardial infarct size and cardiac hypertrophy. Finally, activating the Nrf2/ARE pathway may important in ischemic preconditioning, in which the heart is subjected to one or more episodes of nonlethal myocardial ischemia-reperfusion before the sustained coronary artery occlusion [423, 465]. It has also been shown that EGCG can protect against oxidative stress that is caused by cerebral ischemia by activating the Nrf2/ARE signaling pathway [423, 460]. It also acts as a mechanosensitive transcription factor that can protect endothelial cells from fluid shear stress that can lead to stroke and other cardiovascular diseases [423, 466].

However, Nrf2 can have a detrimental effect on the heart when autophagy is impaired [423, 467]. One mechanism of autopoiesis is autophagy, in which unnecessary or dysfunctional proteins, organelles and cells are degraded. When autophagy is impaired in the heart, the Nrf2/ARE signaling pathway can become dysfunctional, leading to heart damage [423, 466]. So, the relative physiological effects of green tea and extracts enriched in EGCG can depend on the health of the individual.

As mentioned previously, many phenolic compounds (especially EGCG) are antioxidants that can help prevent smoldering inflammation which can lead to cardiovascular and neurodegenerative diseases [423, 456]. In general, phenolic compounds in foods may be able to help prevent

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cognitive and neurodegenerative disorders from developing [306, 455, 468, 469].

Moreover, epidemiologic evidence suggests that green tea may help prevent stroke [423, 443]. However, the tendency to think of individual diseases in a reductionist manner can be misleading [1, 51, 100]. Many diseases are linked with each other, leading many researchers to study their interactions in a disease-ome, similar to the genome, metabolome and other –omics [49]. That is, neurodegenerative and cardiovascular diseases can be caused by the complications that emerge due to metabolic syndrome [51, 100]. One of the complications is that the composition of Bacteria in the gut is quite different in obese people than in those who aren't [51]. So, it is important to note that drinking green tea can lead to an increase in the relative amount of *Bifidobacterium* species that are in the gut [469]. As mentioned in Chapter 2, the composition and function of the intestinal microbial flora change when people become obese [470]. One of the changes is that barrier-protective bacterial species (*Bifidobacterium* species) decrease and endotoxin-producing species (*Desulfovibrionaceae*) increase [471, 472]. *Bifidobacterium* species also help the immune system protect against the development of inflammatory diseases. They do this by helping to digest fermentable dietary fiber [423, 470].

Even though epidemiological evidence indicated that green tea extract does not decrease overall mortality due to cancer [423, 461], there are several studies that indicated that it may help prevent and even treat several types of cancer [443, 468, 473]. One way that it may be able to prevent cancer is by preventing angiogenesis [423, 473]. That is, healthy people can have microscopic tumors and remain asymptomatic. Only after the cancer grows and spreads (metastasizes), does it become deadly. To grow and metastasize, new blood vessels need to be formed. If this can be prevented, it could prevent cancer. So, a dietary supplement (Polyphenon E) that contains polyphenols that are extracted from green tea is being evaluated in some clinical trials for treating prostate, bladder, esophageal, lung, head and neck cancers, as well as leukemia, and is already commercially available as a topical ointment [423, 473].

So, green tea and the phenolic compounds (especially EGCG) in it may be able to prevent and even help to treat many types of cancer by activating the Nrf/ARE signaling system [443, 468, 473]. However, when over-activated, the cancer cells may become resistant to many drugs used in chemotherapy [474]. However, if they or EGCG are over-consumed as dietary supplements by patients who are starting chemotherapy, it could lead to multidrug resistant cancer and death. Still, more research is needed to establish just how much EGCG and other supposedly healthy

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compounds are needed to over-activate the Nrf2/ARE and cause multidrug resistant cancer [423].

However, if one drinks green tea it may leave less room in the stomach for other foods, thus limiting caloric intake [423]. So, it is quite unlikely that green tea is toxic. Even if cancer patients are starting chemotherapy, the effects on suppressing the appetite will probably keep them from over-consuming green tea. However, they or their caregivers may read about the potential health effects of green tea extracts and especially EGCG. So, they might think that they should consume dietary supplements enriched in EGCG or even pure 100% EGCG. For people who don't have cancer, there is still another potential toxicity of green tea extract - caffeine. It can be toxic at high doses, so some green tea extracts are decaffeinated [423, 451]. However, caffeine is usually safe when consumed in moderation (500 mg or less daily) and may even help protect against Parkinson's disease [475]. EGCG may also help prevent Parkinson's disease, since it inhibits the aggregation of α-synuclein oligomers that can cause not only Parkinson's disease, but also multiple system atrophy [476].

There is another dietary supplement that has shown beneficial effects in Parkinson's disease and Alzheimer's disease – cytidine 5'-diphosphocholine, also known as Citicoline and CDP-choline [477]. It may also be effective in treating glaucoma [477]. It might also be a good source of choline for vegetarians who don't eat eggs [478]. Citicoline could be especially good for people who have a kidney disease or are highly susceptible to cardiovascular disease. They may want to restrict their consumption of red meat and egg yolks, as well as avoid lecithin dietary supplements, since lecithin (also known as phosphatidylcholine) can be metabolized to form trimethylamine *N*-oxide (TMAO), a known kidney toxin and a biomarker for kidney diseases. In addition, citicoline may improve memory, including verbal memory in patients with age related cognitive impairment, as well as improving cognitive function in elderly patients with vascular related mild cognitive impairment [477-482].

Still, Citicoline has not gone through the extensive clinical trials that are required for prescription drugs. So, even though it may be safe, its efficacy has not been clearly established.

3.2.3 A complex, nonlinear dynamic systems perspective on Ayurveda and Ayurvedic research

Long before there were any dietary supplements, there were several forms of traditional medicine – many of which are still being used effectively. One of the most popular botanicals used in traditional,

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Ayurvedic medicine is turmeric, which was first discussed in this book in section 3.2.1. So, it is interesting that the fields of complexity theory and nonlinear dynamic systems (NDS) are becoming relevant for analyzing the theory and practice of Ayurvedic medicine from a Western scientific perspective [483]. NDS theory hypothesizes that complexity is a property of a healthy system and that a decrease in complexity can degrade information processing. Many diseases are characterized by a regularity of pattern responses that show that the system has become somewhat rigid. Examples include the cyclic breathing patterns characteristic of heart failure or the increasing regularity of respiratory rate as a coma deepens. Moreover, complexity is linked to adaptability. It degrades as we age and/or suffer from diseases. A state of good health is characterized by ‘‘correlations that extend over many scales of space or time” [483]. For example, one’s heart rate is not just affected by immediately preceding conditions, but also by things that happened in the past. If a person has suffered an allergic response to a bee sting, the mere sight of a bee can be terrifying. Also, a healthy immune system is a complex, adaptive system that is flexible and responds to different external stimuli in various ways, rather than responding to each stimulus in a standardized and ultimately less effective way. At the same time, living systems are nonlinear. Small changes can lead to large effects that can be difficult to predict. In addition, complex nonlinear dynamics exist in living systems that are nested within other interdependent systems. The components of the system don’t exert their effects in a linear, additive way. They are nonlinear and transformative. Despite this unpredictability, nonlinear systems can undergo abrupt nonlinear transitions or bifurcations. This is consistent with the Ayurvedic paradigm, in which multiple scales and their interactions are evaluated simultaneously. This includes both internal (psychophysiologic) and external (social–environmental) factors. “Health is an emergent property of the interaction among multiple, many-layered systems” [483]. In addition, Ayurvedic treatments are specific for each individual. All of this means that new ways of collecting and evaluating data are needed. This includes evaluating the overall benefit to one’s health or phenotype [483]. As discussed in section 3.1.14, phenotypic screening is being emphasized in modern, systems-based medicinal chemistry and new drug development.

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3.2.4 Discovery and development of innovative drugs from traditional medicine by integrated chinmedomics strategies in the post-genomic era

Like Ayurvedic medicine, traditional Chinese medicine (TCM) often uses a combination of several herbs (called formulae) when treating a person's diseases [484]. To help identify new bioactive compounds in TCM, a new platform called chinmedomics was developed. It combines blood serum pharmacochemistry with metabolomics to determine the effectiveness and molecular mechanisms of the bioactive ingredients in TCM. This could be very important, since the WHO estimates that 80% of the world's people rely on herbs for their primary health care needs and TCM is used by over a billion people in China, Korea and Japan. However, conventional strategies based on reductionist thinking are limited. The goal of this type of approach is to isolate single components from an herb and testing them one at a time. Instead, chinmedomics analyzes a patient's blood, since most TCM remedies are given orally and can undergo metabolism in the liver before reaching the blood. That way, the actual *in vivo* bioactive substances can be identified. As a result, chinmedomics has been used to discover bioactive ingredients in some traditional Chinese formulae, such as Yinchenhao Tang. It consists of *Artemisia annua* L., *Gardenia jasminoides* Ellis, and *Rheum Palmatum* L. Chinmedomics was used to identify 19 bioactive compounds and two metabolites. This showed the feasibility of this approach [484].

3.2.5 A complex systems science perspective for whole systems of complementary and alternative medicine research

Complementary and alternative medicines and treatments are being studied with a whole systems complementary and alternative medicine (WS-CAM) approach [485]. The United States National Center for Complementary and Alternative Medicine (NCCAM) in the NIH defined WS-CAM as a "complete systems of theory and practice that have evolved independently from or parallel to allopathic (conventional) medicine. Many are traditional systems of medicine that are practiced by individual cultures throughout the world" [485]. WS-CAM recognizes the importance of interconnectedness and looks for emergent, non-linear outcomes of treatments that include both local and global changes in the human condition. It uses the contextual view that humans are inseparable from and responsive to their environments. In the process, interventions that are complex, synergistic, and interdependent emerge. This is quite

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different from reductionist approaches that assume that there is a one-dimensional or simple cause for every disease. This led former researchers to isolate and test individual components that are just single aspects of the WS-CAM system. Instead, WS-CAM uses Complex Adaptive Systems (CAS) models to provide working hypotheses to explain the persistence of changes that can last for weeks to months between treatments and/or after cessation of treatment. It can also look for nonlocal and whole systems changes that occur after therapy and crises that can emerge during treatment. More importantly, the complex systems approach offers an alternative perspective on cause, beyond the simple reductionist ideas. This is leading to important research methods such as agent-based modeling, network analysis, state space analysis and several time-series analyses for measuring complexity and patterns. This entire field is known as complex systems science [485].

3.2.6 A Pilot Feasibility Study of whole-systems Ayurvedic medicine and yoga therapy for weight loss

A systems approach is also useful in evaluating the synergistic benefits of combining exercise (yoga) with Ayurvedic medicine [486, 487]. Yoga has been used with Ayurvedic medicine for thousands of years. Historically, malnutrition was a major health concern, but these days, obesity, metabolic syndrome and attendant diseases is just as important. So, recent research has been done that showed that the combination of Ayurvedic medicine and yoga, with emotional and physical group support can help female yoga practitioners and novices lose weight [486, 487]. It is important to include the concepts of model validity and effectiveness research to make the research more relevant [488]. Whole systems of healing and overall wellness should address the complex relationships involved. Similar to TQM, this approach must be patient-centered (or customer-centered in the nomenclature of TQM). In addition, complex, dynamic systems theory should be used to design studies, choose outcomes and analyze data. That way, the nonlinear, often cyclic trajectories of therapy can be properly documented. This can be combined with practice-based research networks within the community of practitioners of yoga and Ayurvedic medicine. Ideally, this collaboration will extend to mainstream clinical researchers to form more extensive collaborative networks [488].

Moreover, yoga has become quite popular and effective in the USA and other western countries that used to scorn it. In systems thinking, today's problems can come from yesterday's solutions. That is, the USA

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and her allies won the World War II in part by giving their soldiers, sailors and Marines strength and endurance training. In the process, the ideal body of a man was defined by the size of his muscles – especially the arms. The popular sports for men in the USA (boxing, baseball, football and basketball) all required great upper body and leg strength. Athletes typically psyched themselves up by making themselves angry at their opponents before a contest began. This was contrary to Martial Arts in the East (such as karate and judo), that emphasize relaxing. This is also consistent with yoga in which one must remain calm in the middle of a storm. That is, while one part of the body undergoes stress (such as the legs in yoga poses), the rest of the body (such as facial muscles) relaxes. Though it was not recognized by fans, successful boxers also had to learn how to relax during a competition to prevent them from becoming exhausted after just one round. Similarly, modern life in so-called developed societies is full of stress that can be quite unhealthy – regardless of one's diet, genetics or epigenetics. Fortunately, yoga has become quite popular since it can help people learn to relax. In addition, well-developed abdominal muscles (such as a 'six-pack') have become much more fashionable than big arm muscles. At the same time, yoga and tai-chi have become quite popular among the elderly – not just because it helps them relax, but also because it greatly increases flexibility. Improved flexibility can prevent dangerous falls that can destroy a person's quality of life and lead to depression and an early death. So, yoga, karate, Tae-Kwon-Do and judo are being practiced by more people – not as a means of improving one's combat readiness, but for improved self-esteem and overall health. In addition, as a practitioner improves the strength of his or her abdominal muscles, it can help prevent back pain and control one's appetite – especially for unhealthy foods (like red meat) and sweetened beverages. The Martial Arts are often misunderstood as being good for one's self defense against a human aggressor. However, almost all instructors and any experienced practitioner knows that you are almost always your own worst enemy. So, the respect of others that one learns, as well as the self-confidence, self-discipline and flexibility can be quite useful in establishing good health habits. They can also help a practitioner control his or her anger and stress, so conflict can be avoided.

3.2.7 Prescription drugs and weight loss supplements for weight loss

So, the brain is not separate from the body [263, 489]. To lose weight and body fat (especially in the abdominal region), one's attitude and

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support from others can be crucial. Our bodies (including our brains) can usually regulate caloric intake, energy balance and body weight. However, social pressure and advertising can lead people to consume emotionally gratifying foods. Many of us elderly people can remember when we were rewarded as children with candy when we were good. Fortunately, this practice is going out of favor. For example, children who take lessons in Tae Kwon Do are told to never eat candy or red meat and never drink sweetened beverages, such as soda pop. Sweetened beverages that have lots of fructose, glucose or sucrose and no nutrients rapidly increase blood glucose concentrations to dangerously high levels [51, 451]. The excess glucose is converted to fat and is stored in adipose cells. Adipose tissue is an active endocrine and immune organ. It secretes lipid hormones (adipokines). So, adipocytes are not just storage cells for triglycerides, but complex cells that must be properly regulated. When caloric intake exceeds energy consumed, adipocytes proliferate. If there is sufficient adipogenesis, metabolic syndrome can be avoided. When adipogenesis is impaired, adipocytes can become dysfunctional and lead to diseases related to metabolic syndrome. This is often called adiposopathy, or sick fat. This fat is especially dangerous when it accumulates in the abdomen. Adipose tissue also produces aromatases, which catalyze reactions that are in the biosynthetic pathway for estrogen. That is important because estrogens have important roles in bone homeostasis. In post-menopausal women, circulating estrogen levels decrease, which leads to an increase in the number of adipocytes and decrease in osteoblasts, which can lead to osteoporosis. Adipocytes, osteoblasts and myoblasts originate from a common progenitor, pluripotent mesenchymal stem cells, or MSCs. So, there is a bone-adipose axis, with bones and adipose tissue acting as endocrine organs [51, 451].

There is even some important research being done to learn about "the neural correlates of hedonic-motivated ingestive behavior, weight gain, weight loss, and metabolic status" [490]. That is, systems thinking is being used to recognize that "the determinants of ingestive behavior and weight regulation are frequently thought to involve homeostatic, hedonic, and somatosensory systems, which interact with the external environment to influence food intake and ultimately metabolic status" [490]. We maintain our energy balance and body weight through signaling by peptides such as leptin, ghrelin, insulin, glucagon-like peptide 1 (GLP-1) that affect one's apetite. They act on neurons in the brain and vagal nerve to influence peptides in the hypothalamus. At the same time, somatosensory and gustatory brain regions (oral somatosensory cortex, anterior insula/frontal operculum) help control one's response to taste and mouth feel (or texture)

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of foods. Hedonic systems (pleasure/reward) modulate the homeostatic and somatosensory systems. With sufficient brainwashing from advertisers and others, these systems can override the homeostatic need to stop eating once you are satiated. This can lead to over-eating and gaining weight while increasing one's body weight and body mass index (BMI). To help understand this better, neuroimaging is used. Several brain regions work together to control food intake. So, neuroimaging has shown that many obese people have increased neural responses to images of food that they feel are delicious – even if they are loaded with calories and deficient in nutrients. In addition, certain cues can make one anticipate eating certain foods and beverages. Obese people tend to show greater response in gustatory and somatosensory brain regions as well as regions associated with visual processing and attention. So, ingestive behavior is both dynamic and complex. Obesity is associated with neuroadaptations that are linked with food cues and altered neuroendocrine function [490].

This is ignored by advertisers who use reductionist thinking to tell people that their dietary supplement will make people lose weight without changing their diet. Supposedly, many of these supplements contain 'fat burners' that will make your fat just melt away – no matter how many calories that you consume. Some advertisers even say that if you lose too much weight that you should reduce the number of 'fat burning' pills that you take each day. The goal is not to keep people from losing too much weight, but to let them think that their weight loss product can make people lose more weight than anyone would expect.

Unfortunately, there are no dietary supplements that safely and effectively cause weight loss. Moreover, some of them are adulterated with banned prescription drugs as well as narcotics, stimulants, antidepressants, anorectics, laxatives and diuretics [451]. Even though many supplements don't contain any of these potentially deadly adulterants, many do contain caffeine and/or green tea extract at such high concentrations that they can also be quite toxic. Still, there are five prescription drugs that are FDA-approved as an adjunct to counseling, diet and exercise for maintaining weight loss. They are orlistat (Xenical® or Alli when sold over-the-counter), lorcaserin (BELVIQ®), phentermine plus extended-release topiramate (Qsymia®), naltrexone plus bupropion (Contrave®) and liraglutide (Saxenda®). Unlike dietary supplements, they were all developed in research programs that were conducted through good laboratory practices (GLP) and manufactured by current good manufacturing practices (cGMP). So, they have been shown to be safe and effective in most obese people. Every step in the manufacturing process is monitored and documented to ensure the purity of the drugs. Finally, post-

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market monitoring of potential adverse side effects is done on these drugs. Still, it should be noted that these prescription weight loss drugs are not very effective unless they are used as together with counseling, diet and exercise [451].

3.2.8 Prescription drugs: strict protocols and quality management systems, take much longer to reach the market

Unlike dietary supplements, strict protocols and quality management systems are used to develop and sell prescription drugs. Still, the first step in developing a dietary supplement and a prescription drug can be quite similar. It often starts with a testable hypothesis. As described in section 3.1, the process for prescription drugs starts with selecting a therapeutic target or a desired phenotype. Some dietary supplements, like CoQ10 start with a testable hypothesis as well. However, other supplements start with an idea that is never tested, but which sounds good – especially to the layman who is uninformed about the sciences of pharmacology and toxicology. For example, even though it is well established that consuming fresh fruits and vegetables is quite healthy, nobody can eat every one that is available. So, instead of trying to eat hundreds of different fruits and vegetables, supposedly it is quite healthy to consume a dietary supplement that contains extracts of countless (or proprietary numbers) of fruits and vegetables. There is no scientific evidence to support this idea, even though there have been some publications that attempt to describe the potential health benefits of one such supplement, including a double blind trial on preventing preeclampsia [491]. The people who market and sell this supplement are encouraged to tell their friends and family members that this study proved that this supplement is safe and effective. However, the results of this trial showed that "there was no difference observed between the phytonutrient supplement group and the placebo group" [491]. Moreover, this trial was not approved (or disapproved) by the FDA or any other government's regulatory agency. It would be very expensive to get this or any clinical trial of a dietary supplement approved. As described in section 3.1, INDs that may undergo clinical trials and become approved prescription drugs need to be registered with the FDA, if they are being developed in the USA. There must be a manufacturing process that is compliant with cGMP. This includes identifying the IND. A dietary supplement that contains a proprietary mixture of components does not meet the cGMP standards required for prescription drugs, even though the supplements might be safe and effective for many people. In addition, pharmacokinetic studies are seldom done on dietary supplements (they are

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quite expensive to conduct). Even if pharmacokinetic studies were to be conducted, Phase I, II and III clinical trials would have to be done if they were to become FDA-approved. When such studies are done on prescription drugs, the trial number that was registered with the FDA or another government's regulatory agency is given when the results are published.

There are other serious differences between how dietary supplements and prescription drugs are sold and marketed. Once a new prescription drug is approved, physicians work with pharmaceutical companies to report any adverse side effects that might occur. This is required by law. In contrast, once a dietary supplement starts to be sold, customers are encouraged and are often paid to give their personal testimonials about how the supplement helped them and maybe even saved their lives. People who may suffer some adverse side effects are seldom encouraged to tell anyone about it. Almost no dietary supplement will be advertised the same way that prescription drugs are. That is, advertisements for prescription drugs will always contain warnings about potential side effects. Such warnings seldom accompany advertisements for dietary supplements.

In addition, lay people and those who market dietary supplements frequently misuse the terms "prove" and "proven". They often say things like "it has been proven to be safe and effective in clinical trials". As mentioned at the beginning of this book, scientists are very reluctant to use the word proof – unless they are talking about a mathematical proof. Instead, they usually say that the data support (not prove) their hypothesis. Moreover, they use systems thinking to realize that no drug will be completely safe or effective in everybody who takes it. For example, weight loss drugs that are taken as a miracle cure for obesity won't work if the person taking them lives a sedentary lifestyle and consumes lots of sugar and red meat. Such people may suffer from the adverse effects of their poor lifestyle or diet. Moreover, it can be very difficult to prove that a drug or supplement was the single or even major cause of a health problem. Pharmaceutical companies that sell prescription drugs are required by law to err on the side of safety. People who sell dietary supplements are not. Many of them will even claim that their supplement must be safe because it is 'natural'.

This does not mean that pharmaceutical companies or scientists are infallible. For example, there was an article published in the very prestigious *Journal of the American Medical Association – Neurology* that claimed to show that proton pump inhibitors (PPIs) increase the likelihood of dementia [492]. PPIs like Nexium® are used by many people to treat gastrointestinal diseases – especially those caused by acid reflux. That is,

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our stomach lining contains proton (H^+) pumps that make the lining acidic. The article in question used a huge database from the largest German statutory health insurer, Allgemeine Ortskrankenkassen (AOK) [492]. The study looked at 73 679 participants 75 years of age or older. Of these, 2950 used PPI medication and had a significantly increased risk of incident dementia compared with the patients not receiving PPI medication (n = 70 729). The conclusion was "The avoidance of PPI medication may prevent the development of dementia". As in any good study like this, the authors accounted for many potential confounding factors, including age, sex, comorbidities, and polypharmacy. However, they completely ignored the fact that 70-75 years before the study was done (from August to November 2015) many Germans (especially women) suffered from undiagnosed and untreated depression. The authors ignored the fact that a war was happening and that depression could be mistaken for defeatism, which was a capital offense – punishable by death. To me, that seems like an extremely confounding issue. That is, depression early in life is a big risk factor for neurodegenerative diseases, including Alzheimer's disease (in which patients suffer from severe dementia) [493]. Moreover, PPIs can prevent the extreme pain that comes with acid reflux and other gastrointestinal disorders. This pain, if not prevented or cured, can easily lead to a feeling of hopelessness and depression. Similarly, wars that are started by arrogant, aggressive men who are filled with hate cause immeasurable suffering and depression in women and their children, who are powerless to prevent it or stop it.

3.3 Immunotherapy – using one's own body to cure one's self

One of the principles of systems-based thinking and traditional medicine is to use natural therapies whenever possible. Proper diet that leads to a healthy microbiome, as well as exercise and meditation are important. Our protective immunity and response to vaccines is affected by the microbiomes of our gut, mouth and nose [494-500]. Still, when diseases like viral infections and cancer emerge, natural substances can be added to the immune system to help it work better. In immunotherapy one uses one's own body to cure one's self. In cancer immunotherapy the ability of one's own immune system to fight cancer is restored or enhanced. For example, interferons (IFNs) are used in some therapies. They are signaling proteins that are released in response to pathogenic viruses, Bacteria and other parasites, as well as cancer cells. They were named for their ability to interfere with viral replication. IFN alpha 2a,

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IFN beta 1a and 1b, IFN gamma 1b, PEGylated IFN2a and 2b, PEGylated IFN alpha 2 plus ribavirin as well as Human leukocyte Interferon-alpha (HuIFN-alpha-Le) are all used in approved therapies. In addition, IFN production can be stimulated by pattern recognition receptor agonists like Bacillus Calmette–Guérin (BCG), monophosphoryl lipid A and imiquimod, which are FDA-approved for treating cancer [501]. However, many IFN therapies had toxic side effects. So, they are being investigated for use at lower doses in combination anticancer therapies, including drugs that target the PD-1 ligand and its receptor, as well as radiotherapy [501].

There are three major types of IFNs [501]. Type I IFNs include 13 types of IFNα, plus IFNβ, IFNε, IFNκ and IFNω. There is just one type II IFN – IFNγ. There are four type III IFNS: IFNλ1, IFNλ2 and IFNλ3 (also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively) and IFNλ4. The expression of genes coding for types I and II IFNs is activated by pattern recognition receptors (PRRs), while IFNγ is induced by cytokines and myokines. Type I IFNs are produced by most types of human eukaryotic cells, with plasmacytoid dendritic cells (pDCs) having the highest basal expression of them. IFNs regulate the expression of many genes that code for proteins that directly affect the growth, proliferation, differentiation, survival, migration and other specialized functions of tumor cells. They also have extrinsic effects, such as regulating angiogenesis, producing osteoclasts and improving immunity. However, the effects of pDCs depend on the type of tumor. They produce high concentrations of type I IFN that leads to immunity in mouse models of melanoma, but their accumulation predicts poor prognosis in breast cancer. In addition, malignant cells themselves can produce their own type I IFNs that suppress the immune system and stimulate metastasis [501]. This is consistent with systems thinking that teaches that the same biochemical (IFN in this case) can have very different physiological effects in different environments or systems. This is also seen in the effect of mRNA vaccines on CD8+ T cell immunity [502]. They induce type I IFNs, which can either stimulate or suppress CD8+ T cell immunity, depending on the timing and intensity of IFN induction relative to the activation of T cell receptors [502].

So, we have both an innate and an adaptive immune system that are important parts of the neuroendocrine immune network [5]. The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) that are common in Bacteria, viruses and fungi as well as damage- or danger-associated molecular patterns (DAMPs) released from damaged cells. It protects us from infections non-specifically. It does this by recruiting immune cells to the site of infection, activating the

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complement cascade, identifying and removing foreign substances and activating the adaptive immune system through antigen presentation. That is, the innate immune system recognizes Bacteria, fungi and other organisms, breaks them down, identifies a characteristic protein on them (an antigen) and attaches it to the surface of specific cells, which present it to the adaptive immune system for destruction. The innate immune system does not have a memory and does not offer specific resistance against organisms that have invaded the host in the past. For this, the adaptive immune system is needed. It is activated by the innate immune system and is made of many parts. Invading viruses and bacterial cells are killed by phagocytosis which uses a subset of leukocytes that circulate in the blood as monocytes. They are converted into macrophages, which enter inflamed tissues. Their phenotypes, rates of homeostatic turnover and function are different in various tissues. Dendritic cells (DCs) are a distinct lineage of mononuclear phagocytes. They specialize in presenting antigens to T cells while initiating and controlling immunity. DCs are crucial for activating and conditioning virus-specific T cells. They link innate and adaptive immunity and have additional roles in shaping the immune response to pathogens, vaccines and tumors. DCs have many thin, long arms. They are found throughout the body and are particularly abundant in interfaces between the external and internal environments, such as the skin and the lining of the nose, lungs and GIT, where they can encounter invading pathogens. DCs can migrate into the blood and reach infected tissues. When they sense a tagged cell that is harmful, they are activated so they can ingest and destroy the various constituents of the cell. They contain a multi-protein complex called the proteasome, which can catalyze the hydrolysis of proteins that are tagged with ubiquitin. The small peptide fragments produced by the hydrolysis are transported to the surface of the DCs, where they are held in place by human leukocyte antigens (HLAs), also called major histocompatibility complex (MHC) proteins. The tagged dendritic cells then migrate to a lymph node where they encounter T-cells that have a matching receptor that binds the HLA-peptide complex. The dendritic cells bind to CD4+ T cells. The dendritic cell then functions as an antigen presenting cell (APC), which activates the CD4+ T-cells [5].

Classical DCs (cDCs) process antigens and present them to T-cells [5]. They have high phagocytic activity when they are immature cells and high cytokine-producing capacity after they mature. They move from tissues to the T cell and B cell zones of lymphoid organs. They regulate T cell responses during both normal homeostasis and during infection. They are usually short-lived and are replaced by blood-borne precursors. Plasmacytoid DCs (pDCs) differ from cDCs in that they are relatively

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long-lived and some of them carry characteristic immunoglobulin rearrangements. They are present in the bone marrow and all peripheral organs. PDCs respond to viral infection by producing type I interferons (IFNs). However, they also can function as antigen-presenting cells and control T cell responses [5].

Dendritic cells (DCs) are deficient in number and function in many patients with various types of cancer [5, 503]. Activated DCs have been used in the immunotherapy of cancer for over a decade [5, 504, 505]. DC products have been delivered to more than 1000 cancer patients in over 150 clinical trials. Cancer-specific antibodies can be isolated from the cancer cells in patients. DCs can be treated with these antibodies, which are presented on the cell surface. The activated, mature dendritic cells can activate tumor-specific cytolytic T lymphocytes, which can kill the cancer cells in the patient. In 2010, the FDA approved Sipuleucel-T (Provenge®), in a new immunotherapy for treating asymptomatic metastatic castrate-resistant prostate cancer [5, 504]. "It consists of dendritic cells from peripheral blood mononuclear cells. They are pulsed *ex vivo* with PA2024, a recombinant fusion protein consisting of granulocyte macrophage colony-stimulating factor and prostatic acid phosphatase (a prostate antigen), as an immunogenic agent" [5, 505].

Recently, scientists and doctors have eliminated leukemia in several patients by converting their cytotoxic T cells into specific killers of cancer cells [5, 506]. They used a lentiviral vector to express chimeric antigen receptors (CARs) in patients' cytotoxic T cells. Extracellular and intracellular domains were fused into a new, synthetic receptor for the CD8+ T cells. The gene for the CAR was delivered by a lentivirus vector, which was made by using the backbone of the HIV-1 virus that was no longer pathogenic and couldn't cause AIDS. Because the natural host for HIV-1 is the T-lymphocyte, the lentivirus vector specifically targeted the cancer cells and multiplied in them. The patients received between 15 million and one billion cytotoxic T-lymphocytes (CTLs). These CTLs rapidly multiplied about 100-fold [5, 506].

Some B cells can be used to make therapeutic monoclonal antibodies [1]. Most B lymphocytes produce a mixture of antibody proteins in response to a single toxic antigen. These are polyclonal antibodies. They can bind specifically to biopolymers on the surface of foreign cells in the blood, from Bacteria to human cancer cells. This specificity and activity can be used by medicinal chemists when they fuse a nearly immortal cell line of myeloma B cells with antibody-producing spleen cells from a mouse. These hybridomas can produce large amounts of a single monoclonal antibody (mAb) because they can survive and grow in cell

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cultures. These mAbs can be directed against specific sites (epitopes) on antigens that are produced by cancer cells. They are also useful in diagnosis. Some of them can be used in in home pregnancy tests, while many others become therapeutic drugs and/or research tools [1]. Some of them are even approved medications such as Herceptin®, and T-DM1® for breast cancer; Rituxan® for B-cell lymphomas; Campath® for leukemia and three others: Erbitux®, Vectibix® and Avastin® for colorectal cancer [51].

As mentioned before, one important aspect of systems thinking is that today's solutions can come from yesterday's problems. That is, pathogenic viruses that have killed billions of people in the past are being modified to produce oncolytic viruses. They can infect and lyse (destroy) cancer cells, while simultaneously activating pro-inflammatory, antiviral biochemical pathways. This was discussed in section 3.1.15. In addition, monoclonal antibodies (mAbs) and fusion proteins are revolutionizing the practice of medicine [507]. Secukinumab (Cosentyx®), Obinutuzumab (Gazyvaro®), trastuzumab emtansine (Kadcyla® also known as T-DM1) brentuximab vedotin, glembatumumab vedotin, Adcetris® and Kadcyla® were discussed previously in this chapter. The 30th anniversary of the first mAb that was approved by the FDA was in 2016 [506]. Since 1986, mAbs have been used to treat and sometimes cure autoimmune diseases, cancers, hyperlipidemia and blood disorders. Consistent with systems thinking, they have several mechanisms of action, as they affect several interacting signaling mechanisms at the same time [507].

In systems thinking, it can be important to think globally while acting locally – especially when dealing with pathogens [508]. That is, it is no longer sufficient to view microbial pathogenicity as a simple linear process that is similar to combat. In reductionist thinking, the pathogen is thought to attack the host. Subsequently, the host reacts and deploys defenses, to which the pathogen reacts by engaging its own defenses and counterattacking. Instead, systems thinking shows that both the pathogen and host have sensory mechanisms that quickly detect the presence of each other. They use signaling pathways that lead to complex bidirectional responses. The pathogen attempts to make the host respond in ways that will favor its survival, such as providing a supply of essential nutrients. At the same time, the host tries to protect itself from harm, while maintaining homeostasis and ultimately eliminating or neutralizing the pathogen. The concept of the 'damage-response framework' is appropriate. The outcome may be an emergent property of the interaction between host and pathogen. It may not be easily predictable from their individual characteristics [508].

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For example, the bacterium *Neisseria gonorrhoeae* causes gonorrhea, but doesn't induce a state of protective immunity against repeat infections like many other pathogens do [508]. Conventional reductionist thinking believed that the lack of protective immunity was due to the extraordinary capacity of *N. gonorrhoeae* for antigenic variation. However, recent research and systems thinking showed that *N. gonorrhoeae* can proactively direct the pattern of host immune response in favor of its own survival while being the detrimental to the host. Thus, *N. gonorrhoeae* selectively elicits T_H17-driven innate defense mechanisms that suppress T_H1- and Th2-driven adaptive immune responses that might be effective in eliminating it. It does this by inducing the immunoregulatory cytokines TGF-β and IL-10 and possibly even the type 1 regulatory T (Treg1) cells. These Treg1 cells suppress adaptive immune responses produced by by T_H1 and T_H2 cells. These effects can be reversed by antibodies that neutralize TGF-β and/or IL-10 or by giving IL-12 in a sustained-release formulation. These treatments induce specific immune responses that are driven by T_H1 cells. This includes making antibodies in the blood serum and genital secretions that act against *N. gonorrhoeae*. They also establish immune memory. Similarly, *Bordetella pertussis* and *Chlamydia trachomatis* induce immune suppression by eliciting IL-10. However, genital gonococcal infection should be treated with local application, low doses, and sustained-release formulations. The goal is to avoid adverse side effects that can occur if a bolus of IL-12 or other cytokines is given. Finally, the safety of such treatments in humans still needs to be demonstrated clearly. That is because prolonged alterations of the cytokine profile in women may affect physiological functions, including pregnancy. They may also affect the normal, healthy microbiota [508].

At the same time, everybody's immune network is unique and subject to changes caused by epigenetics, nutrition, lifestyle and exposure to environmental toxins. This is also true for tumor-associated antigens that can be targeted by personalized therapies [51]. Most tumor-specific antigens are ideal targets for immunotherapy since they emerge from somatic mutations in the tumor cells and are patient-specific. Mutations that are specific to cancer cells are identified by comparing the whole genome of cancer cells with those of the original cells [51, 509, 510]. Still, whole-exome sequencing (WES) is more affordable for larger sample sizes, since only 1% of the genome needs to be sequenced. WES found several mutations in cancer of the breast, ovaries, kidneys, head and neck. Recently, mutated antigens that are recognized by anti-tumor T cells were identified by mining whole exome sequence data to identify the mutated proteins that were expressed on patients' tumors. Mutated T cell epitopes

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were identified using an MHC binding algorithm. They were synthesized and tested to see if they were recognized by tumor infiltrating T-lymphocytes (TIL). Mutated antigens expressed on autologous tumor cells were identified as targets of three TIL that were associated with tumor regressions following adoptive transfer of the lymphocytes. This simplified approach, which avoids the need to generate and laboriously screen cDNA libraries from tumors, may become quite useful in identifying mutated T cell antigens that are expressed in many types of tumors [51, 510].

Viral antigens are also tumor-specific [51, 509]. It is relatively easy to prime T cells to be active against them. For example, the human papilloma virus (HPV) produces two oncoproteins (E6 and E7) that are possible targets for cancer immunotherapy. There are two prophylactic HPV vaccines that are available that protect against cervical cancer and other diseases that are associated with HPV [51, 509].

Once tumor antigens are identified, active and passive immunotherapies can be devised [51, 509]. Active immunotherapies induce a tumor-directed immune response by vaccinating patients with tumor antigens. Passive immunotherapies can use cytokines, mAbs and/or adoptive T cells to stimulate the immune system. For example, the cytokine IL-2 is a growth factor for T-cells. It was approved by the FDA because it caused complete cancer regression in 8% of the patients with metastatic renal cancer or melanoma. There is also a type of mAb that targets the immunosuppressive proteins CTLA-4 and PD-1. They block immunoinhibitory pathways, which may enhance normal, endogenous anti-tumor responses. CTLA-4 acts as a checkpoint to down-regulate the activation of T cells. Ipilimumab is a human mAb that blocks CTLA-4, so it is called a checkpoint inhibitor. This enhances the proliferation and effector activity of T cells, thus disrupting the way that cancer cells avoid detection. The FDA approved Ipilimumab in 2011 for the treatment of patients with metastatic melanoma. As mentioned in section 3.1.3, PD-1 and its ligand PD-L1 are promising targets for antibody therapy. When PD-L1 binds to its PD-1 receptor on T cells, it inhibits the activation of T cells and induces apoptosis in them. PD-L1 is expressed on antigen presenting cells and several solid tumors such as renal cell, colon, breast, ovarian and hematological cancers. Nivolumab (Keytruda®) is an anti-PD-1 mAb that is approved for treating non-small cell lung carcinoma [51, 509].

Passive immunotherapy can also use adoptive T cell therapy (ACT), in which patients are treated with tumor-reactive T cells that are selected and expanded *ex vivo* [51, 509]. Both effector and memory T cells can induce complete remission in patients that can't be cured by other anticancer

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therapies. The two major sources of T lymphocytes for ACT are the tumor itself and the peripheral blood of the patient. When a tumor is infiltrated by anti-tumor T cells, these cells can be isolated from the resected tumor mass. For example, TILs were isolated from 93 metastatic melanoma patients who had failed chemotherapy and other immunotherapies such as IL-2 and anti-CTLA-4 treatments. The T cells isolated from the resected tumor were first selected for anti-tumor reactivity and then expanded. Twenty patients (22%) achieved a complete regression with ongoing complete responses in 19 of them after three years. Despite this success, a major limitation of TIL therapy is the absence of sufficient numbers of TILs in other types of cancer. To extend this type of therapy to other types of cancer, genes coding for the antigen-specific T cell receptor (TCR) can be transferred into lymphocytes that are isolated from the patient's peripheral blood. The T cell transduction is done with retro- or lentiviral vectors to produce T cells that recognize tumor antigens and eradicate tumor cells that express them in many patients. There were definite, positive clinical responses in patients with metastatic synovial cell sarcoma and melanoma who were treated with TCRs that targeted NY-ESO-1 antigens. Two melanoma patients out of the 11 in the clinical trial were in complete regression that persisted for more than one year [51, 509].

Also, T cells can be modified by chimeric antigen receptors (CARs) [51, 509]. In contrast to TCRs that are specific for a certain HLA-peptide complex, CARs can recognize antigens without depending on them. This may make CARs useful for patients with different HLA haplotypes, and increase the number of potential targets, including carbohydrates and glycolipids. Although one of the first successful CAR therapies with complete remissions was reported on a solid neuroblastoma, they may also be effective against hematological malignancies. The most investigated CAR target is CD19, which is expressed on normal B cells as well as on most B cells in leukemias and lymphomas. Several clinical studies on patients with B cell malignancies (such as CLL) caused partial and complete responses in subsets of patients. Also, two children with relapsed and refractory acute lymphoblastic leukemia were treated successfully with CARs that targeted CD19. One child was reported to have an ongoing complete regression, while the other one relapsed due to the growth of blast cells that no longer expressed CD19. Still, CD19 CARs killed aggressive leukemia cells. So, T cells with CARs against other targets in combination with CD19 CARs may be useful on tumors that downregulate the CD19 CAR target. Based on the success of CAR therapies in hematological malignancies, the expansion of this therapy to solid tumors

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may be possible. However, it will be more challenging due to the immunosuppressive tumor microenvironment that can make cell-based immunotherapy ineffective [51, 509]. Despite this, an FDA panel recommended the approval of CAR-T gene therapy (Tisagenlecleucel-T, Kymriah®) on July 14, 2017 for pediatric and young adult patients with B-cell acute lymphoblastic leukemia (ALL) and it was approved on August 30 [511]. The extracellular portion of the CAR protein consists of an antibody fragment that targets CD19. The intracellular portion contains T-cell signaling and co-stimulatory domains that activate T-cells and enable them to persist, while exerting anti-tumor activity. Once the CAR protein binds to the CD19 target, the intracellular domains promote the T-cell expansion, while triggering subsequent effector functions that eliminate the targeted B-cells, which are malignant [511]. So, blood cells are removed from the patient at an appropriate medical center and shipped to the manufacturer (Novartis) for processing. Then, they are sent back to the medical center and administered to the patient.

The tumor microenvironment can create barriers such as impeding T cell infiltration and inhibiting the stimulation of T cells [51, 509]. It can up-regulate suppressive pathways in immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T_{regs}). MDSCs are a heterogeneous population of immune cells that promote tumor growth by suppressing the immune system. This includes suppressing the activation of anti-tumor T cells. Also, T_{regs} inhibit the anti-tumor functions of T cells. So, they are eliminated by lymphodepleting regimens prior to adoptive T cell transfer of either TIL or TCR/CAR engineered T cells. Despite some clinical benefits there can be toxic side effects. One way to overcome this toxicity is to use genetic engineering on isolated anti-tumor T cells so that they can produce their own pro-inflammatory or pro-proliferative cytokines. Another option is to attach interleukin-loaded nanoparticles onto the cell membrane of adoptively transferred T cells. The sustained release of the interleukins from the stably attached particles can deliver immunostimulatory agents directly to the cells [51, 509].

Although immunostimulating cytokines expand and activate anti-tumor T cells while lymphodepletion eliminates T_{regs}, several other immunosuppressive pathways may be able to impede cancer immunotherapy [51, 509]. Blocking these pathways with immunomodulating antibodies may be effective. These inhibitory pathways are also known as immune checkpoints. They maintain self-tolerance while regulating the amount and the duration of immune responses. Several immunoinhibitory pathways are involved in tumor development. So, a combination of antibodies may enhance and

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prolong the anti-tumor effects. For example, a combination of CTLA-4 and PD-1 pathway inhibition caused a synergistic effect that further enhanced the activation of anti-tumor T cells. This improved the long-term survival rates compared to the inhibition of both pathways alone. Moreover, the tumor rejection was further improved by including a cancer vaccine [51, 509].

However, patients can still have different immune responses against cancer, based on mutations or polymorphisms in immune regulatory genes or exposure to environmental toxins [51, 509]. So, researchers are looking for both prognostic and predictive biomarkers. Predictive biomarkers can be used to help make therapeutic decisions. For example, indoleamine 2,3-dioxygenase (IDO) and FoxP3 are two suggested biomarkers for patients being treated with ipilimumab. IDO is an immunosuppressive enzyme that is expressed by immune cells and cancer cells, while FoxP3 is a marker of T_{Regs}. It is a transcription factor that enables T_{Regs} to limit immune responses and favor self-tolerance, including in cancer. The increased expression of both of them was associated with a positive clinical response to ipilimumab. In another study, the pre-existence of antibody responses and the presence of tumor antigen-specific T cells isolated from the peripheral blood of a patient were found to be predictive biomarkers for treatment with ipilimumab. This is because the presence of these biomarkers was highly correlated with the clinical benefit the anti-CTLA-4 treatment. Also, the PD-L1 ligand was suggested to be a potential predictive biomarker for the antibody therapy against the PD-1 pathway. In general, an inflamed microenvironment around T cells can be a predictive biomarker for all cancer immunotherapies. This is because patients that have T cells in the tumor site are more likely to benefit from additional immunotherapies such as blocking the PD-1 pathway. Therefore, the presence of TILs and PD-L1 on the tumor cells is a better predictive biomarker for patients receiving PD-1 immune checkpoint blockers than PD-L1 expression alone. Consistent with systems thinking, this means that a combination of predictive biomarkers is better for making clinical decisions [51, 509].

Perhaps the best-known patient to be successfully treated by blocking PD-1 is President Jimmy Carter, who had metastatic melanoma [511]. In 2015, it was announced that his cancer was in remission [511]. As of 2018, he is still alive, healthy and active.

All this work has led to a new term being coined – immuno-oncology [512]. It uses antibodies, peptides, proteins, small molecules, adjuvants, cytokines, oncolytic viruses, bi-specific molecules and cellular therapies. Ever since the approval of the first mAb, ipilimumab, this field has

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experienced a paradigm shift that includes systems-based thinking. It rejects dogma and introduced progress in both preclinical and clinical science, including the development of new methods of investigation. Immunotherapeutic drugs don't attack tumors directly, but activate the neuroendocrine immune system to produce positive clinical results. The first two anticancer immune-oncology therapies to be approved (ipilimumab and tremelimumab) were evaluated using the old chemotherapeutic drug development paradigm. Response rates were low and there were inflammatory side effects. While these drugs were being investigated, the Cancer Immunotherapy Consortium (CIC) of the Cancer Research Institute (CRI) started a new immunotherapy development paradigm. This led to two important insights. First, immunotherapies can produce some mixed or new types of responses that can't be captured by conventional chemotherapy criteria such as the Response Evaluation Criteria in Solid Tumours (RECIST) and the criteria set out by the WHO. Second, the effects of immunotherapy on time-to-event end points such as survival may take several months to manifest and this should be accounted for when evaluating randomized clinical trials. These insights helped get ipilimumab approved. It also became the basis for using new criteria to assess clinical response, termed immune-related response criteria (irRC). This included new clinical end points, such as immune-related overall response rate (irORR), immune-related disease control rate (irDCR), immune-related progression-free survival (irPFS) and milestone survival. It also characterized immunotherapy toxicities, called immune-related adverse events (irAEs). Their clinical management was defined in treatment algorithms. These algorithms subsequently provided a model creating other safety-management processes for next-generation immunotherapies. The CIC's initiatives led to a broad collaboration in the immunotherapy community and provided information that was used in regulatory guidance documents issued by the FDA and the European Medicines Agency (EMA) [512].

About four years after the approval of ipilimumab, second generation checkpoint (PD-1) inhibitors entered clinical trials [512]. The first drugs to target PD-1 – pembrolizumab (Keytruda®) and nivolumab (Optdivo®) - were approved by the FDA and the EMA in 2014 (pembrolizumab) and 2015 (nivolumab). Atezolizumab (MPDL3280A, Genentech/Roche) and durvalumab (MEDI-4736, AstraZeneca/ MedImmune) are in clinical trials. Pembrolizumab was approved in record time, after only four years in the clinic. In addition, blinatumomab, a bi-specific T cell engager (BITE) that targets CD19+ B cell malignancies, was approved in 2015. Finally, autologous cell therapies that target CD19 using chimeric antigen receptor

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(CAR)-transduced T cells are being developed. In the spirit of TQM and using systems-based thinking, a CIC workshop brought together the four pharmaceutical companies that were working on these drugs. They assessed the usefulness of this new model and its clinical endpoints, irORR, irDCR and overall survival. In 2013, *Science* magazine designated cancer immunotherapy as the breakthrough of the year. Then, in 2014 and 2015, immuno-oncology technologies and business deals made based on them were called extremely valuable at a J.P. Morgan investment banking conference. Moreover, health-care analysts have predicted a market size for this sector of US$35 billion by 2023 [512, 513].

In addition to these, there is also a third generation of immuno-oncology drugs that target the innate immune system [512]. This includes "cytokines, chemokines, cell therapies (including genetically engineered cells), checkpoint modulatory antibodies, cancer vaccines, BITEs that direct T cells to cancer cells (connectors), dual-specific antibodies that integrate two targeting moieties into one molecule, small molecules, oncolytic viruses and immune adjuvants (for example, toll-like receptor (TLR) agonists)" [512].

Overall, immuno-oncology drugs are changing standards of care, improving end points and research methods, discovering new biomarkers and providing new possibilities for combination therapies [512].

Synthetic biology can also be used to produce engineered therapeutic T cells [514]. They are in clinical trials for treating cancer, solid organ transplants and autoimmune diseases. However, the design and manufacturing of these T cells are not standardized and are usually done in academic settings. Moreover, reliable methods for determining appropriate doses and evaluating pharmacokinetics need to be developed and approved by regulatory agencies. "The goal of T cell engineering is to augment the natural functions of T cells through *ex vivo* manipulation" [514]. Unmanipulated allogeneic T cells have been used for some time to treat hematopoietic malignancies in patients who have received hematopoietic stem cell transplants [514, 515]. In addition, autologous tumor-infiltrating lymphocytes (TILs) were obtained from 20 patients with metastatic melanoma and adoptively transferred back to them together with IL-2 [514, 516, 517]. Eleven of these patients had positive, objective responses and some had complete remission of their tumors [514, 516, 517]. However, success with melanoma may not mean that other tumors that are less immunogenic can be treated this way [514]. So, approaches using genetic engineering are being investigated. T cell receptors that recognize tumor antigens have been cloned from TILs [514]. The receptors were subsequently inserted into autologous T cells, which were infused into

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patients with metastatic melanoma and other cancers [514, 518, 519]. Although many patients in these studies showed cancer regression, normal melanocytes were destroyed in some patients [514]. To avoid this, polyclonal autologous T cells that express CARs have been used to successfully treat patients with B cell–derived tumors that did not respond to standard therapies [514, 520-522]. Even though most of the effort on T cell therapies has been directed against cancer, some work is being done on using them to treat inflammatory and autoimmune diseases [514]. This is because T_{reg} cells enforce tolerance to many self and non-self antigens. A deficiency in these T_{reg} cells can lead to inflammatory and autoimmune diseases. So, *ex vivo* expanded T_{reg} cells are in clinical trials to treat type-1 diabetes and to prevent rejection of solid organ transplants [514].

Genetic engineering is being investigated for its use in producing new T cell therapies [514]. However, the emerging field of synthetic biology may prove to be even more useful. Unlike genetic engineering, synthetic biology uses methods that are popular among most engineers in other fields. Its goal is to make cellular engineering a systematic process in which methods are designed "to meet quantitative performance specifications set in advance, separating design from fabrication, using modular biological parts, and using standardized cell populations to allow for consistent results" [514].

Another goal is to avoid much of the reductionist thinking as well as trial and error processes that were used in the 25 years that it took to optimize CARs therapy [514]. That is, all the individual parts of of these receptors were optimized by a time-consuming and expensive trial and error process. Instead, synthetic biology aims to produce logic gates, small molecule controls and docking systems to develop new therapies. Currently used CARS combine the binding affinity of an antibody with the signaling domains of the ς-chain of the T cell receptor complex and costimulatory receptors. However, the cancer cells that are targeted can only sense a single antigen in these forms of CARs therapy. So, synthetic biology aims to produce CARs that can sense two antigens to make an AND gate that opens only when both antigens are detected. At the same time, synthetic biology is also trying to gain control of T cell behavior by using small molecules. For example, GPCRs that respond only to synthetic small-molecule ligands have been developed for each class of GPCRs. Another approach uses RNA aptamers in the 3' untranslated region of genes. When they are bound to a small-molecule ligand, the RNA transcript should be stabilized, and the gene product will be translated. In the absence of ligand, the RNA is destroyed. Finally, there is also an effort being made to control CAR T cell activity and specificity by making "a

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universal CAR T cell whose CAR receptor can be docked with an antibody or antibody fragment that gives the cell different specificity" [514].

3.4 Synthetic biology and CRISPR technology in new drug development

Recent advances in synthetic biology and other technologies have inspired new collaborations that are based on teamwork, TQM and systems thinking [523]. In the past, pharmaceutical companies have been reluctant to start research programs that often lead to failure. So, research institutes and foundations that focus on individual diseases have filled this pre-clinical gap. Through venture philanthropy, foundations have formed partnerships with the pharmaceutical industry and regulatory agencies (such as the FDA) to share financial risks, shorten the time it takes to complete preclinical studies and do research that focuses on benefits to humanity instead of profit. These collaborations are producing important results in treating cystic fibrosis, multiple myeloma and type-1 diabetes [523].

Synthetic biology can also be used to make hundreds of thousands of NMEs that could become effective drugs [524]. One way to do this is to make new Bacteria that can produce natural products. Bacteria and plants that already exist have been sources of natural products that have been used as prescription drugs to treat infections and cancers, as well as being used as immunosuppressive agents. Many organisms that biosynthesize natural products use genes that are all located in compact biosynthetic gene clusters. DNA sequencing and bioinformatics are being used to identify these clusters. Some people claim that natural products are an almost limitless resource because of the incredible biological and chemical diversity that exists in Gaia. It seems limitless in part due to the relatively low throughput methods that are currently available to evaluate these natural products and their sources. However, improved bioinformatics and a rapid increase in the number of genomes that have been solved are closing this gap. As the cost of sequencing and synthesizing DNA continues to decrease, this gap will narrow. However, this will not be easy since many factors must be considered and understood better. This includes DNA transcription, translation of mRNA into proteins, protein-protein interactions, export and bacterial resistance. So, synthetic biology is being used to improve the properties of proteins (protein engineering) and to understand how several enzymes can self-assemble into metabolic pathways (metabolic engineering). Applied microbiology and strain

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engineering are being used to identify existing organisms and make new organisms that produce more natural products. Synthetic biology focuses on using tools to accelerate and increase the productivity of genetic engineering. This includes creating genetic parts such as promoters that induce the desired level of gene expression. It also includes using advanced methods to synthesize DNA that contains 20 – 100 kbases that are needed to produce a gene cluster. New computer-aided design models are also being developed. Genetic 'circuits' have been made that can function as logic gates, timers, switches and oscillators. It is also useful to make many changes in an organism's genome by using CRISPR-Cas9. Since systems-based thinking rejects dogma, it also recognizes that there are times when some reductionist thinking can be useful. So, large biosynthetic gene clusters are being broken down into 'genetic parts'. That is, these gene clusters often contain several genes that exist as operons that can face either direction in the genome. The 'genetic parts' have modular functions. So, some genes can be 're-written' or 're-factored' to make them easier to work with in bioengineering. This leads to a "modular genetic architecture, which facilitates part-swapping and combinatorial optimization" [524].

At the same time, synthetic biology has been used to develop 'insulators' that increase the reliability of 'genetic parts' when placed in different organisms [524]. Still, the design and synthesis of DNA libraries have improved to the point to where screening and understanding the results have become the bottlenecks. Currently available methods in analytical chemistry can analyze each sample in under 3 min. This means that about 1000 samples can be analyzed per day. Alternatively, *in vivo* biosensors are being made that can measure the concentrations of natural products by producing light or fluorescence. For example, the fluorescence of the natural product astaxanthine has been used to determine the titers in single cells by flow cytometry and cell sorting. This led to the production of over-producing strains that made 10 000 times as much astaxanthine than other techniques [524].

This type of increased production can be accomplished by modifying the natural regulatory elements that limit the amount of natural products that a wild-type organism can produce [524]. They use a complex regulatory network consisting of protein-protein interactions, RNA and DNA that perform the equivalent of a computational operation. In contrast, CRISPR is being used to create artificially connected regulatory proteins that build cascades, bistable switches, pulse generators, oscillators, feedback/feedforward motifs, and logic gates. By replacing the native biosynthetic circuits with synthetic circuits created using CRISPR

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technology, the necessary feedback and dynamics can be created. They bypass the native regulatory elements that limit the production of the natural products that humans need for their survival, but the organisms don't need at such high levels to survive in their native ecosystems [524].

Synthetic circuits can act as 'control valves' to redirect metabolic pathways [524]. This control must be dynamic to keep from producing organisms whose growth is limited by producing too many natural products all the time (constitutively). The timing of production can be controlled by bistable switches and engineered timing circuits. In addition, genetic logic gates enable many inputs to be integrated before a metabolic pathway is turned on or accelerated. Once the synthetic multi-input circuits are connected, cells can detect cofactors, metabolic precursors, dissolved O_2 and energy (ATP) levels before they commit to making the desired natural products [524].

In addition, changes in the genetics of organisms outside the biosynthetic gene clusters are needed to produce higher titers (amounts) of natural products [524]. In the recent past, genetic diversity in strains of Bacteria used in industrial-scale production has been generated by random mutagenesis. Screening methods identified the most useful mutations. These days, synthetic biology can generate genetic diversity in a multiplex way. Also, genomes can be made by *de novo* chemical synthesis. Multiplexed genetic engineering can target hundreds of genes for over- or under-expression in parallel. When this process is automated, it is called MAGE (Multiplexed Automated Genome Engineering). This enables combinatorial exploration of mutations in a continuously evolving population of Bacteria and yeast. A related technique is called TRMR (trackable multiplex recombineering). It was developed for use in mapping >95% of the genes in *E. coli* to specific traits. It uses array-based DNA synthesis to make barcoded oliogonucleotides that can target >4000 genes for either over- or underexpression [524].

At the same time, synthetic biology is teaching us much about biological systems [525]. This was described in several articles in a special issue of *Synthetic Biology*. The ways that biologists are building genetic circuitry were described, while considering their differences between human-made electronic circuits. It also discussed library-based approaches that complement rational drug design for constructing and optimizing DNA circuitry and metabolic pathways. It uses computational modeling based on a competitive growth equation to learn how synthetic systems can respond effectively to changes in their environment. Moreover, "synthetic biology can be used to increase our understanding of biological processes from a systems-level perspective" [525]. It simplifies biological

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phenomena by redesigning minimal components. The fundamental elements of a given process are identified and quantified. This has led to a better understanding of "multistable, stochastic, oscillatory, and pattern forming dynamics in bacteria" [525]. It has also provided a better understanding of the intra- and extracellular organization of mammalian cells. Synthetic biology also bridges the gap between geneticists and biologists, who are identifying the parts and establishing their interrelationships while building complex living systems from these parts. It is also converting cells into test tubes in which hypotheses can be tested. This is similar to synthetic quantum devices that are being used by physicists to "reveal fundamental quantum properties that are usually nonexistent in nature due to their stripped down simplicity" [525]. Similarly, the well-characterized *lac* operon (for regulating bacterial transcription of DNA) is analogous to the hydrogen atom in physics since it can be characterized quantitatively. In addition, positive and negative feedback mechanisms can be identified. The special issue of *Synthetic Biology* also described some applications of synthetic biology, such as those being used in cancer immunotherapy. One goal is to provide additional levels of control beyond CAR-T therapy to genetically engineer a patient's own T cells to express receptors on cancer cells and deliver them back to the patient. Another goal is to make components and metabolic pathways of pathogenic organisms that can be used to develop screening methods for new drugs without having to use the complete organism. Others are recoding the UAG codon in *E. coli* to make it code for a sulfotyrosine in proteins like the one that promotes resistance to the bacterial pathogen *Xanthomonas oryzae* in rice. Still others are genetically modifying bacteriophages to detect and suppress pathogenic Bacteria, package nanomaterials and fight infections in agricultural crops. There are also people who are altering soil Bacteria such as *Pseudomonas putrida* so they can biodegrade environmental pollutants such as *m*-xylene [525]. Finally, some groups are using systems thinking to combine synthetic biology, complexity, and tissue engineering to build and study organs and organoids [525, 526]. They have mapped the diverse collection of organs and organoids into a morphospace (the space of possible structures). It has three axes, similar to the x-, y- and z-axes in Euclidean space. Instead, the three axes are physical state, developmental complexity, and cognitive complexity. It revealed a large, unexplored region that can guide the discovery and production of new organs not found in nature. Systems thinking is combining our knowledge of organogenesis, organotypic cultures, tissue bioprinting and synthetic biology to build artificial organs and organoids. A unifying framework connects the concept of

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morphospace with synthetic biology and tissue engineering. The goal is to use our understanding of both evolutionary and architectural constraints to explore diverse design principles to build these artificial organs and organoids [535, 526].

So, CRISPR and multiplexed engineering of genomes have produced scientists that communicate in the languages of both electrical engineering and biology. This collaborative, interdisciplinary effort is quite consistent with systems thinking. However, one should be cautioned against thinking that genetic engineering produces machines instead of living organisms [525, 527]. Instead, it is producing "non-machines". That is, living organisms are not made by external agents. Humans might design some new organisms, but they end up making themselves. Moreover, "biological components show massive overlapping and functional degeneracy, standard-free complexity, intrinsic variation and context dependent performances" [527].

CRISPR is actually a naturally-occurring, ancient defense mechanism that many Bacteria use to prevent viral (bacteriophage) infections. These Bacteria have genomes that contain base sequences that are repeated many times, with unique sequences in between the repeats. They are called "clustered regularly interspaced short palindromic repeats" or CRISPR. It is a part of the bacterial immune system that keeps pieces of viral genomes around so they can recognize them and defend the host against future infections. The second part of the defense mechanism is a set of enzymes called Cas (**C**RISPR-**as**sociated proteins), which can precisely excise the viral DNA sequence and destroy the invading viruses. Fortunately, the genes that code for Cas enzymes are always located near the CRISPR sequences. The best known and most useful Cas enzyme is Cas9, which comes from *Streptococcus pyogenes*, which causes strep throat. When combined with CRISPR, it becomes the CRISPR/Cas9 system. This is often simply abbreviated as CRISPR. So, Cas9 hydrolyzes the DNA, while CRISPR shows it where to do the hydrolysis. As a result, scientists can show Cas9 the right sequence, called a guide RNA. The desired base sequences can be cut out and pasted anywhere into the host's genome. In the CRISPR-Cas9 system, the enzyme Cas9 catalyzes the excision of viral DNA at a location specified by an RNA molecule - the CRISPR RNA (crRNA). It is bound to another RNA, the trans-activating crRNA, or tracrRNA. Faulty genes can be cut out and replaced with the desired, functioning gene. Alternatively, genes can simply be removed to determine their effects on phenotypes. This has been done for many years using mice whose genes have been 'knocked out'. However, it can take years to establish new lines of genetically-altered mice this way. Instead, it

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only takes a few months to make genetically modified mice (or other organisms) using CRISPR. So, CRISPR can be used to make completely new organisms and potentially to bring back formerly extinct species, such as the woolly mammoth [528].

In contrast, when the expression of a gene is inhibited, it is called CRISPRi [524]. In this technique, a catalytically inactive (or dead) Cas9 (called dCas9) is expressed and directed to the desired target when the single guiding RNA is transcribed. This regulates the expression of genes on a transcriptional level, as opposed to a translational level, which is used in RNA interference by siRNAs. CRISPr can also be used to activate the expression of a gene by fusing a transcriptional activator to dCas9. Several single guide RNAs can be used to control many genes simultaneously. Unlike siRNAs, CRISPRi can also target ncRNAs, microRNAs, antisense transcripts and RNAs that are in the cell nucleus.

CRISPR has been used to reverse mutations that cause blindness, stop cancer cells from multiplying and make white blood cells impervious to the HIV virus [529]. Agronomists have made strains of wheat that are not affected by deadly fungi, such as powdery mildew. Other researchers are removing defective genes from pigs and cattle and replacing them with healthy genes. Similar research could produce engineered staple crops and livestock that may be able to feed a population of 9 billion on a warmer form of Gaia. CRISPR has also been used to make genetically modified yeasts that can consume plant matter and excrete ethanol, which could help end our addiction to fossil fuels. This technique has been called revolutionary, but like many revolutions, it may become dangerous. This was not the first time that genetic engineering has been called potentially perilous [529].

In 1975, geneticists, biochemists and others went to Asilomar, California to discuss the potential problems that could be caused by research on cloning and recombinant DNA [529]. Many people were afraid of what some called a “God-like power” to insert new genes from one organism into another. If used safely and wisely, it could produce Bacteria and other genetically-modified organisms that could manufacture new, lifesaving prescription drugs. However, it could also be used to possibly make genetically modified humans and even ‘super babies’ or ‘super soldiers’ with blond hair and blue eyes or any other trait that parents or society might find fashionable or useful in warfare. There was also the fear that such genes could be transferred from one bacterial species to another through horizontal transfer. So, after long discussions, the scientists at the Asilomar conference wrote a consensus statement that described the kinds of experiments that should not be done, such as

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creating dangerous pathogens or pathogenic organisms. Few, if any worried about modifying human embryos to make a 'super race' with traits that could be passed on to future generations. They thought that it would be too difficult to be done in the foreseeable future [529].

Well, the future has changed. Viable human preimplantation embryos were modified by an international team using the CRISPR technique to correct pathogenic gene mutations [530, 531]. They targeted a mutation in the *MYBPC3* gene that causes myocytes to thicken (hypertrophic cardiomyopathy), which is a leading cause of death in young athletes. It is a dominant mutation, so a child only needs one copy of it to suffer from this condition. They also overcame two safety problems that had limited previous efforts: off-target mutations and mosaicism. No unintended genetic targets were modified and no genetic mosaicism occurred. They also went around current regulations in the USA that prevent federal money from being used for research on human embryos by using private funding. Such regulations don't exist in several other countries, so some Chinese scientists have already reported using CRISPR to alter genes in human embryos. So, CRISPR technology has advanced far enough for people to consider using it to correct inheritable mutations in human embryos after they are discovered in preimplantation genetic analysis [530, 531].

Still, just two years before the journal article that described modifying a human embryo was published, some of the same scientists that met at Asilomar subsequently met at the Carneros Inn in Napa Valley [529]. All the attendees had access to the CRISPR technology. They discussed the moral and legal implications of genetic engineering [529]. After the conference, an article was published in the prestigious journal *Nature* (which also published the report of the *MYBC3* gene being targeted) titled "Don't edit the human germ line" [532]. Many countries (but not the USA or China) have laws that specifically forbid genetic engineering in humans, including 15 of 22 nations in Western Europe. Still, the US National Institutes of Health's Recombinant DNA Advisory Committee stated that it "will not at present entertain proposals for germ line alterations" [532]. However, people who say that something can't be done or shouldn't be done are often being passed by other people who are doing it.

One example of this is the low-cost detection of Zika virus [533]. It uses paper-based sensors in a cell-free system. Scientists linked isothermal RNA amplification with toehold RNA sensors and coupled this to a CRISPR-Cas9 module. "Toehold switch sensors are programmable synthetic riboregulators that control the translation of a gene via the

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binding of a *trans*-acting trigger RNA" [533]. This method was able to distinguish between different strains of the Zika virus in an infected macaque with single base resolution. The freeze-dried platform enables it to be used in the field and is potentially applicable to many diverse pathogenic viruses [533]. Another example is to sensitize pathogens to antibiotics [534]. Bacterial populations can be edited to eliminate genes that make them resistant to antibiotics. So, a CRISPR system was delivered into Bacteria using a temperate bacteriophage that has a lysogenic life cycle. The CRISPR system was programmed to destroy plasmids that have DNA coding for antibiotic resistance and to protect against specific lytic phages (viruses). This linked bacteriophage protection with antibiotic sensitization. It applies selective pressure against antibiotic-resistant Bacteria and in favor of Bacteria that are sensitive to antibiotics. This was accomplished by using temperate phages in the CRISPR system and then delivering lethal phages to the Bacteria [534].

There are many other potential applications of CRISPR in new drug development [535]. It is being used to make new mammalian model systems to accelerate functional genomics, which studies the interactions between genes and functions of the proteins which they encode. This is leading to new discoveries about cellular mechanisms for identifying or validating new therapeutic targets. It is also being used to make new animal models for human diseases. Potential clinical applications of CRISPR are not restricted to genetic diseases. It is being used to make customized autologous cells for personalized therapies. This includes T-cells that bind to a patient's cancer cells and reprogrammed iPSCs. This could lead to new therapies based on the CAR-T technology. For now, CAR-T cells are made from each patient's own T-cells, so they are called autologous. This is difficult and time consuming. So, research is being done to develop universal donor CAR-T cells that can be stored and pulled off the shelf when needed by any patient. To prevent graft-versus-host toxicity, researchers are trying to find ways to decrease the expression of histocompatibility antigens on the engineered donor T-cells. Others are looking for ways to increase the efficacy of CAR-T cells by knocking out the genes that code for receptors that inhibit T cells or signaling molecules. Examples include the cytotoxic T lymphocyte-associated protein 4 (CTLA4) or programmed cell death protein 1 (PD1). For example, the US NIH Recombinant DNA Advisory Committee (RAC) recently approved a clinical trial in which Cas9 will be used to knock out the genes encoding PD1 and the endogenous T cell receptor in melanoma-targeting CAR T cells. Chinese workers recently began the first clinical trial of CRISPR–Cas. They are using Cas9 to knock out *PD1* in T cells of

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people who have lung cancer, although no CAR will be introduced in that trial. CRISPR-Cas is also being studied for its possible use in *ex vivo* gene editing of peripheral blood samples. Once the desired genes are removed or inserted, the blood can be transfused back into the patient. For example, attempts are being made to induce the expression of the fetal hemoglobin γ-subunit (γ-globin) in patients who have sickle cell disease or β-thalassaemia. The transcription factor BCL11A represses the expression of fetal γ-globin in adults. So, two companies, Biogen and Sangamo Therapeutics have combined their efforts to focus on the enhancer region of BCL11A [535].

Finally, CRISPR-Cas is being used to produce completely new organisms and to try to bring back extinct species [528]. For example, CRISPR-Cas is being used to make mutants of the mosquitoes that causes malaria (*Anopheles* genus), yellow fever, dengue and chikunguya and Zika (*Aedes aegypti*), as well West Nile and encephalitis (*Culex quinquefasciatus*). Together, these mosquitoes have killed more people than any other animal on Earth – including us humans with all our wars. CRISPR-Cas9 is being used to identify genes in human hosts that can reduce viral infection when they are edited [536]. Researchers from China and the USA did a genome-wide CRISPR-Cas9 screen that targeted 19050 genes. The goal was to identify those human host genes that could reduce flavivirus infection. This included the West Nile, Zika, Japanese encephalitis, Dengue serotype 2 and yellow fever viruses. They found nine genes that flaviviruses needed to be infective. They were associated with the function of the endoplasmic reticulum. Genes coding for signal peptidase complex (SPC) proteins were found to be required for the proper breakdown (hydrolysis) of flavivirus structural proteins and the secretion of viral particles. When the expression of these genes was suppressed, it not only reduced viral infections, but also had no adverse side effects on the human host cells. One or more of these SPC proteins could become effective therapeutic targets for new prescription drugs to protect against infections by flaviviruses [536].

Another potentially controversial approach is to create genetically modified mosquitoes that have properties that will kill or neutralize mosquitoes that carry deadly diseases. One approach is to make genetically engineered males that are either sterile or carry a dominant lethal mutation that kills any potential offspring [537]. However, creating and releasing many genetically modified mosquitoes is expensive and not all countries have the necessary infrastructure to do it. Instead, synthetic gene-drive systems can enable super-Mendelian inheritance of a transgene that could potentially reduce mosquito populations without having to

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create millions of genetically modified mosquitoes and releasing them into the environment. Instead, the gene drive technology can simply cause a desired gene or genes to be released [538]. The idea is that they will spread on their own. Gene drives are 'selfish' genetic elements that spread through wild populations even though they confer no survival or reproductive advantage in the individuals that carry them. They can even make those individuals and their offspring less fit [538]. So, CRISPR-Cas9 was used to produce a gene drive system in *A. gambiae*, the carrier of malaria [539]. It targeted the reproduction of female mosquitoes. The goal is to reduce mosquito populations to such low levels that they can't spread malaria effectively [539].

However, some people may question the wisdom of using gene drives that cause genes to spread relatively uncontrollably in the environment. Such concerns already exist on the use of genetically modified crops, like soybeans that are more commonly referred to as genetically modified organisms (GMOs) [263]. However, unlike these GMOs that can spread their seeds and generate many new offspring, the goal of producing genetically modified mosquitoes is to NOT produce more offspring and possibly even eradicate them completely. Still, some may question the wisdom of possibly driving any species into extinction – even the deadly mosquito. In contrast, the attempt to resurrect currently extinct species like the woolly mammoth may not raise such concerns. Instead, the goal is to produce enough woolly mammoths and other large but extinct animals to convert the frozen tundra in Siberia from forests to its former grasslands that will be more environmentally friendly and reduce the emission of methane gas as the frozen tundra melts. However, the woolly mammoths and other extinct species will not be exactly the same as the animals that existed in the Pleistocene epoch. They will be different holobionts with different microbiota because they will be grown *in utero* in currently existing species, such as elephants for the woolly mammoth. Still, if they can adapt to their new microbiota, they could grow to be big enough to convert the vegetation in the Siberian tundra to grass, which will reflect more sunlight than the trees that are there now. However, there is so much that we don't know about the Siberian ecosystem that some people are saying we should be cautious or maybe not even allow such things to be done. Still, people who say it shouldn't be done are often being passed by people who are doing it. In fact, a new version of the CRISPR system has been developed that can target not only DNA, but also RNA [540]. In contrast to CRISPR-Cas9, Cpf1 can process the pre-crRNA on its own, and then using the processed RNA to target and cut DNA specifically [541]. Not requiring a host-derived RNase and the tracrRNA makes this

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the most minimalistic CRISPR immune system yet. The mechanism of combining two separate catalytic modalities in one creates new possibilities for sequence specific genome engineering, and enables researchers to target several sites at once or do multiplexing [541].

3.5 Rapid prototyping by 3D printing

3.5.1 Introduction

Rapid prototyping has been used in the automotive and aerospace industries for decades [234]. Before an assembly line started making thousands of copies of parts that went into a car, truck or airplane, plastic models (prototypes) were made to be sure that all the parts could fit together properly. Rapid prototyping was also used in the Human Genome Project to make plastic models of biopolymers, based on their 3D structures in the Protein Databank. Some of these models were used to help teach structural biology and are in the Brookhaven National Laboratory. Rapid prototyping, or 3D printing, has now improved so that it can be used with systems thinking to make personalized devices such as a bioresorbable tracheal splint for an infant who was critically ill [542]. 3D printing is now able to make food and is transforming the supply chain for restaurants. Even the U.S. Army is trying to use 3D printing to customize food for every soldier. At the same time, 3D models of images of tissues and bones from computed tomography (CT) or magnetic resonance imaging (MRI) enable surgeons to know what to expect before they begin surgery. Models made this way can also be used by medical students and residents to learn how to do difficult surgeries. At the same time, 3D models can make it much easier for oncologists to deliver radiotherapy to the precise, proper locations in tumors. In 3D printing, one or more materials are printed one layer at a time in a precise pattern that is controlled by a computer [543]. These patterns can be obtained from CT scans (for biomedical applications) or from the Cartesian coordinates of the atoms in a protein or other biopolymer that have been deposited into a protein data bank (for education about protein, DNA and RNA structures). In addition, self-organizing cells can be coated on, or embedded with live cells and growth factors to make tissues [543]. The field of 4D printing has been reviewed [544].

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3.5.2 Biodegradable scaffolds

3D printing has been able to make biodegradable scaffolds (called AngioChips) with with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis [545]. They were made from biodegradable poly(octamethylene maleate (anhydride) citrate), or POMaC. It is polymerized by exposure to UV light and is more elastic than polyesters that have already been approved by the FDA. AngioChips support "the assembly of parenchymal cells on a mechanically tunable matrix surrounding a perfusable, branched, three-dimensional microchannel network coated with endothelial cells" [545]. It decouples the choice of materials for the network of blood vessels from the cell seeding in the parenchyma. This enables remodeling while maintaining an open-vessel lumen. Nanopores and micro-holes are incorporated into the walls of the blood vessels, making them permeable and enabling intercellular crosstalk. Also, monocytes and endothelial cells can undergo extravasation when the system is stimulated. Hepatic and cardiac tissues made this way can process prescription drugs and INDs that are delivered through the vasculature. When AngioChip cardiac tissues were implanted into the femoral vessels of rat hindlimbs, they perfused blood immediately. So, this platform could enable *in vitro* test results to be translated into *in vivo* validation of these tests and even the development of devises for regenerative medicine [545].

3.5.3 Other applications of 3D and 4D printing

The FDA has approved several naturally derived polymeric biomaterials for clinical use [546]. This includes collagens, silks, elastins, alginates, and fibrins that all have acceptable biocompatibility and bioactivity, as well as tunable mechanical and degradation kinetics. They don't release toxic degradation products and are often processed using environmentally friendly aqueous-based methods. Collagen hydrogels, silk proteins, alginates are being developed for bone replacement. Calcium phosphates such as hydroxyapatite are used as bone cements and filler materials. Research is also being done to develop materials for regenerating muscle and nerve tissue. Biomaterials based on type-1 collagen are being developed for repairing peripheral nerves and soft tissues. FDA-appproved materials for repairing peripheral nerves that use type-1 collagen include NeuroMatrix™, NueroFlex™ and NeuroMend™. TissueMend™ is approved for repairing tissues. Research is also being done to develop hydrogels and decellularized extracellular matrix

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solutions that can be injected into the heart to repair cardiac tissue. Polysaccharides such as alginate and chitosan are currently being evaluated. Liquid alginate solutions containing sodium alginate and calcium gluconate are undergoing clinical trials. Gelatin-based sheets are also being used to make cardiac patches to treat ischemic cardiomyopathy. Fibrin-based scaffolds are being tested *in vitro* for cardiac repair. To repair damaged cornea in the eyes, cross-linked recombinant human type III collagen is being developed. Finally, research is also being done to produce "pre-vascularized and reinnervated tissues to promote angiogenesis and neovascularization, lymphangiogenesis, and innervation in order to create fully functioning tissues" [546].

So, 3D printing is making a huge impact on regenerative medicine, an interdisciplinary field that applies engineering and life science principles to promote regeneration [547]. The goal is to restore diseased and injured tissues as well as whole organs, instead of having to use organ transplants. 3D polymer scaffolds promote the expansion of chondrocytes in cartilage repair and provide a scaffold for fibroblasts for treating venous ulcers. In addition, growth factors that promote healing and/or regeneration are being inserted into biomaterials. For example, PDGF (platelet-dependent growth factor) is used in biomaterials that promote wound healing and bone formation. Another approach is to use organs from which all the cells have been removed. This includes immunogenic cells and biochemicals that could cause the organ to be rejected by the host. After removing the unwanted cells, the cells that are needed can be added back to the organs before transplantation. Decellularized tissues to which no new cells have been added back can be used as medical devices to repair large defects in skeletal muscles. In addition, blood vessels can be engineered or modified and then decellularized before being administered to patients who need a kidney transplant. 3D bioprinting can be used to make structures that combine high resolution control of the properties of the biomaterials and the appropriate placement of cells. For example, inkjet bioprinting was used to make 3D cartilage by alternating layer-by-layer depositions of electrospun polycaprolactone fibers and chondrocytes that were suspended in a fibrin–collagen matrix. The cells produced collagen II and glycosaminoglycans after they were implanted. Microextrusion printing has also been used to make aortic valve replacements using cells embedded in a 3D hydrogel made from a mixture of alginate plus gelatin. Both smooth muscle and interstitial cells were printed into two separate regions, comprising the valve root and leaflets, respectively. Microextrusion printing of inks with different gelation temperatures produced complex 3D tubular networks. They were seeded with

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endothelial cells to mimic vasculature. Autonomous cellular self-assembly can also help to make tissues. For example, vascular cells aggregated into multicellular spheroids and were printed alongside agarose rods. This produced structures that resembled a vascular network. Given the appropriate cues and initial cell composition, even complex structures self-assemble. For example, intestinal structures resembling intestinal crypts can be grown in 3D culture that has increased Wnt signaling activities. It is also possible to include angiogenic growth factors in implants to ensure that they are properly vascularized, which is essential for them to function properly. That is, VEGF, bFGF (basic fibroblast growth factor), angiopoietin and PDGF produce robust angiogenesis and can rescue ischemic limbs from necrosis. Alternatively, endothelial cells and tissue-specific cells can be combined on a scaffold before transplantation. It's also important for transplanted 3D printed organs to be properly innervated. This can be done by including appropriate growth factors in hydrogels. Since angiogenesis and nerve growth share common signaling pathways, controlled delivery of VEGF using biomaterials can promote axon regrowth in regenerating skeletal muscles [547].

Silk fibers have been used as a scaffold for regenerating the anterior cruciate ligament (ACL) [548]. Injuries to the ACL have an estimated annual cost in the USA of about $6 billion. About 350 000 ACL surgical reconstructions are performed each year. Since silk has remarkable mechanical strength and toughness as well as a very slow rate of degradation, it could be quite useful. Moreover, silk fibers can produce a scaffold that matches the mechanical properties of normal, healthy ACLs. However, it is important to remove proteins called sericins from the silk before using them to make tham into scaffolds. By doing so, silk scaffolds were able to stimulate the regeneration of ACL in an animal model [548].

However, there are many applications in which one doesn't want a stable 3D construct, but one that can modify its shape over time – as the patient grows and/or recovers. So, when time is added as a fourth dimension, this becomes 4D printing [549]. 4D constructs transform, change shape and reconfigure themselves over time. 3D printers are still used, but with appropriate materials. The materials can self-assemble without human or machine intervention and without using added energy. This is a fundamental principle of biology, chemistry, and materials science. That's the way that precision, functionality and all higher-order structures emerge. They are due to local interactions between components and their relationship to the surrounding environment. As an example, researchers from the University of Wollongong in Australia 4D printed a valve that automatically opens and contracts in the presence of water and

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temperature. Also, a team working at the CS Mott Children's Hospital at the University of Michigan printed a splint for three boys who had tracheobronchomalacia, which causes the windpipe to collapse when they breathe. It was made of polycaprolactone, a biodegradable plastic that can be sewn around the trachea and bronchi to keep them open. The splints eventually dissolved as the children's tracheas grew stronger. So, with 4D printing one prints a structure, deposits it in the body and then it adjusts to local forces and responds to the local environment by changing its shape into whatever it needs to be [549].

4D printing is also being used to make stereolithographic models from computed tomography (CT) data [550]. The bones of the thumb ray were 3D printed while the thumb was moving to show the position of metacarpals over time. This can help with pre-operative planning. 4D bioprinting has also been used to make vascular grafts that can reduce thrombosis [551]. It can also be used to print 'multisomes' that can precisely control the delivery of drugs to the right location in the body [552]. They are made of small oil droplets that encapsulate water droplets that contain release drugs in a programmable way [552].

3.5.4 Other personalized medical devises

A bihormonal bionic pancreas has been made that continuously monitors and controls the glucose concentration in the blood of patients with type-1 diabetes who are living at home [553]. It monitored insulin and glucagon and delivered the appropriate amounts of them at the right time. It regulated the glucose concentration better than conventional and sensor-augmented insulin pump therapy without having to count dietary carbohydrates [553].

There are also many natural-based nanocomposites that can be used in regenerative medicine and to engineer bone tissue [554]. They are made up of biopolymeric matrices and bioactive nanosized fillers that change the properties of the polymeric matrix. This enables improved biomaterials to be engineered so that they obtain properties that the individual components don't have. The nanoparticles have large surface areas, so they can bind tightly with the polymeric matrices. This gives them better mechanical properties, while maintaining the favorable osteoconductivity and biocompatibility of the fillers. This influences protein adsorption, cellular adhesion, cell proliferation and differentiation into new tissues. The different types of biopolymers and calcium phosphates were described in the review. In addition, processing methods used to engineer nanocomposites were discussed [554].

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In conclusion, drug development using systems thinking is a nonlinear, iterative process that requires feedback and open communication between all stakeholders. The different steps in new drug development and marketing of approved drugs have been described. Even though many approved drugs were developed based on their abilities to affect a single therapeutic target, we now know that many of them can be re-positioned to treat other diseases. In addition, new drug combinations are showing synergistic healing properties. At the same time, some of the therapeutic targets that were effectively targeted are now known to have formerly unexpected biological roles. More recently, phenotypic screening has reemerged as a pre-eminent way of finding new lead compounds. However, few of these ideas or techniques are being used in developing or marketing dietary supplements – some of which are fraudulent and/or adulterated with deadly poisons and former prescription drugs that have been banned and pulled off the market. So, important differences between prescription drugs, dietary supplements and 'superfoods' were described. Subsequently, new applications of immunotherapy that enable one's body to heal itself were described. Different ways that CRISPR-Cas9 technology can be used in immunotherapy and other medical applications were described. Finally, rapid prototyping by 3D and 4D printing was briefly discussed.

References

1. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 2nd ed*. Bentham Science, Sharjah, U.A.E. **2014**.
2. FDA, Developing Products for Rare Diseases & Conditions, http://www.fda.gov/ForIndustry/DevelopingProductsforRareDiseasesConditions/default.htm
3. Gong S, Jin S. Current progress in the management of rare diseases and orphan drugs in China. *Intractable Rare Dis. Res*. 2012, *1(2)*, 45-52.
4. Armstrong W. Pharma's Orphans. *Pharmaceutical Executive* May **2010**.
5. US FDA. Search Orphan Drug Designations and Approvals. http://www.accessdata.fda.gov/scripts/opdlisting/oopd/index.cfm
6. Bill & Melinda Gates Foundation, http://www.gatesfoundation.org/Pages/home.aspx
7. Wellcome Trust. Orphanet portal for rare diseases and orphan drugs **2016**.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

http://www.orpha.net/consor/cgi-bin/Directory_Professionals.php?lng=EN&data_id=27858&MISSING%20CONTENT=WELLCOME-TRUST&title=WELLCOME-TRUST&search=Directory_Professionals_Simple

8. Jupe S, Akkerman JW, Soranzo N, Ouwehand WH. Reactome - a curated knowledgebase of biological pathways: megakaryocytes and platelets. *J. Thromb. Haemost*. **2012**, *10*, 2399–402.
9. Xu K, Coté TR. Database identifies FDA-approved drugs with potential to be repurposed for treatment of orphan diseases. *Brief. Bioinform*. **2011**, *12*, 341-345.
10. Li J, Zheng S, Chen B, Butte AJ, Swamidass SJ et al. A survey of current trends in computational drug repositioning. Briefings in Bioinformatics, **2015**, 1–11, doi: 10.1093/bib/bbv020.
11. Volmar C-H, Wahlestedt C, Brothers SP. Orphan diseases: state of the drug discovery art. *Wien. Med. Wochenschr*. **2016**, DOI 10.1007/s10354-015-0423-0.
12. Novick PA, Ortiz OF, Poelman J, Abdulhay AY, Pande VS. SWEETLEAD: an *in silico* database of approved drugs, regulated chemicals, and herbal isolates for computer-aided drug discovery. *PLoS One* **2013**, *8 (11)*, Article e79568.
13. Zhou H, Gao M, Skolnick J. Comprehensive prediction of drug-protein interactions and side effects for the human proteome. *Sci. Rep*. **2015**, *5*, article 11090.
14. Litterman NK, Rhee M, Swinney DC, Sean Ekins S. Collaboration for rare disease drug discovery research. *F1000 Res*. **2014**, *3*, Article 261, 15 pp.
15. Sisk JM, Frieman MB. Screening of FDA approved drugs for treatment of emerging pathogens. *ACS Infect. Dis*. **2015**, *1*, 401-402.
16. Zheng W, Thorne N, McKew JC. Phenotypic screens as a renewed approach for drug discovery. *Drug Disc. Today* **2013**, *18*, 1067-1073.
17. Eder J, Sedrani R, Wiesmann C. The discovery of first in-class drugs: origins and evolution. *Nature Rev. Drug Disc*. **2014**, *13*, 577-587.
18. Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. *Neurol*. **1998**, *50*, 136-145.
19. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* **2001**, *292*, 1160-1164.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

20. Buchdunger E, Zimmermann J, Mett H, Meyer T, Müller M et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res*. **1996**, *56*, 100-4.
21. Pan X-S, Ambler J, Mehtar S, Fisher LM. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in Streptococcus pneumoniae. *Antimicrob. Agents Chemother*. **1996**, *40*, 2321-2326.
22. Cihlar T, Ray AS. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. *Antiviral Res*. **2010**, *85*, 39-58.
23. Presta LG, Chen H, O'Connor SJ, Chisolm V, Meng YG et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res*. **1997**, *57*, 4593-9.
24. Li X-Q, Andersson TB, Ahlström M, Weidolf L. Comparison of inhibitory effects of the proton pump-inhibiting drugs omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole on human cytochrome P450 activities. *Drug Metab. Dispos*. **2004**, *32*, 821-827.
25. Kim D, Wang L, Beconi M, Eirmann GJ, Fisher MH et al. (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine: a potent, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. *J. Med. Chem*. **2005**, *48*, 141-151.
26. López-Sendón J, Swedberg K, McMurray J, Tamargo J, Maggioni AP et al. Expert consensus document on β-adrenergic receptor blockers. *Eur. Heart J*. **2004**, *25*, 1341-1362.
27. Farkas IJ et al. Network-based tools for the identification of novel drug targets. *Sci. Signal*. **2011**, *4 (173)*, 1-5.
28. Endo A. The origin of the statins. *Int. Congr. Ser*. **2004**, *1262*, 3-8.
29. Becker DJ, Gordon RY, Halbert SC, French B, Morris PB et al. Red yeast rice for dyslipidemia in statin-intolerant patients. *Ann. Int. Med*. **2009**, *150*, 830-839.
30. Consumer Lab. Red Yeast Rice Supplements Review, **2016**, https://www.consumerlab.com/reviews/Red-Yeast-Rice-Supplements-Review/Red_Yeast_Rice/
31. Vasudevan DM, Sreekumari S, Vaidyanathan K. *Textbook of Biochemistry for Medical Students*. JP Medical Ltd, London, **2013**.
32. Cook PN, Ward WHJ, Petrash JM, Mirrlees DJ, Sennitt CM et al. Kinetic characteristics of Zeneca ZD5522, a potent inhibitor of

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

human and bovine lens aldose reductase. *Biochem. Pharmacol*. **1995**, *49*, 1043-1049.
33. Cui Q, Karplus M. Allostery and cooperativity revisited. *Prot. Sci*. **2008**, *17*, 1295-1307.
34. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions: A plausible model. *J. Mol. Biol*. **1965**, *12*, 88–118.
35. Pauling L. The oxygen equilibrium of hemoglobin and its structural interpretation. *Proc. Natl. Acad. Sci*. **1935**, *21*, 181–191.
36. Koshland DE, Nemethy G, Filmer D. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochem*. **1966**, *5*, 365–385.
37. McCammon JA, Gelin BR, Karplus M. Dynamics of folded proteins. *Nature* **1977**, *267*, 585–590.
38. Brooks BR, Karplus M. Harmonic dynamics of proteins: Normal modes and fluctuations in bovine pancreatic trypsin inhibitor. *Proc. Natl. Acad. Sci*. **1983**, *80*, 6571–6575.
39. Protein Data Bank, PDB http://www.rcsb.org/pdb/home/home.do
40. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. *Nature* **1970**, *228*, 726–739.
41. Boehr DD, McElheny D, Dyson HJ, Wright PE. The dynamic energy landscape of dihydrofolate reductase catalysis. *Sci*. **2006**, *313*, 1638–1642.
42. Grallert H, Buchner J. Review: A structural view of the GroE chaperone cycle. *J. Struct. Biol*. **2001**, *135*, 95–103.
43. Ferreon ACM, Ferreon JC, Wright PE, Deniz AA. Modulation of allostery by protein intrinsic disorder. *Nature* **2013**, *498*, 390-394.
44. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. *Annu. Rev. Biochem*. **2000**, *69*, 183–215.
45. Popovych N, Sun S, Ebright RH, Kalodimos, C.G. Dynamically driven protein allostery. *Nat. Struct. Mol. Biol*. **2006**, *13*, 831–838.
46. Gunasekaran K, Ma B, Nussinov, R. Is allostery an intrinsic property of all dynamic proteins. *Proteins* **2004**, *57*, 433–443.
47. Cooper A, Dryden DTF. Allostery without conformational change. *Eur. Biophys. J*. **1984**, *11*, 103–109.
48. Kar G, Keskin O, Gursoy A, Nussinov R. Allostery and population shift in drug discovery. *Curr. Opin. Pharmacol*. **2010**, *10*, 715-722.
49. Tsai C-J, del Sol A, Nussinov R. Protein allostery, signal transmission and dynamics: A classification scheme of allosteric mechanisms. *Mol. Biosyst*. **2009**, *5*, 207-216.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

50. Nussinov R, Tsai C-J, Csermely P. Allo-network drugs: harnessing allostery in cellular networks. *Trend. Pharmacol. Sci*. **2011**, *32*, 686-693.
51. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 3rd ed*. Bentham Science, Sharjah, U.A.E. **2015**.
52. Bedi O, Dhawan V, Sharma PL, Kumar P. Pleiotropic effects of statins: new therapeutic targets in drug design. *Naunyn-Schmiedeberg's Arch. Pharmacol*. **2016**, *389*, 695–712.
53. Ledford H. Cholesterol limits lose their lustre. *Nature* **2013**, *494*, 410-411.
54. Libby P. Inflammation and cardiovascular disease mechanisms. *Amer. J. Clinical Nutr*. **2006**, *83*, 456S-460S.
55. Liao JK, Laufs U. Pleiotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol*. **2005**, *45*, 89–118.
56. Kavalipati N, Shah J, A, Vasnawala H. Pleiotropic effects of statins. *Indian J. Endocrinol. Metab*. **2015**, *19*, 554–562.
57. Janicko M, Drazilova S, Pella D, Fedacko J, Jarcuska P. Pleiotropic effects of statins in the diseases of the liver. *World J. Gastroenterol*. **2016**, *22*, 6201-6213.
58. Bedi O, Dhawan V, Sharma PL, Kumar P. Pleiotropic effects of statins: new therapeutic targets in drug design. *Naunyn-Schmiedeberg's Arch. Pharmacol*. **2016**, *389*, 695–712.
59. Karlson BW, Palmer MK, Nicholls SJ, Lundman P, Barter PJ. A VOYAGER meta-analysis of the impact of statin therapy on low-density lipoprotein cholesterol and triglyceride levels in patients with hypertriglyceridemia. *Am. J. Cardiol*. **2016**, *117*, 1444-1448.
60. Stein EA, Lane M, Laskarzewski P. Comparison of statins in hypertriglyceridemia. *Am. J. Cardiol*. **1998**, *81 (4A)*, 66B–69B.
61. Kudo S, Satoh K, Nogi M, Suzuki K, Sunamura S et al. SmgGDS as a crucial mediator of the inhibitory effects of statins on cardiac hypertrophy. *Hypertension* **2016**, *67*, 878-889.
62. Theusch E, Kim K, Stevens K, Smith JD, Chen Y-D et al. Statin-induced expression change of *INSIG1* in lymphoblastoid cell lines correlates with plasma triglyceride statin response in a sex-specific manner. *Pharmacogenom. J*. **2016**, doi:10.1038/tpj.2016.12.
63. Mullard A. 2016 FDA drug approvals. *Nature Rev. Drug Disc*. **2017**, *16*, 73-76.
64. Zamponi GW. Targeting voltage-gated calcium channels in neurological and psychiatric diseases. *Nature Rev. Drug Disc*. **2016**, *15*, 19-34.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

65. Gonzales-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV et al. Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* **2008**, *452*, 93-97.
66. Lin JS, Dauvilliers Y, Arnulf I, Bastuji H, Anaclet C et al. An inverse agonist of the histamine H(3) receptor improves wakefulness in narcolepsy: studies in orexin-/- mice and patients. *Neurobiol. Dis*. **2008**, *30*, 74-83.
67. Syed YY. Pitolisant: First Global Approval. *Drugs* **2016**, *76*, 1313-1318.
68. Szakacs Z, Dauvilliers Y, Mikhaylov V, Poverennova I, Krylov S et al. Safety and efficacy of pitolisant on cataplexy in patients with narcolepsy: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol*. **2017**, *16*, 200-207.
69. Thurmond RL, Chen B, Dunford PJ, Eckert WA, Karlsson L et al. Pharmacology and clinical activity of Toreforant, a Histamine H_4 receptor antagonist. *Ann. Pharmacol. Pharmaceut*. **2017**, *2*, Article 1013.
70. Cox JC, Coulter AR. Adjuvants-a classification and review of their modes of action. *Vaccine* **1997**, *15*, 248-256.
71. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunol*. **2001**, *2*, 675-680.
72. Speeckaert R, van Geel N, Lambert J, Cleays L, Delanghe JR, Speeckaert MM. Secukinumab: IL-17A inhibition to treat psoriatic arthritis. *Drugs Today* **2016**, *52*, 607-616.
73. Miossec P, Kolls JK. Targeting IL-17 and T_H17 cells in chronic inflammation. *Nature Rev. Drug Disc*. **2012**, *11*, 763-776.
74. Novartis. Cosentyx (Secukinumab), **2017**. https://www.cosentyx.com/index.jsp?site=PS002901&irmasrc=CCSWB24097&dclid=COPt0q3MhtQCFea4swod2qkANw&usertrack.filter_applied=true&NovaId=2935377070802455721
75. Holbeck SL, Collins JM, Doroshow JH. Analysis of Food and Drug Administration-approved anticancer agents in the NCI60 panel of human tumor cell lines. *Mol. Cancer Ther*. **2010**, *9*, 1451-1460.
76. Mullard A. EMA greenlights second gene therapy. *Nature Rev. Drug Disc*. **2016**, *5*, 299.
77. Signori E, Lurescia S, Massi E, Fioretti D, Chiarella P et al. DNA vaccination strategies for anti-tumour effective gene therapy protocols. *Cancer Immunol. Immunother*. **2010**, *59*, 1583-1591.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

78. Tiptiri-Kourpeti A, Spyridopoulou K, Pappa A, Chlichlia. DNA vaccines to attack cancer: Strategies for improving immunogenicity and efficacy. *Pharmacol. Therapeut*. **2016**, *165*, 32-49.
79. Mutwiri G Gerdts V. Strategies to improve neonatal vaccines for the neonate. *Supplement to Biopharm. Internl*. **2008**, 24-29.
80. Morrison C. DNA vaccines against Zika virus speed into clinical trials. *Nature Rev. Drug Disc*. **2016**, *16*, 521.
81. Chu H, Kohane DS, Langer R. RNA therapeutics - The potential treatment for myocardial infarction. *Regen. Therapy* **2016**, *4*, 83-91.
82. Ng EW, Shima DT, Calias P, Cunningham ET Jr., Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat. Rev. Drug Discov*. **2006**, *5*, 123–132.
83. Sahin U, Kariko K, Tureci O. mRNA-based therapeutics - developing a new class of drugs. *Nature Rev. Drug Disc*. **2014**, *13*, 759-771.
84. Zangi L, Lui KO, von Gise A, Ma Q, Ebina W. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nature Biotechnol*. **2013**, *31*, 898-907.
85. Li B, Luo X, Dong Y. Effects of chemically modified messenger RNA on protein expression. *Bioconjugate Chem*. **2016**, *27*, 849−853.
86. Gallach S, Calabuig-Fariñas S, Jantus-Lewintre E, Camps C. MicroRNAs: Promising new antiangiogenic targets in cancer. *BioMed Res. Int*. **2014**, Article ID 878450, 14 pp.
87. Zhao Y, Alexandrov PN, Lukiw WJ. Anti-microRNAs as novel therapeutic agents in the clinical management of Alzheimer's disease. *Front. Neurosci*. **2016**, *10*, Article 59, doi: 10.3389/fnins.2016.00059, 7 pp.
88. Ng EW, Shima DT, Calias P, Cunningham ET Jr., Guyer DR, Adamis AP. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat. Rev. Drug Discov*. **2006**, *5*, 123–132.
89. Csermely P, Korcsmáros T, Kiss HJM, London G, Nussinov R. Structure and dynamics of molecular networks: A novel paradigm of drug discovery. A comprehensive review. *Pharmacol. Therapeut.* **2013**, *138,* 333-408.
90. Meng H, Liu Y, Lai L. Diverse ways of perturbing the human arachidonic acid metabolic network to control inflammation. *Acc. Chem. Res*. **2015**, *48*, 2242-2250.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

91. Collins FS, Gray GM, Bucher JR. Toxicology: Transforming environmental health protection. *Science* **2008**, *319*, 906-907.
92. Silvestre HL, Blundell TL, Abell C, Ciulli A. Integrated biophysical approach to fragment screening and validation for fragment-based lead discovery. *Proc. Natl. Acad. Sci*. **2013**, *110*, 12984–12989.
93. Du G, Fang Q, den Toonder JMJ. Microfluidics for cell-based high throughput screening platforms – A review. *Anal. Chim. Acta* **2016**, *903*, 36-50.
94. Prestwitch GD. Evaluating drug efficacy and toxicology in three dimensions: using synthetic extracellular matrices in drug discovery. *Acc. Chem. Res*. **2008**, *41*, 139-148.
95. Kenny HA, Lal-Nag M, White EA, Shen M, Chiang C-Y et al. Quantitative high throughput screening using a primary human three-dimensional organotypic culture predicts *in vivo* efficacy. *Nat. Commun*. **2015**, *6*, Article 6220.
96. Werkele H., Meinl E. An old drug plays new tricks. *Science* **2013**, *512*, 314-315.
97. Winslow RL, Trayanova N, Geman D, Miller MI. Computational medicine: Translating models to clinical care. *Sci. Trans. Med*. **2012**, *4 (158)*, 158rv11.
98. Lai X, Wolknhauer O, Vera J. Understanding microRNA-mediated gene regulatory networks through mathematical modelling. *Nucleic Acids Res*. **2016**, *44*, 6019-6035.
99. Krauss M, Hofmann U, Schakmayer C, Igel S, Schlender J et al. Translational learning from clinical studies predicts drug pharmacokinetics across patient populations. *NPJ Syst. Biol. Appl*. **2017**, *3*, Article 11.
100. Smith RE, Tran K, Richards KM. Systems thinking for medicinal chemists. *Jacobs J. Med. Chem*. **2015**, *1 (1)*, Article 004.
101. Houston JG, Banks MN, Binnie A, Brenner S, O'Connell J, Petrilo EW. Case study: impact of technology investment on lead discovery at Bristol–Myers Squibb, 1998–2006. *Drug Disc. Today* **2008**, *13*, 44-51.
102. Zhang L, Cvijic ME, Lippy J, Myslik J, Brenner SL et al. Case study: technology initiative led to advanced lead optimization screening processes at Bristol-Myers Squibb, 2004–2009. *Drug Disc. Today* **2012**, *17*, 733-740.
103. Cvijic ME, Sum CS, Alt A, Zhang L. GPCR profiling: from hits to leads and from genotype to phenotype. *Drug Disc. Today* **2015**, *18*, 30-37.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

104. George ML, Rowlands D, eds. *What is Lean Sigma?* McGraw-Hill: New York, **2004**.
105. Inglese J, Johnson RL, Simeonov A, Xia M, Austin CP, Auld DS. High throughput screening assays for the identification of chemical probes. *Nature Chem. Biol*. **2007**, *3*, 466-479.
106. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. *BioTech*. **2008**, *44*, 619-626.
107. Schwartz et al. High throughput lipidomic profiling of schizophrenia and bipolar disorder brain tissue reveals alterations of free fatty acids, phosphatidylcholines, and ceramides. *J. Proteome Res*. **2008**, *7*, 4266-4277.
108. Liang S, Xu Z, Xu X, Zhao X, Huang C, Wei Y. Quantitative proteomics for cancer biomarker discovery. *Combinator. Chem. High Throughput Screen*. **2012**, *15*, 221-231.
109. Wiklund ED et al. Coordinated epigenetic repression of the miR-200 family andmiR-205 in invasive bladder cancer. *Intl. J. Cancer* **2011**, *128*, 1327-1334.
110. Hoffmann J, Schmidt TL, Heckel A, Brutschy B. Probing the limits of liquid droplet laser desorption mass spectrometry in the analysis of oligonucleotides and nucleic acids. *Rapid Commun. Mass Spectrom*. **2009**, *23*, 2176–2180.
111. Soleilhac E, Nadon R, Lafanechere L. High-content screening for the discovery of pharmacological compounds: advantages, challenges and potential benefits of recent technological developments. *Expert Opin. Drug Discov*. **2010**, *5*, 135-144.
112. Lorentzen A, Kinkhabwala A, Rocks O, Vartak N, Bastiaens PIH. Regulation of Ras localization by acylation enables a mode of intracellular signal propagation. *Sci. Signal*. **2010**, *3 (140)*, 1-8.
113. Heal WP, Wickramasinghe SR, Tate EW. Activity based chemical proteomics: Profiling proteases as drug targets. *Curr. Drug Disc. Technol*. **2008**, *5*, 200-212.
114. Xiang M, Cao Y, Fan W, Chen L, Mo Y. Computer-aided drug design: Lead discovery and optimization. *Comb. Chem. High T. Screen*. **2012**, *15*, 328-337.
115. Todorov NP, Dean PM. Evaluation of a method for controlling molecular scaffold diversity in de novo ligand design. *J. Comput. Aided Mol. Des*. **1997**, *11 (2)*, 175-192.
116. Stahl M, Todorov NP, James T, Mauser H, Boehm H-J, Dean PM. A validation study on the practical use of automated de novo design. *J. Comput. Aided Mol. Des*. **2002**, *16 (7)*, 459-478.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

117. Amaro RE, Li WW. Emerging methods for ensemble-based virtual screening. *Curr. Top. Med. Chem*. **2010**, *10*, 3-13.
118. Liu Q, Masek B, Smith K, Smith J. Tagged fragment method for evolutionary structure-based de novo lead generation and optimization. *J. Med. Chem*. **2007**, *50*, 5392-5402.
119. Hartenfeller M, Proschak E, Schuller A, Schneider G. Concept of combinatorial de novo design of drug-like molecules by particle swarm optimization. *Chem. Biol. Drug Des*. **2008**, *72*, 16-26.
120. Swanson JM, Henchman RH, McCammon JA. Revisiting free energy calculations: A theoretical connection to MM/PBSA and direct calculation of the association free energy. *Biophys. J*. **2004**, *86*, 67-74.
121. Aquist J, Marelius J. The linear interaction energy method for predicting ligand binding free energies. *Comb. Chem. High Throughput Screen*. **2001**, *4*, 613-626.
122. Menikarachchi LC, Gascón JA. QM/MM approaches in medicinal chemistry research. *Curr, Top. Med. Chem*. **2010**, *10*, 46-54.
123. Rastelli G, Del Rio A, Degliesposti G, Sgobba M. Fast and accurate predictions of binding free energies using MM-PBSA and MM-GBSA. *J. Comput. Chem*. **2010**, *31*, 97-810.
124. Zhang L, Tsai K-C., Du L, Fang H, Li M, Xu W. How to generate reliable and predictive CoMFA models. *Curr. Med. Chem*. **2011**, *18 (6)*, 623-930.
125. Semichem, AMPAC 10 Semiempirical Quantum Mechanics with State-of-the-Art Graphics. Shawnee Mission, KS, **2017**. www.semichem.com
126. Semichem, CODESSA III. Shawnee Mission, KS, **2017**. www.semichem.com
127. Bienstock RJ. Computational drug design targeting protein-protein interactions. *Curr. Pharmaceut. Design* **2012**, *18*, 1240-1254.
128. Higueruelo AP, Schreyer A, Bickerton GR, Pitt WR, Groom CR, Blundell TL. Atomic interactions and profile of small molecules disrupting protein-protein interfaces: the TIMBAL database. *Chem. Biol. Drug Des*. **2009**, *74*, 457-67.
129. Bourgeas R, Basse MJ, Morelli X, Roche P. Atomic analysis of protein-protein interfaces with known inhibitors: The 2P2I database. *PLoS One* **2010**, *5*, e9598.
130. Loving K, Alberts I, Sherman W. Computational approaches for fragment-based and *de novo* design. *Curr. Top. Med. Chem*. **2010**, *10*, 14-32.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

131. Chu L-H, Chen B-S. Construction of a cancer-perturbed protein-protein interaction network for discovery of apoptosis drug targets. *BMC Syst. Biol*. **2008**, *2*, 1-17.
132. Shen Y, Liu J, Estiu G, Isin B, Ahn YY et al. Blueprint for antimicrobial hit discovery targeting metabolic networks. *Proc. Natl. Acad. Sci*. **2010**, *107*, 1082-1087.
133. Sharma A, Burridge PW, McKeithan WL, Serrano R, Shukla P et al. High-throughput screening of tyrosine kinase inhibitor cardiotoxicity with human induced pluripotent stem cells. *Sci. Transl. Med*. **2017**, *9 (377)*, doi:10.1126/scitranslmed.aaf2584.
134. Patel AK, Tibbitt MK, Celiz AD, Davies MC, Langer R et al. High throughput screening for discovery of materials that control stem cell fate. *Curr. Opin. Solid State Mat. Sci*. **2016**, *20*, 202-211.
135. Vinayagam A, Gibson TE, Lee H-J, Yilmazel B, Charles Roesel C et al. Controllability analysis of the directed human protein interaction network identifies disease genes and drug targets. *Proc. Natl. Acad. Sci*. **2016**, *113*, 4976-4981.
136. Kuenemann MA, Olivier Sperandio O, Labbé CM, Lagorce D, Miteva MA, Villoutreix BO. In silico design of low molecular weight protein-protein interaction inhibitors: Overall concept and recent advances. *Progr. Biophys. Mol. Biol*. **2015**, *119*, 20-32.
137. Chattopadhyay A, O'Connor CJ, Zhang F, Galvagnion C, Galloway WRJD. Discovery of a small-molecule binder of the oncoprotein gankyrin that modulates gankyrin activity in the cell. *Sci. Rep*. **2016**, *6*, Article 23732.
138. Chen D, Liu X, Yang Y, Yang H, Lu P. Systematic synergy modeling: understanding drug synergy from a systems biology perspective. *BMC Syst. Biol*. **2015**, *9*, Article 56.
139. Nussinov R, Tsai CJ. Unraveling structural mechanisms of allosteric drug action. *Trends Pharmacolog. Sci*. **2014**, *35*, 256-63.
140. London N, Raveh B, Schueler-Furman O. Druggable protein-protein interactions – from hot spots to hot segments. *Curr. Opin. Chem. Biol*. **2013**, *6*, 952-959.
141. Basse M-J, Betze S, Morelli X, Roche P. 2P2Idb v2: update of a structural database dedicated to orthosteric modulation of protein–protein interactions. *Database* **2016**, *2016*: Article ID baw007; doi:10.1093/database/baw007.
142. Wu L, Tang L, Li M, Wang J, Wu F. Biomolecular network controllability with drug binding information. *IEEE Trans. NanoBioscience* **2017**, DOI: 10.1109/TNB.2017.2705106

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

143. Kuenemann MA, Olivier Sperandio O, Labbé CM, Lagorce D, Miteva MA, Villoutreix BO. In silico design of low molecular weight protein-protein interaction inhibitors: Overall concept and recent advances. *Progr. Biophys. Mol. Biol*. **2015**, *119*, 20-32.
144. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev*. **1997**, *23*, 3–25.
145. Lipinski CA. Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discovery Today*: *Technol*. **2004**, *1*, 337-341.
146. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **2007,** *70,* 461-477.
147. Santos GB, Ganeson A, Emery FS. Oral administration of peptide-based drugs: beyond Lipinski's rule. *Chem. Med. Chem*. **2016**, *11*, 2245–2251.
148. Lipinski CA. Rule of five in 2015 and beyond: Target and ligand structural limitations, ligand chemistry structure and drug discovery project decisions. *Adv. Drug Deliv. Rev*. **2016**, *101*, 34-41.
149. Smith RE, Tran K, Smith CC, McDonald M, Shejwalkar P, Hara K. The role of the Nrf2/ARE antioxidant system in preventing cardiovascular diseases. *Diseases* **2016**, *4*, Article 34.
150. Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM et al. Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Rad. Mol. Biol. Med*. **2015**, *88*, 108-146.
151. Kensler TW. Wakabayashi N. Biswal B. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol*. **2007**, *47*, 89–116.
152. Chapple SJ, Siow RCM, Mann GE. Crosstalk between Nrf2 and the proteasome: Therapeutic potential of Nrf2 inducers in vascular disease and aging. *Int. J. Biochem. Cell Biol*. **2012**, *44*, 1315-1320.
153. Korsmeyer SJ. *BCL-2* gene family and the regulation of cell death. *Cancer Res*. **1999**, *59*, S1693-S1670.
154. Ashkenazi A, Fairbrother WJ, Leverson JD, Souers AJ. From basic apoptosis discoveries to advanced selective BCL-2 family inhibitors. *Nature Rev. Drug Disc*. **2017**, *16*, 273-284.
155. Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD et al. Targeting BCL2 with Venetoclax in relapsed chronic lymphocytic leukemia. *N. Engl. J. Med*. **2016**, *374*, 311-22.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

156. Adler EM. Signaling Breakthroughs of the Year. *Sci. Signal*. **2010**, *3(103)*, Article eg1.
157. Turner KM, Deshpande V, Beyter D, Koga T, Rusert J et al. Extrachromosomal oncogene amplification drives tumour evolution and genetic heterogeneity. *Nature* **2017**, doi:10.1038/nature21356
158. Gandini S, Massi D, Mandala M. PD-L1 expression in cancer patients receiving anti PD-1/PD-L1 antibodies: A systematic review and meta-analysis. *Crit. Rev. Oncol. Hematol*. **2016**, *100*, 88-98.
159. FDA. FDA approves first cancer treatment for any solid tumor with a specific genetic feature. **2017**. https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm560167.htm
160. Kazandijian D, Suzman DL, Blumenthal G, Mushti S, He K et al. FDA approval summary: Nivolumab for the treatment of metastatic non-small cell lung cancer with progression on or after platinum-based chemotherapy. *Oncologist* **2016**, *21*, 634-642.
161. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nature Rev. Cancer* **2016**, *16*, 275–87.
162. Smith RE, Tran K, Vocque, R. Network Medicine and High throughput Screening. *Curr. Drug Disc. Technol*. **2013**, *10*, 182-194.
163. Vuillon L, Lesieur C. From local to global changes in proteins: a network view. *Curr. Opin. Struct. Biol*. **2015**, *31*, 1–8.
164. Tsai C-J, Nussinov R. A unified view of "how allostery works". *PLoS Comp. Biol*. **2014**, *10*, Article e1003394.
165. Motlagh HN, Wrabl JO, Hilser VJ. The ensemble nature of allostery. *Nature* **2014**, *508*, 331-339.
166. Nussinov R, Tsai C-J. Allostery in disease and drug discovery. *Cell* **2013**, *153*, 293-305.
167. Nussinov R, Tsai C-J, Ma B. The still underappreciated role of allostery in the cellular network. *Annu. Rev. Biophys*. **2013**, *42*, 169–89.
168. Huang Z, Mou L, Shen Q, Lu S, Li C, *et al*. ASD v2.0: update content and novel features focusing on allosteric regulation. *Nucleic Acids Res*. **2014**, *42*, D510-D516.
169. Zhang Z. Allosteric Database. **2015**, http://mdl.shsmu.edu.cn/ASD/
170. Pei J, Yinn N, Ma X, Lai L. Systems biology brings new dimensions for structure-based drug design. *J. Am. Chem. Soc*. **2014**, *136*, 11556-1565.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

171. Engin HB, Gursoy A, Nussinov R, Keskin O. Network-based strategies can help mono- and poly-pharmacology drug discovery: A systems biology view. *Curr. Pharmaceut. Design* **2014**, *20*, 1201-1207.
172. Soundararajan V, Aravamudan M. Global connectivity of hub residues in oncoprotein structures encodes genetic factors dictating personalized drug response to targeted cancer therapy. *Sci. Rep*. **2014**, *4*, Article 7294, DOI: 10.1038/srep07294.
173. Szalay KZ, Nussinov R, Csermely P. Attractor structures of signaling networks: consequences of different conformational barcode dynamics and their relations to network-based drug design. *Mol. Inform*. **2014**, *33*, 463-468.
174. Szilágyi A, Nussinov R, Csermely P. Allo-network drugs: Extension of the allosteric drug concept to protein-protein interaction and signaling networks. *Curr. Top. Med. Chem*. **2013**, *13*, 64-77.
175. Joensuu H, Dimitrijevic S. Tyrosine kinase inhibitor imatinib (STI571) as an anticancer agent for solid tumors. *Ann. Med*. **2001**, *33*, 451-455.
176. Fallacara AL, Tintori C, Radi M, Schenoni S, Botta M. Insight into the allosteric inhibition of Abl kinase. *J. Chem. Inf. Model*. **2014**, *54*, 1325-1338.
177. Skora L, Mestan J, Fabbro D, Schenone S, Botta M. NMR reveals the allosteric opening and closing of Abelson tyrosine kinase by ATP-site and myristoyl pocket inhibitors. *Proc. Natl. Acad. Sci*. **2013**, *110*, E4437-E4445.
178. Blanc J, Geney R, Menet C. Type II kinase inhibitors: An opportunity in cancer for rational design. *Anticancer Agents Med. Chem*. **2013**, *13*, 731-747.
179. Nussinov R, Tsai C-J, Liu J. Principles of allosteric interactions in cell signaling. *J. Am. Chem. Soc*. **2014**, *136*, 17692−17701.
180. Grewal RK, Roy S. Modeling proteins as residue interaction networks. *Protein Pept. Lett*. **2015**, *22*, 923-933.
181. Loscalzo J, Barabási A-L, Silverman, eds. *Network Medicine. Complex Systems in Human Disease and Therapeutics*. Harvard press, Harvard, **2017**.
182. Di Paola L, Giuliani A. Protein contact network topology: a natural language for allostery. *Curr. Opin. Struct. Biol*. **2015**, *31*, 43–48.
183. Farkas IJ et al. Network-based tools for the identification of novel drug targets. *Sci. Signal*. **2011**, *4*, 1-5.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

184. Smith RE, Tran K, Richards KM, Luo R. Using NMR to develop new allosteric and network drugs. *Curr. Drug Disc. Technol*. **2015**, *12,* 193-204.
185. RCSB Protein Data Bank http://www.rcsb.org/pdb/home/home.do
186. Jeeves M, Quill L, Overduin M. Structure-based drug design using NMR. *eMagRes*. **2015**, *4*, 231-240.
187. Hajduk, PJ, Olejniczak ET, Fesik SW. One dimensional relaxation- and diffusion-edited NMR methods for screening compounds that bind to macromolecules. *J. Am. Chem. Soc*. **1997**, *119*, 12257-12261.
188. Meyer B, Klein J, Mayer M, Meinecke R, Moller H et al. Saturation transfer difference NMR spectroscopy for identifying ligand epitopes and binding specificities. *Ernst Schering Res. Found. Workshop* **2004**, *44*, 149-170.
189. Shuker, SB, Hajduk, PJ, Meadows RP, Fesik SW. Discovering high-affinity ligands forproteins: SAR by NMR. *Science* **1996**, *274*, 1531-1534.
190. Dalvit C, Fogliatto GP, Stewart A, Veronesi M, Stockman B. WaterLOGSY as a method for primary NMR screening; Practical aspects and range of applicability. *J. Biomol. NMR* **2001**, *21*, 349-359.
191. Huang R, Bonnichon R, Claridge TDW, Leung IKH. Protein-ligand binding affinity determination by the waterLOGSY method: An optimized approach considering ligand rebinding. *Sci. Rep*. **2017**, *7*, article 43727.
192. Skora L, Jahnke W. Contributions of biomolecular NMR to drug discovery. *Chimia* **2015**, *69*, 421-424.
193. Cobbert JD, DeMott C, Majumder S, Smith EA, Reverdatto S et al. Caught in action: selecting peptide aptamers against intrinsically disordered proteins in live cells. *Sci. Rep*. **2015**, *5*, 9 pp.
194. Boulton S, Akimoto M, Selvaratnam R, Bashiri A, Melacini G. A tool set to map allosteric networks through the NMR chemical shift covariance analysis. *Sci. Rep*. **2014**, *4*, 7306, 11 pp.
195. Akimoto M, Moleschi K, Boulton S, van Schouwen B, Selvaratnam R et al. Allosteric linkers in cAMP signaling. *Biochem. Soc. Trans*. **2014**, *42*, 139–144.
196. Axe JM, Yezdimer EM, O'Rourke KF. Amino acid networks in a (β/α)8 barrel enzyme change during catalytic turnover. *J. Am. Chem. Soc*. **2014**, *136*, 6818−6821.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

197. Xiao, Y, Liddle JC, Pardi A, Ahn NG. Dynamics of protein kinases: Insights from nuclear magnetic resonance. *Acc. Chem. Res*. **2015**, *48*, 1106-1114.
198. Kay LE, Keifer P, Saarinen T. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc*. **1992**, *114*, 10663-10665.
199. Williamson MP. Using chemical shift perturbation to characterise ligand binding. *Prog. Nucl. Mag. Res. Sp*. **2013**, *73*, 1–16.
200. Gobl C, Madl T, Simon B, Sattler M. NMR approaches for structural analysis of multidomain proteins in solution. *Prog. Nucl. Magn. Res*. **2014**, *80*, 26-63.
201. Selvaratnam R, Chowdhury S, VanSchouwena B, Melacini G. Mapping allostery through the covariance analysis of NMR chemical shifts. *Proc. Natl. Acad. Sci*. **2011**, *108*, 6133-6138.
202. Cembran A, Kim J, Gao J, Veglia G. NMR mapping of protein conformational landscapes using coordinated behavior of chemical shifts upon ligand binding. *Phys. Chem. Chem. Phys*. **2014**, *16*, 6508–6518.
203. Schulze-Sünninghausen D, Becker J, Luy B. Rapid heteronuclear single quantum correlation NMR spectra at natural abundance. *J. Am. Chem. Soc*. **2014**, *136*, 1242-1245.
204. Castañarr, Parella T. Recent advances in small molecule NMR: Improved HSQC and HSQMBC experiments. *Ann. Rep. NMR Spectr*. **2015**, *84*, 163-232.
205. Huang Z, Zhu L, Cao Y et al. ASD: a comprehensive database of allosteric proteins and modulators. *Nucleic Acids Res*. **2011**, *39*, D663-D669.
206. Perez-Lopez AR, Szalay KZ, Türei D et al. Targets of drugs are generally, and targets of drugs having side effects are specifically good spreaders of human interactome perturbations. *Sci. Rep*. **2015**, *5*, 1-9.
207. Majumder S, de Mott CM, Burtz DS, Shekhtman A. Using singular value decomposition to characterize protein-protein interactions by in-cell NMR. *Chembiochem*. **2014**, *15*, 929-933.
208. Zhou B. Adverse outcome pathway: Framework, application, and challenges in chemical risk assessment. *J. Env. Sci*. **2015**, *35*, 191-193.
209. NRC (National Research Council). *Toxicity Testing in the 21st century: A Vision and a Strategy*. National Academies Press, Washington, DC, **2007**.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

210. Sauer JM, Kleensang A, Peitsch MC, Hayes AW. Advancing risk assessment through the application of systems toxicology. *Toxicol. Res*. **2016**, *32*, 5-8.
211. Phillips MB, Leonard JA, Grulke CM, Chang DT, Edwards SW et al. A workflow to investigate exposure and pharmacokinetic influences on high-throughput *in vitro* chemical screening based on adverse outcome pathways. *Environ. Health Perspect*. **2016**, *124*, 53–60.
212. Adeleye Y, Melvin Andersen M, Clewell R, Davies M, Dent M et al. Implementing toxicity testing in the 21st Century (TT21C): Making safety decisions using toxicity pathways, and progress in a prototype risk assessment. *Toxicol*. **2015**, *332*, 102-111.
213. Kerns M. Foods with the highest content of quercetin. **2015**. http://www.livestrong.com/article/301326-foods-with-the-highest-content-of-quercetin/
214. Sun GY, Chen Z, Jasmer KJ, Chuang DY, Gu Z et al. Quercetin attenuates inflammatory responses in BV-2 microglial cells: Role of MAPKs on the Nrf2 pathway and induction of heme oxygenase-1. *PLoS One* **2015**, *10 (10)*, e0141509.
215. Ajit, D., Simonyi, A., Li, R., Chen, Z., Hannik, M et al. Phytochemicals and botanical extracts regulate NF-kB and Nrf2/ARE reporter activities in DI TNC astrocytes. *Neurochem. Int*. **2016**, *97*, 49-56.
216. Klaasen CD, ed. *Casarett and Doull's Toxicology. The Basic Science of Poisons, 7th ed*. McGraw-Hill, New York, **2008**.
217. Zhang Q, Bhattacharya S, Andersen ME. Ultrasensitive response motifs: basic amplifiers in molecular signalling networks. *Open Biol*. **2013**, *3*, Article 130031.
218. Bale SS, Vernetti L, Senutovitch N, Jindal R, Hegde M. In vitro platforms for evaluating liver toxicity. *Exp Biol Med*. **2014**, *239*, 1180–1191.
219. Judson R, Richard A, Dix D, Houck K, Elloumi F et al. ACToR–aggregated computational toxicology resource. *Toxicol. Appl. Pharmacol*. **2008**, *233*, 7–13.
220. Lim E, Pon A, Djoumbou Y, Knox C, Shrivastava S et al. T3DB: a comprehensively annotated database of common toxins and their targets. *Nucleic Acid Res*. **2010**, *38*, D781–6.
221. Ribas J, Sadeghi H, Manbachi A, Leijten J, Brinegar K et al. Cardiovascular organ-on-a-chip platforms for drug discovery and development. *Appl. In vitro Toxicol*. **2016**, *2*, DOI: 10.1089/aivt.2016.0002.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

222. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ et al. Heart disease and stroke statistics - 2015 update: a report from the American Heart Association. *Circulation* **2014**, *131*, e29-e322.
223. Weinberger F, Mannhardt E, Eschenhagen T. Engineering cardiac muscle tissue. A maturating field of research. *Circ Res*. **2017**, *120*, 1487-1500.
224. Bian W, Jackman CP, Bursac N. Controlling the structural and functional anisotropy of engineered cardiac tissues. *Biofabrication* **2014**, *6*, Article 024109.
225. Grosberg A, Alford PW, McCain ML, et al. Ensembles of engineered cardiac tissues for physiological and pharmacological study: Heart on a chip. *Lab Chip* **2011**, *11*, 4165.
226. Tandon N, Cannizzaro C, Chao PH, Maidhof R, Marsano A et al. Electrical stimulation systems for cardiac tissue engineering. *Nat. Protoc*. **2009**, *4*, 155–173.
227. Wong KH, Chan JM, Kamm RD, et al. Microfluidic models of vascular functions. *Annu. Rev. Biomed. Eng*. **2012**, *14*, 205–230.
228. Kim S, Lee H, Chung M, Jeon NL. Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab on a Chip* **2013**, *8*, 1489-1500.
229. Tsvirkun D, Grichine A, Duperray A, Misbah C, Bureau L. Microvasculature on a chip: study of the endothelial surface layer and the flow structure of red blood cells. *Sci. Rep*. **2017**, *7*, article 45036.
230. Bertassoni LE, Cardoso JC, Manoharan V, et al. Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. *Biofabrication* **2014**, *6*, 024105.
231. Richards D, Jia J, Yost M, Markwald R, Mei Y. 3D bioprinting for vascularized tissue fabrication. *Ann. Biomed. Engr*. **2017**, *45*, 132-147.
232. Ma X, Qu X, Zhu W, Li Y-S, Yuan S et al. Deterministically patterned biomimetic human iPSC-derived hepatic model via rapid 3D bioprinting. *Proc. Natl. Acad. Sci*. **2016**, *113*, 2206-2213.
233. Knowlton S, Yenilmez B, Tasoglu S. Towards single-step biofabrication of organs on a chip via 3D printing. *Trens Biotechnol*. **2016**, *34*, 685-688.
234. Yourtee D, Emery J, Smith RE, Hodgson B. Stereolithographic models of biopolymers. *J. Mol. Graph. Model*. **2000**, *18*, 26-28.
235. Scalera F, Corcione CE, Montagna F, Sannino A, Maffezzoli A. Development and characterization of UV curable

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

epoxy/hydroxyapatite suspensions for stereolithography applied to bone tissue engineering. *Ceram. Int*. **2014**, *40*, 15455–15462.
236. Eick JD, Smith RE, Pinzino CS, Kostoryz, EL. Stability of silorane monomers in aqueous systems. *J. Dent*. **2006**, *34*, 405-410.
237. Kesti M, Müller M, Becher J, Schnabelrauch M, D'Este M et al. A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation. *Acta Biomater*. **2015**, *11*, 162–172.
238. Corey EJ, Czako B, Kürti L. Molecules and Medicine. John Wiley & Sons, New York, **2007**.
239. Daylight. www.daylight.com
240. OpenEye Scientific Software. www.eyesopen.com
241. ChemAxon. www.chemaxon.com
242. The Chemistry Development Kit102. http://sourceforge.net/projects/cdk/files/cdk
243. RDkit. http://www.rdkit.org
244. Dragon descriptors103. http://www.talete.mi.it
245. Batch Modules for ACD/Percepta. http://www.acdlabs.com/products/percepta/batch.php
246. R Project for Statistical Computing. http://www.r-project.org
247. Bioclipse59. http://bioclipse.net
248. JMP statistical discovery software. http://www.jmp.com
249. Pipeline Pilot. http://accelrys.com/products/pipeline-pilot
250. Knime104. http://www.knime.org
251. Mathews EJ, Kruhlak ML, Benz RD, Contrera JF. A comprehensive model for reproductive and developmental toxicity hazard identification: II. Construction of QSAR models to predict activities of untested chemicals. *Regulat. Toxicol. Pharmacol*. **2007**, *47*, 136-155.
252. Barenholz Y. Doxil® - the first FDA approved nono-drug: lessons learned. *J. Contr. Rel*. **2012**, *160*, 117-134.
253. Bell IR, Ives JA, Jonas WB. Nonlinear effects of nanoparticles: Biological variability from hormetic doses, small particle sizes, and adaptive interactions. *Dose-Response* **2014**, *12*, 202-254.
254. Xu X, Ho W, Zhang Q, Bertrand N, Farokhzad O. Cancer nanomedicine: from targeted delivery to combination therapy. *Trends Mol. Med*. **2015**, *21*, 223–232.
255. Sosale NG, Ivanovska II, Tsai RK, Swift J, Hsu JW et al. "Marker of Self" CD47 on lentiviral vectors decreases macrophage-mediated clearance and increases delivery to SIRPA-expressing

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

lung carcinoma tumors. *Mol. Ther. Meth. Clin. Develop*. **2016**, *3*, Article 16080.
256. Sakamoto JH, van de Ven AL, Godin B, Blanco E, Serda RE et al. Enabling individualized therapy through nanotechnology. *Pharmacolog. Res*. **2010**, *62*, 57-89.
257. Chen H, Khemtong C, Yang X, Chang X, Gao J. Nanonization strategies for poorly water- soluble drugs. *Drug Disc. Today* **2011**, *16*, 354-360.
258. Oerlemans C, Bult W, Bos M, Storm G, Nijsen FW, Heenink WE. Polymeric micelles in anticancer therapy: targeting, imaging and triggered release. *Pharm. Res*. **2010**, *27*, 2569–2589.
259. Mochida Y, Cabral H, Kataoka K. Polymeric micelles for targeted tumor therapy of platinum anticancer drugs. *Expert Opin. Drug Deliv*. **2017**, DOI: 10.1080/17425247.2017.1307338
260. Jhaveri AM, Torchilin VP. Multifunctional polymeric micelles for delivery of drugs and siRNA. *Front. Pharmacol*. **2014**, *5*, article 77.
261. Doppalapudi S, Jain A, Domb AJ, Khan W. Biodegradable polymers for targeted delivery of anti-cancer drugs. *Expert Opin. Drug Disc*. **2016**, *13*, 891-909.
262. Mozhi A, Ahmad I, Okeke CI, Li C, Liand X-J. pH-sensitive polymeric micelles for the co-delivery of proapoptotic peptide and anticancer drug for synergistic cancer therapy. *RSC Adv*. **2017**, *7*, 12886-12896.
263. Capra F, Luisi PL. *A Systems View of Life: A Unifying Vision*. Cambridge University Press, Cambridge, UK, **2014**.
264. Pavan GM, Danani A. Supporting the design of efficient dendritic DNA and siRNA nano-carriers with molecular modeling. *Curr. Drug Discov. Technol*. **2011**, *8*, 314-438.
265. Merkle RC. Nanotechnology and Medicine, **2017**. http://www.zyvex.com/nanotech/nanotechAndMedicine.html
266. Etheridge ML, Campbell SA, Erdman AG, Haynes CL, Wolf SM, McCoullough J. The big picture on small medicine: The state of nanomedicine products approved for use or in clinical trials. *Nanomed*. **2013**, *9 (1)*, 1–14. doi:10.1016/j.nano.2012.05.013.
267. Tan A, Chawla R, Natasha G, Mahdibeiraghdar S, Jeyaraj R et al. Nanotechnology and regenerative therapeutics in plastic surgery: The next frontier. *J. Plast. Reconstr. Aesthet. Surg*. **2016**, *69 (1)*, 1-13.
268. Jahangirian H, Lemraski EG, Webster TJ, Rafiee-Moghaddam R, Abdollahi Y. A review of drug delivery systems based on

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

nanotechnology and green chemistry: green nanomedicine. *Int. J. Nanomed.* **2017**, *12*, 2957-2978.
269. Muyldermans S. Nanobodies: Natural single-domain antibodies. *Annu. Rev. Biochem*. **2013**, *82*, 775–97.
270. Revets H, De Baetselier P, Muyldermans S. Nanobodies as novel agents for cancer therapy. *Expert Opin. Biol. Ther*. **2005**, *5*, 111-124.
271. Steeland S, Vandenbroucke RE, Libert C. Nanobodies as therapeutics: big opportunities for small antibodies. *Drug Disc. Today* **2016**, *21*, 1076-1113.
272. Van Heeke G, Allosery K, De Brabandere V, De Smedt T, Detalle L et al. Nanobodies® as inhaled biotherapeutics for lung diseases. *Pharmacol. Therapeut*. **2017**, *169*, 47-56.
273. Zhang N, Guo H, Zheng W, Wang P, Ma X. The small and powerful nanobodies (Nbs) originating from peculiar animals used for therapy of cancers. *Int. J. Cancer Stud. Res*. **2016**, *S4*, 001, 1-7.
274. Audenhove IV, Gettemans J. Nanobodies as versatile tools to understand, diagnose, visualize and treat cancer. *EBioMed*. **2016**, *8*, 40–48.
275. Ji X, Peng Z, Li X, Yan Z, Yang Y et al. Neutralization of TNFα in tumor with a novel nanobody potentiates paclitaxel-therapy and inhibits metastasis in breast cancer. *Cancer Lett*. **2016**, *386*, 24-34.
276. Nikkhoi SK, Rahbarizadeh F, Ahmadvand D. Oligo-clonal nanobodies as an innovative targeting agent for cancer therapy: New biology and novel targeting systems. *Prot. Expression Purification* **2017**, *129*, 115-121.
277. Catalent Pharma Solutions. Considering Patient's Voice in Drug Design. Pharmaceutical Technology, London, **2016**.
278. FDA. Size, Shape, and Other Physical Attributes of Generic Tablets and Capsules, **2015**. https://www.federalregister.gov/documents/2015/06/19/2015-15076/size-shape-and-other-physical-attributes-of-generic-tablets-and-capsules-guidance-for-industry
279. He T-T, Ung COL, Hu H, Wang Y-T. Good manufacturing practice (GMP) regulation of herbal medicine incomparative research: China GMP, cGMP, WHO-GMP, PIC/S andEU-GMP. *Eur. J. Integr. Med*. **2015**, *7*, 55-66.
280. US FDA. CFR – Code of Federal Regulations Title 21. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=210

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

281. Mead WJ. *Dietary Supplement Good Manufacturing Practices. Preparing for Compliance*. Informa Health Care, New York, **2012**, Chapter 1, pp. 1-16.
282. Longwell and Associates. Unites States of America v. Barr Labs, Inc. 812F. Supp. http://fdclaw.com/cases/gmp/
283. Wang L, Zheng H, Ren X, Sun H. Trend analysis of FDA warning letters issued to medical products about violations to current Good Manufacturing Practices (CGMP) between 2007 and 2014. *Ther. Innov. Regul. Sci*. **2016**, *50*, 312-318.
284. U.S. FDA. FDA Form 484 Frequently Asked Questions, **2015**. https://www.fda.gov/iceci/inspections/ucm256377.htm
285. Lee SL, O'Connoer TF, Yang X, Cruz CN, Chatterjee S et al. Modernizing pharmaceutical manufacturing: from batch to continuous production. *J. Pharm. Innov*. **2015**, *10*, 191–199.
286. U.S. The Code of Federal Regulations. 21 CFR 210.3(2). Washington, DC: The federal government, **2009**.
287. Roschanger F, Colberg J, Dunn PJ, Gallou F, Hayler JD et al. A deeper shade of green: inspiring sustainable drug manufacturing. *Green Chem*. **2017**, *19*, 281-285.
288. Cross R, Hargadon A, Parise S, Thomas R. Critical Connections: Driving Rapid Innovation with a Network Perspective, Sloan Management Review and Wall Street Journal Weekend Edition, http://www.robcross.org/pdf/roundtable/networks_and_innovation.pdf, **2008**.
289. Gerrits E, Woerdenbag HJ, Luurtsema G, Hooge MNL, Boersma HH. The added value of Good Manufacturing Practices (GMP) in the production of radiopharmaceuticals, in *Quality in Nuclear Medicine*, Glaudemans AWJM et al, eds., Springer International, Switzerland, **2017**, pp. 59-67.
290. Clément N, Grieger JC. Manufacturing of recombinant adeno-associated viral vectors for clinical trials. *Mol. Ther. Meth. Clin. Develop*. **2016**, *3*, article 16003.
291. Mazo-Barbara A, Mirabel C, Nieto V, Reyes B, García-López J et al. Qualification of computerized monitoring systems in a cell therapy facility compliant with the good manufacturing practices. *Regen. Med*. **2016**, *11*, 521–528.
292. Pasqualini FS, Emmert MY, Parker KK, Hoerstrup SP. Organ chips: quality assurance systems in regenerative medicine. *Clin. Pharmacol. Therapeut*. **2017**, *101*, 31-34.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

293. Pamies D, Bal-Price A, Simeonov A, Tagle D, Allen D et al. Good cell culture practice for stem cells and stem-cell-derived models. *ALTEX*, **2017**, *34 (1)*, doi.org/10.14573/altex.1607121
294. FDA. Guidance for Industry: Current Good Manufacturing Practice in Manufacturing, Packaging, Labeling, or Holding Operations for Dietary Supplements; Small Entity Compliance Guide, **2016**, https://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/dietarysupplements/ucm238182.htm
295. Kumar M. Strategies for implementing the new GMPs for dietary supplements, **2011**. www.raps.org/WorkArea/DownloadAsset.aspx?id=3562
296. U.S. FDA. Guidance for Industry E6 Good Clinical Practice: Consolidated Guidance, **1996**, https://www.fda.gov/downloads/drugs/guidances/ucm073122.pdf
297. US FDA. CFR - Code of Federal Regulations Title 21, **2016**, https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRsearch.cfm?CFRPart=312
298. Schulz MMP, Reisen F, Zgraggen S, Fischer S, Yuen D *et al*. Phenotype-based high-content chemical library screening identifies statins as inhibitors of *in vivo* lymphangiogenesis. *Proc. Natl. Acad. Sci*. **2012**, *109*, E2665- E2674.
299. SigmaAldrich LOPAC® Library of Pharmacologically Active Compounds, **2017**, http://www.sigmaaldrich.com/catalog/product/sigma/sc001?lang=en&region=US
300. Banfi C, Baette R, Gianazza E, Tremoli E. Technological advances and proteomic applications in drug discovery and target deconvolution identification of the pleiotropic effects of statins. *Drug Disc. Today* **2017**, *22*, 848-869.
301. Hopkins AL. Network pharmacology: the next paradigm in drug discovery. *Nat. Chem. Biol*. **2008**, *4*, 682–690.
302. Medina-Franco JL, Giulianotti MA, Welmaker GS, Houghten RA. Shifting from the single to the multitarget paradigm in drug discovery. *Drug Disc. Today* **2013**, *18*, 495-501.
303. Xu K, Coté TR. Database identifies FDA-approved drugs with potential to be repurposed for treatment of orphan diseases. *Brief. Bioinform*. **2011**, *12*, 341-345.
304. Kamal A, Nekkanti S, Shankaraiah N, Sathish M. Future of drug discovery, in *Drug Resistance in Bacteria, Fungi, Malaria, and Cancer*, Arora G, Andaleeb S, Chandra V, eds. Springer International Publishing, Cham, Switzerland, **2017**.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

305. Mo C, Wang L, Zhang Z, Numazawa S, Tang H et al. The crosstalk between Nrf2 and AMPK signal pathways is important for the anti-inflammatory effect of berberine in LPS-stimulated macrophages and endotoxin-shocked mice. *Antioxid. Redox Signal*. **2014**, *20*, 574-588.
306. Scapagnini G, Sonya V, Nader AG, Calogero C, Zella D, Fabio G. Modulation of Nrf2/ARE pathway by food polyphenols: A nutritional neuroprotective strategy for cognitive and neurodegenerative disorders. *Mol. Neurobiol*. **2011**, *44*, 192–201.
307. Grossman R, Ram Z. The dark side of Nrf2. *World Neurol*. **2013**, *80*, 284-285.
308. Kansanen E, Kuosmanen SM, Leinonen H, Levonen, A-L. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biol*. **2013**, *1*, 45–49.
309. Cebula M, Schmidt EE, Arnér ESJ. TrxR1 as a potent regulator of the Nrf2-Keap1 response system. *Antioxid. Redox Signal*. **2015**, *23*, 823-853.
310. Zolot RS, Basu S, Million RP. Antibody–drug conjugates. *Nature Rev. Drug Disc*. **2013**, *12*, 259-260.
311. Webb S. Pharma interest surges in antibody drug conjugates. *Nature Biotechnol*. **2011**, *29*, 297-298.
312. LoRusso PM, Weiss D, Guardino E, Girish S, Sliwkowski MX. Trastuzumab emtansine: A unique antibody-drug conjugate in development for human epidermal growth factor receptor 2–positive cancer. *Clin. Cancer Res*. **2011**, *17*, 6437–47.
313. Krop IE, LoRusso P, Miller KD, Modi S, Yardley D et al. A Phase II study of trastuzumab emtansine in patients with human Epidermal growth factor receptor 2–positive metastatic breast cancer who were previously treated with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine. *J. Clin. Oncol*. **2012**, *30*, 3234-3241.
314. Singh R, Zhang Y, Pastan I, Kreitman RJ. Synergistic antitumor activity of anti-CD25 recombinant immunotoxin LMB-2 with chemotherapy. *Clin. Cancer Res*. **2011**, *18*, 152–60.
315. Teicher BA, Chari RVJ. Antibody conjugate therapeutics: Challenges and potential. *Clin. Cancer Res*. **2011**, *17*, 6389–97.
316. Shusterman S, London WB, Gillies SD, Hank JA, Voss SD et al. Antitumor activity of Hu14.18-IL2 in patients with relapsed/refractory neuroblastoma: A children's oncology group (COG) Phase II study. *J. Clin. Oncol*. **2010**, *28*, 4969-4975.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

317. Yao Y-D, Sun T-M, Huang S-Y, Dou S, Lin J et al. Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis. *Sci. Transl. Med*. **2012**, *4 (130)*, 1-10.
318. Sharma SK, Bagshawe KD. Translating antibody directed enzyme prodrug therapy (ADEPT) and prospects for combination. *Expert Opin. Biol. Ther*. **2017**, *17*, 1-13.
319. Chua HE, Bhomick SS, Tucker-Kellogg L. Synergistic target combination prediction from curated signaling networks: machine learning meets systems biology and pharmacology. *Methods* **2017**, DOI: http://dx.doi.org/10.1016/j.ymeth.2017.05.015
320. Smith GR. Angiotensin and systems thinking: wrapping your mind around the big picture. *Oschner J*. **2013**, *13*, 11-25.
321. Bromage DI, Yellon DM. The pleiotropic effects of metformin: time for prospective studies. Metformin clinical trial to increase longevity – pleiotropic effects. *Cardiovasc. Diabetol*. **2015**, *14*, 109.
322. De Kreutzenberg SV, Ceolotto G, Cattelan A, Pagnin E, Mazzucato M et al. Metformin improves putative longevity effectors in peripheral mononuclear cells from subjects with prediabetes. A randomized controlled trial. *Nutr. Metab. Cardiovasc. Dis*. **2015**, *25*, 686-693.
323. Wu L, Zhou B, Oshiro-Rapley N, Li M, Paulo JA et al. An ancient, unified mechanism for metformin growth inhibition in *C. elegans* and cancer. *Cell* **2016**, *167*, 1705–1718.
324. Barzilai N, Crandall JP, Kritchevsky SB, Espeland MA. Metformin as a tool to target aging. *Cell Metab*. **2016**, *23*, 1060-1065.
325. Cuyás E, Fernández-Arroyo S, Joven J, Menendez JA. Metformin targets histone acetylation in cancer-prone epithelial cells. *Cell Cycle* **2016**, *15*, 3355-3361.
326. Linton R. AMPK and aging. *Life Extension Magazine* **2015**, http://www.lifeextension.com/magazine/2015/11/ampk-and-aging/page-01
327. Wang C-P, Lorenzo C, Habib SL, Jo B, Espinoza SE. Differential effects of metformin on age related comorbidities in older men with type 2 diabetes. *J. Diabetes Complications* **2017**, *31*, 679-686.
328. Clark WR. *A Means to an End: The Biological Basis of Aging and Health*. Oxford University Press, Oxford, **2002**, pp. 3-20.
329. Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am. J. Physiol*. **1999**, *277 (1 Pt 1)*, E1-10.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

330. U.S. National Institutes of Health. Metformin in Longevity Study (MILES). ClinicalTirals.gov, **2017**, https://clinicaltrials.gov/ct2/show/NCT02432287.
331. Novick PA, Ortiz OF, Poelman J, Abdulhay AY, Pande VS. SWEETLEAD: an *in silico* database of approved drugs, regulated chemicals, and herbal isolates for computer-aided drug discovery. *PLoS One* **2013**, *8 (11)*, Article e79568.
332. Li J, Zheng S, Chen B, Butte AJ, Swamidass SJ et al. A survey of current trends in computational drug repositioning. *Brief. Bioinform*. **2015**, 1–11, doi: 10.1093/bib/bbv020.
333. Nguyen L, Thomas KL, Lucke-Wold BP, Cavendish JD, Crowe MS et al. Dextromethorphan: An update on its utility for neurological and neuropsychiatric disorders. *Pharmacol. Therapeut*. **2016**, *159*, 1-22.
334. Visk DA. The Reemergence of Phenotypic Screening. *Genet. Eng. Biotechn. N*. **2016**, *36(3)*, http://www.genengnews.com/gen-articles/the-reemergence-of-phenotypic-screening/5675/
335. Eder J, Sedrani R, Wiesmann C. The discovery of first in-class drugs: origins and evolution. *Nature Rev. Drug Disc*. **2014**, *13*, 577-587.
336. Nierode GJ, Perea BC, McFarland SK, Pascoal JF, Clark DS. High-throughput toxicity and phenotypic screening of 3D human neural progenitor cell cultures on a microarray chip platform. *Stem Cell Rep*. **2016**, *7*, 970-982.
337. Kang J, Hsu C-H, Wu Q, Liu S, Coster AD et al. Improving drug discovery with high-content phenotypic screens by systematic selection of reporter cell lines. *Nature Biotechnol*. **2016**, *34*, 70-77.
338. Moffat JG, Vincent F, Lee JA, Eder J, Prunotto M. Opportunities and challenges in phenotypic drug discovery: an industry perspective. *Nat. Rev. Drug Disc*. **2017**, *16*, 531-543.
339. Moffat JG, Rudolph JR, Bailey D. Phenotypic screening in cancer drug discovery - past, present and future. *Nature Rev. Drug Disc*. **2014**, *13*, 588-602.
340. Futamura Y, Yamamoto K, Osada H. Phenotypic screening meets natural products in drug discovery. *Biosci. Biotechnol. Biochem*. **2017**, *81*, 28-31.
341. Gonzalez-Munoz AL, Minter RR, Rust SJ. Phenotypic screening: the future of antibody discovery. *Drug Disc. Today* **2016**, *21*, 150-156.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

342. Zheng W, Thorne N, McKew JC. Phenotypic screens as a renewed approach for drug discovery. *Drug Disc. Today* **2013**, *18*, 1067-1073.
343. Cooper DJ, Zunino G, Bixby JL Lemmon VP. Phenotypic screening with primary neurons to identify drug targets for regeneration and degeneration. *Mol. Cell Neurosci*. **2016**, http://dx.doi.org/10.1016/j.mcn.2016.07.001.
344. Hovlid ML, Winzeler EA. Phenotypic screens in antimalarial drug discovery. *Trend. Parasitol*. **2016**, *32*, 697-707.
345. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nature Rev. Drug Disc*. **2007**, *6*, 273-286.
346. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* **2005**, *438*, 932-936.
347. Kumar MD, Dravid A, Kumar A, Sen D. Gene therapy as a potential tool for treating neuroblastoma – a focused review. *Cancer Gene Ther*. **2016**, *23*, 115–124.
348. Lin S, Ching LT, Chen J, Cheung PCK. Antioxidant and anti-angiogenic effects of mushroom phenolics-rich fractions. *J. Funct. Foods* **2015**, *17*, 802-815.
349. Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S et al. Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **2007**, *131*, 463–475.
350. Briquez PS, Clegg LE, Martino MM, Gabhann FM, Hubbell JA. Design principles for therapeutic angiogenic materials. *Nature Rev. Materials* **2016**, *1*, 1-15.
351. Chang J, Kwon HJ. Discovery of novel drug targets and their functions using phenotypic screening of natural products. *J. Ind. Microbiol. Biotechnol*. **2016**, *43*, 221–231.
352. Gilbert IH. Drug discovery for neglected diseases: Molecular target-based and phenotypic approaches. *J. Med. Chem*. **2013**, *56*, 7719-7726.
353. Cabrera AC, Lucena-Agell D, Redondo-Horcajo M, Barasoain I, Díaz JF. Aggregated compound biological signatures facilitate phenotypic drug discovery and target elucidation. *ACS Chem. Biol*. **2016**, *11*, 3024-3034.
354. Zhang H, Wheeler W, Hyland PL, Yang Y, Shi J et al. A powerful procedure for pathway-based meta-analysis using summary statistics identifies 43 pathways associated with type II diabetes in European populations. *PLoS Genet*. **2016**, *12 (6)*, Article e1006122.
355. Lamparter D, Marbach D, Rueedi R, Kutalik Z, Bergmann S. Fast and rigorous computation of gene and pathway scores from SNP-

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

based summary statistics. *PLoS Comp. Biol*. **2016**, *12*, article e1004714.
356. Brown MC, Gromeier M. Cytotoxic and immunogenic mechanisms of recombinant oncolytic poliovirus. *Curr. Opin. Virol*. **2015**, *13*, 81-85.
357. Dolgin E. The billion dollar biotech. *Nature* **2015**, *522*, 26-28.
358. Li B, Luo X, Dong Y. Effects of chemically modified messenger RNA on protein expression. *Bioconjugate Chem*. **2016**, *27*, 849−853.
359. Scrom E, Huber M, Aneja M, Dohmen C, Emrich D et al. Translation of angiotensin-converting enzyme 2 upon liver- and lung-targeted delivery of optimized chemically modified mRNA. *Mol. Ther. Nucl. Acid*. **2017**, *16*, 350-365.
360. Sahin U, Kariko K, Tureci O. mRNA-based therapeutics - developing a new class of drugs. *Nature Rev. Drug Disc*. **2014**, *13*, 759-771.
361. Li B, Luo X, Dong Y. Effects of chemically modified messenger RNA on protein expression. *Bioconjugate Chem*. **2016**, *27*, 849−853.
362. Hadas Y, Katz MG, Bridges CR, Zangi L. Modified mRNA as a therapeutic tool to induce cardiac regeneration in ischemic heart disease. *WIREs Syst. Biol. Med*. **2017**, *9*, Article e1367.
363. Zangi L, Lui KO, von Gise A, Ma Q, Ebina W. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nature Biotechnol*. **2013**, *31*, 898-907.
364. Guan S, Rosenecker J. Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. *Gene Ther*. **2017**, *24*, 133-143.
365. Ottesen EW. ISS-N1 makes the first FDA-approved drug for spinal muscular atrophy. *Translat. Neurosci*. **2017**, *8*, 1-6.
366. Khorkova O, Wahlestedt C. Oligonucleotide therapies for disorders of the nervous system. *Nature Biotechnol*. **2017**, *35*, 249-263.
367. Kumar MD, Dravid A, Kumar A, Sen D. Gene therapy as a potential tool for treating neuroblastoma – a focused review. *Cancer Gene Ther*. **2016**, *23*, 115–124.
368. Liu H, Kurtoglu M, León-Annicchiarico CL, Munoz-Pinedo C, Barredo J. Combining 2-deoxy-D-glucose with fenofibrate leads to tumor cell death mediated by simultaneous induction of energy and ER stress. *Oncotarget* **2016**, *7 (24)*, article 36461.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

369. Halicka HD, Garcia J, Li J, Zhao H, Darzynkiewicz, Z. Synergy of 2-deoxy-D-glucose combined with berberine in inducing the lysosome/autophagy and transglutaminase activation-facilitated apoptosis. *Apoptosis* **2017**, *22*, 229-238.
370. Chattopadhyay A, O'Connor CJ, Zhang F, Galvagnion C, Galloway WRJD. Discovery of a small-molecule binder of the oncoprotein gankyrin that modulates gankyrin activity in the cell. *Sci Rep*. **2016**, *6*, Article 23732.
371. Bolouri H. Network dynamics in the tumor microenvironment. *Semin. Cancer Biol*. **2015**, *30*, 52–59.
372. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* **2011**, *20*, 576-590.
373. U.S. FDA. Now available without a prescription, **2016**, https://www.fda.gov/drugs/resourcesforyou/consumers/ucm143547.htm
374. MacDonald S. Aspirin use to be banned in under 16-year olds. *Br. Med. J*. **2002**, *325*, 988.
375. Samman S, Sivarajah G, Man JC, Ahmad ZI, Petocz P, Caterson ID. A mixed fruit and vegetable concentrate increases plasma antioxidant vitamins andfFolate and lowers plasma homocysteine in men. *J. Nutr*. **2003**, *133*, 2188-2193.
376. Gal KL, Ibrahim MX, Wiel C, Sayin VI, Akula MK et al. Antioxidants can increase melanoma metastasis in mice. *Sci. Transl. Med*. **2015**, *7 (308)*, Article 308re8.
377. Wang H, Liu X, Long M, Huang Y, Zhang L. NRF2 activation by antioxidant antidiabetic agents accelerates tumor metastasis. *Sci. Transl. Med*. **2016**, *8 (334)*, Article 334ra51.
378. Hawk MA, McCallister C, Schafer ZT. Antioxidant activity during tumor progression: a necessity for the survival of cancer cells? *Cancers* **2016** *8*, Article 92.
379. Smith RE, Tran K, Shejwalkar P, Hara K. The role of the Nrf2/ARE antioxidant system in preventing cardiovascular diseases. *Diseases* **2016**, *4*, Article 34.
380. Prolla TA, Denu JM. NAD^+ deficiency in age-related mitochondrial dysfunction. *Cell Metab*. **2014**, *19*, 178-180.
381. Yoshino J, Mills KF, Yoon MJ, Imai S-I. Nicotinamide mononucleotide, a key NAD^+ intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab*. **2011**, *14*, 528–536.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

382. Heilbronn LK. Clinical trials corner. *Nutr. Healthy Aging* **2017**, *4*, 193-194.
383. Saw CLL, Guo Y, Yang AY, Paredes-Gonzales X, Ramirez C et al. The berry constituents quercetin, kaempferol, and pterostilbene synergistically attenuate reactive oxygen species: Involvement of the Nrf2-ARE signaling pathway. *Food Cehm. Toxicol*. **2014**, *72*, 303-311.
384. Nagao K, Jinnouchi T, Kai S, Yanagita T. Pterostilbene, a dimethylated analog of resveratrol, promotes energy metabolism in obese rats. *J. Nutr. Biochem*. **2017**, *43*, 151-155.
385. Human Longevity, Inc.™ **2017**, http://www.humanlongevity.com/about/overview/
386. Telenti A, Pierce LCT, Biggs WH, di Lulio J, Wong EHM et al. Deep sequencing of 10,000 human genomes. *Proc. Natl Acad. Sci*. **2016**, *113*, 11901-11906.
387. Health Nucleus, **2017**, https://www.healthnucleus.com/faq
388. Calico, **2017**, https://www.calicolabs.com/
389. McDonald D, Glusman G, Price ND. Personalized nutrition through big data. *Nature Biotechnol*. **2016**, *34*, 152-154.
390. Arivale. Your scientific path to wellness, **2017**, https://www.arivale.com/
391. Day Two, Balance your personalized nutrition app based on your unique microbiome, **2017**. https://www.daytwo.com/
392. Google Baseline, Project Baseline, **2017**, https://verily.com/projects/precision-medicine/baseline-study/
393. American Gut, **2017**, http://americangut.org/
394. Enterome Bioscience, **2017**, http://www.enterome.fr/
395. Second Genome, **2017**, http://www.secondgenome.com/
396. Seres Health, **2017**, http://www.serestherapeutics.com/
397. Vedanta Bioscience, **2017**, http://www.vedantabio.com/
398. Sikka T. Contemporary superfood cults. Nutritionism, neoliberalism and gender, in *Food Cults: How Fads, Dogma, and Doctrine Influence Diet*. Cargill K, ed., Rowan & Littlefield, **2017**, 87-108.
399. Siro I, Kapolna E, Kapolna B, Lugasi A. Functional food: product development, marketing and consumer acceptance – a review. *Appetite* **2008**, *51*, 457.
400. Loyer J. *The Social Lives of Superfoods*. Ph.D. thesis, University of Adelaide, Australia, **2016**.
401. Aronson JK. Defining 'nutraceuticals': neither nutritious nor pharmaceutical. *Br. J. Clin. Pharmacol*. **2017**, *83*, 8-19.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

402. Wodak R. Critical discourse analysis, in *Qualitative Research Practice*, Seale C, Gobo G, Gubrium JF, Silverman D, eds. SAGE, Thousand Oaks, CA, **2004**, 3.
403. McNally, Alex. Superfoods market set to double by 2011. NutraIngredients. com-Europe, **2007**. http://nufruits.com/documents/news/Superfoods%20market%20set%20to%20double%20by%202011.pdf
404. Curll J, Parker C, MacGregor C, Petersen A. Unlocking the energy of the Amazon? The need for a food fraud policy approach to the regulation of anti-ageing health claims on superfood labelling. *Fed. Law Rev*. **2016**, *44*, 419-449.
405. Schauss A. Advances in the study of the health benefits and mechanisms of action of the pulp and seed of the Amazonian palm fruit, *Euterpe oleracea* Mart., known as "Açai", Chapter 10 in *Fruits, Vegetables and Herbs: Bioactive Foods in Health Promotion*, Watson RR, Preedy VR, eds., Elsevier, Amsterdam, **2016**.
406. Kim H-K, Kwon M-K, Kim J-N, Kim C-K, Lee Y-J et al. Identification of novel fatty acid glucosides from the tropical fruit *Morinda citrifolia* L. *Phytochem. Lett*. **2010**, *3*, 238-241.
407. Richards, K.M., Tran, K., Levine, R.A., Luo R., Maia, G.M et al. Improved extraction of soluble solids from some Brazilian and North American fruits. *Nat. Prod. J*. **2014**, *4*, 201-210.
408. Santini A, Tenore GC, Novellino E. Nutraceuticals: A paradigm of proactive medicine. *Eur. J. Pharm. Sci*. **2017**, *96*, 53-61.
409. Singletary K. Cinnamon: overview of health benefits. *Nutr. Today* **2008**, *43*, 263-266.
410. Ranasinghe P, Galappaththy P. Health benefits of Ceylon cinnamon (*Cinnamomum zeylanicum*): a summary of the current evidence. *Ceylon Med. J*. **2016**, *61 (1)*, 1-5.
411. Quiroz-Iturra L, Rosas-Saaverda C, Klein CS. Functional fruits through metabolic engineering, Chapter 3 in *Superfood and Functional Food - The Development of Superfoods and Their Roles as Medicine*, Shiomi N, Waisundara V, eds., InTech, Rijeka, Croatia, **2017**.
412. Kocaadam B, Sanlier N. Curcumin, an active component of turmeric (*Curcuma longa*), and its effects on health. *Crit. Rev. Food Sci. Nutr*. **2017**, *57*, 2889-2995.
413. Wu A, Noble EE, Tyagi E, Ying Z, Zhuang Y et al. Curcumin boosts DHA in the brain: Implications for the prevention of anxiety disorders. *Biochim. Biophys. Acta* **2015**, *1852*, 951-961.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

414. Rasul CH. Alarming situation of food adulteration. *Bang. Med. J. Khulna*. **2013**, *46*, 1-2.
415. Messner DJ, Robinson T, Kowdley KV. Curcumin and turmeric modulate the tumor-promoting effects of iron in vitro. *J. Cancer Nutr*. **2017**, *69*, 481-489.
416. Roelen CCJ, van Riel A, de Vries I. Serious health problems after use of a dietary supplement for weight-loss and sports enhancement. Clin. Toxicol. **2014**, *52*, 78-79.
417. US FDA. Dietary Supplements. http://www.fda.gov/Food/DietarySupplements/
418. Cohen PA. American roulette – Contaminated dietary supplements. *New Eng. J. Med*. **2009**, *361*, 1523-1525.
419. Cohen PA, Maller G, DeSouza R, Neal-Kababick J. Presence of banned drugs in dietary supplements following FDA recalls. *J. Am. Med. Assoc*. **2014**, *312*, 1691-1693.
420. US Food and Drug Administration, Safety: recalls, market withdrawals, & safety alerts, **2015**. http://www.fda.gov/Safety/Recalls/ucm165546.htm.
421. Smoker M, FDA, personal communication, **2015**.
422. Tsai H-J, Hsu YH, Huang Y-W, Chang YK, Liu JS, Hsu CC. Use of non-steroidal anti-inflammatory drugs and risk of chronic kidney disease in people with Type-2 diabetes melitus, a nationwide longitudinal cohort study. *Diabet. Med*. **2015**, *32*, 382–390.
423. Smith RE, McDonald M, Smith C. Pleiotropic effects of green tea. *Ann. Med. Chem. Res*. **2016**, *2*, 1014.
424. Levin J, Mass S, Schuberth M, Respondek G, Paul F et al. The PROMESA-protocol: progression rate of multiple system atrophy under EGCG supplementation as anti-aggregation approach. *J. Neural. Transm*. **2016**, *123*, 439-445.
425. Koder AE, Atef H, Said E, El Kashef HA, Salem HA. Implication of Nrf2/HO-1 pathway in the coloprotective effect of coenzyme Q10 against experimentally induced ulcerative colitis. *Inflammopharmacol*. **2017**, *25*, 119–135.
426. Littarru GP, Bruge F, Tiano L. The biochemistry of coenzyme Q10, Chapter 2 in *Antioxidants in Andrology*, Balercia G, Gandini L, Lenzi A, Lombardo F, eds. Springer, Switzerland, **2017**.
427. Maladkar M, Patil S, Sood S. Coenzyme Q10: the cardiac bio-energizer in cardiovascular diseases. *J. Cardiol. Cardiovasc. Ther*. **2016**, *1 (4)*, Article 555560.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

428. Peres HA, Foss MCF, Pereira LGL. The role of coenzyme Q10 supplementation with statin drug use and chronic diseases. *J. Infect. Dis. Prevent. Med*. **2017**, *5 (2)*, Article 1000157.
429. Taylor BA, Lorson L, White CM, Thompson PD. A randomized trial of coenzyme Q10 in patients with confirmed Statin myopathy. *Atherosclerosis* **2015**, *238*, 329-335.
430. Bliznakov EG, Hunt GL. *The Miracle Nutrient Coenzyme Q10*. Bantam Books, New York, 1987.
431. Lockwood K, Moesgaard S, Yamamoto T, Folkers K. Progress on therapy of breast cancer with vitamin Q10 and the regression of metastases. *Biochem. Biophys. Res. Commun*. **1995**, *212*, 172-177.
432. Gaby AR. The role of coenzyme Q10 in clinical medicine. *Altern. Med. Rev*. **1996**, *1*, 168-172.
433. Langsjoen PH, Folkers K. Long term efficacy and safety of coenzyme Q10 therapy for idiopathic dilated cardiomyopathy. *Amer. J. Cardiol*. **1989**, *65*, 521 – 523.
434. Ylikoski T et al. The effect of coenzyme Q10 on the exercise performance of cross-country skiers. *Mol. Asp. Med*. **1997**, *18 (Suppl)*, S283-290.
435. Folkers K, Simonsen R. Two successful double-blind trials with coenzyme Q10 (vitamin Q10) on muscular dystrophies and neurogenic atrophies. *Biochim. Biophys. Acta* **1995**, *1271*, 281-286.
436. Littaru GP. A Letter from the Institute of Biochemistry, **2001**.
437. Langsjoen PH. The clinical use of HMG CoA-reductase inhibitors (statins) and the associated depletion of the essential co-factor coenzyme Q_{10}: a review of pertinent human and animal data. https://www.fda.gov/ohrms/dockets/.../02p-0244-cp00001-02-exhibit_a-vol1.pdf
438. Lee BJ, Tseng Y-F, Yen C-Y, Lin P-T. Effects of coenzyme Q10 supplementation (300 mg/day) on antioxidation and anti-inflammation in coronary artery disease patients during statins therapy: a randomized, placebo-controlled trial. *Nutr. J*. **2013**, *12*, Article 142.
439. Banach M, Serban C, Sahebkar A, Ursoniu S, Rysz J et al. Effects of coenzyme Q10 on statin-induced myopathy: a meta-analysis of randomized controlled trials. *Mayo Clin. Proc*. **2015**, *90*, 24-34.
440. Buettner C, Greenman RL, Ngo LH, Wu JS. Effects of Coenzyme Q10 on skeletal muscle oxidative metabolism in statin users assessed using ^{31}P magnetic resonance spectroscopy: a randomized controlled study. *J. Nat. Sci*. **2016**, *2 (8)*, Article e212.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

441. Marcoff L, Thompson PD. The role of coenzyme Q10 in statin associated myopathy: a systematic review. *J. Am. Coll. Cardiol*. **2007**, *49*, 2231-2237.
442. Centro de Investigación Biomédica en Red, Ubiquinol, designated as an orphan drug for treatment of primary coenzyme Q10 deficiency, **2016**, http://icqaproject.org/wp-content/uploads/2016/12/Press-release-Ubiquinol-EMA-decision.pdf
443. Schneider C, Segre T. Green tea: Potential health benefits. *Am. Fam. Physician* **2009**, *79*, 591-594.
444. Most J, van Can JGP, van Dijk J-W, Goossens GH, Jocken J, Hospers JJ, Bendik I, Blaak EE. A 3-day EGCG-supplementation reduces interstitial lactate concentration in skeletal muscle of overweight subjects. *Sci Rep*. **2015**, *5*, 17896, 9 p.
445. Mageed MJ, Saliem SS. Antibacterial effects of green tea extracts on *Aggregatibacteractinomycetemcomitans* (i*n-vitro* study). *J. Bagh. College Dent*. **2015**, *27*, 102-108.
446. Wang YF, Shao SH, Xu P, Yang XQ, Qian LS. Catechin-enriched green tea extract as a safe and effective agent for antimicrobial and anti-inflammatory treatment. *Afr. J. Pharm. Pharmacol* **2011**, *5*, 1452-1461.
447. Peng Y, Yu B, Liu F. Epigallocatechin-3-gallate promotes osteoblastic activity in human osteoblast-like cells. *Trop. J. Pharmaceut. Res*. **2016**, *15*, 313-317.
448. Min S-Y, Yan M, Kim SB, Ravikumar S, Kwon S-R, Vanarsa K, et al. Green teaepigallocatechin-3-gallate suppresses autoimmune arthritis through indoleamine-2,3-dioxygenase expressing dendritic cells and the nuclear factor, erythroid 2-like 2 antioxidant pathway. *J. Inflam*. **2015**, *12*, 53.
449. Chen I-J, Liu C-Y, Chiu J-P, Hsu C-H. Therapeutic effect of high-dose green tea extract on weight reduction: A randomized, double-blind, placebo-controlled clinical trial. *Clin. Nutr*. **2016**, *35*, 592-599.
450. Meltzer SM, Monk BJ, Tewari KS. Green tea catechins for treatment of external genital warts. *Am. J. Obstet. Gynecol*. **2009**, *200*, Article 233-e1.
451. Tran K, Richards KM, Smith RE. Prescription drugs and dietary supplements for weight loss. *Adv. Obes. Weight Manag. Control* **2015**, *3*, Article 00045.
452. Schöntal AH. Adverse effects of green tea extracts. *Mol. Nutr. Food Res*. **2011**, *55*, 874-885.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

453. Sarma DN, Barrett ML, Chavez ML, Gardiner P, Ko R, et al. Safety of green tea extracts. A systematic review by the US Pharmacopeia. *Drug Safety* **2008**, *31*, 469-484.
454. Cabrera C, Artacho R, Giménez R. Beneficial effects of green tea - a review. *J. Am. Coll. Nutr*. **2006**, *25*, 79-99.
455. Zhang S, Shan L, Li Q, Wang X, Li S, Zhang Y, et al. Systematic analysis of themultiple bioactivities of green tea through a network pharmacology approach. *Evid. Based Complement. Alt. Med*. **2014**, *2014*, Article ID 512081, 11 p.
456. Kerimi A, Williamson G. At the interface of antioxidant signalling and cellular function: Key polyphenol effects. *Mol. Nutr. Food. Res*. **2016**, 1–19, DOI 10.1002/mnfr.201500940.
457. Na H-K, Surh Y-J. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food Chem. Toxicol*. **2008**, *46*, 1271–1278.
458. Chen J, Zhang Z, Cai L. Diabetic cardiomyopathy and its prevention by Nrf2: current status. *Diabetes Metab. J*. **2014,** *38*, 337-345.
459. Wang Y, Wang B, Du F, Su X, Sun G, Zhou G. Epigallocatechin-3-gallate attenuates oxidative stress and inflammation in obstructive nephropathy via NF-κB and Nrf2/HO-1 signalling pathway regulation. *Basic Clin. Pharmacol. Toxicol*. **2015**, *117*, 164–172.
460. Han J, Wang M, Jing X, Shi Y, Ren M, Lou H. (2)-Epigallocatechin gallate protects against cerebral ischemia-induced oxidative stress via Nrf2/ARE signaling. *Neurochem. Res*. **2014**, *39*, 1292–1299.
461. Saito E, Inoue M, Sawada N, Shimazu T, Yamaji T, Iwasaki M, et al. Association of green tea consumption with mortality due to all causes and major causes of death in a Japanese population: the Japan public health center-based prospective study (JPHC Study). *Ann. Epidemiol*. **2015**, *25*, 512-518.
462. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, Nishino Y, et al. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan. *J. Am. Med. Assoc*. **2006**, *296*, 1255-1265.
463. Thielecke F, Boschmann M. The potential role of green tea catechins in the prevention of the metabolic syndrome – A review. *Phytochem*. **2009**, *70*, 11-24.
464. Momose Y, Maeda-Yamamoto M, Nabetani H. Systematic review of green tea epigallocatechin gallate in reducing low-density

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

lipoprotein cholesterol levels of humans. *Int. J. Food Sci. Nutr*. **2016**, *67*, 606-613.
465. Cominacini L, Mozzini C, Garbin U, Pasini A, Stranieri C, Solani E et al. Endoplasmic reticulum stress and Nrf2 signaling in cardiovascular diseases. *Free Rad. Biol. Med*. **2015**, *88*, 233-242.
466. McSweeney SR, Warabi E, Siow RCM. Nrf2 as an endothelial mechanosensitive transcription factor going with the flow. *Hypertension* **2016**, *67*, 20-29.
467. Dodson M, Redmann M, Rajasekaran NS, Darley-Usmar V, Zhang J. KEAP1–NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity. *Biochem J*. **2015**, *469*, 347–355.
468. Afzal M, Safer AM, Menon M. Green tea polyphenols and their potential role in health and disease. *Inflammopharmacol*. **2015**, *23*, 151–161.
469. Jin J-S, Touyama M, Takayoshi Hisada T, Benno Y. Effects of green tea consumption on human fecal microbiota with special reference to *Bifidobacterium* species. *Microbiol. Immunol*. **2012**, *56*, 729–739.
470. Turnbaugh PJ, Gordon JI. The core gut microbiome, energy balance and obesity. *J. Physiol.* **2009**, *587*, 4153–4158.
471. Zhang C, Zhang M, Wang S, Han R, Cao Y et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *ISME J.* **2010**, *4*, 232–241.
472. Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. *J. Clin. Invest*. **2011**, *121*, 2126-2132.
473. Albini A, Tosetti F, Li VW, Noonan DM, li WW. Cancer prevention by targeting angiogenesis. *Nat. Rev. Clin. Oncol*. **2012**, *9*, 498–509.
474. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. *Pharmacol. Res*. **2008**, *58*, 262–270.
475. Jankovic J. Are adenosine antagonists, such as istradefylline, caffeine, and chocolate, useful in the treatment of Parkinson's disease? *Ann. Neurol*. **2008**, *63*, 267-269.
476. Levin J, Mass S, Schuberth M, Respondek G, Paul F et al. The PROMESA-protocol: progression rate of multiple system atrophy under EGCG supplementation as anti-aggregation approach. *J. Neural. Transm*. **2016**, *123*, 439-445.
477. Roberti G, Tanga L, Michelessi M, Quaranta L, Parisi V et al. Cytidine 5'-diphosphocholine (citicoline) in glaucoma: Rationale

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

of its use, current evidence and future perspectives. *Int. J. Mol. Sci*. **2015**, *16*, 28401–28417.
478. Smith RE, Rouchotas P, Fritz H. Lecithin (phosphatidylcholine): healthy dietary supplement or dangerous toxin? *Nat. Prod. J*. **2016**, *6,* 242-249.
479. Alvarez XA, Laredo M, Corzo D, Fernández-Novoa, L, Mouzo R et al. Citicoline improves memory performance in elderly subjects. *Methods Find. Exp. Clin. Pharmacol*. **1997**, *19*, 201-10.
480. Spiers PA, Myers D, Hochanadel GS, Lieberman HR, Wurtman RJ. Citicoline improves verbal memory in aging. *Arch. Neurol*. **1996**, *53*, 441-8.
481. Cotroneo AM, Castagna A, Putignano S, Lacava R, Fantò F,et al. Effectiveness and safety of citicoline in mild vascular cognitive impairment: the IDEALE study. *Clin. Interv. Aging* **2013**, *8*, 131-7.
482. Grieb P. Citicoline: A food that may improve memory. *Med. Sci. Rev*. **2015**, *2*, 67-72.
483. Rioux J. A complex, nonlinear dynamic systems perspective on Ayurveda and Ayurvedic research. *J. Altern. Complement. Med*. **2012**, *18*, 709-718.
484. Wang X, Zhang A, Sun H, Han Y, Yan G. Discovery and development of innovative drug from traditional medicine by integrated chinmedomics strategies in the post-genomic era. *Trend. Anal. Chem*. **2016**, *76*, 86-94.
485. Koithan M. A complex systems science perspective for whole systems of complementary and alternative medicine research. *Forsch. Komplementarmed*. **2012**, *19*, 7-14.
486. Rioux J, Thomson C, Howerter A. A pilot feasibility study of whole-systems Ayurvedic medicine and yoga therapy for weight loss. *Global Adv. Health Med*. **2014**, *3*, 28-35.
487. Braun TD, Park CL, Gorin AA, Garivaltis H, Noggle JJ, Conboy LA. Group-based Yogic weight loss with Ayurveda-inspired components: a pilot investigation of female Yoga practitioners and novices. *Intl. J. Yoga Ther*. **2016**, *26*, 55-72.
488. Rioux J. Yoga Therapy Research: A whole-systems perspective on comparative effectiveness and patient-centered outcomes. *Intl. J. Yoga Ther*. **2015**, *25*, 9-19.
489. Capra F. *The Web of Life*. Anchor Books. New York, **1997**.
490. Burger KS, Shearrer GE, Sanders AJ. Brain-based etiology of weight regulation. *Curr. Diab. Rep*. **2015**, *15*, 1-9.
491. Parrish MR, Martin JN, Ellis B, Parrish SA, Owen MY, May WL. Randomized, placebo controlled, double blind trial evaluating early

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

pregnancy phytonutrient supplementation in the prevention of preeclampsia. *J. Perinatol*. **2013**, *33*, 593-599.
492. Gomm W, von Holt K, Thomé F, Broich K, Maier W et al. Association of proton pump inhibitors with risk of dementia. A pharmacoepidemiological claims data analysis. *JAMA Neurol*. **2016**, *73*, 410-416.
493. Snowden D. *Aging with Grace: What the Nun Study Teaches Us about Leading Longer,Healthier and More Meaningful Lives*, Bantam Books, New York, **2001**.
494. Collins N, Belkaid Y. Do the microbiota influence vaccines and protective immunity to pathogens? Engaging our endogenous adjuvants. *Cold Spring Harbor Persp. Biol*. **2017**, Article a028860.
495. Salk HM, Simon WL, Lambert ND, Kennedy RD, Grill DE et al. Taxa of the nasal microbiome are associated with influenza-specific IgA response to live attenuated influenza vaccine. *PLoS One*, **2016**, *11 (9)*, Article e0162803.
496. Pulendran B. Systems vaccinology: Probing humanity's diverse immune systems with vaccines. *Proc. Natl. Acad. Sci*. **2014**, *111*, 12300-12306.
497. Nakaya HI, Bruna-Romero O. Is the gut microbiome key to modulating vaccine efficacy? *Expert Rev. Vaccines* **2015**, *14 (6)*, 777–779.
498. Valdez Y, Brown EM, Finlay BB. Influence of the microbiota on vaccine effectiveness. *Trend. Immunol*. **2014**, *35*, 526-537.
499. Ferreira RBR, Antunes LCM, Finlay BB. Should the human microbiome be considered when developing vaccines? *PLoS Pathogen*. **2010**, *6 (11)*, Article e1001190.
500. Harris VC, Armah G, Fuentes S, Korpela KE, Parashar U et al. Significant correlation between the infant gut microbiome and rotavirus vaccine response in rural Ghana. *J. Infect. Dis*. **2017**, *215*, 34-41.
501. Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. *Nature Rev. Cancer* **2016**, *16*, 131-144.
502. De Bueckelaer A, Grooten J, de Koker S. Type I interferons modulate CD8+ T cell immunity to mRNA vaccines. *Trend. Mol. Med*. **2017**, *23*, 216-226.
503. Veglia F, Gabrilovich DI. Dendritic cells in cancer: the role revisited. *Curr. Opin. Immunol*. **2017**, *45*, 43-51.
504. Schlom J. Therapeutic cancer vaccines: Current status and moving forward. *J. Natl. Cancer Inst*. **2012**, *104*, 599–613.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

505. Melief CJM, van Hall T, Arens R, Ossendorp F, van der Burg S. Therapeutic cancer vaccines. *J. Clin. Invest*. **2015**, *125*, 3401-3412.
506. Radic M. Armed and accurate: engineering cytotoxic T cells for eradication of leukemia. *BMC Biotechnol*. **2012**, *12 (6)*, 1-4.
507. Rodgers KR, Chou RC. Therapeutic monoclonal antibodies and derivatives: Historical perspectives and future directions. *Biotechnol. Adv*. **2016**, *34*, 1149-1158.
508. Russell MW. Thinking globally, acting locally: Harnessing the immune system to deal with recalcitrant pathogens. *mBio* **2015**, *6 (3)*, Article e00382-15.
509. Wayteck L, Breckpot K, Demeester J, de Smedt S.C., Raemdon K. A personalized view oncancer immunotherapy. *Cancer Lett*. **2013**, *352*, 113-125.
510. Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C et al. Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nature Med*. **2013**, *19*, 747–752.
511. U.S. FDA. Tisagenlecleucel, FDA Briefing Document, **2017**. https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/OncologicDrugsAdvisoryCommittee/UCM566166.pdf
512. Hoos A. Development of immuno-oncology drugs - from CTLA4 to PD1 to the next generations. *Nature Rev. Drug Disc*. **2016**, *16*, 235-247.
513. Baum AS. Immunotherapy - the beginning of the end for cancer. Citibank, **2013** https://www.citivelocity.com/citigps/OpArticleDetail.action?recordId=209
514. Esenten JH, Bluestone JA, Lim WA. Engineering therapeutic T cells: from synthetic biology to clinical trials. *Annu. Rev. Pathol. Mech. Dis*. **2017**, *12*, 305-330.
515. Depil S, Deconinck E, Milpied N, Sutton L, Witz F, et al. Donor lymphocyte infusion to treat relapse after allogeneic bone marrow transplantation for myelodysplastic syndrome. *Bone Marrow Transplant*. **2004**, *33*, 531–534.
516. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, et al. Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. **1988**, *N. Engl. J. Med. 319*, 1676–1680.
517. Feldman SA, Assadipour Y, Kriley I, Goff SL, Rosenberg SA. Adoptive cell therapy—tumor infiltrating lymphocytes, T-cell

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

receptors, and chimeric antigen receptors. *Semin. Oncol.* **2015**, *42*, 626–39.
518. Robbins PF, Kassim SH, Tran TL, Crystal JS, Morgan RA, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin. Cancer Res*. **2015**, *21*, 1019–1127.
519. Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat.Med*. **2015**, *21*, 914–921.
520. Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood* **2015**, *125*, 4017–4023.
521. Garfall AL, Maus MV, Hwang WT, Lacey SF, Mahnke YD, et al. Chimeric antigen receptor T cells against CD19 for multiple myeloma. *N. Engl. J. Med*. **2015**, *373*, 1040–47.
522. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* **2015**, *385*, 517–528.
523. Ramsey BW, Nepom GT, Lonial S. Academic, foundation, and industry collaboration in new therapies. **2017**, *376*, 1762-1769.
524. Smanski MJ, Zhou H, Claesen J, Shen B, Fischbach M, Voigt CA. Synthetic biology to access and expand nature's chemical diversity. *Nature Rev. Microbiol*. **2016**, *14*, 135-149.
525. Garcia-Ojalvo J, Khalil AS, McCarthy J. Biological insights from synthetic biology. *Integr. Biol*. **2016**, *8*, 380-382.
526. Ollé-Villa A, Duran-Nebreda S, Conde-Pueyo N, Montañez R, Solé R. A morphospace for synthetic organs and organoids: the possible and the actual. *Integr. Biol*. **2016**, *8*, 495-503.
527. Porcar M, Peretó J. Nature *versus* design: synthetic biology or how to build a non-machine. *Integr. Biol*. **2016**, *8*, 451-455.
528. Mezrich B. *Woolly: The True Story of the Quest to Revive One of History's Most Iconic Extinct Creatures*. Atria Books, New York, **2017**.
529. Regalato A. *Engineering the Perfect Baby*, MIT Technology Review, Cambridge, MA, **2015**. https://www.technologyreview.com/s/535661/engineering-the-perfect-baby/
530. Ledford H. CRISPR fixes embryo error. *Nature*, **2017**, *548*, 13-14.

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

531. Ma H, Marti-Gutierrez N, Park S-W, Wu J, Lee Y et al. Correction of a pathogenic gene mutation in human embryos. *Nature*, **2017**, *548*, 413-419.
532. Lanphier A, Haecker SE, Werner M, Smolenski J. Don't edit the human germ line. *Nature*, **2015**, *519*, 410-411.
533. Pardee K, Grre AA Rapid. Low cost detection of Zika virus using programmable biomolecular components. *Cell* **2016**, *165*, 1255-1266.
534. Goren M, Yosef I, Qimron U. Sensitizing pathogens to antibiotics using the CRISPR-Cas system. *Drug Resist. Updates* **2017**, *30*, 1-6.
535. Fellman C, Gowen BG, Lin P-C, Doudna JA, Corn JE. Cornerstones of CRISPR-Cas in drug discovery and therapy. *Nature Rev. Drug Disc*. **2017**, *16*, 89-100.
536. Zhang R, Miner JJ, Gorman MJ, Rausch K, Ramage H et al. A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature* **2016**, *535*, 164-168.
537. Harris AF, McKemey AR, Nimmo D, Curtis C, Black A et al. Successful suppression of a field mosquito population by sustained release of engineered male. *Nature Biotechnol*. **2012**, *30*, 828-830.
538. Alphey L. Can CRISPR-Cas9 gene drives curb malaria? *Nature Biotechnol*. **2016**, *34*, 149-150.
539. Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C et al. A CRISPR-Cas9 Gene Drive System Targeting Female Reproduction in the Malaria Mosquito vector *Anopheles gambiae*. *Nature Biotechnol*. **2016**, *34*, 78-83.
540. Zetsche B, Zhang F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **2015**, *163*, 759-771.
541. GE News. New CRISPR System Targets Both DNA and RNA, April 25, **2016**. http://www.genengnews.com/gen-news-highlights/new-crispr-system-targets-both-dna-and-rna/81252652/
542. Hamburg MA, Paving the Way for Personalized Medicine: FDA's Role in a New Era of Medical Product Development, US FDA, Bethesda, MD, **2013**.
543. Gao B, Yang Q, Zhao X, Jin G, May Y, Xu F. 4D bioprinting for biomedical applications. *Trend. Biotechnol*. **2016**, *34*, 746-756.
544. Momeni F, Liu X, Ni J. A review of 4D printing. *Mater. Des*. **2017**, *122*, 42-79.
545. Zhang B, Montgomery M, Chamberlain MD, Ogawa S, Korolj A et al. Biodegradable scaffold with built-in vasculature for organ-on-a-

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chip engineering and direct surgical anastomosis. *Nat. Mat*. **2016**, *15*, 669-678.
546. Stoppel WL, Ghezzi CE, McNamara SL, Black III LD, Kaplan DL. Clinical applications of naturally derived biopolymer-based scaffolds for regenerative medicine. *Ann. Biomed. Eng*. **2015**, *43*, 657-680.
547. Mao AS, Mooney DJ. Regenerative medicine: Current therapies and future directions. *Proc. Natl. Acad. Sci*. **2015**, *112*, 14452-14459.
548. Teuschl A, Heimel P, Nürnberger S, van Griensven M. Reidl H et al. A novel silk fiber–based scaffold for regeneration of the anterior cruciate ligament histological results from a study in sheep. *Am. J. Sports* **2016**, DOI: 10.1177/0363546516631954.
549. GE News. 4D Printing Now on Life Sciences' Radar. **2016**, *36*, http://www.genengnews.com/gen-articles/4d-printing-now-on-life-sciences-radar/5683/
550. Chae MP, Hunter-Smith DJ, De-Silva I, Tham S, Spychal RT, Rozen WM. Four-dimensional (4D) printing: a new evolution in computed tomography-guided stereolithographic modeling. Principles and application. *J. Reconstr. Microsurg*. **2015**, *31*, 458–463.
551. Kolesky DB, Truby RL, Gladman A, Busbee TA, Homan KA, Lewis JA. 3D Bioprinting of vascularized, hetero-geneous cell-laden tissue constructs. *Adv. Mater*. **2014**, *26*, 3124–3130.
552. Villar G, Heron AJ, Bayley H. Formation of droplet networks that function in aqueous environments. *Nat. Nanotechnol*. **2011**, *6*, 803–808.
553. El-Khatib FH, Balliro C, Hillard MA, Magyar KL, Ekhlaspour L et al. Home use of a bihormonal bionic pancreas versus insulin pump therapy in adults with type 1 diabetes: a multicenter randomized crossover trial. *Lancet* **2016**, *389*, 369-380.
554. Pina S, Oliveira JM, Reis RL. Natural-based nanocomposites for bone tissue engineering and regenerative medicine: A review. *Adv. Mat*. **2015**, *27*, 1143-1169.

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APPENDIX

PRIMER ON PHYSICAL AND ANALYTICAL CHEMISTRY

A1.1 Introduction

Analytical chemistry is the branch of chemistry that analyzes matter to determine what and/or how much of different substances (analytes) are in it. Matter is anything that has mass and takes up space. It can contain carbon, hydrogen, oxygen, sulfur and phosphorus (CHONPS), which are the primary elements in living beings. However, elemental H, O and N seldom occur in nature. Instead, they exist as diatomic gases, H_2, O_2 and N_2 and as parts of molecules, such as H_2O and NO. In molecules, two or more atoms are bonded together so whole new properties emerge. Molecules are the smallest fundamental unit of a chemical compound. Note that the bonds between the H-H, O-O, N-N, H-O-H and N-O are covalent bonds that are not easily broken. The H, O and N in the diatomic gases share their electrons equally, so H_2, O_2 and N_2 are nonpolar. However, the H, O and N in H-O-H and N-O are not shared equally. O and N are more electronegative than H. That is, O and N have a stronger tendency to attract the covalently bonded electrons than H. So, there is a partial positive charge on the H and a partial negative charge on the O in water. There is a partial positive charge on the N in nitric oxide (NO) and a partial negative charge on the O. As a result, water molecules also bind to each other through noncovalent hydrogen bonds that form between the H and O, as shown in Figure 1. In addition, there are hydrogen bonds between adenosine and thymine, as well as guanine and cytosine. Figure 2 shows the hydrogen bonds between adenine and thymine.

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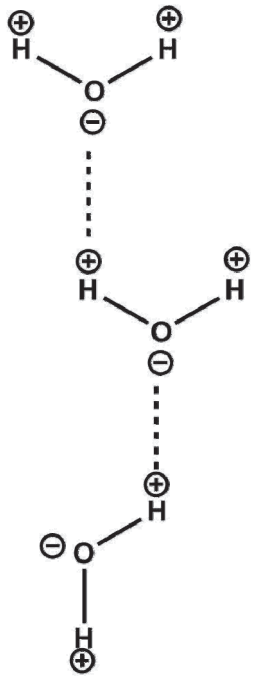

Figure 1. Illustration of hydrogen bonds (dashed lines) between water molecules, where the (+) and (-) represent partial positive and negative charges, which are often written as δ^+ and δ^-.

Similar hydrogen bonds are formed between hydrogens and oxygens as well as hydrogens and nitrogens in the bases in DNA. The hydrogen bonds between adenine and thymine are shown in Figure 2.

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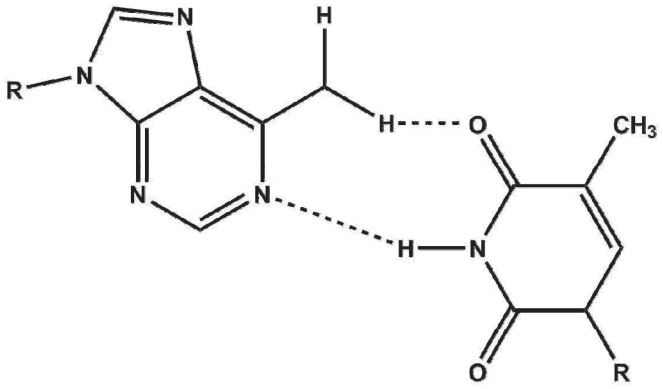

Figure 2. Illustration of hydrogen bonds (dashed lines) between adenine and thymine. The R represents the rest of the DNA structure.

H, O and N also exist as ions or parts of polyatomic ions, such as H^+, OH^- and NO_3^-. However, ions don't exist in isolation. Analysts can't buy primary standards made of just H^+, OH^- or NO_3^-. Instead, they can buy acids, like HCl (hydrochloric acid), HNO_3 (nitric acid), sulfuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) and bases like NaOH and KOH. HCl, HNO_3, H_2SO_4 and H_3PO_4 are strong acids. When they are dissolved in water, they dissociate to form a hydrogen ion, H^+, which is simply a proton, plus an anion (a negatively charged ion). The H^+ adds to water to form the hydronium ion, H_3O^+, as shown in reactions 1-5. The anions Cl^-, NO_3^-, HSO_4^- and $H_2PO_4^-$ are also attracted to water, which forms a noncovalent bond with them. So, when a mixture of anions in a sample are separated by ion exchange, the water that is bound to the anions must dissociate (be removed) from them before they can bind to the column. At the same time, the water that is attached to the quaternary ammonium anion exchange sites on the anion exchange column (R_4N^+) must also be removed. This takes energy, but is favored by the stronger energy of noncovalent binding between the positively charged quaternary ammonium and the negatively charged anions.

Reaction 1: $HCl + H_2O \rightarrow H_3O^+ + Cl^-$
Reaction 2: $HNO_3 + H_2O \rightarrow H_3O^+ + NO_3^-$
Reaction 3: $H_2SO_4 + H_2O \rightarrow H_3O^+ + HSO_4^-$
Reaction 4: $HSO_4^- + H_2O \rightarrow H_3O^+ + SO_4^{2-}$
Reaction 5: $H_3PO_4 + H_2O \rightarrow H_3O^+ + H_2PO_4^-$
Reaction 6: $H_2PO_4^- + H_2O \rightleftharpoons H_3O^+ + HPO_4^{2-}$

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Note that reactions 1-5 have a single arrow (→) in them. That means that if they are dissolved in water, the reaction is one way – towards the formation of H_3O^+ and an anion (called a conjugate base). So, HCl, HNO_3, H_2SO_4, HSO_4^- and H_3PO_4 are called strong acids. They dissociate completely. However, it is much more common to write them without the water or hydronium ions, as shown in reactions 7-12. In contrast, reactions 6 and 12 have arrows going both directions because they are equilibria. That means that not only $H_2PO_4^-$ and H_2O can react to produce H_3O^+ and HPO_4^{2-}, H_3O^+ and HPO_4^{2-} can react to produce $H_2PO_4^-$ and H_2O.

Reaction 7: $HCl \rightarrow H^+ + Cl^-$
Reaction 8: $HNO_3 \rightarrow H^+ + NO_3$
Reaction 9: $H_2SO_4 \rightarrow H^+ + HSO_4^-$
Reaction 10: $HSO_4^- \rightarrow H^+ + SO_4^{2-}$
Reaction 11: $H_3PO_4 \rightarrow H^+ + H_2PO_4^-$
Reaction 12: $H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$

Note that $H_2PO_4^-$ is a weak acid, so only some of the $H_2PO_4^-$ dissociates into H^+ and HPO_4^{2-}. Other examples of weak acids include hydrofluoric acid (HF), carbonic acid (H_2CO_3), acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$) and fatty acids, such as palmitic acid, $CH_3(CH_2)_{14}COOH$. However, to an industrial hygienist, HF is not weak – it is the most dangerous of all the acids that are used in industry. If a worker is exposed to HF when he or she is not wearing protective equipment, the HF will deaden the nerve cells on the skin and pass through all the way to the bones. The worker will not feel any pain (like he or she would if some HCl were to splash onto their skin). He or she will not even realize the danger as the HF penetrates all the way to the bones and does severe damage. Also, HF fumes are quite deadly because they severely damage the lungs. Still, the H in HF, H_2CO_3 and -COOH groups doesn't dissociate completely when these weak acids are dissolved in water. So, their dissociation reactions are shown as an equilibrium, with arrows pointed in each direction (⇌). Some of them are shown in reactions 13-15.

Reaction 13: $HF \rightleftharpoons H^+ + F^-$
Reaction 14: $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$
Reaction 15: $CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$

Note that the COO^- is called the carboxylate anion, while the undissociated COOH is called a carboxylic acid. The relative

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concentrations of acid (HF, H_2CO_3 and CH_3COOH) and H^+ can be calculated from the acid dissociation constants, abbreviated as K_a. As in other equilibrium constants, they are calculated by the ratio of products divided by reactants, as shown in equations 1-3.

Equation 1: K_a for HF = $\frac{[H^+][F^-]}{[HF]} = 7.2\text{x}10^{-4}$, $pK_a = 3.14$

Equation 2: K_a for H_2CO_3 = $\frac{[H^+][HCO_3^-]}{[H_2CO_3]} = 4.3\text{x}10^{-7}$, $pK_a = 6.37$

Equation 3: K_a for CH_3COOH = $\frac{[H^+][CH_3COO^-]}{[CH_3COO^-]} = 1.76\text{x}10^{-5}$, $pK_a = 4.75$

Note that brackets represent concentrations and pK_a is the negative log of K_a, just like pH is the negative log of the hydrogen ion concentration, $[H^+]$. That is, water also dissociates, in what is called autodissociation, as shown in equation 4:

Equation 4: $H_2O \rightleftharpoons H^+ + OH^-$ $K_w = 10^{-14}$, $pK_w = 14$

Since pure water is neutral (neither an acid nor a base) (both H^+ and OH^- are produced), this is not an acid dissociation. So, the autodissociation constant for water is abbreviated as K_w and its negative log is pK_w. That is, in pure water the concentrations of H^+ and OH^- are both 10^{-7} M (moles/L). Note that compounds that have an ionizable -OH group in them, like sodium hydroxide (NaOH), are called bases. When they are dissolved in water, they raise the pH, to form an alkaline solution. Since pH is the negative log of the concentration of H^+, the pH of pure, unbuffered water is 7. However, water in living cells and in the blood stream has CO_2 dissolved in it. There is another equilibrium, shown in reaction 16.

Reaction 16: $CO_2 + H_2O \rightleftharpoons H_2CO_3$

The H_2CO_3 (carbonic acid) is a weak acid, as shown in reaction 17.

Reaction 17: $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, $K_a = 4.3\text{x}10^{-7}$, $pK_a = 6.37$

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So, the mixture of H_2CO_3 and HCO_3^- acts as part of the essential buffering system in the blood and in the cytoplasm of cells. The other key component of the buffering system is the mixture of $H_2PO_4^-$ and HPO_4^{2-}, which are in equilibrium, as shown in reaction 18.

Reaction 18: $H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$, $K_a = 6.23x10^{-6}$, $pK_a = 7.21$

Buffers are resistant to changes in pH. That is, if a small amount of acid or base is added to pure, unbuffered water, it will lower or raise the pH very much. However, if a small amount of acid or base is added to blood, the cytosol of a cell or any other buffered aqueous solution, it will react with either HCO_3^- or HPO_4^{2-} (if it is an acid), or with H_2CO_3 or $H_2PO_4^-$ (if it is a base) and the pH will not change very much.

The reaction between an acid and a base produces a salt plus water. For example, one molecule of the base, NaOH, will react with one molecule of H_3PO_4 to produce one molecule each of water and NaH_2PO_4. However, one molecule of NaOH weighs less than one molecule of H_3PO_4. So, $6x10^{23}$ molecules (1 mol) of NaOH weigh less than $6x10^{23}$ molecules (1 mol) of H_3PO_4. Since the atomic weights of Na, O, H and P are 23, 16, 1 and 32 amu, respectively, the gram molecular weights of NaOH and H_3PO_4 are 40 g/mol and 120 g/mol, respectively.

Chemists want to predict not only how many grams or moles of a reactant will be consumed and how much product will be produced. They also want to predict if a reaction will produce heat (exothermic) or if it absorbs heat from the environment (endothermic). This is the realm of thermodynamics. It is the study of the relations between heat and other forms of energy. It teaches us that there is a relationship between the equilibrium constant and Gibbs free energy (G), which is the enthalpy of the system (H) minus the product of the temperature times the entropy (S) of the system, as shown by equation 5. Entropy is defined mathematically as the product of the Boltzmann constant times the number of available quantum states. The Boltzmann constant (k) relates the kinetic energy of particles in a gas to the temperature of the gas, the gas constant, R, and Avogadro's number, N, as shown in equation 6.

Equation 5: $G = H - (T)*(S)$

Equation 6: $k = \frac{R}{N}$

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The gas constant, R, is equivalent to the Boltzmann constant (which is $1.38x10^{-23}$ J·°K), but it has units of energy per temperature increment. The value of R is 8.314 kg m^2 s^{-2} $^oK^{-1}$, where ^oK is the temperature in degrees Kelvin. This is a much better unit for temperature because something that is at 2 ^oK is twice as hot (twice the thermal energy) as when it is at 1 ^oK, just as 200 ^oK is twice as hot as 100 ^oK. In contrast, a substance that has a temperature of 2 ^oC (275 ^oK) is not anywhere near twice as hot as a substance that has a temperature of 1 ^oC (274 ^oK).

Enthalpy is the total heat content of the system, where entropy is the unavailability of a system's thermal energy for conversion into mechanical work, often interpreted as the degree of disorder or randomness in the system. If the Gibbs free energy is negative, the science of thermodynamics tells us that the reaction is spontaneous. That doesn't mean that the reaction will happen fast. It just means that the reaction will occur, given enough time or if either energy or a catalyst is added. So, the Gibbs free energy of the reaction between H_2 and O_2 to produce water is negative. However, if you were to put hydrogen and oxygen gas in a sealed bottle, it could take over a million years for them to react completely. That is, the reaction has a large activation energy that must be overcome. It acts as a barrier to slow down the reaction. On the other hand, if you were to add a spark of fire to the mixture, it will react explosively. Similarly, there are many biochemical reactions that would take millions of years if there were no catalyst present. So, living systems have enzymes and ribozymes that catalyze these reactions. They lower the activation energy of the reaction in both directions. For example, esterase enzymes in the blood and liver will catalyze the hydrolysis of esters, such as UVR6105, which was described in Chapter 3. The generalized reaction is shown in reaction 19.

Reaction 19: $R_1COOR_2 + H_2O \rightleftharpoons R_1COOH + R_2OH$

Esterases, like all enzymes, can catalyze the reaction in either direction. When there is plenty of water available, esterases catalyze the hydrolysis reaction. However, esterases can also be used in some nonaqueous solvents to catalyze the reverse reaction between a carboxylic acid (R_1COOH) and an alcohol (R_2OH) to form an ester plus water. This property of enzymes is a basic principal of physical biochemistry.

When organizations make their NMEs and prescription drugs, it's important for them to report percent yields at every step of the synthesis. For example, aspirin (acetylsalicylic acid) is synthesized in a reaction between salicylic acid and acetic anhydride. To get a 100% yield, excess

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acetic anhydride is used. Salicylic acid is the limiting reagent. The excess acetic anhydride is removed before the purified aspirin can be sold. However, not all organic reactions produce a single product. Some produce a mixture of isomers. If the NME or prescription drug is just one of several products, the percent yield will be less than 100%. For example, aromatic organic compounds that have substituted phenyl groups that can undergo reactions in which more than one compound is produced. For example, the natural product, apocynin, can react with itself to produce a dehydro dimer. That is, one hydrogen is removed from each of the apocynin molecules (dehydro), before diapocynin (the dimer) is produced. Apocynin has a methoxy (-OCH_3) and a hydroxyl (-OH) group. Hydroxyl is an *ortho* director and methoxy is a *para* director. That is, any group that adds to the apocynin (like a dehydroapocynin) has a strong tendency to add to the position that is *ortho* to the hydroxyl and *para* to the methoxy. However, a small percentage of the dehydro apocynin does add to the other positions, so the yield is not 100%. The major reaction is shown in Figure 3.

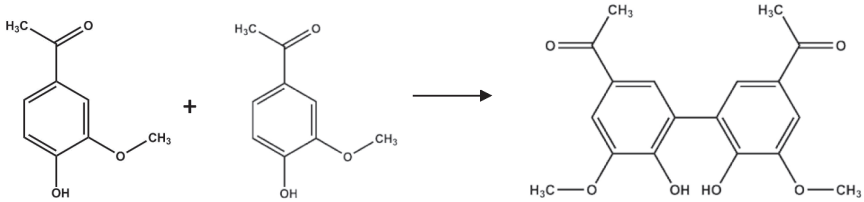

Figure 3. Reaction that produces diapocynin from apocynin.

The molecular weights of apocynin and diapocynin are 166 and 330 g/mol, respectively. It takes two moles of apocynin to make one mole of diapocynin. So, if one starts with 16.6 g of apocynin and obtains 30.0 g of diapocynin, the yield would be 90.9% (since 33.0 g would be needed for a 100% yield).

In reactions that have more than one reactant or product, it's important to know the ratios in which they will react. Stoichiometry is the study of such ratios. That is, chemicals react in exact proportions. For example, two molecules of H_2 will react with exactly one molecule of O_2 to make water, as shown in reaction 20.

Reaction 20: $2H_2 + O_2 \rightarrow 2H_2O$

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When writing chemical reactions like this, the coefficient in front of each molecular formula indicates how many molecules are needed. If there is no coefficient (as in O_2 above), it is understood that only one molecule is needed. To calculate how many grams of water will be produced in a reaction using any given amount of reagent, the gram molecular weights are needed. The gram molecular weights of H_2, O_2 and H_2O are 2, 32 and 18 g/mol, respectively. That is, there are 4 g of H_2 in two moles, or $12.04x10^{23}$ molecules of H_2, 32 g of O_2 in one mole ($6.02x10^{23}$ molecules) and 36 g of H_2O in two moles of H_2O. So, if one mixes 40 g of H_2 with 330 g of O_2, the O_2 will be in excess and H_2 will be the limiting reagent. The theoretical yield of water will be 360 g.

The tools of analytical chemistry are needed to determine percent yields of products and to quantify the biochemical and pollutants that are present in samples. Methods used in analytical chemistry vary from simply weighing materials (gravimetric methods) to measuring pH to doing elaborate sample preparation and instrumental analysis. The simple pH meter with its electrode is perhaps the most elegant and sensitive tool available to analysts. It can measure H^+ concentrations as low as 10^{-14} moles per liter (10^{-14} M). The atomic mass of the H^+ is 1 g/mol. So, the pH meter can detect as little as 10^{-14} g/L of H^+.

Analysis is the key to cGMP and many types of –omics studies. However, analytical chemistry depends on both reductionist and systems thinking. Reductionist thinking is used when an analyst separates each analyte from a mixture and then quantifies them one at a time. Systems thinking is used to realize that it's impossible to know exactly how much of each analyte is in a sample. Even though analytical methods are validated by measurements of spike-recovery, there is always some uncertainty. The analyst carefully weighs a certified standard, dilutes it into an appropriate solvent and adds a portion of it to the sample. This is called spiking the sample. Then, the spiked sample is extracted with a suitable solvent and the analyst quantifies it using the method that he or she is trying to validate. However, no standard or solvent is 100% pure. Moreover, when a standard is spiked into a solid sample or a suspension of water and oil (or fat), it often stays on the surface of the sample. As a result, it can be easily extracted – even though analytes that are buried inside the sample may not be extracted. So, if the analyte is spiked at a much higher level than the amount of the analyte that was present in the original, unspiked sample, the analyst will mistakenly think that the method is capable of obtaining nearly 100% recovery. If most of the analytes are actually buried deep inside the sample, they might not be extracted completely. For example, when analyzing fatty foods for

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lipophilic pesticides, the analyst can probably get good recovery of a standard that is spiked on the surface of the sample by using any solvent that they want to use for the extraction. Instead, it's often better to extract lipophilic compounds with a solvent like hexane that has low polarity and maybe even use sonication or heat to extract the lipophilic analytes completely.

This leads to the first rule of analytical chemistry – the analysis is only as good as the sample preparation. It also reinforces the idea that the analyst can never know exactly how much of each analyte is in the sample. That is, few (if any) foods or physiological samples (like blood) are homogeneous. So, the FDA and other governments' regulatory agencies grind up all solid food samples as much as they can to try to make the sample as nearly homogeneous as possible. For many analytes, this is sufficient. However, for toxins that are produced by a fungus (mycotoxins), there can be hot spots, in which there is a much higher concentration of the toxin in some very small parts of the sample. So, the analyst will need to analyze the ground-up sample at least three times to see if the results on that sample are reproducible. However, the food sample is almost always taken from a much larger container (like a railroad car or large truck) that has not been ground up. In that case, the best that the analyst can do is to report the results that he or she finds and focus on customer service, in the spirit of TQM. That is, the taxpaying public and the government want analysts to analyze as many samples as possible as fast as possible. They don't want the analyst to make his or her work an academic exercise, in which he or she spends as much time as possible to determine the concentration of the toxic analytes as accurately as possible in just a few samples. They don't care if a sample with 10 μg/g of a toxic analyte can be analyzed within an accuracy of ±10% if anything above 1 μg/g is violative and hazardous to one's health.

Note that the EPA and FDA as well as many other governments' regulatory agencies express the concentrations of pollutants or adulterants in food and environmental samples in units of ppm (μg/g or mg/kg for solids and μg/mL or mg/L for aqueous solutions), ppb (ng/g or μg/kg for solids and ng/mL or μg/L for aqueous solutions) or ppt (pg/g or ng/kg for solids and pg/mL or ng/L for aqueous solutions). However, biochemists and medicinal chemists usually prefer to express concentrations based on the number of molecules, or moles (where one mole = 6.02×10^{23} molecules) per L (liters) or mL. The units can be M (for moles/L), mM (for mmoles/L), μM (for μmoles/L) or nM (for nmoles/L). Note that many people use the word mol instead of moles, or mols/L instead of moles/L.

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This brings us to another important point that many biochemists and medicinal chemists overlook. There is a huge difference between concentration and amount, or level. The best example of this is that there is far more gold in the world's oceans than there is in any gold mine or any nation's gold reserves. One might say that the level of gold is very high in the oceans. However, the concentration of gold is extremely low. Throughout this book, I've cited many research articles that stated that the level of an enzyme or some other analyte has increased or decreased due to a disease or the administration of a drug. When reading the experimental section of the original article, it is clear that the authors were actually measuring the concentration. So, I changed the word level to concentration. In addition, when a lab analyzes a patient's blood for sodium, potassium, cholesterol and other substances, they report the results as concentrations, such as mg or μg per dL (deciliter). To determine the total amount of the analyte in the patient's blood, they would have to know the total blood volume, but this is not done. The total volume of blood in the body can change depending on the degree to which the patient is hydrated. For example, a patient suffering from chronic kidney disease and anemia might have blood concentrations of creatinine and hemoglobin of 3.9 mg/dL and 9.3 g/dL, respectively. His or her physician might rush the patient to the hospital for treatment. Once sufficient fluids are administered intravenously, the creatinine and hemoglobin concentrations could drop significantly. This probably doesn't mean that the patient's kidneys have improved or that the anemia has worsened. The total amount of each is probably about the same as before, but the additional water has diluted them. Similarly, in the Lineweaver-Burke plots of enzyme activity vs concentration of the substrate that were shown in Chapter 3, the enzyme concentration (not level) is kept constant. In fact, it is the concentration of an enzyme that determines how fast it catalyzes a reaction in a cell – not the amount or level.

To determine concentration and to fulfill legal requirements that a manufacturer of a drug or dietary supplement have methods to verify the identity of their product, there are several analytical methods that can be used. Two of the easiest and most cost effective – especially for dietary supplements, are ultraviolet-visible (UV-Vis) spectrophotometry and fluorescence spectroscopy. However, UV-Vis spectrophotometry is not very specific. That is, many compounds have very similar UV-Vis spectra. Fluorescence is more specific, but most compounds don't fluoresce. So, most manufacturers of prescription drugs invest a few hundred thousand dollars in more informative analytical methods to protect their billion dollar investments in all the initial drug development and subsequent

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clinical trials that are needed. Almost all these methods use one form or another of spectroscopy, which is the study of the interaction between electromagnetic energy and matter. Electromagnetic energy travels as a wave, with a characteristic wavelength (λ) and frequency (ν) that are related by equation 7.

Equation 7: $E = h\nu = \frac{hc}{\lambda}$

Where E is the energy, h is Planck's constant (6.626176×10^{-34} joule-s, where s is seconds and a joule is $kg \cdot m^2 \cdot s^{-2}$) and c is the speed of light (2.998×10^8 $m \cdot s^{-1}$). So, the higher the frequency, the higher the energy and the lower the wavelength. That is, highly energetic gamma rays have much higher frequencies $>10^{19}$ Hz (cycles per second) and shorter wavelengths <1 nm (or $<1 \times 10^{-9}$ m) than visible light. In contrast, visible light has a wavelength between about 390-700 nm, while UV light has a wavelength between about 100-390 nm. UV light is also subdivided into UVA (315-390 nm), UVB (280-315 nm) and UVC (100-280 nm). Infrared (IR) electromagnetic radiation has a wavelength between about 800 nm to 1 mm, or 0.8-1000 μm, often abbreviated as just μ. By convention, UV and visible light are expressed in units of wavelength (as nm), while IR light can be expressed as either wavelength (as μ) or frequency. However, as in much of science, chemists who measure IR spectra also use their own, unique units for frequency (cycles per cm), cm^{-1}, which is also called wave numbers. IR light is often classified as near-IR (1400-4000 cm^{-1}), mid-IR (4000-400 cm^{-1}) and far-IR (400-10 cm^{-1}). The corresponding wavelengths are 0.8-2.5 μm (near-IR), 2.5-25 μm (mid-IR) and 25-1000 μm (far-IR). On the other hand, radio waves have wavelengths between 1 mm and 100 km (frequencies from 300 GHz to 3 kHz, or 300×10^9 to 3×10^3 Hz).

Spectrophotometry is a subset of spectroscopy. It is a method by which one quantifies a substance by measuring how much light is absorbed or how much is transmitted through the substance. Mass spectrometry is the method by which one measures the masses of analytes once they are converted into ions (such as $[M-H]^+$ ions in Table 4 in Chapter 3). When a compound is almost 100% pure, it can be characterized by its spectra. Even if the compound doesn't absorb UV or visible light, it will interact other forms of electromagnetic radiation. That is, UV and visible light are just small portions of the electromagnetic spectrum. Organic compounds absorb IR light and when placed in a strong magnetic field, they absorb radio frequencies.

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However, a mixture of organic compounds (or biochemicals) in physiological samples produce spectra that are a sum of all the spectra that would be produced by each isolated compound. So, to quantify one or more of the compounds, they must first be separated from each other. Even though this idea of separating individual components of a mixture is a form of reductionist thinking, it is essential for proper analysis. The area of science that is devoted to separating individual components is called chromatography. If the samples being analyzed are gases (like one's breath), gas chromatography (or GC) is used. If the samples being analyzed are liquids (like blood, urine, fruit juices or beverages), liquid chromatography (or LC) is usually used. Liquids that are volatile and stable when heated (like acetone in diabetic patients suffering from ketoacidosis) can be analyzed by GC. Solids can be dissolved in a suitable solvent and analyzed by either GC (if they are thermally stable) or LC (if they are ionic or not thermally stable). Ions (like arsenate, AsO_4^-, and arsenite, AsO_3^-) can be separated from each other using an ion exchange column. When NMEs or prescription drugs are being analyzed for purity, chromatography is coupled to one of the forms of spectroscopy as in LC-MS and GC-MS.

In the next sections, several forms of spectroscopy are described.

A1.2 Ultraviolet-Visible (UV-Vis) Spectrophotometry and Fluorescence Spectroscopy

Many organic compounds absorb UV-Vis light [1]. The energy excites electrons in the compounds to higher electronic energy levels. By convention, the wavelengths of UV and visible light are expressed in units of nm. The exact wavelength of the light that is absorbed depends on the structure of the compound. Organic compounds, such as benzene, with a few conjugated C=C double bonds, absorb well in the UV region. Organic chemists and biochemists use the term conjugated when others might say alternating. The structure of benzene with conjugated carbon-carbon double bonds is shown in Figure 4. The structure of benzene with all the carbons and hydrogens shown explicitly is in Figure 5. However, both of these structures are over-simplifications. In organic chemistry, it is well-known that the carbon-carbon single bond is longer than a carbon-carbon double bond. So, the structures in Figures 4 and 5 imply that three of the bonds are longer than the other three. Actually, the lengths of the bonds between the carbons in benzene are all the same. They are intermediate between carbon-carbon single bonds and carbon-carbon double bonds. They are stabilized by resonance. So, a more accurate structure of benzene

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shows all the bonds as a circle, as in Figure 6. Note that the benzene ring can be covalently attached to a variety of functional groups, in which case the benzene ring is called a phenyl group.

Figure 4. 2D **s**tructure of benzene, with conjugated, or alternating carbon-carbon double bonds, but with hydrogens not shown.

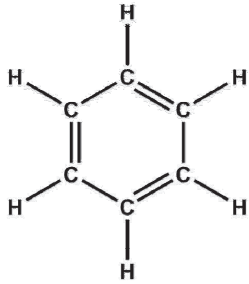

Figure 5. 2D structure of benzene, with conjugated C=C bonds and with all carbons and hydrogens shown explicitly.

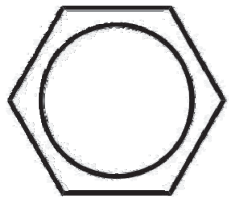

Figure 6. 2D resonance energy structure of benzene, showing all carbon-carbon bonds with equal lengths.

When there are two substituents on a phenyl, they can be adjacent to each other, or either one or two carbons away. For example, there are three aminobenzoic acids, as shown in Figure 7. When they are adjacent to each other, the compound is *ortho*-aminobenzoic acid. Its official IUPAC (International Union of Pure and Appled Chemists) name is 2-aminobenzoic acid, because the amino group is on carbon number two (the

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COOH is attached to carbon number one). When they are two carbons away from each other, it is *meta*-aminobenzoic acid. Its official IUPAC name is 3-aminobenzoic acid, because the amino group is on carbon number three. When they are three carbons away from each other, it is *para*-aminobenzoic acid or 4-aminobenzoic acid. Note that the amino and COOH groups are as far away from each other as they can be. So, organic chemists and biochemists use the prefix *para* just the opposite as everyone else. That is, in the English language, the prefix *para* means close to – not far away. That is, a paralegal is someone who is close to being a lawyer, or almost a lawyer. A paramedic is someone who is close to being a medical doctor (or physician).

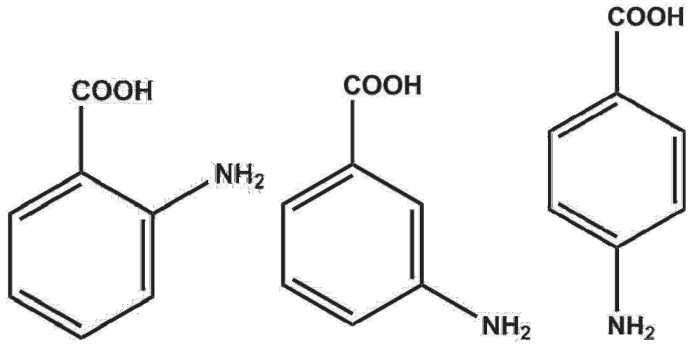

Figure 7. From left to right, structures of *ortho*-, *meta*- and *para*-aminobenzoic acid.

Compounds such as doxorubicin, with many C=C bonds, also absorb in the visible region. Its structure is shown in Figure 8. Note that bonds shown as dashed lines are below the plane of the paper, while bonds shown in dark are above the plane of the paper.

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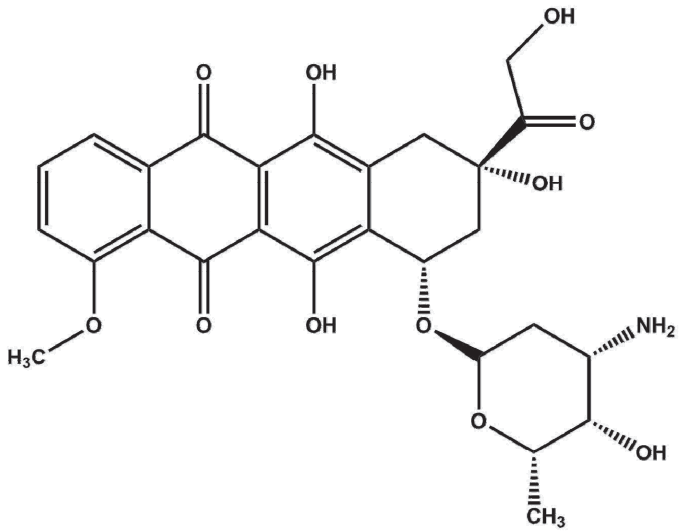

Figure 8. 2D structure of adriamycin, also known as doxorubicin.

UV-Vis spectra are measured with a spectrophotometer [1]. It has two sources of light, a tungsten lamp for the visible region and a deuterium lamp for the UV region. Samples are dissolved in water or some other solvent and placed in a quartz cuvette. It should be noted that O_2 that is dissolved in the solvent will absorb UV light below 205 nm, so a spectrometer must be continuously purged with N_2 to obtain accurate UV data at the lowest wavelengths. The light source is split into two beams. One beam passes through the sample cuvette and the other passes through the cuvette holding the solvent. A photodiode or charge coupled detector (CCD) is placed just past the cuvettes. It is used to detect the amount of light that gets transmitted through the sample cuvette and compares it to the amount that gets through the cuvette holding the solvent only. The percent transmittance, or % T, is automatically converted to absorbance, A, as shown in equation 8 [1].

Equation 8: $A = - \log(\%T)$

UV-Vis spectra contain relatively broad peaks [1]. So, the spectra of many organic compounds with double bonds can look very similar. As a result, other methods are usually needed to verify that a compound is present. Alternatively, the other compounds present in the sample can be

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separated by liquid chromatography (LC) and detected by their UV and/or visible spectra. Once this is done, the amount of each substance in the sample that absorbs light can be determined from the amount of light that is absorbed using Beer's Law, equation 9.

Equation 9: $A = \varepsilon bc$

In this equation ε is the absorptivity of the sample, b is the path length of the light in the spectrophotometer or UV-Vis detector and c is the concentration of the sample. If the concentration is in units of moles/L, or M, and the path length is 1 cm, ε has units of $M^{-1}cm^{-1}$. If a compound contains a phenyl group or other type of conjugated C=C bonds, it will have a molar extinction coefficient, ε, that is at least 10^4 at the wavelength at which maximum absorbance occurs (λ_{max}). Some UV-Vis spectrophotometers have a maximum detectable absorbance of 1, so a compound with $\varepsilon = 10^4$ $M^{-1}cm^{-1}$ must be diluted to a concentration of 10^{-4} M (10^{-1} mM) or less. Thus, ε is sometimes expressed in units of $mM^{-1}cm^{-1}$ or even $\mu M^{-1}cm^{-1}$ (sometimes called the micromolar extinction coefficient) [1].

Some compounds that absorb UV or Vis light will glow in the dark after the light is turned off [1]. This is called fluorescence if light is emitted almost immediately and phosphorescence if light is emitted after a short delay. In either case, the UV or Vis light excites an electron in the sample to a higher energy level. The electron then relaxes to a lower energy level and emits a photon of light at a longer wavelength than the light that was absorbed. The detector is placed at a 90^o angle from the incoming light. Instead of measuring a difference in the light intensity between a reference and a sample, the absolute amount of light being emitted at a specified wavelength (λ_{emit}) when the sample is excited with light at a lower wavelength (λ_{excite}) is measured [1].

As a result, fluorescence is more sensitive than UV-Vis absorbance spectrophotometry [1]. However, it is important that the analyte not be too concentrated when trying to determine its concentration by fluorescence. The absorbance spectrum is measured first. The solution containing the analyte should be diluted until the absorbance at λ_{emit} is <0.10. If it is >0.10, a significant amount of the emitted light will be re-absorbed, giving an artificially low reading. Thus, calibration curves from fluorescence data are usually linear over one order of magnitude (such as 1 – 10 μM). On the other hand, calibration curves from UV-Vis data can be linear over three or more orders of magnitude, such as 0.01 – 10 μM [1].

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Fluorescence is more selective than UV-Vis spectra, since fewer compounds fluoresce [1]. However, the fluorescence and/or UV-Vis absorbance will be the sum of all the compounds in the sample. So, analytes should be separated by LC when doing quantitative analysis. A fluorescence detector will detect the fluorescence of any fluorophore that elutes off the LC column. A UV-Vis a diode detector can measure the absorbance at all wavelengths simultaneously of each chromophore as it comes off the LC column. The analyst observes the spectrum at the beginning of the peak, its apex and at the end of the peak. If the spectrum does not change, it is most likely produced by a single compound. If the spectrum changes, then the peak is due to two or more components that have not been separated completely by the LC column [1].

A1.3 HPLC and UPLC

LC is a general term for the form of chromatography in which the mobile phase is a liquid. The sample is dissolved in a solvent and then is passed through a column that is packed with a substance that binds the analytes or the substance that one is trying to purify [1]. That is, when preparing a significant quantity (as much as hundreds of grams) of an NME or prescription drug, a large glass column (sometimes up to 2 m in length) can be packed manually with silica or alumina that has been pre-soaked with a suitable solvent and deaerated. That is, it is crucial that there be no air bubbles in the packed column. The column will have a large spherical reservoir on top, in which the analyst can put the solvent (also called eluent or mobile phase) that will be used to wash the analytes off (or elute them off) the column using gravity to force it through. This is called preparative-scale LC because the goal is to prepare (or purify) large quantities of the NME, IND or prescription drug. It can also be used to separate and purify compounds (like artemisinin) from a botanical (such as the plant *Artemisia annua*). Alternatively, smaller columns can be used in an automated, preparative-scale LC that can still purify gram quantities of the compound of interest. When the column is packed with silica or alumina, non-aqueous solvents like hexane, acetone and/or methanol are used. This is called normal phase LC. Alternatively, the column can be packed with something like octadecyl silica, which is relatively nonpolar. In that case, the mobile phase usually contains at least 5% water and one or more water-miscible solvents (such as acetonitrile and/or methanol). That is called reverse phase LC [1].

Once the NME, prescription drug (active pharmaceutical ingredient, API) or active ingredient from a botanical comes off the preparative-scale

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column, the solvent used as the mobile phase can be removed by distillation and the compound of interest analyzed for purity [1]. The analyst takes just a few mg of the (hopefully) purified compound and re-dissolves it in either the same or a different solvent, or mixture of solvents. Then, it is analyzed using a smaller column in what we call analytical-scale LC. This is called high performance LC or high pressure LC, because the mobile phase is not forced through the column using gravity, but with a pump that can handle relatively high pressures. This is abbreviated as HPLC. HPLC columns are packed with materials that have particle sizes of 2.5-10 μm. To achieve better separation of analytes, smaller particle sizes (<2.5 μm) are used and this is called ultra-performance liquid chromatography, or UPLC [1].

In both HPLC and UPLC samples are usually injected onto the separator column using a syringe that is part of an autosampler [1]. The sample is gradually eluted off the column by pumping the mobile phase through it. The amount of time that it takes for analytes to elute depends on the type of column and mobile phase that are used, as well as the physical properties of the analytes and mobile phase. As the mobile phase is pumped through the column, the individual components come off the column and are usually detected by UV-Vis, fluorescence, conductivity or MS. So, an HPLC (or UPLC) will have a pump (to pump the mobile phase), an autosampler, an injector, a column (sometimes two columns), a detector (sometimes two detectors) and a computer to acquire, store and analyze the chromatograms that are produced [1].

That is, the data that are produced is called a chromatogram [1]. The first part of the chromatogram usually looks like a straight line, because none of the chemicals in the sample have come off the column yet. Moreover, it takes a bit of time for the injected sample to pass through the tubing that connects the pump to the column. This is the void volume or void time, abbreviated as t_0. However, there is always a small amount of random noise. After the void time, the analytes start eluting off the column and produce a signal. The amount of time that it takes for each analyte to be eluted is called the retention time for that analyte. It is abbreviated as t_r. So, the chromatogram shows the detector response over time. One part of method validation is to determine the detection limit, or limit of detection (LOD). That is, when the analyte is present at a very low concentration, it will produce a signal that is not much bigger than the average signal produced by the noise. The ratio of signal to noise is abbreviated as S/N. Somewhat arbitrarily, the LOD is often defined as the concentration of the analyte that produces an S/N of three. It's also important to determine the limit of quantitation (LOQ). This is also defined arbitrarily – usually by

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the customer or the regulatory agency when analyzing foods and beverages. Some define it as the analyte concentration that produces an S/N of 10. However, many others define it as the lowest concentration of the analyte on the calibration curve [1].

So, to determine the concentrations of the analytes in the sample, the analyst must first inject some standards on the LC (or GC if trying to analyze a drug for residual solvents) [1]. The US Pharmacopeia, or USP, has many standards of APIs, so many manufacturers will use USP standards for this. However, if the API is a new, the USP will not have it. In that case, the standard must be produced by the original manufacturer. It is often called an in-house standard. Either way, the analyst should dissolve an accurately weighed standard in a carefully measured volume of solvent, using a volumetric flask. It is important that the solvent be no stronger than the mobile phase, to prevent extra-column effects from changing the t_r of the analyte. That is, in reverse-phase HPLC or UPLC, if the mobile phase is a mixture of 80% methanol in water (v/v), then the analytes should be dissolved in a solvent that contains no more than 80% methanol mixed with water. For example, the standard operating procedure (SOP) might tell the analyst to weigh about 100 mg of the API accurately (to the nearest 0.1 mg or 0.0001 g) and dissolve it in 100 mL of a properly chosen solvent, to prepare a stock solution. The SOP may then tell the analyst to dilute the stock solution (containing about 1 mg/mL or 1000 μg/mL of API) five different times to produce diluted standards containing about 1, 2, 3, 4 and 5 μg/mL of the API. The concentrations of standards that are analyzed will depend on the sensitivity of the detector. It is important that the lowest concentration standard produces a large enough peak that the S/N is >10. It is also possible that standards can be too concentrated so that they overload the column and/or detector. To give an everyday example of this, if your bathroom scale only goes up to 150 kg, it can't accurately weigh a person who weighs over 150 kg. The scale (or detector) is overloaded [1].

So, the SOP might tell the analyst to inject each standard on an HPLC, UPLC, or GC and have the computer printout the peak areas that are obtained [1]. The following (Table 1) data might be obtained:

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Table 1: Possible Example of Calibration Data

Concentration (µM)	Peak Area
1.01	126
2.02	261
3.03	372
4.04	523
5.05	629

The SOP might tell the analyst to use Microsoft Excel™ to fit the data to a straight line, such as the one shown in Figure 9.

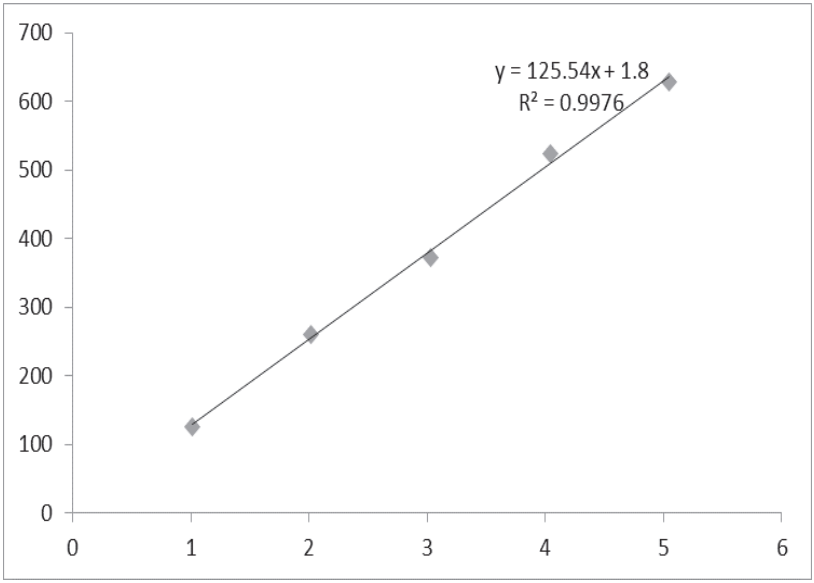

Figure 9. Calibration 'curve' showing a fit to a straight line, the equation of the line and the correlation coefficient (R^2).

Note that the five points do not fit exactly on the straight line that was calculated using Microsoft Excel™ [1]. The degree to which the points fit to a straight line is indicated by the correlation coefficient, abbreviated as R^2. If R^2 is 1.000, then the points all fall exactly on the line. This almost never happens. So, an SOP might specify that the R^2 must be $\geq$0.997, in which case, the calibration just barely passes. The SOP might tell the analyst to re-inject one of the standards, such the one in the middle (3.03

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ppm), and analyze it as if it were an unknown. Suppose it produces a peak area of 369. The equation of the calibration curve, or straight line is calculated by Excel^{TM} and is shown in Figure 9 to be y = 125.54 x + 1.8, where y is the peak area and x is the concentration in μM. Since the standard is being analyzed as if it were a sample, concentration can be calculated x by equation 29, to give 2.92 μM

Equation 10: $369 = 125.54\ x + 1.8$

The SOP might specify that the re-analyzed standard be accurate to within ± 10%, in which case the analysis would pass. Thus, the HPLC, UPLC, or GC has been certified as being accurate enough to analyze actual samples. The SOP might tell the analyst to re-analyze (re-inject) the same standard after every 10 samples, to ensure that the method is still valid. It is important to do this, because the column used in the analysis can degrade after injecting several samples. The different types of columns that can be used have been described in a previous book [1].

A1.4 Gas Chromatography, GC

If a compound is stable at higher temperatures and if it can be volatilized, GC can be used [1]. Many drugs are synthesized by dissolving reagents in volatile organic solvents. GC is an excellent way to analyze them to be sure that all the solvent has been removed before giving the drug product to an animal (for veterinary medicines) or a human [1].

GC is used to separate gases and volatile compounds [1]. The analytes are dissolved in a thermally stable, volatile solvent, which is vaporized or volatilized. Next, it is transported to a GC column using an inert gas as the mobile phase. GC can also be used to analyze gas samples directly. This is often done in industrial hygiene to ensure that workers are not exposed to toxic concentrations of solvents. An industrial hygienist can draw some of the lab air into a gas-tight syringe and inject it on the GC that contains either a packed column, or capillary column. A packed GC column can contain either a liquid or solid stationary phase as the absorbent. It can be absorbed onto a solid support, usually made of diatomaceous earth from diatoms. Silica and alumina can also be used. Liquid absorbents can be made of silicone polymers or polyethylene glycol. Packed columns can be used to analyze sample volumes ranging from 0.1 – 20 μL. Capillary columns need much less sample. For capillary GC, the injector can be used in one of two modes: split or splitless. That is, some of the sample can be sent to waste, while only a portion of it is injected (split mode), or it can

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all be sent to the detector (spliless mode). The injector contains a heated chamber with a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes when the injector is in split mode. The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes. A proportion of this mixture passes onto the column, but most exits through the outlet [1].

Most packed columns are 1.5-10 m long and have an internal diameter of 2-4 mm [1]. Capillary columns have an internal diameter of only a few tenths of a millimeter. They can be wall-coated open tubular (WCOT) or support-coated open tubular (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with a liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns [1].

Polysiloxanes are the most popular type of stationary phase in GC columns [1]. They contain a mixture of poly(dimethylsiloxane), poly(phenylmethylsiloxane) and poly(diphenylsiloxanes). The percentage of methyl or phenyl groups varies with the exact type of column. Poly(ethylene glycol), or PEG can also be used as a coating [1].

In LC, analytes are separated based on differences in polarity and the presence of long chain alkyl groups [1]. In GC, analytes are separated based on differences in their volatility or boiling point. So, methanol will elute off a GC column before hexane, because it has a lower boiling point. Nonvolatile compounds are not suited for GC analysis, unless they can be derivatized to form a volatile derivative. An example of this is the derivatization of fatty acids by reacting them with BF_3-methanol to produce fatty acid methyl esters (FAMEs) which are volatile [1].

In LC, analytes are washed off the column by changing the composition of the liquid mobile phase in a gradient elution [1]. In GC, analytes are eluted off a GC column by gradually increasing the temperature of the carrier gas (temperature gradient). Detectors are also quite different in GC. Flame ionization detectors (FID) have been used for a long time for combustible analytes (but not fluorinated, chlorinated or brominated compounds). Other detectors include thermal conductivity (TCD), electron capture (ECD), electrolytic conductivity (ELCD), pulsed flame photometric detectors (PFPD), photoionization detectors (PID), halogen specific detectors (XSD) and mass spectrometry (MS). FIDs are sometimes considered to be an almost universal detector for GC. As analytes elute off a GC column, they are mixed with H_2 and air and are

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sent to a flame, which burns the compound and produces an electrical current. FIDs are relatively easy to use. They are sensitive (0.1 – 10 ng LOD) and linear over a large dynamic range (10^5 to 10^7). However, water doesn't burn, so aqueous solutions shouldn't be analyzed with them. A TCD contains a detector cell that has a heated filament to which an electrical current is applied. As the carrier gas containing volatile analytes passes through the cell, a current is produced. It is not as sensitive as the FID, though. It can detect 5 – 20 ng. Still, it has a large linear dynamic range of 10^5 to 10^6 [1].

PIDs contain a detector cell that is irradiated with light [1]. Compounds with ionization energies lower than the photon energy in the cell are ionized. They are attracted to an electrode and subsequently detected. It is usually used for aromatic and olefinic organics. Sensitivities are usually about 25–50 pg (10^{-9} grams) for aromatic compounds (that have phenyl groups) and 50–200 pg for olefins (compounds with C=C bonds) [1].

Compounds that contain halogens don't burn very well [1]. For these, an ECD is used. It contains a radioactive ^{63}Ni collar, which emits electrons. As halogenated compounds enter the detector, the ECD captures some of the electrons. It is more sensitive than an FID, with LODs of about 0.1 pg ($1x10^{-13}$ g). However, some ECDs can become overloaded when the analyte concentration is too high. So, calibration curves are sometimes valid over only three to four orders of magnitude (10^3 to 10^4) and samples must be diluted to keep them within the range of concentrations of standards used to construct the calibration curve [1].

ELCDs are used for analytes that contain halogens, nitrogen and/or sulfur [1]. Analytes dissolved in a solvent are mixed with a reaction gas and passed through a high temperature reaction tube. Sensitivities are usually about 5 – 10 pg for halogens, 10 – 20 pg for S and 10 – 20 pg for N. Although the ELCD and EC detectors are used widely, they are not completely specific. For this reason, an MS is often used as a GC detector to confirm (or deny) the presence of an analyte that seems to appear with an ELCD or EC [1].

FPDs have a pulsed flame that produces chemilluminescence [1]. A mixture of H_2 and air is ignited, extinguished and re-ignited three to four times per second in the FPD. Light is emitted within a few milliseconds. The PFPD can detect at least 28 elements: S, P, N, As, Sn, Se, Mn, B, Br, Ga, Ge, Pb, Si, Te, V, Al, Bi, Cr, Cu, Eu, Fe, Rh, Ru, W, In and Sb [1].

MS can also be used as a detector [1]. When used with GC, relatively hard ionization methods are used that produce fragments of the analyte. The fragmentation pattern can help identify an unknown compound or

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confirm the presence of a suspected analyte. Molecular ions are sometimes not seen, or are merely relatively small peaks [1].

Many polar and ionic compounds are not volatilized or they decompose at the temperatures used in GC. For these, liquid chromatography (LC) is used.

A1.5 Mass Spectrometry, GC-MS and LC-MS

Mass spectrometry (MS) measures the ratio of the molecular weight (or mass) of ionized analytes to their charge. This is abbreviated as *m/z*. Mass is expressed in units of grams per mole, which is the same as Daltons, abbreviated as Da. So, water has a molecular weight of 18 g/mol, or 18 Da. There are several ways to introduce samples and analytes into the MS. There is a direct probe, in which the sample is placed in the probe and it is heated in a vacuum. This is useful when analyzing solid samples, such as electronic devices, for organic contaminants. For biological samples, ionization at ambient pressure can be done using desorption electrospray ionization (DESI) or direct analysis in real time (DART) with little or no sample preparation [2]. It can be used to detect many analytes in very small volumes, including imaging small molecules that are in tissues. This includes amino acids, peptides, protein digests, and intact proteins. DESI often produces multi-charged species. Since the MS measures the ratio of mass to charge, this enables the detection of higher masses – up to 150 000 Da using a quadrupole time of flight mass spectrometer (Q-TOF) with DESI [2]. Similarly, liquid samples can be loaded onto 96 well plates and directly infused into a MS using chip-based electrospray [1]. This was done to analyze lipids that are important in signal transduction, such as diacylglycerol and phosphoinositides [1, 3].

Samples can also be introduced into an MS by carrying them in a stream of gas in GC-MS, or in a stream of liquid LC-MS [1]. Also, there are several ways to ionize substances. Nonionic compounds are often ionized in GC-MS by electron ionization, or EI (formerly called electron impact). It is a relatively hard ionization technique because the electrons strike the analytes with enough energy to break the analyte into fragments. Molecular ions may not appear at all. Instead, the molecular ion is broken into smaller fragments by the loss of atoms, ions, radicals, molecules or functional groups such as H•, •OH, H_2, Cl•, Br•, HCl, HBr, H_2O, $•CH_3$, CH_3^+, CO, CHO, and many others. When mass spectra are interpreted, the smaller fragments are indicated as Molecular ions (M) minus the fragment that is lost, such as [M-H], [M-OH], [M-Cl], [M-Br], [M-H_2O], etc [1].

This is in contrast with soft ionization techniques used in LC-MS [1]. They don't produce many fragments, but often do produce relatively

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intense molecular ions and adducts with Na^+ and NH_4^+ - even if there are no sodium or ammonium salts present in the sample and none have been added to the mobile phase. This is because the water used in mobile phases always contains trace amounts of NaCl and sometimes even NH_4Cl. In addition, if a previous sample or mobile phase contained any sodium or ammonium salts, they leave trace amounts of them in the MS. So, when analyzing dietary supplements for toxic adulterants, quite often Na^+ and NH_4^+ adducts are seen. Table 2 provides a list of such adducts that have been seen in the adulterants whose $[M+H]^+$ ions were listed in Table 4 of Chapter 3. Note that fragments of these and the $[M+H]^+$ ions can also be produced. The exact fragments and their relative intensities will depend on the ionization potential of the MS that is used by the analyst.

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Table 2. Analytes in drug screen, with the *m/z* values (in Da) of ions, Q_2 and Q_3, formed by adducts in LC-MS [4].

Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
1,3-DMAA	133.16993	$M+NH_4$	138.12532	M+Na
1,3-DMBA	119.15428	$M+NH_4$	124.10967	M+Na
17-α-ethynylestradiol	314.21146	$M+NH_4$	319.16685	M+Na
17-α-Methyltestosterone	320.25841	$M+NH_4$	325.2138	M+Na
17-β-Estradiol	290.21146	$M+NH_4$	295.16685	M+Na
19-Norandrostenedione	290.21146	$M+NH_4$	295.16685	M+Na
1-Androstenediol	308.25841	$M+NH_4$	313.2138	M+Na
1-Androstenedione	304.22711	$M+NH_4$	309.1825	M+Na
1-Testosterone	306.24276	$M+NH_4$	311.19815	M+Na
2,4-DNP	207.00124	M+Na	202.04585	$M+NH_4$
2-Hydroxypropylnortadalafil	451.1976	$M+NH_4$	456.15299	M+Na
4-Hydroxytestosterone	322.23767	$M+NH_4$	327.19307	M+Na
4-para-Nonylphenol	238.21654	$M+NH_4$	243.17194	M+Na
4-tert-Octylphenol	224.20089	$M+NH_4$	229.15629	M+Na
5-HTP	238.11862	$M+NH_4$	243.07401	M+Na
5-HydroxyTryptophan	243.07401	M+Na	238.11862	$M+NH_4$

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
α-Amanitin	936.38799	$M+NH_4$	941.34339	M+Na
Acetaminophen	169.09715	$M+NH_4$	174.05255	M+Na
Acetildenafil	484.30306	$M+NH_4$	489.25846	M+Na
Acetylvardenafil	484.30306	$M+NH_4$	489.25846	M+Na
Aconitine	663.34874	$M+NH_4$	668.30413	M+Na
Aminopterin	458.18949	$M+NH_4$	463.14489	M+Na
Aminotadalafil	408.16663	$M+NH_4$	413.12203	M+Na
Aminotadalafil condensation product	515.17993	$M+NH_4$	520.13533	M+Na
Apomorphine	285.15975	$M+NH_4$	290.11515	M+Na
Aspirin	181.04954	M+H	198.07608	$M+NH_4$
Atorvastatin	576.28683	$M+NH_4$	581.24222	M+Na
Atropine	307.20162	$M+NH_4$	312.15701	M+Na
Avanafil	501.21239	$M+NH_4$	506.16779	M+Na
β-alanaine	107.0815	$M+NH_4$	112.0369	M+Na
Benfluorex	369.17844	$M+NH_4$	374.13383	M+Na
Benzphetamine	257.20123	$M+NH_4$	262.15662	M+Na
Berberine	354.15741	$M+NH_4$	359.1128	M+Na
Bisphenol A	246.14886	$M+NH_4$	251.10425	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Bolandiol	294.24276	$M+NH_4$	299.19815	M+Na
Bolasterone	334.27406	$M+NH_4$	339.22945	M+Na
Boldenone	304.22711	$M+NH_4$	309.1825	M+Na
Boldione	302.21146	$M+NH_4$	307.16685	M+Na
Brompheniramine	336.10699	$M+NH_4$	341.06238	M+Na
Brucine	412.22308	$M+NH_4$	417.17848	M+Na
Bumetanide	382.14312	$M+NH_4$	387.09851	M+Na
Bupropion	257.14152	$M+NH_4$	262.09691	M+Na
Caffeine	212.1142	$M+NH_4$	217.0696	M+Na
Calusterone	334.27406	$M+NH_4$	339.22945	M+Na
Captopril	235.11109	$M+NH_4$	240.06649	M+Na
Cerivastatin	477.27593	$M+NH_4$	482.23132	M+Na
Cetilistat	419.32682	$M+NH_4$	424.28222	M+Na
Chloropretadalafil	444.13207	$M+NH_4$	449.08747	M+Na
Chlorpheniramine	292.1575	$M+NH_4$	297.1129	M+Na
Chlorzoxazone	187.02688	$M+NH_4$	191.98228	M+Na
Cimetidine	270.14954	$M+NH_4$	275.10494	M+Na
Citric Acid	215.01622	M+Na	210.06083	$M+NH_4$

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Clenbuterol	294.11344	$M+NH_4$	299.06884	M+Na
Clenbuterol Hydrochloride	294.11344	$M+NH_4$	299.06884	M+Na
Clostebol	340.20378	$M+NH_4$	345.15918	M+Na
Codeine	317.18597	$M+NH_4$	322.14136	M+Na
Colchicine	417.20201	$M+NH_4$	422.15741	M+Na
Cyclopentynafil	546.2857	$M+NH_4$	551.2411	M+Na
Cyproheptadine	305.20123	$M+NH_4$	310.15662	M+Na
Danazol	355.238	$M+NH_4$	360.1934	M+Na
Dapoxetine	323.21179	$M+NH_4$	328.16719	M+Na
Dehydrochlormethyltestosterone	352.20378	$M+NH_4$	357.15918	M+Na
Desmethylsildenafil	478.2231	$M+NH_4$	483.1785	M+Na
Desoxymethyltestosterone	306.27914	$M+NH_4$	311.23454	M+Na
Desvenlafaxine	281.22235	$M+NH_4$	286.17775	M+Na
Dexamethasone	410.23373	$M+NH_4$	415.18912	M+Na
Dextroamphetamine Sulfate	153.13863	$M+NH_4$	158.09402	M+Na
Dextromethorphan	289.22744	$M+NH_4$	294.18284	M+Na
DHEA	306.24276	$M+NH_4$	311.19815	M+Na
DHEA (minus H_2O)	288.23219	$M+NH_4$	293.18759	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Diclofenac	313.05051	$M+NH_4$	318.00591	M+Na
Diclofenac Sodium Salt	313.05051	$M+NH_4$	318.00591	M+Na
Diethylpropion	223.18049	$M+NH_4$	228.13589	M+Na
Digitoxigenin	392.27954	$M+NH_4$	397.23493	M+Na
Digitoxin	782.46852	$M+NH_4$	787.42391	M+Na
Digoxigenin	408.27445	$M+NH_4$	413.22985	M+Na
Digoxin	798.46343	$M+NH_4$	803.41883	M+Na
Dimethylsildenafil	506.2544	$M+NH_4$	511.2098	M+Na
Dinitrophenol	202.04585	$M+NH_4$	207.00124	M+Na
Diosgenin	437.30262	M+Na	432.34722	$M+NH_4$
Dithiodesmethylcarbodenafil	488.22608	$M+NH_4$	493.18147	M+Na
Drostanolone	322.27406	$M+NH_4$	327.22945	M+Na
Emetine	498.33263	$M+NH_4$	503.28803	M+Na
Ephedrine Hydrochloride	183.14919	$M+NH_4$	188.10459	M+Na
Epigallocatechin gallate	476.11874	$M+NH_4$	481.07413	M+Na
Eserine	293.1972	$M+NH_4$	298.1526	M+Na
Estrone	288.19581	$M+NH_4$	293.1512	M+Na
Ethylestrenol	306.27914	$M+NH_4$	311.23454	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Fenfluramine	249.15731	$M+NH_4$	254.11271	M+Na
Finasteride	390.3115	$M+NH_4$	395.2669	M+Na
Fluoxetine	327.16787	$M+NH_4$	332.12327	M+Na
Fluoxymesterone	354.2439	$M+NH_4$	359.19929	M+Na
Flutamide	294.106	$M+NH_4$	299.0614	M+Na
Fluvastatin	429.21841	$M+NH_4$	434.17381	M+Na
Formebolone	362.23258	$M+NH_4$	367.18798	M+Na
Furazabol	348.26455	$M+NH_4$	353.21995	M+Na
Furosemide	348.04155	$M+NH_4$	352.99694	M+Na
Furosemide (-NH3)	351.94433	M+K	335.97039	M+Na
GammaAminoButyricAcid	126.05255	M+Na	121.09715	$M+NH_4$
Gendenafil	372.20302	$M+NH_4$	377.15841	M+Na
Genipin	244.11795	$M+NH_4$	249.07334	M+Na
Gestrinone	326.21146	$M+NH_4$	331.16685	M+Na
Glibenclamide	511.17764	$M+NH_4$	516.13304	M+Na
Glutamine	169.05836	M+Na	164.10297	$M+NH_4$
Heroin	387.19145	$M+NH_4$	392.14684	M+Na
Homosildenafil	506.2544	$M+NH_4$	511.2098	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Hordenine	183.14919	$M+NH_4$	188.10459	M+Na
Hydrastine	401.17071	$M+NH_4$	406.12611	M+Na
Hydrochlorothiazide	314.9983	$M+NH_4$	319.9537	M+Na
Hydrocodone	317.18597	$M+NH_4$	322.14136	M+Na
Hydrocortisone	385.19855	M+Na	401.17248	M+K
Hydroxyacetildenafil	500.29798	$M+NH_4$	505.25337	M+Na
Hydroxyhomosildenafil	522.24931	$M+NH_4$	527.20471	M+Na
Hydroxythiohomosildenafil	538.22647	$M+NH_4$	543.18187	M+Na
Hyoscyamine	307.20162	$M+NH_4$	312.15701	M+Na
Ibuprofen	224.16451	$M+NH_4$	229.1199	M+Na
Icariin	699.22594	M+Na	694.27055	$M+NH_4$
Isopiperazinonafil	500.29798	$M+NH_4$	505.25337	M+Na
Ketoconazole	548.18259	$M+NH_4$	553.13798	M+Na
L-DOPA	220.05803	M+Na	215.10263	$M+NH_4$
Leucine/isoLeucine	154.08385	M+Na	149.12845	$M+NH_4$
Levorphanol	275.21179	$M+NH_4$	280.16719	M+Na
Lobeline	355.238	$M+NH_4$	360.1934	M+Na
Lodenafil Carbonate	1052.44407	$M+NH_4$	1057.39946	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Lorcaserin	213.1153	$M+NH_4$	218.0707	M+Na
Lovastatin	422.2901	$M+NH_4$	427.2455	M+Na
Lovastatin Hydroxy Acid	440.30066	$M+NH_4$	445.25606	M+Na
Lovastatin Related Compound A	424.30575	$M+NH_4$	429.26115	M+Na
Mazindol	302.10547	$M+NH_4$	307.06086	M+Na
Megestrol (acetate)	402.26389	$M+NH_4$	407.21928	M+Na
Melatonin	255.1104	M+Na	250.155	$M+NH_4$
Meldonium	165.14718	$M+NH_4$	170.10257	M+Na
Mestanolone	322.27406	$M+NH_4$	327.22945	M+Na
Mesterolone	322.27406	$M+NH_4$	327.22945	M+Na
Metenolone	320.25841	$M+NH_4$	325.2138	M+Na
Metformin	147.13527	$M+NH_4$	152.09067	M+Na
Methandienone	318.24276	$M+NH_4$	323.19815	M+Na
Methandriol	322.27406	$M+NH_4$	327.22945	M+Na
Methasterone	336.28971	$M+NH_4$	341.2451	M+Na
Methyl Ephedrine, N-	197.16484	$M+NH_4$	202.12024	M+Na
Methyl Pseudoephedrine, N-	197.16484	$M+NH_4$	202.12024	M+Na
Methyl synephrine (oxilofrine)	199.1441	$M+NH_4$	204.0995	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Methyl-1-testosterone	320.25841	$M+NH_4$	325.2138	M+Na
Methyldienolone	304.22711	$M+NH_4$	309.1825	M+Na
Methylnortestosterone	306.24276	$M+NH_4$	311.19815	M+Na
Methylphenidate	251.1754	$M+NH_4$	256.1308	M+Na
Methyltestosterone	320.25841	$M+NH_4$	325.2138	M+Na
Metribolone (methyltrienolone)	302.21146	$M+NH_4$	307.16685	M+Na
Mibolerone	320.25841	$M+NH_4$	325.2138	M+Na
Minoxidil	227.16149	$M+NH_4$	232.11688	M+Na
Mitragynine	416.25438	$M+NH_4$	421.20978	M+Na
Modafinil	291.11618	$M+NH_4$	296.07157	M+Na
MPEA	153.13863	$M+NH_4$	158.09402	M+Na
Nandrolone	292.22711	$M+NH_4$	297.1825	M+Na
Naproxen	248.12812	$M+NH_4$	253.08352	M+Na
n-Butylnortadalafil	449.21833	$M+NH_4$	454.17373	M+Na
N-desethyl vardenafil	478.2231	$M+NH_4$	483.1785	M+Na
N-Desmethylsibutramine	283.19355	$M+NH_4$	288.14895	M+Na
N-Didesmethylsibutramine	269.1779	$M+NH_4$	274.1333	M+Na
N-Formyl-N,N-didesmethylsibutramine	297.17282	$M+NH_4$	302.12821	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Nitroso-prodenafil	647.25408	$M+NH_4$	652.20948	M+Na
N-Octylnortadalafil	505.28093	$M+NH_4$	510.23633	M+Na
Noopept	336.19178	$M+NH_4$	341.14718	M+Na
Noracetildenafil	470.28741	$M+NH_4$	475.24281	M+Na
Norbolethone	334.27406	$M+NH_4$	339.22945	M+Na
Norclostebol	326.18813	$M+NH_4$	331.14353	M+Na
Norephedrine	169.13354	$M+NH_4$	174.08894	M+Na
Norethandrolone	320.25841	$M+NH_4$	325.2138	M+Na
Norneosildenafil	477.22785	$M+NH_4$	482.18325	M+Na
Orlistat	513.4262	$M+NH_4$	518.38159	M+Na
Ouabain	602.3171	$M+NH_4$	607.2725	M+Na
Oxabolone	308.22202	$M+NH_4$	313.17742	M+Na
Oxandrolone	324.25332	$M+NH_4$	329.20872	M+Na
Oxycodone	333.18088	$M+NH_4$	338.13628	M+Na
Oxymesterone	336.25332	$M+NH_4$	341.20872	M+Na
Oxymetazoline	278.22269	$M+NH_4$	283.17808	M+Na
Oxymetholone	350.26897	$M+NH_4$	355.22437	M+Na
Paroxetine	347.17655	$M+NH_4$	352.13194	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Pentazocine	303.24309	$M+NH_4$	308.19849	M+Na
Phendimetrazine	209.16484	$M+NH_4$	214.12024	M+Na
Phenformin	223.16657	$M+NH_4$	228.12197	M+Na
Phenibut	197.12845	$M+NH_4$	202.08385	M+Na
Phenolphthalein	225.05462	$M+NH_4$	197.05971	M+Na
Phentermine Hydrochloride	167.15428	$M+NH_4$	172.10967	M+Na
Phenylephrine	185.12845	$M+NH_4$	190.08385	M+Na
Phenytoin	270.1237	$M+NH_4$	275.0791	M+Na
Picrotin	328.13908	$M+NH_4$	333.09447	M+Na
Picrotoxinin	310.12851	$M+NH_4$	315.08391	M+Na
Piperazinonafil	500.29798	$M+NH_4$	505.25337	M+Na
Pravastatin	442.27993	$M+NH_4$	447.23532	M+Na
Pravastatin-1,1,3,3-tetramethyl	442.27993	$M+NH_4$	447.23532	M+Na
Pregnenolone	334.27406	$M+NH_4$	339.22945	M+Na
Pregnenolone (minus H_2O)	316.26349	$M+NH_4$	321.21889	M+Na
Primidone	236.13935	$M+NH_4$	241.09475	M+Na
Progesterone	332.25841	$M+NH_4$	337.2138	M+Na
Prostanozol	416.32715	$M+NH_4$	421.28255	M+Na

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Compound	Q_2 (*m/z*)	Adduct	Q_3 (*m/z*)	Adduct
Pseudoephedrine	183.14919	$M+NH_4$	188.10459	M+Na
Pseudovardenafil	477.22785	$M+NH_4$	482.18325	M+Na
Quinbolone	370.27406	$M+NH_4$	375.22945	M+Na
Ricinine	182.0924	$M+NH_4$	187.0478	M+Na
Rimonabant	480.11192	$M+NH_4$	485.06732	M+Na
Rosuvastatin	499.20211	$M+NH_4$	504.15751	M+Na
Scopolamine	321.18088	$M+NH_4$	326.13628	M+Na
Sertraline	323.10763	$M+NH_4$	328.06303	M+Na
Sibutramine	125.01525	$M+NH_4$	89.03883	M+Na
Sildenafil	492.23875	$M+NH_4$	497.19415	M+Na
Simvastatin	436.30575	$M+NH_4$	441.26115	M+Na
Solanine	885.53185	$M+NH_4$	890.48724	M+Na
Spironolactone	434.23596	$M+NH_4$	439.19135	M+Na
Stanozolol	346.28529	$M+NH_4$	351.24068	M+Na
Stenbolone	320.25841	$M+NH_4$	325.2138	M+Na
Strychnine	352.20195	$M+NH_4$	357.15735	M+Na
Sucralose	419.00377	M+Na	414.04838	$M+NH_4$
Sulfoaildenafil	522.23156	$M+NH_4$	527.18695	M+Na

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Compound	**Q_2 (*m/z*)**	**Adduct**	**Q_3 (*m/z*)**	**Adduct**
Sulfosildenafil	508.21591	$M+NH_4$	513.1713	M+Na
Super-4-DHEA	404.35231	$M+NH_4$	409.3077	M+Na
Super-Ra-DHEA	420.34722	$M+NH_4$	425.30262	M+Na
Super-Rb-DHEA	476.40982	$M+NH_4$	481.36522	M+Na
Synephrine	185.12845	$M+NH_4$	190.08385	M+Na
Tadalafil	407.17138	$M+NH_4$	412.12678	M+Na
Testosterone (Propionate)	362.26897	$M+NH_4$	367.22437	M+Na
Tetrahydrogestrinone	330.24276	$M+NH_4$	335.19815	M+Na
Theanine	197.08966	M+Na	192.13427	$M+NH_4$
Theophylline	198.09855	$M+NH_4$	203.05395	M+Na
Thiohomosildenafil	522.23156	$M+NH_4$	527.18695	M+Na
Thioquinapiperifil	466.23836	$M+NH_4$	471.19375	M+Na
Topiramate	357.13261	$M+NH_4$	362.08801	M+Na
Trenbolone	288.19581	$M+NH_4$	293.1512	M+Na
Udenafil	534.2857	$M+NH_4$	539.2411	M+Na
Valine	140.0682	M+Na	135.1128	$M+NH_4$
Vardenafil	506.2544	$M+NH_4$	511.2098	M+Na
Vardenafil hydrolysis product	330.19245	$M+NH_4$	335.14785	M+Na

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Compound	**Q_2 (*m/z*)**	**Adduct**	**Q_3 (*m/z*)**	**Adduct**
Xanthoanthrafil	407.19251	$M+NH_4$	412.14791	M+Na
Yohimbine	372.22817	$M+NH_4$	377.18356	M+Na

Soft ionization techniques include chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB) and matrix assisted laser desorption and ionization (MALDI). Ions are separated in an analyzer, which uses magnetic and/or electric fields to separate them. Quadrupole mass spectrometers have two pairs of magnetic rods per quadrupole. Both direct current (DC) and radio frequency (rf) voltages are applied to each set. For a given amplitude of DC and rf, only the ions with a specific *m/z* will have a stable trajectory and pass the quadrupole to reach the detector. The quadrupole is scanned so one *m/z* at a time is detected. One set of rods has a positive electropotential, the other set is negative. The positive pair of rods filter high masses, while negative rods filter low masses. The quadrupoles (and other mass analyzers) can be used to scan the complete range of masses to get a full scan mass spectrum. Alternatively, they can be set to detect only a selected number of ions, in the selected ion monitoring or SIM mode (also known as single ion monitoring) and multiple reaction monitoring (MRM) [1].

There are also mass spectrometers that have two quadrupoles, separated by a collision cell [1]. They are called triple quadrupoles, or triple quads. Precursor ions and product ions are created and analyzed in different parts of the MS. The first quadrupole selects the precursor ion, which is then fragmented in the collision cell by accelerating the ions in the presence of a collision gas, such as argon or helium. The third quadrupole separates the product ions from each other. The first analyzer, used in the SIM or MRM mode, selects a single precursor ion. The second analyzer is set to look for selected product ions [1].

Ion traps are another form of mass analyzers [1]. Unlike quadrupole mass spectrometers, all the ions are collected at once in an ion trap. Often, they are trapped in a device that consists of a ring electrode and two end cap electrodes. The ions are stabilized in the trap by applying a RF voltage on the ring electrode. By increasing the rf voltage, ions are ejected from the trap and reach the MS detector. All the ions produced are collected in the trap. This includes parent and fragment ions. One can do a full scan, in which ions within the mass range of the trap (such as 100 – 750 amu) can be detected. After collecting the data, the computer that is attached to the MS can show all the ions produced, or just those ions that the analyst selects. This is called a data-dependent scan. To obtain better sensitivity, though, it is better to only select a few ions in the SIM mode [1]. For example, when analyzing food for VOCs (volatile organic compounds), including trichloroethene, one might choose the molecular ion (*m/z* = 132), along with fragment ions with *m/z* = 95, 130 and 132 Da [5].

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Another type of mass analyzer is the time of flight MS, abbreviated as TOF-MS [1]. In the first TOF-MSs, ions formed in the ion source were accelerated by an electric field into an analyzer that consists of a long straight flight tube. The ions passed along the tube until they reached the MS detector. Smaller molecules needed less time to fly through the tube and be detected, thus the name time of flight. These TOF-MSs were used to analyze gases, such as H_2, He, N_2 and O_2, in a residual gas analyzer. The time needed for the gases to reach the detector depended on their mass. Linear TOF-MS spectrometers are still available, but they can now separate much larger molecules, with higher mass resolution (>20 000). Analysts can also do MS/MS, also known as TOF-TOF, in which precursor ions can be fragmented into product ions. More recently, TOF is being used to analyze proteins and polypeptides using the soft ionization technique called matrix-assisted laser desorption, or MALDI. So, MALDI-TOF is very popular in analyzing proteomes. Bacteria, yeast and spores can be identified, based on the pattern of proteins that are detected by MALDI-TOF MS. Protein-protein interactions can also be evaluated. A MALDI-TOF MS can be connected to robotic-controlled autosamplers to prepare samples and to a ChIP chemical inkjet printer to deliver volumes as low as 100 picoliters to the smallest parts of electrophoresis gels or tissues that one might want to analyze [1].

Regardless of the type of MS used, the types of ions produced can provide useful information in identifying a compound [1]. The molecular ion (when present) can give the molecular weight and molecular formula, if the resolution of the mass spectrometer is high enough. Resolution is the smallest difference in *m/z* that can be detected, divided by the mass being detected. For example, a high resolution mass spectrometer may have a resolution of 10^6, meaning that it can separate ions with m/z = 100.0001 and m/z = 100.0002. These ions have a difference in *m/z* of 0.0001, or 10^{-4} amu and the mass being detected is 10^2 Da. The resolution is 10^{-4} divided by 10^2, or 10^{-6}. If masses can be detected with four decimal place accuracy, one can calculate the molecular formula (using a computer program). For example, the atomic mass of ^{12}C is 12.0000, ^{16}O is 15.9999 and ^{14}N is 14.0031. Thus, the compounds C_6H_{12} C_5H_8O and $C_4H_8N_2$ have molecular weights of 84.0939, 84.0575 and 84.0688, respectively. So, high resolution mass spectrometry (HRMS) can be very useful in unequivocally identifying analytes [1].

MS can be used to determine how many carbons are in an analyte, since two isotopes, ^{12}C and ^{13}C are present [1]. The ^{13}C isotope has a natural abundance of 1.1%. Each carbon in a molecule will produce a peak that is 1 Da larger in mass than the ^{12}C-containing compound, but only

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1.1% of the peak area of the ^{12}C isotope. For example, octane, C_8H_{18}, with eight carbons and a monoisotopic molecular weight of 114.140854 Da will produce a molecular ion with *m/z* = 114.140854 Da and a smaller peak with m/z = 115.140854 Da. That is, the monoisotopic molecular weight is the molecular weight of the octane molecules that have only the ^{12}C and ^{1}H isotopes. The smaller peak will be due to those octane molecules that have seven ^{12}C and one ^{13}C. The area of the smaller peak will be about 8.8% of the peak produced by the molecular ion, because 8 x 1.1% = 8.8% [1].

Many mass spectrometers have a library of mass spectra on their computer [1]. The analyst can have the computer search the library and list the compounds that come closest to matching the spectrum of a sample. The computer will look at the *m/z* of the ions and their relative intensities. When an analysis is very important, the analytical method will usually require MS-MS detection. The method will specify the *m/z* of the parent ion and at least three smaller fragments for GC-MS and at least three other adducts and/or fragments for LC-MS. The method should also specify the expected ratios of the ion intensities [1].

Typically, hard ionization techniques, such as electron ionization, are used in GC-MS with the ionization potential set at 70 eV [1]. This often produces a small molecular ion and several fragment ions, depending on the analyte and the MS that is used. For example, the largest peak in the conventional MS of chloroform ($CHCl_3$) is the fragment with *m/z* = 83, due to loss of a ^{35}Cl, but the largest peak in the MS of benzene is the molecular ion, with *m/z* = 78 amu. Long chain hydrocarbons can be difficult to distinguish by the electron ionization. The fragmentation patterns are nearly identical and molecular ions are seldom seen. More recently, researchers in Israel developed a softer ionization technique, called molecular beam ionization [1, 6]. The beam is produced by seeding a carrier gas, such as helium, with a low concentration of analytes and then directing the mixture through a tiny opening and into a vacuum chamber [6]. This process cools the analytes, leaving them with less energy, so they have a lower probability of fragmenting. Molecular ions dominate the mass spectrum of hydrocarbons as large as $C_{84}H_{170}$. This method was also used to detect not just large hydrocarbons, but also carbamate pesticides, which had previously been detected by other methods [6].

Many polar and ionic compounds can't be analyzed by GC [1]. They are not volatilized at the temperatures used in a GC. Others decompose or react at these temperatures. For such analytes, LC is preferred. If an analyte conducts electricity, absorbs in the UV, fluoresces, or can be made to undergo an electrochemical reaction, it can be detected by conductivity,

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UV, fluorescence or electrochemical detection. However, these (especially UV) are all susceptible to interferences [1].

For example, in the spring of 2007, several cats and dogs died from contaminated pet food [1]. Initially, analysts at Cornell University suggested that the contaminant was a rat poison, aminopterin. The FDA and other labs were not able to confirm this. This is because the foods contained folic acid, which can be easily confused with aminopterin when the analysis is done by HPLC with a UV detector, or with MS detection that does not have high resolution. Aminopterin and folic acid elute off a C_{18} column at the same time and they both absorb strongly in the UV. Thus, it was very difficult to tell if a food sample has aminopterin or folic acid. Instead, high resolution MS was needed. It can distinguish between aminopterin and folic acid, because they have slightly different molecular weights and produce different fragments. Folic acid produced a fragment with m/z = 295, along with a positive $[M+H]^+$ ion with m/z = 442. In contrast, aminopterin produced a positive $[M+H]^+$ ion with m/z = 441 and a fragment with m/z = 294 [1]. Upon further analysis of the pet foods, it was found that the adulterant was melamine.

When used properly, an MS can be a very selective and sensitive detector for LC [1]. In LC-MS, unlike GC-MS, soft ionization techniques are that usually produce molecular ions as the largest peaks in the mass spectrum. These include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), fast atom bombardment (FAB) and matrix-assisted laser desorption ionization (MALDI). Both APCI and ESI ionize analytes at atmospheric pressure, where electron ionization in GC is done at a very low pressure. In soft ionization, the solvent must be removed from the mobile phase and leave behind the ionized analytes that are in the gas phase. Both APCI and ESI are used for analytes with molecular weights less than about 1000. Unlike APCI, ESI often produces multi-charged ions. Since the ratio m/z is measured, proteins with high molecular weights can produce ions with charges up to +70 [1].

In ESI, the analytes must be ionized while they are dissolved in a solvent [1]. The surface of the droplets of solvent that contain the ionized analytes will become charged at the tip of the capillary. They can have either a positive or negative charge, depending on the polarity of the applied voltage. The size of the droplets decreases as solvent evaporates, while the charge density increases. The droplet explodes and then ions evaporate. ESI is preferred for ionic or polar analytes that are not stable at high temperatures. APCI is preferred for analytes with lower polarity, but high volatility. Since there is usually more fragmentation with ESI, it can produce very different results than APCI [1].

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FAB, or fast atom bombardment, is useful for many analytes, including those that are polar, ionic, thermally or energetically unstable, as well as those that have high molecular weights [1]. In FAB, the analyte and a non-volatile liquid matrix mixture are ionized with a 4 to 10 keV (kilo electrovolt) particle beam of an inert gas like argon or xenon. Glycerol and 3-nitrobenzyl alcohol are commonly used liquid matrices. Following ionization, the selected positive or negative ions are extracted and accelerated towards the MS, which measures their *m/z* values. The FAB mass spectrum contains peaks that are due to ions coming from the matrix, analyte, impurities and ions of other matrix modifiers (such as trifluoroacetic acid) that can be added to increase the abundance of the analytes. The observed ions are usually adducts formed with either cations (such as $[M+H]^+$ and $[M+Na]^+$), or negative ions due to a loss of hydrogen, $[M-H]^-$. The sample must be soluble in the matrix, which must be have relatively low volatility so that it will not evaporate rapidly in the high vacuum system present in the MS. Proteins and peptides are often analyzed by FAB-MS because the primary ion ejected is $[M+H]^+$. This makes it possible to determine their molecular weights accurately [1].

MALDI, or matrix assisted laser desorption ionization, is also useful for large polar and ionic compounds, such as proteins, DNA and polysaccharides [1]. The sample must be mixed with a matrix that contains a chemical that absorbs in the UV, such as 2,5-dihydroxybenzoic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, or α-cyano-4-hydroxycinnamic acid. The diluted peptide or protein (about 10 μM) and matrix solutions can be premixed or applied directly to a sample plate. It is important that neither the matrix nor the analyte precipitates out when the two are mixed. Once the sample is applied to the sample support, it is evaporated. When MALDI is combined with a time of flight (TOF) mass spectrometer, it is called MALDI-TOF. It is very useful in analyzing proteins and polypeptides - especially in proteomics studies. MALDI-TOF has increased the upper mass limit for mass spectrometric analyses to over 1.5 million Da. It makes the analyses of large biopolymers by MS easier and more sensitive. MALDI-TOF is quite useful in identifying pathogenic Bacteria, based on the proteins that they contain. In addition, cross-linking reagents and buffers can be used to analyze protein-protein interactions, while hydrogel particles can be used to trap and separate biomarkers from albumin and other proteins in blood. The biomarkers can be eluted from the nanoparticles and spotted onto a MALDI plate along with the matrix after they are separated from the blood using automated methods [1].

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A1.6 Quantitative Analysis

When doing quantitative analysis, a primary standard is needed. It is a substance that can be obtained at nearly 100% purity. For example, in the Kjeldahl method of determining protein concentrations in foods, the nitrogens in the sample are converted to ammonia (NH_3), which reacts with NaOH to form NH_4OH. Excess NaOH is always used. The excess, unreacted NaOH is titrated with an acid, like HCl. That value is subtracted from the total amount of NaOH originally added to determine how many moles of NaOH reacted and how many moles of nitrogen were in the sample. However, NaOH is a solid (pellets) that absorbs water and CO_2 from the air. So, it's never 100% pure. Its concentration must be determined by titrating it with a primary standard such as the solid salt, potassium acid phthalate (KHP). It can be obtained at nearly 100% purity. The major impurity is water, which can be removed in a drying oven. The dried KHP can be kept dry in a desiccator and used whenever needed. If the lab's SOP doesn't require the best accuracy that is possible (by using KHP), solutions of NaOH and HCl that have been standardized by titration by the vendor can be used. However, when analyzing a prescription drug for the concentration of its active pharmaceutical ingredient (API), primary USP standards are usually required.

When the EPA wants to validate a new analytical method, they want to see if it's accurate, even though accuracy can never be established with 100% certainty. Instead, the EPA will obtain a primary standard that is almost 100% pure. They will weigh it carefully and add some of it to water or soil. They will send the spiked water or solid to several labs and tell them what method they should use to analyze it. This is called a round robin analysis. Even though this will establish the accuracy by regulatory standards, it doesn't mean that every lab obtained the same results. If almost all the labs report finding concentrations that are within some previously specified limit (like ±10%), then the EPA might consider that sufficient to demonstrate the accuracy. The FDA and other governments' regulatory agencies also have standards that have been prepared in foods that can be used to test a method's accuracy.

In any case, analysts can use either an internal standard or an external standard [1]. External standards are solutions containing different concentrations of that analyte. They are not mixed with the sample. They are external and are analyzed separately to prepare calibration curves. Analysts can prepare solution standards, in which the analyte standard is dissolved in a solvent. Alternatively, analysts can prepare external matrix standards, in which the standard is added to the sample matrix. For

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example, when quantifying analytes in physiological samples, the analyst can prepare external matrix standards by adding the standard to a sample that has doesn't contain any of the analyte [1].

On the other hand, an internal standard is sometimes preferred [1]. The internal standard is added to the samples and standards before they are injected on the instrument being used. The peak area (or peak height) of both the internal standard and the analyte are determined. The ratio of the peaks due to each analyte are divided by the areas (or heights) of the internal standard. For example, a calibration curve might be obtained by preparing standards that contain 2 – 20 μg/g of the analyte with 5 μg/g of internal standard being added to each. The ratios of the peak areas or heights are plotted vs the concentration of the analyte in each mixture. A linear regression analysis is done using a computer program such as Microsoft Excel™. The equation of the straight line and the correlation coefficient are obtained. Then unknown samples are all spiked with the same concentration (5 μg/g) of internal standard and they are analyzed the same way the spiked standards. The calibration equation will be in the form of equation 9.

Equation 9: Peak area ratio = slope (conc of analyte) + y-intercept

The analyst will put the peak area ratio obtained for each sample being analyzed into this equation and solve for it the concentration of the analyte [1].

For LC, the internal standard should absorb UV or visible light at as close to the same wavelength as the analyte as possible, but it should be well-separated from the analyte on the LC column [1]. In MS, the internal standard often contains only the ^{13}C isotope and not ^{12}C. The isotopically labeled standard can co-elute off the LC column with the analyte, since it will produce different ions in the mass spectrometer [1].

A1.7 IR and NMR

Two of the best ways to tell verify the identity of an expected IND, API or active ingredient in a dietary supplement is to analyze them by both IR and NMR [1]. IR spectrometry measures the interaction between matter and electromagnetic radiation in the IR region, with wavelengths between 1–30 μm. NMR, though, deals with the interaction between matter and electromagnetic radiation in the radio frequency (rf) range, with frequencies from 60–1000 MHz [1].

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Unlike UV spectrometry, both IR and NMR use a Fourier transform to analyze the data that they produce [1]. This form of IR is called FTIR, for Fourier transform IR, while NMR is occasionally called FTNMR. The Fourier transform enables the analyst to acquire data faster and with better sensitivity. In calculus, the Fourier theorem states that any periodic function can be broken down into a combination of sine and cosine waves. In Fourier analysis, one transforms one function into another. In spectroscopy, the Fourier transform converts data that is acquired in the time domain (rf intensity vs time) into the frequency domain (rf intensity vs frequency) [1].

FTIR is based on the absorption of IR light, due to the vibrations of chemical bonds in the analyte [1]. Very old IR spectrometers scanned the IR spectrum one wavelength at a time. When computers improved in the 1970s, the Fourier transforms could be used. Samples were irradiated with the full range of IR light simultaneously and this was repeated as many times as needed. This way, analyst can acquire spectra faster. As each spectrum is acquired, some frequencies of IR light are absorbed by the sample. With each subsequent acquisition, the signal increases linearly. That is, when the analyst acquires two spectra, the signal doubles. If the analyst acquires four spectra, it quadruples. At the same time, the noise (which is random) increases by the square root of the number of acquisitions (n). Thus, the signal to noise ratio, or S/N, increased by n divided by the square root of n, as shown in equation 10 [1].

$$\text{Equation 10: S/N} = \frac{n}{\sqrt{n}} = \sqrt{n}$$

Thus, the S/N increases with the $\sqrt{N}$ [1]. If the analyst acquires 16 spectra, the S/N will increase by 4; if 100, the S/N increases by 10 and if 10 000 scans, the S/N will increase by 100. Thus, there is a point of diminishing returns, so the analyst often one stops after 16 or 32 scans when acquiring an FTIR spectrum [1].

The FTIR spectrum can tentatively identify the functional groups present in the sample [1]. The atoms in a functional group vibrate, bend and/or stretch. If these movements cause a change in the dipole moment of the bonds in the functional group, the analyte will absorb in the IR at characteristic frequencies [1].

Unlike UV and visible spectrometry, in which the spectra are displayed with the wavelength (in nm) on the x-axis, IR spectra show frequencies (in wavenumbers, or cm^{-1}) [1]. The y-axis of the spectrum can be either

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percent transmittance or the amount of Absorbance. At those frequencies at which IR light passes through the sample without being absorbed, there is 100% transmittance, or zero absorbance. In absorbance spectra, peaks (or bands) are positive. In transmittance spectra, they are negative. The transmittance (T) is the fraction of IR light that is transmitted through the sample is given by the equation $T = P/P_0$, where P is the amount of light that penetrates the sample and P_0 is the amount that penetrates through dry N_2, containing no sample. The percent transmittance, or %T, is just Tx100. The absorbance, A, is related to the %T by equation 11.

Equation 11: $A = \log_{10} (100/\%T)$

That is, $A = 2 - \log_{10}(\%T)$, so if %T = 10%, $A = 2 - \log_{10}(10)$, or 2 minus 1, which is equal to 1 [1]. So, if only 10% of the IR light at a given wavelength (or wavenumber) is transmitted through a relatively concentrated sample, the absorbance is 1. As predicted by Beer's law, the concentration of a sample is directly proportional to the absorbance, not the %T [1].

There is also a region from about 1500 to 500 cm^{-1} that is unique for each compound [1]. So, it is called the fingerprint region. It can be used to help verify the identity of an analyte. Like an MS, many FTIR spectrometers have a library of spectra on their computer. The analyst can search the library and find the compounds that best to matches the spectrum of a sample [1].

NMR is even more useful for verifying the identity of an organic compound [1]. Although the word nuclear appears in its name, this does not imply radioactivity, even though nuclear implies radioactivity in the field of medicine. To avoid confusion and fear in patients, the type of NMR spectrometer that is used in medicine to produce images is just called magnetic resonance imaging, or MRI. There is no radioactivity in MRI. However, to verify the identity of an analyte or to do metabolomic studies, an NMR spectrum is needed - not an image. NMR spectroscopy is the study of the interaction between matter and electromagnetic radiation in the radio frequency (rf) region [1, 6].

When an organic compound or biochemical is placed in a strong magnetic field, the ^{1}H, ^{15}N and ^{13}C nuclei in it become magnetized so they can absorb rf energy [1, 5]. The frequencies that are absorbed are the chemical shifts. The exact frequencies absorbed depend on the chemical environment of the nuclei that absorb them. The more rf energy that is absorbed, the larger the signals (or peaks) that appear in the spectra. So, rf transmitters can be set at the range of frequencies that ^{1}H, ^{15}N and ^{13}C

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nuclei absorb. In addition, clever sequences of rf pulses can be used to transfer magnetization from one ^{1}H to another ^{1}H and/or to ^{15}N and ^{13}C nuclei that are located next to them or are close to them [1, 6].

Some nuclei, such as ^{1}H, ^{2}H, ^{15}N and ^{13}C, will interact with a strong magnetic field [1, 6]. These nuclei will move like a top. When a top spins, it starts to precess or wobble around the axis of rapid rotation, and so do magnetically susceptible nuclei. Some precess parallel to the magnetic field and are in the ground state (lower energy) and others precess anti-parallel to the magnetic field and are in the excited state [1, 6].

Most nuclei are in the ground state when they align themselves parallel to the magnetic field [1, 6]. Unlike UV and IR, there is only a small difference in energy between the ground and excited states. Almost as many nuclei are aligned anti-parallel to the magnetic field and are in an excited state. UV and IR light have much more energy than NMR. The difference in electronic and vibrational energy levels is much larger the differences in energy levels of nuclei. So, when a sample is irradiated with UV or IR light, many electrons in the analyte will become excited and there is plenty of room in the excited energy level to hold them. However, in NMR far fewer nuclei can be excited before the excited state becomes saturated and no more rf energy can be absorbed. This makes UV and IR relatively sensitive techniques, when compared to NMR. Only a small fraction of the nuclei in a sample can absorb rf before the excited state becomes saturated with energy. As a result, they can absorb no more rf energy [1, 6].

If the rf transmitter is turned on long enough (10 – 20 μs), the nuclei will be tipped 90^{o} and will take a while to relax to the ground state (1 – 8 s for ^{1}H and up to 2 min for ^{13}C) [1, 6]. So, NMR spectroscopists usually use a pulse delay before irradiating the sample with another pulse of rf energy. On the other hand, if the rf transmitter is turned on for less time, the nuclei are tipped at less of an angle. For example, if 10 μs is needed for a 90^{o} pulse, 3.3 μs will be needed for a 30^{o} pulse. When the 30^{o} pulse is used, less time will be needed for the nuclei to relax to the ground state. So, a shorter pulse delay is usually used so more acquisitions (or scans) can be acquired [1, 6].

This means that there is as important difference between FTIR and NMR [1]. In FTIR the analyst can irradiate the sample with IR light several times with no delay between subsequent scans. The electrons rapidly relax to the ground state after the first scan, so they can absorb the same amount of IR light on the second and each subsequent scan. On the other hand, it can take a few seconds or even minutes for the nuclei to relax to the ground state in NMR. So, there should be a delay between rf

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pulses. If the rf transmitter is left on all the time, the sample quickly becomes saturated with rf energy and the nuclei will absorb no more energy [1, 6]. Still, in both FTIR and NMR the S/N increases with the square root of the number of acquisitions [1].

FTIR, MS and NMR are complementary techniques [1]. FTIR can identify functional groups that are present in the analyte. FTIR is especially sensitive for –OH groups. MS can provide the molecular weight and the molecular formula. NMR can tell the number of hydrogens and carbons that are present. In ^{1}H NMR, one observes the hydrogens (or protons), so this is called proton NMR. Different kinds of ^{1}H absorb at different rf. For example, the ^{1}H nuclei in tetramethylsilane (TMS, $Si(CH_3)_4$) absorb lower rf than other ^{1}H in analytes that don't contain silicon. So, TMS is often used as a standard to compare chemical shifts with other ^{1}H nuclei. Methyl groups that are not attached to a Si absorb slightly higher rf. Methylenes (CH_2) absorb slightly higher rf and methines (CH) even higher. If there is a nitrogen, oxygen or other electronegative atom in the molecule, they cause the nearby ^{1}H nuclei to absorb higher energy rf. Hydrogens attached to carbon-carbon double bonds (such as CH_2=CHR) absorb even higher rf energy. Phenyl hydrogens absorb even higher. Hydrogens in an aldehyde or carboxylic acid absorb even higher rf energy [1].

Another important difference between NMR and both UV or IR is the way the spectra are displayed [1]. In all three, the y-axis shows the amount of energy that the sample absorbs. Based on Beer's law, amount of energy absorbed (or the size of the peaks) is directly proportional to the concentration of the sample that is UV or IR active. However, different functional groups and compounds absorb UV and/or IR light with different sensitivities, or absorptivities. So, standards are required for quantitative analysis in UV and FTIR. However, in ^{1}H-NMR, all the nuclei have the same absorptivity (even though it is not called absorptivity in NMR). So, the analyst can determine the relative amount of each type of hydrogen in the sample. That is, in ^{1}H NMR, the peak areas of the signals tells the analyst the relative abundance of each all the hydrogens that are in the sample. However, the x-axis is different in NMR. In UV, the x-axis displays the wavelength, in nm, over a range of about 190 – 340 nm, with the lowest wavelength on the left and the highest on the right. In FTIR the x-axis can be either wavelength (in microns, or μ) or in frequency (also called wavenumbers) in cm^{-1}. If it is displayed in wavelengths, the lowest wavelength is on the left and the highest is on the right of the x-axis. On the other hand, if the x-axis in an IR spectrum is expressed in frequencies,

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the lowest wavenumber (in cm^{-1}) is on the right and the highest is on the left [1].

In NMR, the x-axis shows the frequencies that are absorbed. Unlike UV or IR, in which there is a relatively broad range of wavelengths or frequencies, the range is very small in NMR. The range depends on the strength of the magnet that is used to generate the magnetic field.

When a physicist measures the strength of a magnet, he or she uses units called a Tesla, or T. For example, the strength of a toy magnet is about 0.001 T. The magnet in an NMR can be anywhere between 1.4 and 21.1 T. At 1.4 T, the ^{1}H nucleus will absorb radio frequencies near 60 MegaHerz (MHz), or 60 million cycles per second. So, NMR spectroscopists often call such an instrument a 60 MHz NMR. At 21.1 T, the ^{1}H nucleus will absorb radio frequencies near 900 MHz, so NMR spectroscopists often call such an instrument a 900 MHz NMR. Unlike UV or IR, in which molecules can absorb at wavelengths over a two to ten-fold range (190-280 nm or 1–10 μ), nuclei in NMR absorb over a much, much shorter range of wavelengths or frequencies. For example, if tetramethyl silane, or TMS, absorbs at 900.0000 MHz, a methyl group in an alkane will absorb near 900.0009 MHz, and phenyl hydrogens near 900.0063 MHz. It would be very inconvenient to start the x-axis at 900.0000 and go to 900.0070 MHz. To make them fit on a single spectrum, you would have to write the numbers so small that it would be nearly impossible to read. Instead, NMR spectroscopists (or their computers) calculate frequencies in parts per million, or ppm. Thus, the difference between 900.0000 and 900.0009 MHz is 0.0009, or $9x10^{-4}$ MHz. When you divide this by the frequency at which the methyl groups in TMS absorb, $9x10^{2}$, you get 10^{-6}, or one ppm. Phenyl protons absorb at or near 7 to 8 ppm. The location of the peak is called the chemical shift. That is because the rf that is absorbed is shifted slightly from the standard, TMS. A compound that was on a recent MCAT (Medical College Aptitude Test) test was succinic anhydride. Its structure is shown in Figure 10. Because of molecular symmetry, it produces just one peak in the ^{1}H-NMR and two peaks in the ^{13}C-NMR. That is, there is only one type of hydrogen ($C\mathbf{H}_2$), and two types of carbons ($\mathbf{C}H_2$ and $\mathbf{C}$=O).

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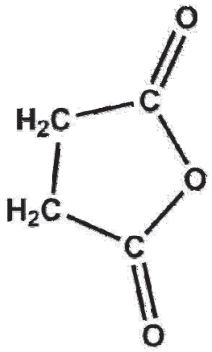

Figure 10. 2D structure of succinic anhydride.

Note also that samples are dissolved in a deuterated solvent before acquiring an NMR spectrum. This is because the NMR spectra of liquid solutions produce much sharper peaks and excellent quantitation, when compared to the spectra that can be obtained for solids. Solids produce much broader peaks than solutions. The medical form of NMR, called magnetic resonance imaging (MRI) takes advantage of the different line widths of fluid water (in blood) and water that is not so fluid (in tumors).

Also, in solutions NMR, the solvent is very important in maintaining the accuracy of the ppm scale of frequencies. As you might imagine, it is very difficult to keep a radio frequency constant to an accuracy of 0.001 ppm (i.e. 900.0000 ± 0.0009 MHz). So, a lock solvent is needed. Thus, one uses a solvent that contains deuterium, or ^{2}H. Deuterium is often abbreviated by the letter D. Thus, D_2O is deuterated water and $CDCl_3$ is deuterated chloroform. When the acronym for the solvent contains the letter D, as in DMSO, or dimethylsulfoxide, the deuterated form is called DMSO-d_6, indicating that all six methyl hydrogens are the deuterium isotope. So, an NMR spectrometer has at least two rf generators. One can be set to the frequency at which ^{1}H absorbs, and the other is set at the frequency that ^{2}H absorbs. The ^{1}H transmitter is pulsed off and on, as described earlier. The ^{2}H transmitter is left on all the time, so the ^{2}H becomes saturated and absorbs the same amount of energy throughout the experiment. The deuterated solvent is called the lock solvent, since it helps lock, or keep constant, the radio frequencies. Thus, to obtain the NMR spectra of liquid solutions one almost always uses a lock solvent.

Another important piece of information that is obtained in a ^{1}H NMR spectrum is the number of hydrogens that are adjacent to each other in a molecule. The structure of sucrose is shown in Figure 11. Its ^{1}H and ^{13}C

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NMR spectra are shown in Figures 12 and 13. Note that the signals in the ^{1}H spectrum are broad and overlapping. Also, the analyst can determine the area under each signal (the integrals) in the ^{1}H spectrum to determine the relative abundances of each type of hydrogen in the sample. The largest signal (at about 4.8 ppm) is not due to any of the hydrogens in sucrose. It is due to the small amount (0.1%) of H_2O in the 99.9% deuterated water that was used to dissolve the sucrose. In contrast, the ^{13}C spectrum contains 12 sharp signals due to the 12 carbons in sucrose. So, ^{13}C NMR spectra are relatively easy to interpret. They tell the analyst how many different types of carbons are in the compound being analyzed. It's also possible to acquire 2D-NMR spectra to obtain more information. For example, heteronuclear ^{1}H-^{13}C NMR spectra can tell the analyst which hydrogens are attached to which carbons. A homonuclear 2D ^{1}H spectrum based on the NOEs is called a NOESY spectrum. It can tell the analyst which hydrogens are coupled with each other because they are attached to adjacent carbons. When this information is coupled with the exact molecular weight and molecular formula obtained from MS, the analyst can unequivocally identify the compound.

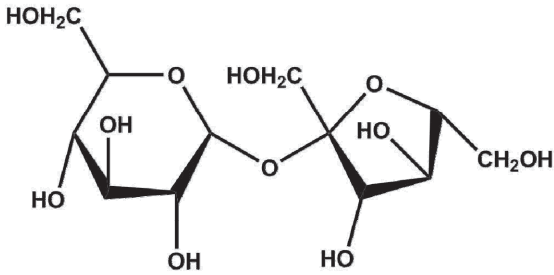

Figure 11. 2D structure of sucrose.

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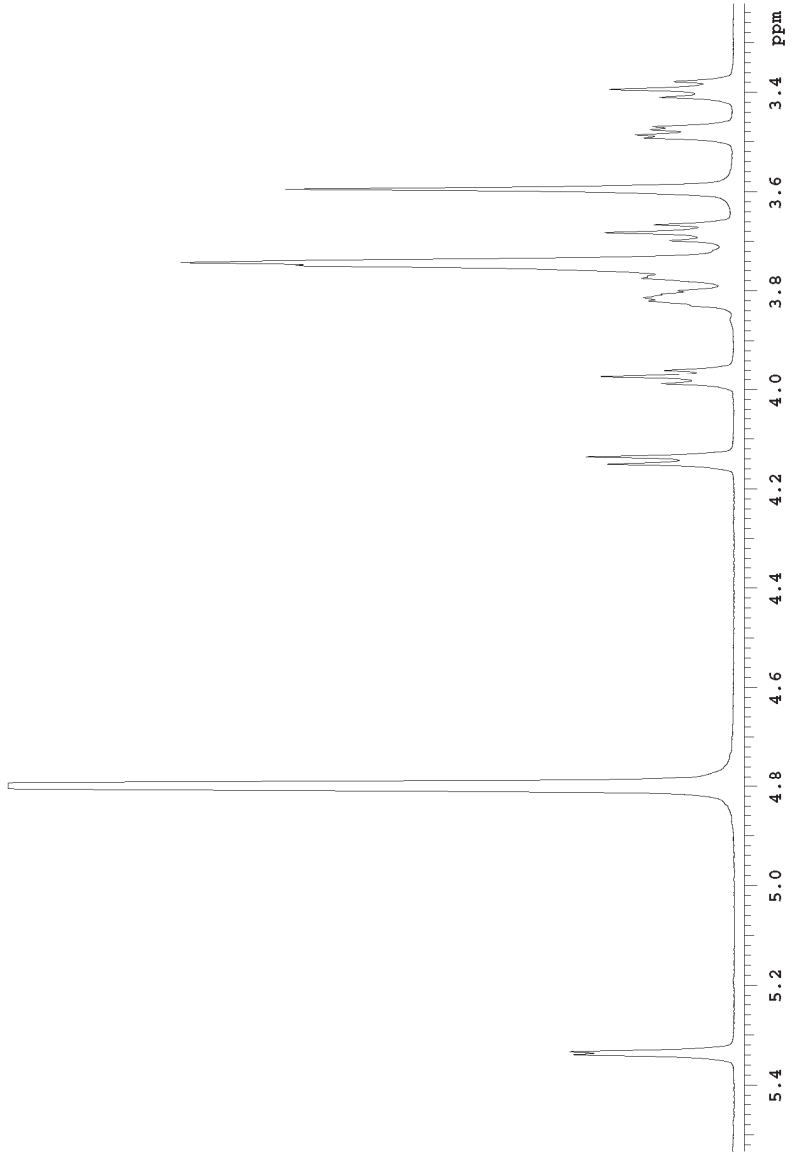

Figure 12. ^{1}H-NMR spectrum of sucrose in 99.9% deuterated water (D_2O), provided by Professor Rensheng Luo from the University of Missouri – St. Louis.

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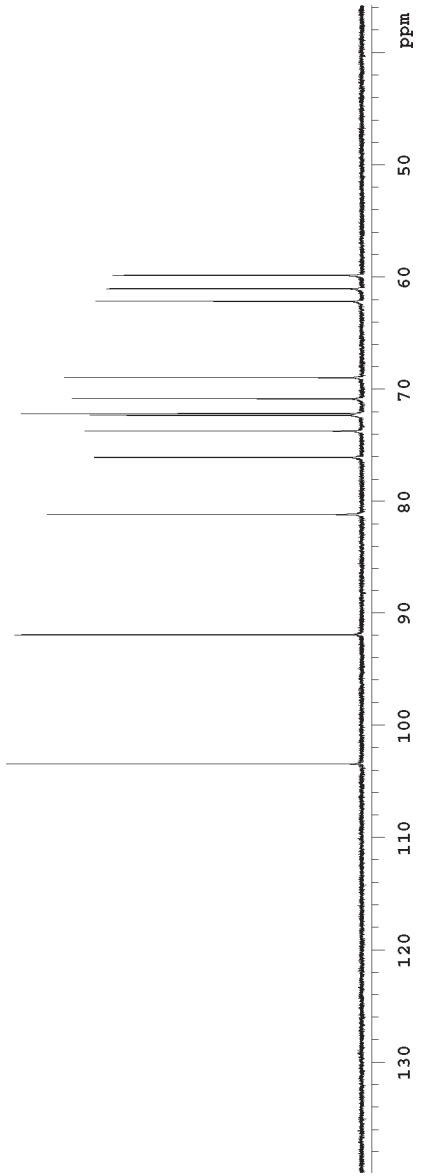

Figure 13. ^{1}H decoupled ^{13}C-NMR spectrum of sucrose in 99.9% deuterated water (D_2O), provided by Professor Rensheng Luo from the University of Missouri – St. Louis.

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The exact location of the signals produced by hydrogens other than any –OH groups in a sample can also vary a little bit, depending on the concentration of the sample and the ionic strength if an aqueous solution is being analyzed. The location of peaks due to hydrogens in –OH groups will vary considerably, depending on the solvent and moisture content. It can depend on the concentrations of ions and salts in the sample. This can make the identification of compounds difficult. For example, one important use of ^{1}H NMR is the analysis of urine for metabolites. Since urine from different people or different animals will have different concentrations of salts, ^{1}H peaks can move around a little. To correct for this, the analyst looks for large peaks from compounds like creatinine, that are in every urine sample. Instead of using TMS (which is insoluble in water) as a standard to set the frequencies, or chemical shifts, one uses creatinine. This can be confusing to analysts who have only done GC, HPLC, GC-MS, LC-MS or UV spectrophotometry. Such analysts are used to following methods that require that each standard and each sample be analyzed in exactly the same way. For example, an HPLC method may require that the analyst determine the size of a peak that elutes at exactly 10.52 minutes. She might be required to set the computer to start integrating the peak at 10.32 min and stop the integration at 10.72 min. Every sample will be analyzed this way, since the HPLC peak will not move. If this same analyst is asked to analyze urine or blood samples by NMR, though, the peak of interest can move around slightly. Thus, the method should be written so that one starts the integration at about 0.1 ppm before the peak begins and stop the integration 0.1 ppm after the peak ends.

Just like rf transmitters used in radio communications and entertainment, the rf transmitter in an NMR can be set to transmit different frequencies. Thus, one can set the transmitter to the frequency for ^{1}H or for the frequency of ^{13}C. Because ^{13}C is a heavier nucleus and has a smaller magnetic moment, it doesn't precess as fast as ^{1}H, so the transmitter must be set at a lower frequency. The magnetic moments of ^{1}H and ^{13}C are 0.7022 and 2.7927, respectively. So, ^{13}C nuclei in a 100 MHz NMR will absorb at or near 25.144 MHz, while ^{1}H nuclei will absorb near 100.000 MHz.

Similar to ^{1}H NMR, hydrogens can be coupled to ^{13}C and ^{13}C nuclei can be coupled to each other. Since the natural abundance of ^{1}H is over 99%, coupling between hydrogens and carbons is readily apparent. A CH_3 will become a quartet in a proton-coupled ^{13}C-NMR spectrum. A CH_2 will be a triplet, a CH a doublet and a carbon with no hydrogens attached will be a singlet [1, 6]. However, this produces many peaks, which can make

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interpretation difficult and can decrease the sensitivity. So, usually ^{1}H-decoupled ^{13}C spectra are obtained, like the one shown in Figure 13. In contrast, the natural abundance of ^{13}C is only 1.1%. Thus, only 1.1% of the carbons will be detected. Still, modern instruments can detect this coupling. Unlike ^{1}H NMR in which both peaks of a doublet are the same size, multiplets due to ^{13}C carbons coupling to other ^{13}C carbons produce a single large peak and additional peaks that are only about 1.1% of the size of the larger peak, so they are seldom seen [1, 6].

Usually, one does not want to see carbon peaks being split by attached hydrogens [1, 6]. Not only does this make the spectrum more complicated, but it makes the sensitivity worse. For example, a CH_3 peak produces four peaks when protons are coupled, but only one peak when the protons are decoupled. So, NMR spectroscopists usually acquire ^{1}H-decoupled ^{13}C NMR spectra. An rf transmitter is set to the frequency that ^{1}H absorbs and is left on throughout the acquisition of spectra, so a CH_3 produces only one peak. Also, when the ^{1}H is decoupled, it transfers its magnetization to the carbon, increasing the size of the carbon peak. This is called Nuclear Overhauser Enhancement, or NOE. It can increase the size of the peak by a factor of 2-3, depending on the number of hydrogens (or protons) attached to the carbon. Thus, ^{1}H decoupling can increase the size of the peak due to CH_3 by a factor of twelve (four times three), since the signal that would be split into four (in ^{1}H-coupled spectra) is all one signal and there is a NOE of about three [1, 6].

NOE can also be used to identify hydrogens that are close to each other in a complex structure, such as a protein or a piece of DNA [1, 6]. In the 1960s and 1970s, when computer memory was measured in kilobytes, scientists could only transmit a very narrow range of radio frequencies that would be absorbed by only one of the hydrogens in a compound. This saturated that hydrogen with rf energy, so that it was no longer coupled to the other hydrogens. The nearby hydrogens produced larger peaks, due to NOE. By doing one experiment at a time, spectroscopists could tell which hydrogens were next to which other hydrogens. As computers and rf transmitters improved, it became possible to do a two-dimensional NMR experiment in which all hydrogens (^{1}H) were irradiated with rf pulses at the same time and there were appropriate pulse delays. This experiment is called Nuclear Overhauser Enhancement Spectroscopy, or NOESY. It is very useful when determining the structures of proteins, DNA, RNA and other larger molecules. The Protein Data Bank has many structures of proteins that were determined by NMR [1, 6].

A slight modification of the NOE is saturation-transfer difference (STD) NMR [1, 6]. It is used to study the binding of a ligand or potential

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drug to a protein receptor or enzyme. If the ligand binds rather weakly (K_D 10^{-8} to 10^{-3} M^{-1}), there will be an exchange between the bound and free ligand. The ^1H nuclei in the receptor are selectively saturated by an rf transmitter in a region of the ^1H-NMR spectrum that contains resonances (or peaks) due to the protein. If the ligand binds, the magnetic saturation will be transferred to the part of the ligand that does the binding. A control spectrum is also acquired by saturating a region of the spectrum that does not contain any resonances. Then the spectrum of the saturated signals is subtracted from the control. If other compounds are present, but do not bind to the target protein, there will be no difference in their NMR spectrum. So, the difference spectrum will only show peaks (resonances) due to the part of the ligand that binds to the protein. The target protein is present at a much lower concentration than the ligand, so its resonances do not appear in the spectrum. So, it can be a very useful method for studying the binding of relatively small molecules (molecular weight <300) to target proteins. This makes STD NMR a powerful tool for fragment-based drug discovery, which was discussed in Chapter 3. This method can be automated with modern NMRs, making it very useful for high throughput screening of many compounds. It was shown recently to be able to screen many compounds for their abilities to inhibit important protein-protein interactions [1, 6].

A variant of STD saturates water instead of the target protein. It is called water ligand observed gradient spectroscopy or WaterLOGSY [7]. Magnetization is transferred from bulk water to the ligand through hydrogens (^1H) both within and far from the binding site of the ligand, as well as from tightly-bound waters in the binding site on the protein [7]. Only those ligands that bind to the protein receive magnetization from both types of water.

Also, when trying to identify an unknown compound it is very useful to know how many hydrogens are attached to each carbon [1, 6]. Instead of acquiring a ^1H coupled spectrum, one does spectral editing. Methods such as the attached proton test (APT) and distortionless enhancement by polarization transfer (DEPT) are used [1, 6].

There are also some two-dimensional NMR experiments that can be done to determine which protons are connected to which carbons [1, 6]. These are called heteronuclear (i.e. ^1H-^{13}C) correlation, or HETCOR experiments. It is also possible to do similar experiments with ^{14}N, so that one can tell which nitrogens in a protein are attached to which carbons. This helps in determining the structure of the protein, DNA or RNA.

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^{13}C NMR is especially useful in telling the analyst how many different kinds of carbons there are in a compound. For example, bisphenol A is a symmetric compound. The structure of BPA is shown in Figure 14.

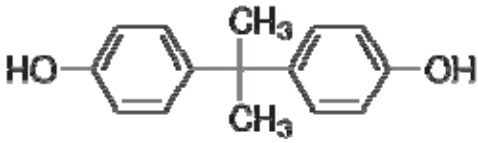

Figure 14. Structure of Bisphenol A, or BPA

There is a single C with no hydrogens at the center of the molecule. It produces one resonance or peak. It has two CH_3 and two substituted phenyl groups attached. The two CH_3 are indistinguishable from each other and produce only one peak, about twice the size of the peak due to the single C. The phenyl groups are also indistinguishable from each other. The phenyl groups each have a hydroxyl (–OH) group on the *para* position. Thus, there is only one type of CH_3 carbon, one C and four types of phenyl carbons, in a mole ratio of 2:1:2:4:4:2. The ^{1}H-decoupled ^{13}C NMR spectrum of bisphenol A will have only six peaks. The relative sizes of these peaks will be about 2:1:2:4:4:2, if the spectrum is acquired carefully, in a quantitative way.

To do quantitative ^{13}C-NMR, one must be concerned with NOE and the long relaxation time needed for ^{13}C nuclei [1, 6]. As mentioned before, the size of carbon peak will increase if there are hydrogens attached to it. So, if the ^{1}H-decoupler is turned on all the time, the peaks due to CH_3 will be larger than predicted by stoichiometry alone. Also, it takes much longer for a ^{13}C nucleus to relax to the ground state that a ^{1}H nucleus and some ^{13}C atoms relax faster than others. In quantitative ^{1}H NMR, a delay of 1–8 seconds is usually sufficient, to ensure that all nuclei relax to the ground state between pulses of radio frequency. However, ^{13}C nuclei require much longer, up to 2 min [1, 6].

One way around this is to turn the ^{13}C radio frequency transmitter on for a shorter time, turn on the ^{1}H decoupler only during acquisition, and turn it off during the pulse delays [1, 6]. This is called gated decoupling. When the ^{13}C radio frequency transmitter is turned on for about 10 μseconds, the ^{13}C nuclei are flipped 90^{o} so that they precess antiparallel to the magnetic field. During the pulse delay, these nuclei relax to the ground state. If the ^{13}C radio frequency transmitter is turned on for less time, it will tip the nuclei by a smaller angle and it will take them less time to

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relax to the ground state. Shorter pulse delays will be needed to do this. Thus, one often uses a 20 – 45^{o} pulse width and a pulse delay of about 0.5 sec to obtain nearly quantitative results, as long as gated decoupling is used [1, 6].

Thus, ^{1}H NMR provides important information about the hydrogens and ^{13}C NMR about the carbons [1, 6]. Other nuclei of interest to medicinal chemists that can be seen by NMR include ^{14}N, ^{15}N, ^{19}F, ^{29}Si and ^{31}P. If nitrogen, fluorine, silicon or phosphorus atoms are present in the sample, an NMR spectrum can help determine the structure of the compound. When this information is combined with the molecular formula (from mass spectrometry) and the information about functional groups (from FTIR), it is often easy to identify the compound. Also, most modern FTIR and mass spectrometers have libraries of spectra on the computer's hard drive. All the analyst has to do is point and click to find compounds that match most closely the spectra that have just been acquired. Also, one can do computer calculations of NMR spectra, to predict chemical shifts. Some calculations don't even require a computer. Such calculations are based on additivity constants. That is, the ^{13}C chemical shift of benzene is 128.5 ppm. If a functional group such as a –OCH_3, an –OH or a ketone is attached to a benzene, each group will affect the chemical shifts of the carbons on the substituted benzene. Empirically determined additivity constants have been reported and they are based on libraries containing the spectra of many compounds [6]. The additivity constants for a ketone, such as the aceto group (–$COCH_3$) in apocynin are: C1 +9.1, *ortho* +0.1, *meta* 0.0 and *para* +4.2. That is, the aceto group increases the chemical shift of the carbon to which it is attached (C-1) by 9.1 ppm. It increases the chemical shift of both of the *ortho* carbons by 0.1 ppm. It doesn't change the chemical shift of the *meta* carbons and it increases chemical shift of the *para* carbon by 4.2 ppm. The additivity constants for a phenolic OH are: "C1 +26.9, *ortho* -12.7, *meta* +1.4, *para*-7.3. The additivity constants for a methoxy (-OCH_3) are: +31.4 (C1, or the carbon to which –OCH_3 is attached), -14.4 (*ortho*), +1.0 (*meta*) and -7.7 (*para*) [6].

NMR can be used to help determine the structures of drugs and proteins targets for the drugs [1, 8, 9]. If a protein is very large (molecular weight over 40 000), X-ray crystallography is needed to determine its structure. For larger structures, such as viruses, electron microscopy at low temperatures (cryo-EM) is often used. Thus, the protein data bank contains the structures of many biopolymers and larger structures that were solved by X-ray crystallography or cryo-EM [1, 6].

NMR can also be used to study interactions between drugs and proteins [8, 9]. That is, when a test compound binds to an enzyme or

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receptor, it will change the chemical shifts of the drug and can change the line width of the peaks produced. Narrower line widths are due to more flexible compounds. The line width can increase when test compounds bind to a target protein. Moreover, magnetization can be transferred from hydrogens on the test compound to those on the binding site of the protein and observed by NOE. This can be used to detect, characterize and quantitate binding sites and identify flexible parts of proteins [8, 9]. In fact, parts of some proteins (such as ubiquitin) have regions that are quite flexible, so they can bind many different ligands. NMR can also be used to dissect ligand binding into fragments and look at diverse compounds with slight alterations to each fragment, to predict binding. This approach has been used to identify drugs that are in current clinical trials [9].

A1.8 Magnetic Resonance Imaging, MRI

A form of NMR called MRI is used to look at living cells, organisms, and even human patients [1]. MRI observes ^{1}H nuclei and detects the most abundant chemical in the body, H_2O. MRI is used by physicians to image tumors and other irregularities in patients. The ^{1}H-NMR spectrum of H_2O is extremely simple. If analyzing aqueous solutions in a test tube (or NMR tube), H_2O will produce a single sharp peak. However, in living systems, not all H_2O molecules are identical. Much or the water in our bodies is quite fluid and mobile, like the water in blood. On the other hand, water in a tumor is less fluid. Free, liquid water with low viscosity produces sharper NMR (or MRI) signals than water that is more viscous in tumors. So, MRI is an excellent technique for diagnosing cancer and monitoring the size of a tumor in clinical studies of anticancer drugs. For example, some anti-cancer drugs work by restricting blood flow to or from the tumor. Cancer cells can't survive without the O_2 that blood provides. Angiogenesis can be inhibited by anti-angiogenic agents, which inhibit blood flow into the tumor. On the other hand, vascular disrupting agents target existing blood vessels and disrupt blood flow out of the tumor. Both can be monitored by a new form of MRI called dynamic contrast enhancement (DCE)-MRI. First, the tumor is located by conventional MRI. Then, DCE-MRI is used to provide doctors with two- or three-dimensional images that are taken as the patient is being given an IV solution of a paramagnetic contrast agent, based on the paramagnetic metal, gadolinium. Continuous DCE-MRI scans are made every five to ten seconds for 15 minutes. Gadolinium concentrates at the tumor. As it circulates through the tumor's blood vessels, images of blood flow appear.

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Blood flow can be quantitated and used as a biomarker for progress in treating or curing the cancer [1].

MRI can also be used to obtain images of brains that don't have tumors in them [1, 10]. MRI can be used with magneto-encephelography (MEG), which measures the tiny magnetic fields (femtoTesla range) created by active areas in the brain. The combination of MRI and MEG is being used to study cognitive function and brain activities in people as they perform different tasks and have a variety of thoughts or mental images. In addition, functional MRI (fMRI) can study brain activity when people are either resting, performing certain activities, or thinking about an emotional subject [1, 10].

References

1. Smith RE. *Medicinal Chemistry – Fusion of Traditional and Western Medicine, 2nd ed*. Bentham Science, Sharjah, U.A.E. **2014**.
2. Ferguson CN, Benchaar SA, Miao Z, Loo JA, Chen H. Direct ionization of large proteins and protein complexes by desorption electrospray ionization-mass spectrometry. *Anal. Chem*. **2011**, *83*, 6468-6473.
3. Haag M, Schmidt A, Brügger B. Quantification of signaling lipids by nano-electrospray ionization tandem mass spectrometry (Nano-ESI MS/MS). *Metabolites* **2012**, *2*, 57-76.
4. Smoker M. Personal communication, FDA, **2015**.
5. Heikes DL, Jensen SR, Fleming-Jones ME. Purge and trap extraction with GC-MS determination of volatile organic compounds in table-ready foods. *J. Agric. Food Chem*. **1995**, *43*, 2869-2875.
6. Wehrli FW, Marchand AP, Wehrli S. *Interpretation of Carbon-13 NMR Spectra, 2nd ed*. John Wiley and Sons, New York **1988**.
7. Dalvit C, Fogliatto GP, Stewart A, Veronesi M, Stockman B. WaterLOGSY as a method for primary NMR screening; Practical aspects and range of applicability. *J. Biomol. NMR* **2001**, *21*, 349-359.
8. Mailavaram RP, Devarakonda M. NMR-A gateway to drug discovery. *Struct. Activ. Relat. Drug. Devel. NMR Spectr*. **2011**, *1*, 1-35.
9. Consonni R, Veronese M. NMR Screening methods in fragment-based drug discovery. *Drug Devel. NMR Spectr*. **2011**, *1*, 67-101.
10. Taylor JR, Williams N, Cusack R, Auer T, Shafto MA, Dixon M et al. The Cambridge Centre for Ageing and Neuroscience (Cam-

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CAN) data repository: Structural and functional MRI, MEG, and cognitive data from a cross-sectional adult lifespan sample. *NueroImage* **2017**, *144*, 262-269.

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ABBREVIATIONS AND ACRONYMS USED

Note: Some abbreviations stand for very different things, so both definitions are given below

1,25-D: 1α,25-dihydroxyvitamin D3
(1,25(OH)2D): 1α,25-dihydroxyvitamin D3
2-AG: 2-arachidonoylglycerol
2-DG: 2-deoxy-D-glucose
5-HT: 5-hydroxytryptamine
25D: 25-hydroxyvitamin D3
A: Absorbance
A: Adenine
AA: Arachidonic acid
ABC: ATP-binding cassette transporter, ABCF2
ABP: Activity-based proteomics
ACE: Angiotensin converting enzyme
ACEIs: ACE inhibitors
Ach: Acetyl choline
AchE: Acetylcholine esterase
ACOG: American College of Obstetricians and Gynecologists
ACS GCI PR: American Chemical Society's Green Chemistry Institute Pharmaceutical Roundtable
ACTH: Adrenocorticotropic hormone
ACToR: Aggregated Computational Toxicology Resource
AD: Alzheimer's disease
ADC: Antibody-drug conjugate
ADEPT: Antibody directed enzyme prodrug therapy
ADHD: Attention deficit hyperactivity disorder
ADMA: Asymmetric dimethylarginine
ADME: Absorption, distribution, metabolism and elimination
ADMS: Antimicrobial defense molecules
ADP: Adenosine diphosphate
AEA: Anandamide
AGEs: Advanced glycation end products
AhR: Aryl hydrocarbon receptor
AKI: Acute kidney injury
Al: Aluminum

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ALCL: Anaplastic large cell lymphoma
ALCOA: Attributable, Legible, Contemporaneous, Original and Accurate
ALL: Acute lymphoblastic leukemia
ALS: Amyotrophic lateral sclerosis
AMA: American Medical Association
AMD: Age-related macular degeneration
AMP: Anti-microbial peptides or adenosine monophosphate
AMPK: AMP-activated protein kinase
ANF: Atrial natriuretic factor
ANG-1: Angiopoietin-1
ANS: Autonomic nervous system
AngII: Angiotensin II
AT1R, AT2R: AngII type 1 and type 2 receptors
AOK: Allgemeine Ortskrankenkassen
AOPs: Adverse Outcome Pathways
APC: Antigen presenting cell
APCI: Atmospheric pressure chemical ionization
API: Active pharmaceutical ingredient
APM: Advanced Alternative Payment Model
ARB: Antibiotic resistance breakers
ARB: AT1R blockers
ARE: Antioxidant response element
As: Arsenic
ASAP-HSQC: HSQC as soon as possible
ASD: Autism spectrum disorder
ASO: Antisense oligonucleotide
ASPE: Assistant Secretary for Planning and Evaluation
ASVD: Atherosclerotic vascular disease
AT: Adipose tissue
AT1R and AT2R: AngII type 1 and AngII type 2 receptors
ATM: Ataxia telangiectasia-mutated
b: Path length of the light in the spectrophotometer
B: Boron
BADGE: Bisphenol A diglycidyl ether
BAK: Bcl-2 antagonist killer 1
BAT: Brown adipose tissue
BBB: Blood-brain barrier
Bcl-2: B-cell lymphoma 2
BCNU: bis-chloroethylnitrosourea
Bcr-Abl: Breakpoint cluster and Abelson protein kinase
BDNF: Brain-derived neurotrophic factor

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Be: Beryllium
BEST: Biochemical Efficacy and Safety Trial
bFGF: basic Fibroblast Growth Factor
BF_3: Boron trifluoride
B&MGF: Bill & Melinda Gates Foundation
BID: BH3-interacting domain death agonist
BIM: Bcl-2-interacting mediator of cell death
BITE: Bi-specific T cell engager
BMI: Body mass index
BMP: Bone morphogenic protein
BNP: Brain natriuretic peptide
BPH: Benign prostate hyperplasia
BPN: NIH Blueprint for Neuroscience Research
Br: Bromine
BSSL: Bile salt stimulating lipase
C: Cytosine
^{o}C: Degrees Centigrade
C1INH: C1 esterase inhibitor
CAD: Computer-aided design
CADD: Computer-aided drug discovery
CAFO: Concentrated animal feeding operations
CAM: Computer-aided manufacturing
CAMK: Ca^{2+}/calmodulin-dependent kinase
CAP: Catabolite activator protein
CAPA: Corrective Actions and Preventive Actions
CAR: Chimeric antigen receptor
CAR-T: CAR-Tisagenlecleucel
Cas: CRISPR-**as**sociated
CAS: Complex Adaptive Systems
CB_1 and CB2: Cannabinoid receptors
CBER: Center for Biologics Evaluation and Research (part of the FDA)
CBP: cAMP binding protein
CCD: Charge coupled detector
CCHN: Community Child Health Network
CCK: Cholecystokinin
CCP: Critical control points
CCR5: C-C (cysteine-cysteine) chemokine receptor type 5
Cd: Cadmium
CDA: Critical Discourse Analysis
CDB: cAMP binding domain
cDC: classic Dendritic Cells

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CDC: Centers for Disease Control
$CDCl_3$: Deuterated chloroform
CDER: Center of Drug Evaluation and Research (part of the FDA)
cDNA: complementary DNA
CE: Capillary electrophoresis
CFR: Code of the Federal Register
CGAP: Cancer Genome Anatomy Project
CGCI: Cancer Genome Characterization Initiative
CGEMS: Cancer Genetic Markers of Susceptibility
cGMP: current Good Manufacturing Practices
cGMP: cyclic Guanosine Monophosphate
cGRPP: Current Good Radiopharmacy Practices
CHESCA: Chemical shift covariance analysis
CI: Chemical ionization
CIC: Cancer Immunotherapy Consortium
CKD: Chronic kidney disease
CLL: Chronic myelogenous leukemia
cm: centimeters
cm^{-1}: Cycles per cm
CMap: Connectivity Map
CMH: Ceramide monohexoside
CML: Chronic myelogenous leukemia
CMS: Centers for Medicare & Medicaid Services
CML: Chronic myelogenous leukemia
CMV: Cytomegalovirus
CNP: C-type natriuretic peptide
CNS: Central nervous system
Co: Cobalt
CO Carbon dioxide
CO_2: Carbon dioxide
CO_3^{2-}: Carbonate cation
CoMFA: Comparative molecular field analysis
COPD: Chronic obstructive pulmonary disease
COX: Cyclooxygenase
CoQ10: Coenzyme Q10
CPI: Critical Path Initiative
CPIA: Clinical practice improvement activities
CPR: Candidate phyla radiation group
C-Path: Critical Path Institute
CRF: Corticotropin-releasing factor
CRH: Corticotropin-releasing hormone

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Cr: Chromium
CR: Chromatin regulator
CRF: Corticotropin-releasing factor
CRI: Cancer Research Institute
CRISPR: Clustered regularly interspaced short palindromic repeats
CRP: C-reactive protein
Cryo-EM: Electron microscopy at low (cryogenic) temperatures
CSF: Cerebral spinal fluid
CT: Computed tomography
CTL: Cytotoxic T-lymphocyte
CTLA4: Cytotoxic T-lymphocyte protein 4
CTRPs: C1q/TNF-related proteins
CTTI: Clinical Trials Transformation Initiative
CVD: Cardiovascular disease
CYP: Cytochrome P450
DACHPt: (*trans-l*-1,2-diaminocyclohexane)platinum(II)
DAMP: Danger-associated molecular patterns
DART: Direct analysis in real time
DAXX: Death domain-associated protein
DC: Dendritic cell
DC: Direct current
DCA: Deoxycholic acid
DCDB: Drug Combination Database
DCE-MRI: Dynamic contrast enhancement MRI
DDRNA: Dicer and Drosha-dependent RNAs
DEPT: Distortionless enhancement by polarization transfer
DESI: Desorption electrospray ionization
DGEBA: Diglycidyl ether of bisphenol A
DHA: Docosahexaenoic acid
DHAP: Dihydroxyacetone phosphate
DHF: Dihydrofolate reductase
DMF: Digital microfluidics
DNA: Deoxyribonucelic acid
DNase: DNA hydrolase
DPP: Diabetes Prevention Program
DPP-4: Dipeptidyl peptidase-4
DPT: Diphtheria–pertussis–tetanus
DRG: Dorsal root ganglia
DRI: Dietary Reference Intakes
dsDNA: double strand DNA
dsRNA: double strand RNA

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ε: Absorptivity
ecDNA: extrachromosomal circular DNA
EC: Enterochromaffin cell
ECD: Electron capture detector
ECHHA: 3,4-epoxycyclohexanoic acid
ECHM: 3,4-epoxycyclohexylmethanol
ECM: Extracellular matrix
ED_{50}: Effective dose on 50% of the test animals or human organs on a chip
EDKB: Endocrine disruptor knowledge base
EECs: Enteroendocrine cells
EGCG: Epigallocatechin-3-gallate
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ELCD: Electrolytic conductivity detector
EMA: European Medicines Agency
EMEA: European Medicines Evaluation Agency
EMP: Earth Microbiome Project
ENCODE: Encyclopedia of DNA elements
ENDC: Enteric neural crest–derived cell
ENS: Enteric nervous system
EOCs: Early outgrowth cells
EPA: Environmental Protection Agency and eicosapentaenoic aid (an omega-3 fatty acid)
EPAC: Exchange protein activated by cAMP
EPO: Erythropoietin
EPR: Enhanced permeability and retention
ER: Endoplasmic Reticulum
ERG: ETS-related gene
ERK: Extracellular signal-related kinase
ESC: Embryonic stem cells
ESI: Electrospray ionization
ETBF: Enteric *Bacterioides fragilis*
ETC: Electron transport chain
ETH: Eidgenössiche Technische Hochschule
ETS: Erythroblast transformation-specific family of transcription factors
Eu: Europium
EU: European Union
eV: Electrovolt
EV: Expression variance
F^-: Fluoride

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FAAH: Fatty acid amide hydrolase
FAB: Fast atom bombardment
FGID: Functional gastrointestinal disorders
FDA: Food and Drug Administration
FD&C Act: Food, Drug and Cosmetic Act
FEP: Free energy perturbation
FFLs: Feed forward loops
FHS: Framingham Heart Study
FID: Flame ionization detectors
FKBP: FK506-binding protein
fMRI: functional MRI
FPP: Farnesyl pyrophosphate
FRET: Fluorescence resonance energy transfer
FSH: Follicle stimulating hormone
FTIR: Fourier transform infrared
FTNMR: Fourier transform NMR
G: Gibbs free energy
ΔG: Change in Gibbs free energy
G: Guanine
Ga: Gallium
GABA: Gamma-amino butyric acid
GAL: Green Aspiration Level
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GBA: Gut-brain axis
GCCP: Good Cell Culture Practice
GCP: Good Clinical Practice
Ge: Germanium
GGPP: geranylgeranyl pyrophosphate
GelMA: Gelatin methacryloyl
GGPP: Geranylgeranyl pyrophosphate
GHz: GigaHerz (million cycles per second)
GIP: Glucagon-like insulinotropic peptide and gastric inhibitory peptide
GIT: Gastrointestinal tract
GLP: Good Laboratory Practices
GLP-1: Glucagon-like peptide-1
GMO: Genetically modified organism
GnRH: Gonadotropin-releasing hormone
GPCR: G-protein-coupled receptor
GR: Glutathione reductase
Grx: A small redox enzyme that uses glutathione as a cofactor
GSK: GlaxoSmithKline

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GTPase: Guanosine triphosphatases
GWAS: Genome-wide association studies
h: Planck's constant
H: Histidine
H_2: Hydrogen gas
HCO_3^-: Bicarbonate anion
H_2CO_3: Carbonic acid
H_2CrO_4: Chromic acid
HAART: Highly active antiretroviral therapy
HACCP: Hazard Analysis and Critical Control Points
HATs: Histone acetylases
HbA_{1c}: Glycated hemoglobin
HCAbs: Heavy (H)-chain antibodies
HCS: High content screening
HCV: Hepatitis C virus
HDACs: Histone deacetylases
HDL: High-density lipoprotein
HGC: High gene count (in the gut microbiome)
HER2: Human epidermal growth factor receptor 2
hESCs: Human embryonic stem cells
HETCOR: Heteronuclear correlation
Hg: Mercury
HGT: Horizontal gene transfer
hiPSCs: Human induced pluripotent stem cells
HITS-CLIP: High throughput sequencing of cross linked immunoprecipitation
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
hnRNA: heterogeneous nuclear RNA
HPA axis: Hypothalamic-pituitary-adrenal axis
HPLC: High performance liquid chromatography
HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
HMO: Human milk oligosaccharides
HMO: Health Maintenance Organization
HMP: Human Microbiome Project
HPO_4^{2-}: Monohydrogen phosphate anion
$H_2PO_4^-$: Dihydrogen phosphate anion
hPSCs: Human plutipotent stem cells
HPV: Human papilloma virus
HRMS: High resolution mass spectrometry
HRV2: Human rhinovirus 2 (HRV2

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HSQC: ^{1}H-^{15}N heteronuclear single quantum coherence
HTS: High throughput screening
HUD: Humanitarian Use Device
HUVEC: Humbilical vein endothelial cells
Hz: Cycles per second
I: Isoleucine
I_2: Iodine
IBDs: Inflammatory bowel diseases
IBS: Irritable bowel syndrome
ICG: Initiative for Chemical Genetics
ICP: Inductively coupled plasma spectroscopy
IDH: Isocitrate dehydrogenase
IDP: Intrinsically disordered proteins
IDO: Indoleamine 2,3-dioxygenase
IFN: Interferon
IFN-γ: Interferon-γ
Ig: Immunoglobulin (s in IgE, IgG, IgM)
IκB: Inhibitory protein for NF-κB
IKKs: IκB kinases
IL: Interleukin
ILCs: Innate lymphoid cells
IMPDH: Inosine-5'-monophosphate dehydrogenase
In: Indium
IND: Investigational new drug
IOM: Institute of Medicine
IP_3: Inositol triphosphate
IPA: International Pharmaceutical Abstracts
IPAN: Intrinsic primary afferent neurons
irAEs: immune-related Adverse Events
IRES: Internal ribosomal entry site
irORR: immune-related Overall Response Rate
irDCR: immune-related Disease Control Rate
irPFS: immune-related progression-free survival
irRC: immune-related Response Criteria
iPSCs: induced Pluripotent Stem Cells
IR: Infrared
iRND3: Institute for Rare and Neglected Diseases Drug Discovery
IRB: Internal review board
IRS: Insulin receptor substrate
ISF: Interstitial fluid
ITC: Isothermal calorimetry

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IUPAC: International Union of Pure and Appled Chemists
IVT: *In vitro* transcribed
JNK: Jun N-terminal kinase
k: Boltzmann constant
^{o}K: Degrees Kelvin
Keap1: Kelch-like enoyl-CoA hydratase-associated protein 1
keV: kilo electrovolt
kHz: kilohertz (thousand cycles per second)
K_m: Michaelis-Menten constant in an enzyme-catalyzed reaction
K_w: Autodissociation constant of water
LABS: Linear alkyl benzene sulfonates
LCA: Life Cycle Analysis
LC-HRMS: Liquid chromatography coupled to high resolution mass spectrometry
LC-MS: Liquid chromatography coupled to mass spectrometry
LDIR: Low dose ionizing radiation
LD_{50}: Lethal dose to 50% of the test animals or human organs on a chip
LDL: Low density lipoprotein
L-DOPA: L-3,4-dihydroxypehnylalanine
LED: Light emitting diode light emitting diode
LEARN: Locate, employ, assess, reflect and nominate
LGC: Low gene count (in the gut microbiome)
LH: Luteinizing hormone
LIE: Linear interaction energy method
LILBID: Liquid beam ionization/desorption
lincRNA: long intergenic RNA
LINCS: Library of Integrated Network-Based Cellular Signatures
LINE: Long interspersed element
LMW: Low molecular weight
LNAs: Locked nucleic acids
lncRNA: long non-coding RNA
LOD: Limit of detection
LOPAC®: Library of Pharmacologically Active Compounds
LOQ: Limit of quantitation
LOX: Lipoxygenase
LPS: Lipopolysaccharide
LRTCs: long-range temporal correlations
LT: Leukotriene
LTKB: Liver Toxicity Knowledge Base
M: Methionine
M: Moles per liter

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M2 macrophages: alternatively activated Macrophages
mAb: monoclonal Antibody
MACRA: Medicare Access and CHIP Reauthorization Act
MAGE: Multiplexed Automated Genome Engineering
MALDI-TOF/MS: Matrix assisted laser desorption and ionization with time of flight mass spectrometry
MAO: Monoamine oxidase
MAPK: Mitogen-activated protein kinase
MCAT: Medical College Aptitude Test
MCL: Mast cell leukemia
MCSF: Macrophage colony stimulating factor
MD: Molecular dynamics
MDMA: 3,4-methylenedioxy-methamphetamine
MDSC: Myeloid-derived suppressor cells
MEG: Magneto-encephelography
MEMs: Microelectronic mechanical systems
MEK: Mitogen--activated protein/extracellular signal-regulated kinase
Mg: Magnesium
MGC: Mammalian Gene Collection
MGDG: Monogalactosyldiacylglycerol
mGluR: metabotropic Glutamate Receptor
MHz: MegaHerz
MI: Myocardial infarction
MIE: Molecular initiating event
MILES: Metformin in Longevity Study
MIPS: Merit Based Incentive Programs
MLPCN: Molecular Libraries Probe Production Centers Network
MMAE: Monomethyl auristatin E
MM/PBSA: Molecular mechanics/Poisson-Boltzmann surface area
MPS: Mononuclear phagocyte system
MRM: Multiple reaction monitoring
mRNA messenger RNA
miRNA: micro RNA
MM4TB: More Medicines for Tuberculosis
MMPs: Matrix metalloproteinases
MMR: Measles, mumps and rubella
MMV: Medicines for Malaria Venture
Mn: Manganese
modRNA: modified RNA
MPS: Mononuclear phagocyte system
MRI: Magnetic resonance imaging

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MRSA: Methicillin-resistant *Staphylococcus aureus*
MS: Mass spectrometer and multiple sclerosis
MSC: Mesenchymal stem cell
MSH: Melanocyte stimulating hormone
MTBE: Methyl t-butyl ether
mtDNA: mitochondrial DNA
MTL-CEBPA: dsRNA that is designed to activate the *CEBPA* gene
mTOR: mammalian Target of Rapamycin
mtTFA: mitochondrial Transcription Factor A
MWC: Monod, Wyman and Changeaux
μCCA: Microfluidic microscale cell culture analog
μM: MicroMolar, or 1 μmole per liter of solvent (a unit of concentration of a molecule dissolved in a solvent, where one mole is the molecular weight of the molecule, expressed in grams and is equal to 6.02×10^{23} molecules. So, one μmole is 10^{-6} moles and 1 μM is 1 μmole of a molecule that is dissolved in 1 liter of solvent)
MVN: Metabolic Network Visualizer
N: Avogadro's number
N_2: Nitrogen gas
Na^+: Sodium cation
$Na_2Cr_2O_7$: Sodium dichromate
NAA: *N*-acetyl aspartate
NAD^+: Nicotinamide adenine dinucleotide oxidized form
NADH: Nicotinamide adenine dinucleotide reduced form
Nbs: Nanobodies
NCATS: National Center for Advancing Translational Sciences
NCCAM: National Center for Complementary and Alternative Medicine
NCE: New chemical entity
ncRNA: noncoding RNA-activating (a type of RNA)
NCT: National Center for Toxicogenomics
NCTR: National Center for Toxicological Research
NDA: New Drug Application
NDS: Nonlinear dynamic systems
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Ni: Nickel
NIEHS: National Institute of Environmental Health Sciences
NIH: National Institutes of Health (USA)
NIMS: Network target-based identification of multicomponent synergy
NK cell: Natural killer cell
NKCC1: Na^+-K^+-Cl^- cotransporter
NLM: National Library of Medicine

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NLR: NOD-like receptor
nM: nanoMolar, or 1 nmole per liter of solvent (a unit of concentration of a molecule dissolved in a solvent, where one mole is the molecular weight of the molecule, expressed in grams and is equal to $6.02x10^{23}$ molecules. So, 1 nmole is 10^{-9} moles and 1 nM is 1 nmole of a molecule that is dissolved in 1 liter of solvent)
NMDA: *N*-methyl-D-aspartate
NME: New molecular entity
NMR: Nuclear magnetic resonance spectroscopy
NMS: Non-motor symptoms
NO: Nitric oxide
NO_3: Nitrate anion
NOAEL: No observed adverse effect level
NOC: Number of clusters
NOE: Nuclear Overhauser Enhancement
NOESY: Nuclear Overhauser Enhancement Spectroscopy
NotchIC: Notch intracellular
NPC: Neural progenitor cell line
NPR-A: Natriuretic peptide clearance receptor
NPY: Neuropeptide Y
NR: Nuclear receptor
NR: Nicotinamide riboside
NRC: National Research Council
Nrf2: Nuclear factor-erythroid 2 p45-related factor 2
NSAID: Non-steroid anti-inflammatory drug
NSCLC: Non-small cell lung cancer
nSSL: Nano sterically stabilized liposomes
O_2: Oxygen gas
$O_2^{\cdot-}$: Superoxide anion
OCG: Office of Cancer Genomics
OCl⁻: Hypochlorite
OCPP: Office of Critical Path Programs
ODDT: Open Drug Discovery Teams
ODT: Orally disintegrating tablets
OMIM: On-line Mendelian Inheritance in Man
OOPD: Office of Orphan Products Development
ORACL: Optimal reporter cell lines for annotating compound libraries
ORF: Open reading frame
OS: Oscillatory disturbed shear
OTC: Over-the-counter
OXT: Oxytocin

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P4 medicine: Predictive, preventive, personalized and participatory medicine
PAMAM: Poly(amidoamine)
PAMP: Pathogen-associated molecular patterns (bacterial)
PANDAS: Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections
PANS: Pediatric Acute-onset Neuropsychiatric Syndrome
PARP: Poly (ADP-ribose) polymerase
Pascal: Pathway scoring algorithm
PAT: Process analytical technology
Pb: Lead
PBA: Phenylboronic acid
PBPK: Physiological based pharmacokinetic models
PC: Phosphatidylcholine
PCBs: Polychlorinated biphenyls
pDC: Plasmacytoid Dendritic Cells
PCDDs, also known as dioxins: Polychlorinated dibenzo-*p*-dioxins
PCPP: 1-(1-**phenyl cyclohexyl)piperidine**
PCR: Polymerase chain reaction
PD: Parkinson's disease
PDB: Protein databank
pDC: plasmacytoid Dendritic Cells
PDC: Pediatric Device Consortia
PDGF: Platelet-dependent growth factor
PDGFR: Platelet-dependent growth factor receptor
PDMS: Polydimethylsiloxane
PDSA: Plan, Do, Study and Analyze
PEG: Poly(ethylene)glycol
PFPD: Pulsed flame photometric detectors
PGE2: Prostaglandin E_2
pH: Negative log of H^+ concentration
PH: Plekstrin homology domain
PheWAS: Phenome-wide association studies
PIC/S: Pharmaceutical Inspection Co-operation Scheme
PID: Photoionization detectors
PI3K: Phosphatidyl inositol 3-kinase
PKA: Protein kinase A
PKC: Protein kinase C
PLK1: Polo-like kinase 1
PMI: Precision Medicine Initiative
PNS: Peripheral nervous system

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POMaC: Poly(octamethylene maleate (anhydride) citrate)
PPAR: Peroxisome proliferator-activated receptor
ppb: parts per billion
ppm: parts per million
PPI: Protein-protein interactions
PPI: Proton pump inhibitor
ppt: parts per trillion
PPY: A neuropeptide that contains tyrosine, or Y
PQRS: Physician Quality Reporting System
pre-mRNA: Precursor mRNA (messenger RNA)
pre-miRNA: Precursor mi-RNAs; DNA is transcribed into long pre-miRNA that contains insertion elements (introns) and exons
pri-miRNA: Primary miRNA
PRR: Pattern recognition receptor
PSA: Prostate specific antigen
PSRP: Preconception Stress and Resiliency Pathways
PTH: Parathyroid hormone
PTM: Post-translational modifications
PUFA: Polyunsaturated fatty acids
PV: Poliovirus
PVSRIPO: PV(**S**abin)-**R**hinovirus **I**RES **PV** **O**pen reading frame
QbD: Quality-by-design
QC: Quality control
QM/MM: Quantum mechanics/molecular mechanics
QMQSAR: Quantum mechanics-based QSAR
QMS: Quality management system
QRM: Quality Risk Management
QSAR: Quantitative structure-activity relationship
QSP: Quantitative systems pharmacology
Q-TOF: Quadrupole time of flight mass spectrometer
R: Gas constant
R: Relaxed state of hemoglobin
rAAVs: recombinant Adeno-associated viruses
RAC: Recombinant DNA Advisory Committee
RAS: Renin-angiotensin system
RAS: An oncogene that codes for the oncoprotein ras
RB1: Retinoblastoma protein 1
RCD: Regulated cell death
rDNA: recombinant DNA
RECIST: Response Evaluation Criteria in Solid Tumours
Redox: Reduction and oxidation

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RefSeq: Reference sequences
RES: Reticuloendothelial system
rf: Radio frequency
Rh: Rhenium
RIG: Residue interaction graph
RNP: Ribonucleoprotein
rRNA: ribosomal RNA
RONS: Reactive oxygen, nitrogen and halogen species
ROS: Reactive oxygen species (the word species is used here in a way that biologists might not approve since ROS do not mate and produce fertile offspring)
RNA: Ribonucleic acid
RNASE: RNA hydrolase
RPG: Relative Process Greenness
RTKs: Receptor tyrosine kinases
RT-PCR: Reverse transcriptase PCR
Ru: Ruthenium
RuBisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase
S1P: Sphingosine-1-phosphate receptor
SAH: S-adenosylhomocysteine
SAM: S-adenosylmethionine
SAR: Structure-activity relationship
saRNA: small-activating RNAs
Sb: Antimony
SCFA: Short chain fatty acids
SCID: Severe combined immunodeficiency
SCN: Suprachiasmatic nucleus
SDS: Sodium dodecylsulfonate
Se: Selenium
SERM: Selective estrogen receptor modulator
SG: Steryl glucoside
SGC: Structural Genomics Consortium
SHC: Sarcoma homology family that functions as a redox enzyme that is linked to apoptosis
shRNA: short hairpin RNA
SIM: Single ion monitoring
SINE: Short interspersed nuclear element
siRNA: Small inhibitory RNAs
SIRTs (also known as sirtuins): Sir proteins
SMA: Spinal muscular atrophy
SMN: Survival Motor Neuron

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Sn: Tin
snRNA: small nuclear RNA
S/N ratio: Signal to noise ratio
snoRNA: small nucleolar RNA
SNPs: single-nucleotide polymorphisms
SODA: Sequential operation droplet array
SOP: Standard Operating Procedure
SR: Scavenger receptor
ssDNA: single strand DNA
ssRNA: single strand RNA
SSRI: Selective serotonin reuptake inhibitor
STD-NMR: Saturation transfer difference NMR
STINT-NMR: Structural interaction-NMR
STK: Serine/threonine kinase
SUMO: Small ubiquitin-like modifier
SVD: Single value decomposition
T: Tense state of hemoglobin
%T: Percent transmittance
T1D: Type-1 diabetes
T2D: Type-2 diabetes
T3DB: Toxin and Toxin-Target Database
Tc: critical Temperature
T cells: Thymus-derived lymphocytes
TAME: Targeting Aging with Metformin
TBT: Tributyl tin
TCGA: The Cancer Genome Atlas
TCM: Traditional Chinese medicine
TCR: T-cell receptor
TF: Transcription factor
T_H: T-helper cells
THF: Tetrahydrofolate
TI: Thermodynamic integration
TIL: Tumor infiltrating lymphocytes
TIGR: The Institute for Genomic Research
TJ: Tight junction
TLR: Toll-like receptor
TMA: Trimethylamine
TMAO: Trimethylamine-*N*-oxide
T-MCA: Tauro-muricholic acid
TMS: Tetramethylsilane
TNF-α: Tumor necrosis factor-α

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TQL: Total quality leadership
TQM: Total quality management
TR: Thyroxine receptor
TROSY: Transverse relaxation optimized spectroscopy
Tr1: Type 1 regulatory T cell
T_{reg}: Regulatory T cells
TRP: Transient receptor potential (phytochemical receptors, ion channels)
tRNA transfer RNA
TSP: 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt
TT21C: Toxicity Testing in the 21st Century
TTFL: Transcription-Translation Feedback Loop
UPLC-QTOF-MS/MS: Ultraperformance liquid chromatography-Q-time of flight Mass Spectrometry/Mass Spectrometry
UPR: Unfolded protein response
USAID: US Agency for International Development
USP: US Pharmacopeia
USPSTF: U.S. Preventative Services Task Force
UV: Ultraviolet
UVA: Ultraviolet-A
UVB: Ultraviolet-B
UVR-6105: Ultraviolet resistant 6105
UV-Vis: UV-visible
VAT: Visceral adipose tissue
VCAM-1: Vascular cell adhesion molecule-1
VDR: Vitamin D receptor
VECs: Valvular endothelial cells
VEGF-A: Vascular endothelial growth factor-A
VHP: Visible Human Project®
VIC: Valvular interstitial cell
VICP: Vaccine Injury Compensation Program
VIP: Vasoactive intestinal peptide
V_{max}: Maximum velocity in an enzyme-catalyzed reaction
VOC: Volatile organic compound
VRML: Virtual Reality Modeling Language
VSMC: Vascular smooth muscle cell
WES: Whole-exome sequencing
WHO: World Health Organization
WS-CAM: Whole systems complementary and alternative medicine
XSD: Halogen specific detectors

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INDEX

1-(1-phenyl cyclohexyl)piperidine, PCPP, 126
^{1}H, 285, 314, 316, 318-321, 536, 542-555
^{13}C, 314, 316, 318, 394, 535, 536, 540, 542, 543, 545-547, 549, 550-555
1α,25-dihydroxyvitamin D3 (1,25-D), 29, 34
2-arachidonoylglycerol, 2-AG, 178-180
2-DG, 2-deoxy-D-glucose, 373, 378
2,3-diaminonaphthalene, 94
21 CFR 210, 347
21 CFR 312, 357
21 CFR 211, 347
2P2Idb v2.0 structural database, 296, 302
2-phenylaminopyrimidine, 311
3,4-epoxycyclohexylmethanol (ECHM), 331, 332
3,4-methylenedioxy-methamphetamine, 126
3D cell cultures, 286
3D cell culture chip, 371
3D hydrogel, 449
3D microtissue spheroid culture, 326
3D bioprinting, 329, 449
3D printing, 4, 326, 329, 335, 336, 447-450
3D polymer scaffolds, 449
3D vascular beds, 328
3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, 242, 250, 253, 267, 358, 370, 380
4D printing, 5, 447-452
5-fluorouracil, 276, 277
5-hydroxytryptamine (5-HT), 41, 180, 181, 271
5-lipoxygenase (5-LOX), 281, 283
25-hydroxyvitamin D3 (25-D), 126
ABC transporters, 276
Abraxane®, 342
Absconditabacteria, 223
Absorbance (A), 94, 256, 509-511
Academy of Opthamology, 142
Açaí, 9, 18, 190, 388, 389
Acanthamoeba polyphaga mimivirus, 47
Accutane®, 274
ACE, angiotensin converting enzyme, 251
ACE inhibitors (ACEIs), 365, 366, 381
Acetaminophen, 172, 325, 381, 382, 396, 521
Acetate, 185, 186, 190
Acetogenins, 140
Acetyl coenzyme A (acetyl CoA), 88
Acetyl-L-carnitine, 96
Acetylon, 131
Acetylsalicylic acid, 250, 252, 282, 336, 500
Acid dissociation constant, 498
Acid reflux, 27, 198, 251, 423, 424
ACOG, 115
ACT, adoptive T cell therapy, 430
ACTH, 29, 176, 185
Activin, 31
Activity-based proteomics (ABP), 294
Actinobacteria, 174, 190, 207, 209
Activated T cells, 71, 72
Active pharmaceutical ingredient (PI), 348, 349, 511, 513, 539, 540
Actron, 381
Acquired immune system, 275

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Adalimumab, 360
Adaptive immunity, 426
Adcetris®, 363, 364, 428
Adenine, 66, 276, 495, 496
Adenosine monophosphate activated protein kinase (AMP-APK), 269
Adenoviridae, 215
ADEPT, 363, 364
Adipocytes, 18, 31-33, 180, 186, 193, 288, 420
Adipokines, 30, 32, 180, 287, 288, 420
Adipose-derived stem cells, 329
Adipose tissue (AT), 30-32, 378, 420
ADME, 290, 322, 324, 357
ADMS, 41
Adjuvants, 202, 274, 277, 339, 433, 435
Adoptive T cell therapy, 430
Adrenaline, 30, 270, 272
Adrenergic receptors, 272
Adrenocorticotropic hormone (ACTH), 29, 176, 185
ADHD, 172
Adrenal cortex, 29
Adrenal gland, 29, 30, 176
Adriamycin, 140, 275, 276, 339, 344, 509
Adulterated dietary supplements, 393, 406
Adulterated pet foods and infant formula, 537
Advair®, 272
Advanced Alternative Payment Model (APM), 402
Advanced glycation end products (AGEs), 193
Advancing Regulatory Science Initiative, 117
Advil, 381
Adverse Outcome Pathways (AOP), 121, 322-324
Advertisements, 121, 122, 388, 389, 423
Advocate Health Care System, 144
Advocate Physician Partners, 144
AEA, 178, 179
Aeromonas, 198
Afferent nerves, 41, 177
Affordable Care Act, 137
AGEs, Advanced glycation end products, 193
Aggregated Computational Toxicology Resource (ACToR), 327
Aggregatibacter actinomycetemcomitans, 196
Agonist, 131, 184, 257, 269-273, 307, 360, 361, 378, 425, 435
ALCL, Anaplastic large cell lymphoma, 362
ALCOA (Attributable, Legible, Contemporaneous, Original and Accurate), 356
Aldactone®, 274
Aldose reductase, 259
Aldosterone, 29, 30
Alemtuzumab, 372
Algae, 67, 68, 220
Alginate, 448, 449
Alistipes, 209, 214
ALL, acute lymphoblastic leukemia, 52, 431, 432
Alleve®, 283
Allgemeine Ortskrankenkassen (AOK), 424
Alli, 421
Allo-network drugs, 1, 58, 266, 280, 309-321
Allosteric drugs, 266, 268, 311-314, 320
Allosteric effect, 259, 260, 263, 264, 312
Allosteric ligands, 293, 301, 310
Allosteric regulation, 311, 318
Allostery, 259-266, 301, 309-321
Allyl isothiocyanate, 401
α-ketoglutarate, 88, 89
α-Synuclein, 37, 184, 200, 408, 415
ALS, 76

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Alternatively activated macrophages, 36
Alu retrotransposons, 84, 85
Alzheimer's disease (AD), 37, 42, 75, 76, 104, 189, 251, 272, 280, 281, 415, 424
Amazonian, 388
Ambien®, 271
American Academy of Pediatrics, 126
American College of Obstetricians and Gynecologists, 115
American Gut, 211, 387
American Medical Association (AMA), 350
Amgen, 352
Amino-acyl-tRNA, 269
Aminoglycosides, 269
AMP, adenosine monophosphate, 90
AMP, Anti-microbial peptides, 36
AMP-activated protein kinase (AMPK), 9, 367, 368, 385, 474
Amphetamine, 124, 394, 397
Amphiphilic, 342, 343
AMPK, 9, 367, 368, 385, 474
Amyotrophic lateral sclerosis (ALS), 76
AMPAC, 295
Analyte, 9, 99, 106, 107, 293, 394, 396-407, 494, 502-505, 510-513, 515-518, 520-533, 535-544
Analytical chemistry, 106, 148, 394, 438, 494-556
Anandamide, 178, 179
Anaplastic large cell lymphoma (ALCL), 362
ANCHOR, 296
Anchors (allosteric drugs), 311
Andrew Wakefield, 138
Androgens, 30, 268, 274, 338
Androstenedione, 396, 520
ANG-1 (angiopoietin-1), 279
AngioChips, 447, 448
AngII, 34, 270
AngII type 1 and AngII type 2 receptors (AT1R and AT2R), 34, 365
Angiogenesis, 64, 124, 279, 328, 359, 372, 373, 414, 425, 449, 450, 555
Angiotensin II, 34, 270
Angiotensin-converting enzyme (ACE), 251
Ankylosing spondylitis, 275
Anopheles genus, 445
ANS, 42
Antagonist, 127, 213, 259, 269, 270, 272, 273, 282, 307, 346, 365
Anterior cingulated cortex, 97
Anthocyanins, 190, 210
Anthracyclines, 275, 276
Antibiotic resistance breakers (ARBs), 360, 361
Antibiotics, 25, 57, 59, 68, 86, 135, 148, 171, 172, 184, 187, 192, 197, 199, 206-208, 214, 219, 268, 286, 297, 360, 361, 444
Antibody, 45, 46, 73, 86, 361-364, 372, 427, 430, 432, 433, 436, 437
Antibody directed enzyme prodrug therapy (ADEPT), 363, 364
Anti-CTLA-4 treatment, 431-433
Anti-Tac(Fv)-PE38 (LMB-2), 363
Antibody-drug conjugates, 361
Anticancer signaling agents, 269
Anticancer vaccines, 277
Antifungal agent, 88, 361
Antigen, 15, 36-39, 41, 45-47, 52, 71-73, 192, 277, 278, 296, 299, 339, 345, 346, 354, 356, 363, 364, 372, 426-431, 433-436, 444
Antigen presenting cell (APC), 37, 426, 430
Antihistamine, 272, 381, 393, 397-398
Antimetabolites anticancer agents, 276

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Antimicrobial defense molecules, 41
Anti-microbial peptides, 36, 90
Antioxidant peroxiredoxin proteins, 81
Antisense oligonucleotides, 278, 376
Apelin, 180, 288
AOK (Allgemeine Ortskrankenkassen), 424
AOP, 121, 322-324
API, active pharmaceutical ingredient, 348, 349, 511, 513, 539, 540
APM, 142
Apocynin, 501, 554
Apoptosis, 29, 46, 47, 52, 53, 71, 75, 80, 102, 184, 186, 190, 286, 297, 303, 306-308, 317, 325, 362, 363, 367, 378, 430
Apoptosis antigens, 47
Apple juice, 94, 137
Aptamers, 278, 436
Aquaporin-4 water channel, 37
Arachidonic acid (AA), 281, 282
ARBs (antibiotic resistance breakers), 360, 361
Archaea, 6, 20, 55, 57, 78, 85, 167-169, 173, 174, 186, 198, 216, 219, 220, 223
Arginine, 88
Argonaute proteins, 77-79
Aricept®, 251
Arivale, 387
Aromatase, 32, 268, 420
Arsenic, 94, 95, 137
Artemisinin, 252, 372, 511
Arteriosclerosis, 187
ASAP-HSQC (HSQC as soon as possible), 320
Ascorbate (Vitamin C), 205, 360
Aspergillus, 221, 222, 254, 266
Asilomar Conference Grounds, 68, 442
ASD, 133, 135, 137, 138, 198-200, 208, 224
Aspergillus terreus, 254, 266
Aspirin, 250, 252, 282, 283, 336, 381, 382, 397, 407, 500, 501, 522
Assistant Secretary for Planning and Evaluation (ASPE), 117
Asthma, 84, 268, 270, 272, 274, 275, 281, 282
AstraZeneca, 130, 131, 434
Astrocytes, 37, 38, 97, 187
ASVD, 203, 204
Ataxia telangiectasia-mutated (ATM), 98
Atezolizumab (Tecentriq®), 72, 308, 434
Atherosclerosis, 36, 102, 170, 184, 187, 194, 196, 197, 203, 281, 327, 367, 372
Atherosclerotic plaques, 124, 267, 359
Atherosclerotic vascular disease (ASVD), 203, 204
AT1R and AT2R, 34, 365, 366
ATM, 98
Atmospheric pressure chemical ionization (APCI), 534, 537
Atorvastatin, 125, 267, 397, 521
Atrial natriuretic factor (ANF), 30
ATP, 3, 53, 64, 90, 92, 218, 251, 262, 276, 311, 313, 364, 368, 439
ATP-binding cassette (ABC) transporter (ABCF2), 276
Attention deficit hyperactivity disorder (ADHD), 172
A-type outward K^+ channels, 180
AUC, area under the curve, 322
Auditory cells in the organ of Corti, 45
Aureobasidium, 222
Autism spectrum disorder (ASD), 133, 135, 137, 138, 198-200, 208, 224
Autodissociation constant of water (K_w), 498

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Autoimmune diseases, 34, 76, 104, 127, 172, 178, 182, 185, 188, 192, 219, 275, 277, 368, 372, 428, 435
Autonomic nervous system (ANS), 42
Autonomous cellular self-assembly, 449
Autophagic cell death, 52
Autophagy, 52, 184, 361, 367, 413
Autopoiesis, 4, 31, 43-52, 273, 299, 332, 333, 413
Avastin®, 251, 428
Avogadro's number (N), 499
Axid AR, 381
Axon, 3, 53, 184, 187, 371, 450
Ayurvedic medicine, 393, 416-419
AZT®, 251
B cells, 72, 73, 192, 201, 296, 302, 426, 427, 431, 434, 436
Bacteria, bacterial, antibacterial, 3, 6, 8, 11, 13-15, 18-20, 26-28, 31, 36, 41, 42, 44, 46, 47, 49, 54, 55, 57, 61, 67, 68, 72, 74, 77, 78, 85-87, 95, 105, 113, 126, 130, 135, 138, 147, 157-223, 242, 250, 262, 263, 269, 275, 277, 291, 292, 297-299, 339, 361-364, 408, 414, 424-427, 437-444, 535, 538
Bacteriophage, 47, 113, 169, 214, 215, 217, 223, 440, 441, 444
Bacteroidetes, 105, 173, 189, 192, 207, 209-211
Bacterioides fragilis (*B. fragilis*), 189, 212
Barbara McClintock, 86
Basal nuclei, 45
basic Fibroblast Growth Factor (bFGF), 372, 450
BASIS^{TM}, 386
Batch Modules for ACD/Percepta (chemoinformatic tool), 338
Batch records, 348
Bazedoxifene, 360
BBB, 36, 37, 142
B-cell lymphoma, 297, 306, 362, 428
B-cells, 31, 38, 297, 306, 360, 362, 428, 432
Bcl-2, 297, 302, 306, 307
BCR-ABL tyrosine kinase, 268
BDNF, 31, 198
Beer's Law, 510, 542, 544
Benign prostate hyperplasia (BPH), 272
Benzene, 137, 506-508, 536, 554
Berberine, 361, 378, 397, 521
BEST, Biochemical Efficacy and Safety Trial, 125
Beta amyloid (β-amyloid), 37
β-adrenergic receptor, 251
β-blockers, 251
β-thalassaemia, 445
β-cells (in the pancreas), 33
Betaproteobactera, 198
Benztropine, 288
Bevacizumab, 251, 372
Bexxar®, 363
Bifidobacterium, 172, 173, 184, 190, 198, 206-208, 414
Bifidobacterium animalis, 207
Bihormonal bionic pancreas, 451
Bile acids, 188
Bill and Melinda Gates Foundation, 131, 247, 277
Biochemical Efficacy and Safety Trial (BEST) of vitamin D, 125
Biochemistry, 12, 23, 70, 221, 243, 312, 500
Biodegradable scaffolds, 447, 448
Biodiversity, 174, 184, 191-193, 200, 205, 209, 211, 212, 219, 220, 224
Biodiversity hypothesis, 209
Bioclipse59 (statistical tool), 338
Biofilms, 169, 185, 203-205, 212, 223
Bioresorbable tracheal splint, 4, 447
Biotechnology, 3, 39, 67, 70, 78, 345, 355

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Bisphenol A diglycidyl ether (BADGE), 329, 330
Bitter orange (*Citrus aurantium* L.), 126
Black Death, 147, 251
Blastocystis, 220
Blautia, 200
Blood-borne lymphocytes, 38
Blood-brain barrier (BBB), 36, 37, 142
Blueberries, 190, 324
BMAL1/CLOCK dimers, 82
B_{max} - the maximum concentration of A-R, or bound ligand, 270
Body mass index (BMI), 173, 185, 421
Boltzmann constant (k), 499, 500
Bone-adipose axis, 33, 420
Bone marrow, 15, 29-34, 44, 72, 286, 342, 427
Bone morphogenic protein (BMP), 31
Bones, 31, 32, 44, 47, 124, 420, 447, 451, 497
Bordetella pertussis, 429
Bottlenecks (in networks), 58, 59, 63, 253, 438
BPH, 272
Brain-derived neurotrophic factor (BDNF), 31, 198
Brain natriuretic peptide (BNP), 30
BRCA-positive ovarian cancer, 268
Breast cancer, 73, 112, 115, 127, 273, 274, 276, 307, 341, 342, 362-364, 369, 410, 425, 428
Breast milk, 206, 208
Brentuximab vedotin (SGN-35), 362, 363
Brian Deer, 138
Bridges (in networks), 58, 59, 62, 64, 253, 265
Bristol-Myers Squibb, 352
Broad Institute, 73
Bromodomains, 91, 102, 103, 130
Bromodomain-containing proteins, 91
Brookhaven National Laboratory, 4, 447
Brown adipose tissue (BAT), 32
Bubonic plague, black death, 147, 251
Butoconazole nitrate, 381
Butyrate/butyric acid, 169, 173, 174, 177, 185, 186, 188-190, 200, 201, 207, 209, 497
C1q/TNF-related proteins (CTRPs), 31
Caffeine, 126, 127, 397, 412, 415, 421, 522
cAbl kinase inhibitor, 251
CAFO, 214, 219
Calcipotriene, 274
Calibration curve, 9, 10, 510, 513, 515, 517, 539, 540
Calico, 387
Calories, 9, 18, 196, 368, 389, 391, 415, 420, 421
Camels, 345, 411
cAMP, 263, 265, 290
cAMP-activated exchange protein (EPAC, exchange protein activated by cAMP), 265
cAMP binding domain (CDB), 265
cAMP binding protein (CBP), 263
Campath®, 428
Canadian Institutes of Health, 277
Cancer, 8, 16, 18, 21, 22, 24, 26, 28, 34, 36, 40, 42, 44-46, 51, 57-59, 62-65, 71-73, 75-77, 80, 82, 8486, 89, 91, 92, 94, 95, 100, 103, 104, 107, 108, 110, 112, 115, 119, 120, 124-127, 132, 133, 140, 144, 147, 170, 184, 187-190, 192, 201, 203, 211, 216, 245, 246, 248, 249, 251, 253, 268, 269, 271, 273-281, 283, 287-289, 293, 294, 296, 297, 299, 300, 302, 304, 307, 308, 310, 317, 321, 324, 329, 332, 339-346, 351, 359-369, 372-375, 377-380, 384-386, 391, 393, 397, 408, 410-412,

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

414, 415, 424, 425, 427-437, 440, 442, 444, 555, 556
Cancer Genetic Markers of Susceptibility (CGEMS), 120
Cancer Genome Anatomy Project (CGAP), 120
Cancer Genome Characterization Initiative, 119-121
Cancer immunity cycle, 71
Cancer immunotherapy, 72, 377, 424, 430, 432, 434, 435, 440
Cancer Immunotherapy Consortium (CIC), 434
Cancer Research Institute (CRI), 434
Cancer stem cells, 44, 51
Candida, 221, 222
Cannabinoid receptors, 178, 179
Candidate phyla radiation group, 222
CAPA (Corrective Actions and Preventive Actions), 353
Capers (*Capparis spinosa*), 324
CAR, chimeric antigen receptors, 427, 431
CAR T cell, 436, 437, 444
Carbohydrates, 9, 18, 45, 80, 97, 104, 169, 173, 190, 205, 207, 209, 211, 216, 219, 220, 221, 389, 391, 431, 451
Carbonic anhydrase, 110, 268
Carboplatin, 276, 360
Carboxylic acids, 221, 389, 498, 500, 544
Carcinogens, carcinogenicity, 87, 95, 120, 170, 334, 338, 392
Cardiac arrhythmias, 251
Cardiomyocytes, 30, 298, 328, 355
Cardiotoxicity, 276, 298, 327, 339, 340
Cardiovascular diseases, 22, 40, 104, 123-125, 147, 187-189, 193-196, 203, 253, 266, 267, 273, 283, 296, 327, 361, 366, 367, 384, 386, 391, 400, 407, 410, 412-415
CARs therapy, 436
Cas, CRISPR-associated protein, 441
trans-activating crRNA (tracrRNA), 441, 446
Casodex®, 274
Caspase, 47, 53, 307
CastP server, 96
Catabolite activator protein (CAP), 263, 265
Catalase, 127
Catalytic RNA, 74, 80
Catechin, 43, 411, 412
Caudovirales bacteriophages, 214
Cavbase, 110
CB_1 and CB2, 178-180
CBER, 6
cBioPortal for Cancer Genomics, 304
CCHN, 118, 119
CCR5 (C-C chemokine receptor type 5) coreceptor, 265, 296
CD8+ T cells, 39, 199, 202, 377, 425, 427
CD20 protein, 360, 363
CD22 protein, 362
CD25 protein, 363
CD30 protein, 362, 364
CD56 protein, 362
CD155 protein, 374
CDA, 388
cDC, classical dendritic cell, 426
CDC, Centers for Disease Control, 135-137
CDER, 6, 116
cDNA, 68, 120, 430
Celebrex®, 283
Celecoxid, 110
Cell-autonomous feedback system, 83
Cell-based assays, 250, 284
Cell-based medicines, 353, 355
Cell cycle arrest, 362
Cell membrane, 45, 265, 273, 287, 300, 301, 306, 311, 340-342, 354, 432

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Cellular network, 45, 55, 57, 49, 61, 252, 266, 280, 309, 311, 313, 315
Cellular senescence, 364-367
Center for Biologics Evaluation and Research (CBER), 6
Centers for Disease Control (CDC), 135-137
Central dogma of molecular biology, 74
Central hit, 280, 281
Central nervous system (CNS), 23, 35-39, 41, 42, 46, 53, 75, 76, 110, 124, 175, 176, 180, 182, 183, 199, 280, 371
Centrality (in networks), 59, 61, 119, 300, 321
Cephalosporins, 361
c-Fos proto-oncogene, 197
CGAP, Cancer Genome Anatomy Project, 120
Center for Biologics Evaluation and Research (CBER), 6
Centers for Medicare & Medicaid Services, 117, 142
Center of Drug Evaluation and Research (CDER), 6, 116
Cerebral cortex, 45, 197
Cerebral spinal fluid (CSF), 37
Cervical lymph nodes, 37-39
Cetuximab, 372
CGCI, Cancer Genome Characterization Initiative, 119-121
CGEMS, Cancer Genetic Markers of Susceptibility, 120
cGMP, current good manufacturing practices, 9, 10, 147, 148, 244, 245, 347-357, 421, 422, 502
cGMP, cyclic guanosine monophosphate, 290
cGRPP, Current Good Radiopharmacy Practices, 353
Charge coupled detector (CCD), 509
Charles Darwin, 12, 242
Chemical screening, 322-328
Chimeric antigen receptors, 427, 431
Chaperone proteins, 101, 111, 262, 346
Checkpoint inhibitors, 71, 308
ChemAxon (chemoinformatic tool), 338
Chembank, 120
Chemically modified mRNA (modRNA), 279, 373-377
Chemical shift covariance analysis (CHESCA), 318-320
Chemical ionization (CI), 534, 537
Chemistry in American Life, 4
Chemogenomics, 111, 130, 359
Chemoinformatic tools, 338
Chemokines, 46, 192, 265, 346, 377, 435
Chemotaxis protein Y (CheY, a bacterial protein), 263
Chemotherapeutic drugs, 268, 343, 408
CHESCA, 318-320
Chikunguya, 445
Childhood Cancer Center, 120
China, 122, 247, 254, 347, 417, 443, 445
Chinmedomics, 113, 417
Chitosan, 448
Chlamydia pneumonia, 196
Choke points, 58, 281
Cholecalciferol, 29, 126
Cholecystokinin (CCK), 175
Cholesterol, 8, 27, 124, 125, 187-189, 194, 197, 204, 242, 243, 251, 253, 266, 267, 340, 358, 370, 377, 378, 381, 407, 409, 412, 504
Cholic acid, 212, 213
Chenodeoxycholic acid, chenodeoxycholate, 213
Chlamydia trachomati, 429s
Chloroform ($CHCl_3$), 340, 389, 536, 546
Choline, 193-196, 415

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Chondroitin sulfate, 336
Chromate, 392
Chromatin networks, 64, 65
Chromatin regulators (CRs), 92
Chromatin structure, 64, 65, 88, 93, 101
Chromosomes, 19, 20, 28, 45, 64-67, 69, 71, 75, 79, 85, 86, 88, 100, 108, 132, 288, 306, 308, 317, 374
Chromosome positive chronic myelogenous leukemia, 132
Chronic autoimmune inflammation, 72
Chronic kidney disease (CKD), 34, 184, 193, 381, 407, 504
Chronic myelogenous leukemia (CML), 132, 251, 317
Chronic obstructive pulmonary disease (COPD), 270, 360
Ciclopirox, 361
Cimetidine, 381, 398, 522
Cinacalcet (Sensipar), 266, 313, 314
Cinnamaldehyde, 41
Cinnamic acid, 41
Cinnamon, 41, 391
Circadian clock, 81-84, 92
Circadian rhythm, 19, 23, 28, 30, 42, 81-84, 184, 193
Circadian rhythmicity, 83, 184
Circular extrachromosomal DNA, 308
Circular RNA and DNA, 85, 86
Citicoline (cytidine diphosphocholine, CDP-choline), 415
Cjoc42, 379
Cladosporium, 221, 222
Claritin®, 272, 381
Classical mechanics, 7
Classical pharmacology, 250
Classified data, 135, 136
Clinical practice improvement activities, 142
Clinical Trials Transformation Initiative, 117
CLL, 306-308, 360, 431
Clostridium bolteae, 198
Clostridium clusters I and XI, 198
Clostridium species, 175, 197
Clozapine, 110, 270
Clustered regularly interspaced short palindromic repeats (CRISPR), 48, 67, 223, 437-447, 452
CMap (Connectivity Map), 248
C_{max}, 322
CML, Chronic myelogenous leukemia, 132, 251, 317
CMS, Centers for Medicare & Medicaid Services, 117, 142
CMV, Cytomegalovirus, 38, 39, 201, 216
CNS, central nervous system, 23, 35-39, 41, 42, 46, 53, 75, 76, 110, 124, 175, 176, 180, 182, 183, 199, 280, 371
Cocaine, 126, 147, 336, 337, 358
Codeine, 126, 271, 337, 398, 523
CODESSA, 295
Coenzyme Q_{10} (CoQ_{10}), 268, 408-411, 422
Cognition, 23, 44, 87, 96, 97, 105, 170
Cognitive genomics, 105
Collagen, 34, 325, 448, 449
Collaboration for rare disease drug discovery research, 165
Collaborations in Chemistry, 131
Collaborative Drug Discovery Inc., 131
Collective dynamics, 24
Colorectal cancer, 40, 127, 184, 188, 190, 211, 321, 372, 428
Colostrum, 206
Columbia University, 131
Combinations of drugs, 22, 110, 249, 360-365
Combine 2-deoxy-D-glucose with fenofibrate, 378
CoMFA, Comparative molecular field analysis, 295

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Commensal Bacteria, 28, 42, 188, 201, 202, 299
Communication, 6, 27, 43, 45, 57, 59, 61, 63, 68, 74, 146, 175-186, 191, 212, 241, 244, 299, 311, 315, 349, 388, 451, 550
Community Child Health Network, 118
Comparative Toxicogenomics Database, 324
Competitive enzyme inhibition, 254, 258, 310, 311
Complementary DNA (cDNA), 68, 120, 430
Complex carbohydrates, 18, 169
Complexity, 2, 20, 25, 62, 65, 416, 418, 440, 441
Complex systems, 2, 22, 59, 417, 418
Computational systems biology pathway (CSBP) models, 324
Computer-aided drug discovery (CADD), 294, 368
Computerized monitoring systems, 353
Computed tomography (CT), 8, 40, 447, 451
Concentrated animal feeding operations (CAFOs), 214, 219
Concentration vs level, 503, 504
Conformational change, positive cooperativity, enthalpy driven proteins, 265
Conjugated estrogens, 274, 360
Connections (of nodes, hubs), 23-25, 28, 44, 55-60, 97, 300, 304
Connectivity Map, 248
Conspiracy, 128, 133, 139, 143, 245, 369, 382, 387
Contrave®, 421
Cooperativity, 80, 260-265
Coordinated chemical shifts behavior (CONCISE), 319
COPD, 270, 360
Coprococcus, 198, 200
Corlanor® (ivabradine), 360
Corn fiber, 210
Cornification, 52
Corrective Actions and Preventive Actions (CAPA), 353
Corpus luteum, 29
Corticotropin-releasing factor (CRF), 30, 176
Corticotropin-releasing hormone (CRH), 197
Corynebacterium, 172, 206
Cosentyx®, 275, 428
COX, COX-1, COX-2, 98, 250, 268, 281-283
CpG, 88, 92, 100, 103, 277, 278
CPI, Critical Path Initiative, 114-119
CPIA, 142
CPR, 222, 223
C-Path, 117
CR, Chromatin regulator, 92
C-reactive protein, 204
Creatine, 125
Creatinine, 504, 550
Creative elements in cellular networks, 253
Creative nodes, 59
CREB (cAMP response element binding protein), 263
Crestor®, 267
Creuzfeldt-Jakob disease, 183, 184
CRF, Corticotropin-releasing factor, 30, 176
CRH, Corticotropin-releasing hormone, 197
CRISPR, CRISPR-Cas9, 48, 67, 223, 437-447, 452
CRISPR RNA, crRNA, 441
Critical Discourse Analysis (CDA), 399
Crohn's disease, 174, 203, 211, 216, 224, 275
Crosstalk, 448
Critical control points (CCPs), 349
Critical Path Initiative, CPI, 114-119
Critical temperature, 24

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Criticality, 22, 23, 111
Cryptococcus, 222
CSF, Cerebral spinal fluid, 37
CS Mott Children's Hospital, 450
CTL4A, 308
CTTI, 117
Curcuma longa, 391, 482
Curcumin, 361, 384, 391, 392
Current Good Manufacturing Practices (cGMP), 9, 10, 147, 148, 244, 245, 347-357, 421, 422, 502
Cyanidin-3-*O*-glucoside, 190
Cyclic demineralization and re-mineralization of teeth, 205
Cyclooxygenase (COX), 98, 250, 268, 281-283
CYP, cytochrome P450, 115, 290, 329, 345
Cyracure® UVR-6105, 329-333, 335
Cysteine-aspartate proteases, 47
Cystic Fibrosis Foundation, 132
Cytidine 5'-diphosphocholine, 415
Cytochrome P450, 115, 290, 329, 345
Cytokines, 21, 22, 31-33, 35, 38, 39, 68, 82, 174, 186, 190, 192, 196, 204, 212, 265, 275, 277, 283, 287, 288, 425, 426, 429, 430, 432, 433
Cytokine signaling, 38
Cytomegalovirus (CMV), 38, 39, 201, 216
Cytosine, 66, 68
Cytotoxic and immunogenic mechanisms of recombinant oncolytic poliovirus, 374
Cytotoxic anticancer drugs, 71
Cytotoxic T-cells, 308
Cytotoxic T-lymphocyte protein 4 (CTLA4), 308, 444
Daniel Koshland, 260
DataChip, 287
Date hubs, 58, 111, 314
Daunorubicin, 275, 276, 341
DAXX, 101
Daylight (chemoinformatic tool), 338
Day Two, 387
DC, dendritic cell, 425-427
DCA, 188
DCDB (Drug Combination Database), 249
DDRNA, 78
Death domain-associated protein, 101
DEC2 gene, 83
Deep ecology, 1, 6, 167-225
Deferrobacteres, 173
δ-cells (in the pancreas), 33
Demethylases, 88-91, 277
Dendrites, 75, 187, 343
Dendritic cells (DCs), 425-427
Dendritic nano-carriers, 343
Dengue, 247, 445
Deoxycholic acid, 188
Deoxyribonucelic acid (DNA), 1, 3, 5, 7-15, 18-21, 28, 39, 44, 45, 47, 48, 51, 54, 55, 57, 63-95, 98-108, 112, 120, 129, 167, 168, 170, 172, 184, 185, 188, 215-220, 240-243, 252, 263-265, 268, 273, 275-280, 288-294, 298, 301, 306-308, 311, 320, 323, 343, 344, 365, 367, 368, 375, 379, 385, 387, 409, 430, 437-447, 495, 496, 538, 551, 552
Department of Defense, USA, 117
Depression, 17, 24, 26, 42, 76, 123, 177, 178, 181, 184, 191, 197, 212, 246, 366, 369, 419, 424
Desulfovibrio, 196, 198
DHA, Docosahexaenoic acid, 121, 122, 177, 283
DHAP, 254, 255
Dialister, 214
Diabetes, 102, 114, 182, 184, 188, 251, 269, 274, 300, 321, 366, 436, 437, 451
Diapocynin, 501, 554

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Diarrhea, 198, 215, 220, 223, 247, 361
Diazepam, 271
Dicer, 75, 77, 78
Dicer and Drosha-dependent RNAs, 78
Dietary supplements, 9, 121-123, 125-128, 139-141, 143, 193, 194, 224, 225, 253, 254, 273, 327, 336, 349, 350, 356-358, 361, 380-387, 391, 393-415, 421-423, 452, 504, 519-532
Digital microfluidics (DMF), 286
Diglycidyl ether of bisphenol A (DGEBA), 329
Dihydrofolate reductase (DHF reductase), 262, 268, 295
Dihydroxyacetone phosphate (DHAP), 254, 255
Dioxins, 99
Dipeptidylpeptidase 4 (DPP-4), 268, 269
Diphtheria–pertussis–tetanus, 134
DirectedPPI database, 304
Directed weighted networks, 303
Disease industry, 140
Division of Cancer Epidemiology & Genetics
DNA, 1, 3, 5, 7-15, 18-21, 28, 39, 44, 45, 47, 48, 51, 54, 55, 57, 63-95, 98-108, 112, 120, 129, 167, 168, 170, 172, 184, 185, 188, 215-220, 240-243, 252, 263-265, 268, 273, 275-280, 288-294, 298, 301, 306-308, 311, 320, 323, 343, 344, 365, 367, 368, 375, 379, 385, 387, 409, 430, 437-447, 495, 496, 538, 551, 552
DNA methylation/DNA methylase, 75, 95, 98, 100, 101, 103, 108, 112
DNA polymerase, 47, 292
DNA repair, 88, 364
Docosahexaenoic acid (DHA), 121, 122, 177, 283
Dogma, 27, 28, 74, 98, 145, 146, 200, 205, 243, 373, 434, 438
Dolichols, 268, 358
Donepexil, 251
Dopamine, 185, 200, 270
Dorsal root ganglia, 175
Dose escalation in clinical trials, 357
Double reciprocal plot, 257-259, 310
double strand DNA (dsDNA), 47
double strand RNA (dsRNA), 47, 377
Dovonex®, 274
Doxil®, 339, 340
Doximity, 132
Doxorubicin, 275, 339, 340, 342, 343, 509
DPP-4, 268, 269
DPT, 134
Dragon descriptors103 (chemoinformatic tool), 338
Driver nodes, 299, 303, 304
Drivers (allosteric drugs), 311
Drosha protein, 75, 77, 78
Drug Combination Database, 249
Drug delivery, 46, 244, 339-347, 363
Drug repurposing, 359
Drug synergy, 22, 300
Drugs that bind to multiple targets, 110
Dry eye, 275
dsDNA, 47
dsRNA, 47, 377
Duavee ®, 360
Duodenum, 40, 211
Duragesic®, 271
Durvalumab, 434
Dynamic contrast enhancement MRI (DCE-MRI), 555
Dynamic modules, 111
Dysplipidemia, 27
Early outgrowth cells (EOCs), 34
Earth Microbiome Project, 171
ecDNA, 86, 308

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

ECM, extracellular matrix, 33, 36, 279, 287, 296, 328, 355, 373, 379, 448
Eco R1 restriction endonuclease, 68
Ecosystems, 1, 3, 4, 6, 20, 22, 26, 47, 48, 50, 54, 55, 57, 71, 93, 98, 113, 167-170, 178, 191, 242, 379, 439, 446
ECs, enterochromaffin cells, 176, 180
ECs, endothelial cells, 279
Ectosymbiosis, 54
Education, 7, 12, 37, 119, 121, 122, 146, 247, 447
EECs, Enteroendocrine cells, 40, 41, 175, 176, 180, 183
EGCG, 9, 127, 361, 384, 399, 407, 408, 411-415
Edentualism, 205
Edges, 3, 56, 57, 59-62, 65, 206, 280, 303, 304, 315
Edgetic drugs, 58
Efavirenz, 314
Effector T cells, 71
Effects of chemically modified mRNA, 374-376
EGCG, 9, 127, 361, 384, 399, 407, 408, 411-415
EGF, 32, 265, 271, 307, 362, 363
EGFR, 269, 271
Eicosanoids, 281, 283
Eicosapentaenoic aid (EPA), 121, 178
Eidgenössiche Technische Hochschule (ETH), 131
Elderberries, 190
Eldermet project, 170
Electrospray ionization (ESI), 518, 534, 537
Eli Lilly, 130, 131, 352, 369
Ellagic acid, ellagitannins, 384
EMA, 277, 411, 434
Embryonic stem cells (ES cells, ESCs), 45, 50, 51, 298, 327
EMEA, 117
Emergent properties, 2, 17, 24-26, 70, 82, 105, 416, 417, 428
EMP, Earth Microbiome Project, 171
Enalapril, 251
ENCODE project, 79, 80
Encephalitis, 445
Encyclopedia of DNA elements, 79, 80
ENDC, Enteric neural crest–derived cell, 183
Endocrine disruptors, 21
Endocrine System, neuroendocrine system, 8, 28, 29, 287, 197-199, 211, 212
Endorphins, 29
ENS, 40, 172-174, 186
Endocannabinoid system, 178-180
Endocrine disruptor knowledge base (EDKB), 338
Endoplasmic reticulum (ER), 58, 89, 307, 378, 413, 445
Endotoxemia, 192, 212
Endurance exercises, 9, 18, 96, 97
Engineered therapeutic T cells, 435
Enhanced permeability and retention (EPR), 340
Enhancers, 73, 88
Enovid, 273
Ensemble, 262, 263, 264, 278, 309, 314, 315
Entamoeba, 220, 223
Enteric *Bacterioides fragilis*, 189, 212
Enteric glial cells, 183, 187
Enteric nervous system (ENS), 40, 172-174, 186
Enteric neural crest–derived cell (ENDC), 183
Enterobacteriaceae, 189
Enterochromaffin cells, 176, 180
Enterococcus, 173, 198
Enteroendocrine cells, 40, 41, 175, 176, 180, 183
Enterome, 387
Enterotypes, 173, 210

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Enthalpy (H), 264, 265, 499, 500
Entropy (S), 265, 499, 500
Environmentome, 112
Enterovirus, 215
Enteroendocrine hormonal signaling system, 40
Entry inhibitors, 360
Environmental Protection Agency (EPA), 106, 121, 177, 327
Enzyme kinetics, 254, 258, 310, 311
Enzyme inhibition, 254, 258, 310, 311
Eosinophils, 31, 283
EPA, eicosapentaenoic acid, 121, 178
EPA, Environmental Protection Agency, 106, 121, 177, 327
Epialleles, 100, 101
Epidermal growth factor (EGF), 32, 265, 271, 307, 362, 363
Epidermal growth factor receptor (EGFR), 269, 271
Epigallocatechin-3-gallate (EGCG)
Epigenetics. 5, 18-21, 71, 73, 78, 87-94, 98, 100-105, 118, 132, 201, 213, 214, 290, 373, 379, 419, 429
Epigenome, 92, 93, 100, 101, 104, 105, 108, 120, 322
Epigenomics, 104
Epitope, 45, 73, 298, 343, 346, 427, 429
EPON™ 828, 329, 330
Equilibrium dissociation constant (K_D), 257, 270
Erbitux®, 372, 428
ERG, 65
Ergocalciferol, 126
ERK, 98
Erlotinib (Tarceva®), 269, 307, 372
Erythrocytes, 31, 44, 46
Erythropoietin (EPO), 29
ES cells, embryonic stem cells (ESCs), 45, 50, 51, 298, 327
Escherichia coli (*E. coli*). 105, 167, 173, 189, 212, 214, 220, 297, 298, 361, 439, 440
Esterases, 251, 266, 268, 313, 324, 330, 335, 500
Estradiol, 268, 273, 274, 341, 396, 420
Estrogen, 32-34, 268, 273, 274, 297, 338, 360, 394, 397, 420
Etanercept, 360
ETBF, 189
ETC, electron transport chain, 367
ETH, 131
ETS-related gene, 65
EU Framework Project, 326
Euchromatin, 101, 102
Eukarya, 20, 57, 167, 169, 186, 219-220
Eukaryotes, 66, 67, 77, 78, 95, 169, 216, 218, 219
European Bioinformatics Institute, 248
European Medicines Agency (EMA), 277, 411, 434
European Medicines Evaluation Agency, 117
European Union (EU), 347
EV, expression variance 111
Evidence based medicine, 114-116
Evista®, 274
Evolution, 11, 12, 86, 87, 168-170, 204, 215, 218, 242, 278, 303, 308, 315, 379, 441
Exercise, 9, 18, 31, 32, 74, 82, 96, 97, 114, 125, 126, 185, 211, 418, 421, 422, 424, 503
Exposome, 108
Expression variance (EV), 111
Exons, 19, 20, 67, 376, 377
External standard, 539
Extracellular matrix (ECM). 33, 36, 279, 287, 296, 328, 355, 373, 379, 448
Extrachromosomal circular DNA (ecDNA), 86

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Extracellular signal-related kinase (ERK), 98
FAAH, Fatty acid amide hydrolase, 180
Facebook, 55, 132
Facial recognition software, 200
Faecalibacterium, 214, 221
Family hubs, 111
Famotidine, 381
Farnesyl pyrophosphate (FPP), 127, 128, 358, 359
Fast atom bombardment (FAB), 534, 537, 538
FastContact 2.0, 296
Fast food diet, 169
Fat, 9, 17, 18, 27, 30-33, 36, 84, 88, 121, 122, 169, 173, 177, 187, 192-194, 198, 200, 201, 209, 210, 214, 219-221, 288, 368, 389, 391, 407, 412, 419-421, 502
Fatty acid amide hydrolase, 180
Fatty acids, 27, 33, 41, 100, 169, 178-180, 185, 186, 192, 193, 201, 209, 221, 222, 281-283, 317, 389, 392, 407, 496, 516
Fatty acid methyl esters (FAMEs), 516
Fatty acyls, 89, 114, 221, 222, 389, 390, 407
Fatty acyl glycosides, 389
Fenofibrate, 373, 378
Fentanyl (Duragesic®), 271
Ferroptosis, 52
FGID, 42
FDA, 6, 13, 43, 51, 52, 57, 94, 98, 99, 115-118, 122, 124, 126-128, 131, 132, 137, 140, 147, 224, 243, 244, 246, 248, 250, 254, 268, 273, 298, 308, 327, 336, 337, 338, 346, 347, 349-352, 357, 358, 359, 362, 363, 365, 368, 370, 372, 376-378, 380-382, 393, 407, 410, 411, 421-428, 430, 432, 434, 437, 448, 503, 537, 539
FDA Form 483, 351
Feedback, 5, 21, 22-25, 30, 32, 43, 48, 50, 81-83, 145, 146, 176, 191, 212, 241, 244, 299, 303, 325, 438, 440, 451
Feed forward loops (FFLs), 248
Femstat 3, 381
Fiber, dietary, 173, 189, 192, 200, 201, 209-211, 219, 391, 414
Fibrin, 448, 449
Find a lead compound, 243, 284-298, 380
Firmicutes, 105, 171, 173, 174, 188-190, 192, 207, 209, 211
Fish, fish oil, 27, 381, 393, 407
Flavivirus, 445
Flavonoids, 34
Flomax®, 272
Flovent®, 272
Fluticasone, 272, 274, 360
Fluorescence, 86, 94, 292, 293, 438, 504, 506, 510-512, 537
Fluorescence resonance energy transfer (FRET, 292)
Folate, 92, 364
Folic acid, 92, 128, 384, 393, 537
Follicle stimulating hormone (FSH), 29
Food and Drug Administration (US FDA), 6, 13, 43, 51, 52, 57, 94, 98, 99, 115-118, 122, 124, 126-128, 131, 132, 137, 140, 147, 224, 243, 244, 246, 248, 250, 254, 268, 273, 298, 308, 327, 336, 337, 338, 346, 347, 349-352, 357, 358, 359, 362, 363, 365, 368, 370, 372, 376-378, 380-382, 393, 407, 410, 411, 421-428, 430, 432, 434, 437, 448, 503, 537, 539
Food, Drug and Cosmetic Act (FD&C Act), 349
Forward chemical biology, 250
Forward pharmacology, 250

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

FoxP3, 433
FPPS (farnesyl pyrophosphate synthase), 128
Fragment-based drug design, 296, 297, 302
Framingham Heart Study (FHS), 26, 253
Francis Crick, 242
Free energy landscape of population shifts, 310
Free fatty acids, 407
Free radicals, 9, 51, 393, 409
Fritjof Capra, 1
Fructose, 18, 122, 389, 420
Fruits, 96, 123, 126, 140, 190, 192, 197, 200, 243, 306, 324, 356, 384, 389, 391, 392, 422
FTFLEX, 296
FTIR, 541-544, 554
FTMAP, 296
FTNMR, 541
Functional gastrointestinal disorders, 42, 182
Functional MRI, 556
Fungi, 36, 54, 78, 167, 182, 220-223, 253, 425, 426, 442
Fusarium, 222
Fusion proteins, 361, 353, 428
Fusobacteria, 171
Fusobacterium nucleatum (*F. nucleatum*), 188, 189
GABA, 97, 197, 271, 400
Gabapentin, 271
Gaia, 43, 48, 49, 168, 219, 223, 437, 442
Gameprost, 273
Gamma-amino butyric acid (GABA), 97, 197, 271, 400
Gankyrin, 373, 379
Gas chromatography (GC), 406, 506, 515-517
Gas constant (R), 499, 500
Gastric inhibitory peptide (GIP), 185, 186
Gastrin, 30, 41
Gastrointestinal tract, 29, 40, 174, 269
Gazyvaro®, Obinutuzumab, 360, 428
GBA, gut-brain axis, 42, 175, 182
GCCP (Good Cell Culture Practice), 355, 356
GC-MS, 506, 518, 536, 537, 550
GCP (Good Clinical Practice), 43, 357
Gefitinib, 269, 307
Gemfibrozil, 274
Gene drives, 446
Gene regulatory network, 63
Gene therapy, 277, 343, 353, 373, 375, 37, 378, 432
Gene therapy as a potential tool for treating neuroblastoma, 377, 378
Genentech, 352, 362, 434
Genistein, 384
Genetic interaction and chromatin networks, 64
Genetic interaction networks, 64, 65
Genetic engineering, 16, 67, 70, 432, 435, 436, 438, 439, 441-443
Genetically engineered adenoviruses, 378
Genetically modified organism (GMO), 48, 446
Genetics, 44, 20, 66-71, 87, 93, 104, 105, 120, 171, 214, 373, 375, 419, 439
Genexol® PM, 342
Genome-wide association studies (GWAS), 62, 73, 100, 106, 304, 373
Genomics, 68, 86, 104-106, 112, 119, 120, 129, 248, 304, 326, 444
Gentuzumab ozogamicin, 362
Geranylgeranyl pyrophosphate (GGPP), 267, 358, 359
Ghrelin, 30, 41, 177, 412, 420
Giant DNA viruses, 215

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Giardia lamblia, 223, 261
Gibbs free energy (G), 310, 499, 500
GIP, Gastric inhibitory peptide, 185, 186
GIT, gastrointestinal tract, 29, 40, 174, 269
Glaucoma, 37, 415
GlaxoSmithKline (GSK), 130
Gleevec®, 110, 132, 251, 269
Glembatumumab vedotin, 363, 428
Glial cells, 3, 37, 53, 183, 184, 187, 199
Glioblastoma multiforme, 38, 39, 102, 216
Glivec®, 269
Global Climate Change, 48, 143, 353
GLP, good laboratory practices, 9, 10, 147, 244, 421
Glucagon, 451
Glucagon-like insulinotropic peptide (GLP-1), 41, 131, 177, 185, 420
Glucocorticoid, 30, 33, 93, 274
Glucose, 30, 32, 82, 89, 108, 114, 122, 180, 189, 210, 253, 258, 269, 364, 366, 368, 378, 389, 420, 451
Glutamate, glutamic acid, 97, 272
Glutamine, 209, 214, 400, 525
Glutathione, 95, 127
Glycans, 104, 367
Glycated hemoglobin (HbA_{1c}), 367
Glycerol 3-phosphate, 254, 258
Glycine, 66, 67, 92, 319
Glycolysis, 8, 113, 252, 254, 255, 307
Glycome, 104
Glycoproteins, 33, 104, 344
Glymphatic system, 37
GMO, 48, 446
Goldenseal (*Hydrastis canadensis*), 361, 378
Good Cell Culture Practice (GCCP), 355, 356
Good Clinical Practice (GCP), 43, 357
Good Laboratory Practices (GLP), 9, 10, 147, 244, 421
Google, 55, 59
Google Baseline, 387
GPCR, G protein-coupled receptor, 179, 185, 264, 270, 271, 274, 291, 436
Gracilibacteria, 223
Granulocytes, 427
Grapes, 190, 215
Graviola (*Annona muricaa*), 140, 200
Green Aspiration Level (GAL), 352
Green Chemistry Institute Pharmaceutical Roundtable, 352
Green tea, 9, 43, 126, 127, 361, 384, 407, 408, 411-415, 421
GridDock, 296
Green scorecard, 352
Gregor Mendel, 241, 242
GroEL, 262
Growth factors, 21, 31, 373, 447, 449, 450
GTPase, 265, 317
Guam, 140
Guadeloupe, 140
Guanosine triphosphatases (GTPases), 265, 317
Guidelines on Current Good Radiopharmacy Practices, 353
Guide RNA, 441, 442
Guanábana, 140
Gut-brain axis (GBA), 42, 175, 182
Gut microbiome dysbiosis, 178, 188, 191-193, 200, 201
Gut microbiota, 39, 42, 174, 176, 178-180, 184-186, 189, 191, 193, 197, 200, 201, 208, 209, 212-214, 219
Gut microbiome, 19, 42, 82, 135, 167, 169-174, 178, 180-193, 196-202, 205, 207-212, 214-216, 220
Guanine, 66, 276

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

GWAS, 62, 73, 100, 106, 304, 373
H^+/K^+ ATPase, 251
H1N1 influenza viral strain, 117
HAART, 360
HACCP, 349
Hannah's Hope Fund, 131
Happiness, 17, 26, 191, 391
Harvard, 73, 117
HATs, 87, 91
Hazard Analysis and Critical Control Points (HACCP), 349
HDACs, 87, 88, 90, 186
HDL, High-density lipoprotein, 196, 204
HDM2 (human protein double minute 2), 296, 302
Health Nucleus, 387
Heart on a chip, 328
Heavy (H)-chain antibodies (HCAbs), 345
Helicobacteria pylori, 26
Helminths, 182, 219, 223-225
Heme A, 358
Hemoglobin, 189, 259-262, 445, 504
Hepatitis, 85, 122, 196, 268, 280, 341
Hepatotoxicity, 325
HER-2 gene, 265, 307, 362, 363
Herceptin®, 115, 361, 362, 428
Hereditary Neuropathy Foundation, 131
Heredity, 20, 241, 242
Heroin, 271, 337, 393, 400, 525
Heterochromatin, 88, 101-103
Heterogeneous nuclear RNA
Heteronuclear single quantum coherence (HSQC), 316-320
Hexanoic acid, 221, 222
Hexosamine pathway, 193
HGC, high gene count, 174, 209
HGT, 20, 87, 170
Hidden connections, 1, 21, 23, 112, 113, 132, 280
High gene count (in the gut microbiome), 174, 209
High performance liquid chromatography (HPLC), 9, 511-515, 537, 550
High throughput screening (HTS)
High throughput sequencing of cross linked immunoprecipitation, 79
Higgs boson, 14
High content screening (HCS), 124, 193, 359
High-density lipoproteins, 204
High intensity interval training, 18
Highly active antiretroviral therapy (HAART), 360
Hippocampus, 84, 96, 412
Histamine, 30, 41, 270, 272, 282
Histone acetylases, 87, 88
Histone deacetylases (HDACs), 87, 88, 90, 186
Histone methylases, 88
Histone proteins, 20, 79, 87
HITS-CLIP, 79
HIV, 17, 110, 202, 222, 251, 252, 266, 268, 296, 297, 360, 427, 442
HIV-1 glycoprotein gp160, 265
HIV reverse transcriptase, 78, 251, 252, 268, 278, 291, 314, 360
HLA, human leukocyte antigen, 46, 299, 356, 426, 431
HL1, 387
HMG-CoA reductase, 242, 250, 253, 267, 358, 370, 380
HMO, 206
HMP, Human Microbiome Project, 170, 196
hnRNA, 74, 76, 77, 79
Hodgkin's lymphoma, 275, 362
Holistic approach, 5, 21, 22, 222
Holobionts, 167, 169, 446
Hologenome, 167, 169
Homeopathic medicine, 98
Homodynamics, 108
Homeopathy, 99
Homeostasis, 8, 31, 32, 34, 36, 38, 39, 49, 52, 53, 81, 83, 90-92, 96,

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

97, 100, 108, 126, 180, 182, 184, 185, 197, 203, 212, 325, 365, 420, 426, 428
Horizontal gene transfer (HGT), 20, 87, 170
Hormesis, 94-98, 121, 341
Hot spots, 296, 305, 315, 503
How prescription drugs, dietary supplements and 'super-foods' are developed and used, 382-387
HPA axis, 176, 182, 197
HPLC, 9, 511-515, 537, 550
HPV (Human papillomavirus), 302, 430
HRMS (high resolution mass spectrometry), 394, 395, 406, 535
HRV2, 374
HSQC, 316-320
Hubs (in networks), 3, 25, 45, 53, 55-65, 109, 111, 252, 253, 260, 281, 297, 304, 309, 314
hu14.18-IL2 fusion protein, 363
HUD (Humanitarian Use Device), 246
Human epidermal growth factor receptor2 positive breast cancer (HER2+), 265, 307, 362, 363
Human genome, 2, 13, 66-69, 73, 74, 76, 79, 84-87, 100, 112, 174, 215, 241, 387, 447
Human Genome Project, 68, 69, 241, 447
Human immunodeficiency virus (HIV), 17, 110, 202, 222, 251, 252, 266, 268, 296, 297, 360, 427, 442
Human induced pluripotent stem cells (hiPSCs), 298, 329
Human leukocyte antigens (HLAs), 46, 299, 356, 426, 431
Human Longevity Inc^{TM} (HL1), 387
HumanMethyl450 Beadchip, 92
Human Microbiome Project (HMP), 104, 170, 196
Human milk oligosaccharides (HMOs), 206
Human papillomavirus, 411
Human Physiome Project (HPP), 289
Human pluripotent stem cells (hPSCs), 355
Human rhinovirus 2 (HRV2), 374
Human umbilical vein endothelial cells (HUVEC), 329
Humanitarian Use Device, 346
Humanized monoclonal antibody, 360
Humoral immune response, 277
Hydrocodone, 393, 400, 526
Hydrogen bonds, 312, 317, 494-496
Hydronium ion, 496, 497
Hydrophilic, 306, 331, 342, 343
Hydrophobic, 301, 305, 306, 312, 340, 342, 343
Hydroxyapatite, 448
Hygiene hypothesis, 178, 208, 219
Hypercycle, 48-50, 80
Hyperparathyroidism, 266, 313
Hypertension, 37, 82, 125, 189, 251, 270, 327, 397, 402
Hypertriglyceridemic, 267
Hypothalamic-pituitary-adrenal axis (HPA axis), 175, 197
Hypochlorite (OCl^-), 205
Hypothalamus, 28, 30, 176, 186, 420
IBDs, 174
IBS, 42, 174, 180, 181, 183, 197, 211, 215, 216, 220
Ibuprofen, 283, 381, 400, 526
IC_{50}, 258, 259, 371
ICG, 120
ICP, 194
Identify a target, 243, 280
IDO, indoleamine 2,3-dioxygenase, 433
IFN-γ, 36
Ig, immunoglobulin, 199, 216
IL, interleukin, 21, 31, 36, 177, 190, 296, 302, 425, 432

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IL-6, 31-33, 190, 196, 283, 287, 288
IL-12, 190, 429
IL-17A, 275
ILCs, Innate lymphoid cells, 35, 36
Illumina, 92
Imatinib, 132, 251, 269, 311
Imatinib mesilate, 269
Immortal jellyfish, 28
Immune dysregulation, 38
Immune-neuroendocrine system, 198, 199
Immune privilege, 35, 36
Immune-related adverse events (irAEs), 434
Immune-related disease control rate (irDCR), 434
Immune-related overall response rate (irORR), 434
Immune-related progression-free survival (irPFS), 434
Immune-related response criteria (irRC), 434
Immune system/network, 21, 28, 29, 31, 32, 35-37, 39, 41, 42, 46, 64, 71, 72, 75, 78, 83, 123, 124, 167, 172, 173, 176, 178, 180, 182-186, 192, 196-199, 201, 202, 208, 211-214, 223, 224, 267, 275, 277, 287, 289, 294, 299, 308, 339, 377, 414, 416, 424-426, 430, 432, 434, 435, 441, 446
Immunoglobulins (Igs), 199, 216
Immunomodulatory drugs, 364
Immuno-oncology, 433, 435
Immunoregulatory Bacteria, 208
Immunostimulation, 21
Immunosurveillance, 188
Immunotherapy, 72, 354, 377, 424-437, 440, 452
Improve the lead compound, 243, 336-338, 380
IND (Investigational New Drug), 244, 347, 353, 357
Induced Pluripotent Stem Cells (iPSC), 50-52, 108, 170, 298, 327, 329, 375, 444
Inductively coupled plasma spectroscopy, 94
Infiltrating myeloid cells, 38
Inflammation, 24, 35, 36, 38, 72, 89, 91, 96, 104, 108, 124, 130, 147, 171, 173-175, 178, 184, 188-190, 192, 193, 196, 197, 199, 200, 202-204, 208, 212, 267, 275, 282, 283, 306, 336, 361, 366-368, 410, 412, 413
Inflammasomes, 196, 201
Inflammatory bowel diseases (IBDs), 174
Infliximab, 360
Influenza virus, 47
Initiative for Chemical Genetics (ICG), 120
Innate immune system, 208, 212, 277, 425, 426, 435
Innate lymphoid cell (ILC), 35, 36
Inositol (1,4,5)-trisphosphate (IP3), 290
Inotuzumab ozogamicin, 362
Insertion elements, 19
Institute of Medicine (IOM), 134
Institute for Rare and Neglected Diseases Drug Discovery, 131
Insulin, 30, 32, 33, 41, 64, 76, 114, 125, 185, 186, 193, 269, 271, 272, 298, 366, 368, 420, 451
Insulin receptor substrate (IRS), 64
Integrase inhibitors, 360
Integrome, 105
Interactome, 105
Interdisciplinary and iterative process, 27, 364
Interferons, 21, 36, 363, 424, 427
Interferon-γ (IFN-γ), 36
Interleukin (IL), 21, 31, 36, 177, 190, 296, 302, 425, 432
Inter-modular hubs, 58, 59
Internal review board (IRB), 357

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Internal ribosomal entry site (IRES), 374
Internal standard, 539, 540
International Consortium for Innovation & Quality in Pharmaceutical Development, 352
International Pharmaceutical Abstracts (IPA) database, 324
International Standards Organization-900 (ISO-9000), 349
Interstitial fluid (ISF), 36, 37
Intestinal worms, 219, 223-225
Intracranial hypertension, 37
Intrinsic neurons, 175, 182
Intrinsic pathway of apoptosis, 306, 307
Intrinsic primary afferent neurons, 41, 175
Intrinsically disordered proteins (IDPs), 263, 318
Introns, 19, 20, 77, 78, 80, 223, 376
Invest in Kids program, 146
Investigational new drug (IND), 244, 347, 353, 357
In vitro transcribed (IVT) mRNA, 374, 376
Ion channel, 41, 252, 269, 271, 290, 291
Ionizable hydrogens, 92, 221, 222, 316
Ionizing radiation, 8, 9, 51, 94, 97, 98
Ion pump,1, 252
IPAN, Intrinsic primary afferent neurons, 41
Ipilimumab (Yervoy®), 308
iPSCs, 50-52, 108, 170, 298, 327, 329, 375, 444
IR (infrared), 505, 541-544, 554
IRB, 357
IRES, 374
Iressa®, 269
Irritable bowel syndrome (IBS), 42, 174, 180, 181, 183, 197, 211, 215, 216, 220
iRND3, Institute for Rare and Neglected Diseases Drug Discovery, 131
Isaac Newton, 7
Ischemia, 413
ISF, Interstitial fluid, 36, 37
ISO-9000, 349
Isopentanyl adenosine, 268, 358
Isoprenoid intermediates, 358
Isothermal titration calorimetry (ITC), 285
Isothiocyanate, 41
Isotretinoin, 274
^{131}I-tositumomab (Bexxar), 363

IUPAC, International Union of Pure and Appled Chemists, 386, 390, 392, 507
Ivabradine (Corlanor®), 360
Ivermectin, 360, 372
IVT mRNA, 374, 375
J. Craig Venter, 69, 387
Jacques Monod, 259
James D. Watson, 242
Janumet ®, 269
Jean-Pierre Changeux, 259
Jeffries Wyman, 259
JMP statistical discovery software, 338
JNK, 98
Joe Biden (Vice President), 133
Johns Hopkins University, 131
Jumpstarting Brain Tumor Drug Discovery Coalition, 132
Jun N-terminal kinase (JNK), 98
Junk DNA, 67, 73, 85
Kalydeco^{TM}, 132
Keap1 (Kelch-like enoyl-CoA hydratase-associated protein 1), 306
KEGG, 324
Kjeldahl method for quantifying protein, 539

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Ketamine, 126
Ketoprofen, 381
Keytruda®, 72, 308, 430, 434
Kidney diseases, 327, 415
Kidneys, 29-31, 33, 52, 346, 365, 429, 504
Kinome, 90, 105
Km, 256, 257
Klebsiella, 361
Knime104, 338
Krembil Foundation, 277
Kupffer cells, 108, 326
Kynurenic acid, 180, 181
L-3,4-dihydroxyphenylalanine (L-DOPA), 140
L1 retrotransposons, 84
Lachnospira, 196
Lachnospiraceae, 221
Lactobacillus bulgaricus, 207
Lactobacillus casei Shirota, 200
Lancet, 138, 139
Laws of heredity, 242
LC-HRMS, 394, 406
LC-MS, 107, 114, 288, 290, 293, 323, 356, 506, 518-538, 550
LD_{50}, 245, 284
LDIR, 97, 98
LDL-cholesterol or LDL-C, 125, 189, 197, 204, 267, 412
L-DOPA, 140, 401, 526
LDL, 125, 189, 197, 204, 267, 412
Lecithin, 193-195, 415
LEARN, 5
Leon Lederman, 14
Leonardo Da Vinci, 40
Leptin, 18, 40, 185, 186, 288, 420
Leukocytes, 39, 44, 46, 196, 267, 282, 283, 299, 356, 425, 426
Leukotrienes, 270, 272, 281-283
Levothyroxine, 274
Lewy body, 37
Library of Integrated Network-Based Cellular Signatures, 249
Library of Pharmacologically Active Compounds (LOPAC®), 124, 359
Licofelone, 283
Licorice, 122
Life Cycle Analysis (LCA), 352
Lifespan, 44, 88, 124, 215, 224, 243, 244, 367, 385, 387
ligENTS algorithm, 249
Lilly, 130, 131, 352, 369
Limit of detection (LOD), 512
Limit of quantitation (LOQ), 512
Limiting reagent, 500, 502
lincRNA, 80
LINCS (Library of Integrated Network-Based Cellular Signatures), 248
LINE, 86
Linear dose-response curve, 9, 94, 95, 390
Lineweaver-Burke plots, 258, 259, 310, 504
Linus Pauling, 260
Lipidomics, 104, 105
Lipids, 21, 45, 80, 90, 104, 114, 188, 254, 267, 273, 293, 294, 311, 339, 376, 407, 431, 518
Lipinski's rule of five, 305
Lipitor®, 267
Lipophilic, 273, 274, 306, 502, 503
Lipopolysaccharide (LPS), 42, 55, 57, 104, 176, 192, 361
Liposomes, 275, 276, 340-343
Lipoxin, 283
Liquid beam ionization/desorption (LILBID), 293
Liquid chromatography coupled to mass spectrometry (LC-MS), 107, 114, 288, 290, 293, 323, 356, 506, 518-538, 550
Liraglutide, 421
Lithography, 326
Liver, 29, 32, 33, 50, 52, 73, 82, 84, 85, 108, 176, 185, 187, 193, 269, 276, 280, 284, 313, 322, 325-330, 342, 343, 345, 381, 417, 500
Liver on a chip, 284, 326
LiverTox website, 327

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Liver Toxicity Knowledge Base (LTKB), 327
Living Food movement, 189
Llamas, 345
lncRNA, long non-coding RNA, 20, 73, 74, 77, 80, 81, 102, 103
Locate, employ, assess, reflect and nominate, 5
Locked nucleic acids (LNAs), 280
Long intergenic RNA (lincRNA), 80
Long interspersed element, 86
long non-coding RNA (lncRNA), 20, 73, 74, 77, 80, 81, 102, 103
Long, precursor-mRNA, 19
Long-range temporal correlations (LRTCs), 23
LOPAC, 124, 359
Loperamide, 361
Lopid®, 274
Loratadine, 270, 272, 381
Lorvotuzumab mertansine
Lovastatin, 254, 266, 401, 527
Low density lipoprotein (LDL), 125, 189, 197, 204, 267, 412
Low dose ionizing radiation (LDIR), 97, 98
Low gene count (in the gut microbiome), 174
LPS, 42, 55, 57, 104, 176, 192, 361
LRTCs, 23
LUNAR™ delivery technology, 103
Luteolin, 384
Lymph nodes, 18, 36-39, 124, 359
Lymphangiogenesis, 124, 359, 449
Lymphatic systems, 37
Lymphocytes, 15, 37, 38, 46, 182, 199, 201, 212, 267, 299, 308, 410, 427, 430, 431, 435, 444
Lysine, 87-89, 91
Lysine demethylases, 91
Lysophosphatidyl cholines, 114
M2 macrophages, 36
Mab (monoclonal antibodies), 72, 275, 360, 362, 364, 427
MACRA, 141, 142
Macrophages, 31, 36, 46, 72, 182, 190, 193, 194, 212, 267, 340, 426, 427
Mad cow disease, 183
MAGE, Multiplexed Automated Genome Engineering, 439
Magnetic resonance imaging (MRI), 447, 542, 546, 555, 556
Magneto-encephelography (MEG), 556
Malaria, 43, 110, 128-130, 202, 246, 247, 251, 252, 268, 294, 372, 445, 446
Malaria Box, 130
Malate, 196
MALDI-TOF/MS, 292, 535, 538
Malignant melanoma, 124, 308, 359
Mamaviridae, 215
Mammalian cDNA Library, 120
Mammalian Gene Collection (MGC), 120
MAPK, 98, 179, 269, 337
Maraviroc (Selzentry), 266, 296, 313, 314
Marseilleviridae, 215
MASCOT, 364, 365
Massachusetts General Hospital, 144
Mass spectrometer, mass spectrometry (MS), 107, 114, 124, 286, 288, 293, 323, 356, 394, 395, 406, 512, 516-538, 540, 542, 544, 547, 550
Mass to charge ratios (*m/z* ratios), 394, 406, 520-538
Mast cells, 34, 365
Mastermind, 265, 266
Matrigel™, 298, 325, 371
Matrix assisted laser desorption and ionization (MALDI), 292, 535, 538
Matrix assisted laser desorption and ionization with time of flight mass spectrometry (MALDI-TOF), 292, 535, 538

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Matrix metalloproteinases (MMPs), 279, 297
Maturation inhibitors, 360
MC4PC computer automated structure evaluation program, 338
MDMA, 126
MDSC, myeloid-derived suppressor cells, 432
Measles, mumps and rubella, 133-136, 138, 139
Meat, 17, 18, 147, 188, 192-195, 204, 209, 214, 219, 393, 415, 419, 420, 423
Medicare, 117, 141, 142, 203
Medicare Access and CHIP Reauthorization Act, 141
Medicines for Malaria Venture (MMV), 129
Meditation, 424
Mediterranean diet, 200
Meiosis, 44, 92
MEK, 314
Melainabacteria, 173
Melamine, 537
Melatonin, 29, 42, 82, 184, 272, 401, 527
Memantine, 272
Memory T cells, 430
Mendelian inheritance, 241, 300, 445
Meninges, 35, 37, 39
Merit Based Incentive Program, 142
Merck, 352
Mesenchymal stem cells (MSCs), 32, 33, 378, 420
Mesentery, 40
messenger RNA (mRNA), 15, 18-21, 47, 62, 63, 66-69, 74-79, 85, 100, 103, 108, 112, 180, 216-218, 242, 262, 269, 275, 278, 279, 288, 290, 291, 293, 373-377, 425, 437
Meta-analyses, 127
Metabolic master switch, 368
Metabolic network, 3, 25, 55-57, 61, 63, 65, 81, 113, 114, 281, 283, 284, 297, 304, 310, 314, 315
Metabolic Field – Schrödinger, 107
Metabolic Network Visualizer (MVN), 34
Metagenomics, 105, 167, 170, 203, 211
Metabolic pathway, 3, 8, 22, 64, 84, 88, 90, 105, 113, 121, 169, 202, 418, 427, 439, 440
Metabolic syndrome, 40, 74, 84, 174, 188, 189, 196, 210, 368, 412, 414, 418, 420
Metabolism, 4, 9, 18, 19, 22, 29, 31, 33, 36, 45, 55, 56, 64, 65, 75, 83, 88-92, 97, 100, 104-107, 109, 113, 114, 126, 169, 173, 181, 182, 196, 197, 210, 211, 214, 217, 218, 254, 263, 267, 272, 284, 287, 290, 307, 322, 325, 327, 329, 354, 357, 378, 385, 408, 411, 412, 415
Mettabolomics, 68, 104-107, 113, 114, 148, 326, 327, 417, 542
Metabonomics, 105
Metastasis, 36, 64, 73, 124, 279, 280, 300, 343, 359, 372, 379, 380, 374, 425
Metastatic melanoma, 72, 431, 433, 435, 436
Metformin (Glucophage®), 269, 364, 366-369, 385, 401, 527
Metformin in Longevity Study (MILES), 359
Methamphetamine, 126
Methanobacteriales, 174
Methaonbrevibacter, 174
Methanol (CH_3OH), 389, 406, 511, 513, 516
Methanosphaera, 174
Methionine, 66, 92
Mevinolin, 254
MGC, 120
Metabolites, 50, 58, 65, 82, 84, 88, 91, 104-108, 114, 171, 175, 183,

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

188, 190, 195, 199, 201, 208, 286, 288, 290, 297, 323, 325, 330, 331, 385, 417, 550
Methotrexate, 277, 360, 364, 369
Methyltransferases, 88, 91, 92, 95
Mevastatin, 242, 253
Michael J. Fox Foundation for Parkinson's Disease Research, 132
Michaelis-Menten equation, 256
Microarrays, 286, 287, 289
Microbial endocrinology, 185
Microbiome, 19, 24, 42, 44, 82, 108, 112, 113, 135, 138, 167-174, 178, 180-193, 196-202, 205-216, 219-224, 387, 424
Microelectronic mechanical systems (MEMS), 345
Microextrusion printing, 449
Microfluidic liver sinusoid model, 326
Microfluidic microscale cell culture analog (μCCA), 326
Microfluids, 286, 287, 326-328
Microglia, 35, 38, 39, 199, 208
Microprocessor complex, 75
microRNAs (miRNAs), 20, 75-79, 85, 103-105, 112, 248, 279, 280, 293, 373
Microtubules, 252, 362
MILES, Metformin in Longevity Study, 359
Mineralocorticoid, 274
Minerals, 218
MIPS (Merit Based Incentive Programs), 142
Miravirsen®, 280
mRNA, 15, 18-21, 47, 62, 63, 66-69, 74-79, 85, 100, 103, 108, 112, 180, 216-218, 242, 262, 269, 275, 278, 279, 288, 290, 291, 293, 373-377, 425, 437
Mifeprex®, 273
Mifepristone, 273
Milk, 169, 170, 172, 206, 208
Mimiviridae, 215
Minoxidil, 381, 402, 528
miRNA, microRNA, 20, 75-79, 85, 103-105, 112, 248, 279, 280, 293, 373
MIT, 73
Mitochondria, 58, 74, 75, 78, 89, 94, 97, 297, 307, 364, 367, 378, 385, 386, 408, 411, 412
Mitochondrial DNA (mtDNA), 74
Mitochondrial complex 1 in the electron transport chain, 367
Mitogen-activated protein kinase (MAPK), 98, 179, 269, 337
Mitogen-activated protein/extracellular signal-regulated kinase (MEK), 314
Mitomycin, 276
Mitosis, 44, 45, 71, 92
MM4TB, 131
MMAE, 362
MMR, 133, 133-136, 138, 139
MMV, 129
Mobile genetic elements, 19, 85-87, 169, 170
Mobilome, 87, 169
modified RNA (modRNA), 279, 373-377
Modular overlaps, 59
Modularity of biological networks, 110-112
Module criticality, 111
Module maps, 112
Molecular circuits, 73
Molecular dynamics (MD), 261, 294, 295, 312, 315
Molecular initiating event (MIE), 324
Molecular Libraries Probe Production Centers Network, 131
Molecular networks, 58
Monacolin K, 254
Monascus pupureus, 254
Monascus ruber, 254
Monoclonal antibody, 72, 275, 360, 362, 364, 427

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Monocytes, 31, 174, 267, 283, 448
Monomethyl auristatin E (MMAE), 362
Mononuclear phagocyte system (MPS), 342
Monozygotic twins, 84, 135
Montelukast sodium, 272
More Medicines for Tuberculosis, 131
Morbidity, 6, 7, 204, 224
Morning after pill to prevent pregnancy, 273
Morphine, 270, 271, 337, 341
Mosaicism in cellular DNA, 108, 443
Motrin, 381
MPS, Mononuclear phagocyte system, 342
MRI (magnetic resonance imaging), 447, 542, 546, 555, 556
MS, mass spectrometry, 107, 114, 124, 286, 288, 293, 323, 356, 394, 395, 406, 512, 516-538, 540, 542, 544, 547, 550
MSC, 32, 33, 378, 420
mtDNA, 74
MTL-CEBPA, 377
mTOR (mammalian target of rapamycin), 366, 267
Mucin, 173
Mulengele (*Vernonia amygdaline*), 42
Multidimensional, non-linear view of diseases, 109, 110
Multi-level marketing, 141
Multi-morbidity, 6, 7
Multiple feedback loops, 48, 50
Multiple sclerosis (MS), 38, 197, 225, 274, 275, 288
Multi-target drugs, 58, 266
Multivitamins, 94
Muscarinic acetylcholine receptors (M1 and M2), 270
Mushrooms, 372, 393, 396
Mutual exclusivity modules, 112
μM, microMolar, 94, 254, 503, 505, 510, 512, 514, 515
MVN, 34
MWC model, 259, 262, 263
Myocet®, 341
Mycobacterium tuberculosis, 268, 285, 321, 361
Mycobiome, 220-222
Myocardial infarction (MI), 59, 125, 204, 242, 278, 279, 327, 353, 375
Myocardium, 355, 413
Myocytes, 30, 31, 289, 298, 328, 355, 443
Myelin sheath, 288
Myeloid cells, 31, 38
Mylotarg® (gentuzumab ozogamicin), 362
Myokines, 31, 425
Myopathy, 125, 411
Myostatin, 31, 32
Myristate, 317
NAD^+, 88, 91, 254, 256, 258, 385, 386, 408, 409
NADH, 88, 91, 254, 256, 258, 408, 409
NADH:ubiquinone oxidoreductase, 408, 409
N- and P/Q-type Ca^{2+} channels, 179
Na^+/H^+-antiporter, 364
Namenda®, 272
Nanobodies (Nbs), 345
Nanobots, 344
Nanodots, 344
Nanoparticles, 98, 341, 342, 346, 377, 432, 451, 538
Nano sterically stabilized liposomes (nSSLs), 340
Nanotechnology, 36, 344, 376
Naproxen, 283, 381, 402, 528
National Brain Tumor Society, 131
National Center for Advancing Translational Sciences (NCATS), 131

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

National Center for Complementary and Alternative Medicine (NCCAM), 417
National Center for Toxicogenomics (NCT), 326
National Center for Toxicological Research (NCTR), 338
National Institute of Environmental Health Sciences (NIEHS), 326
National Institute of Diabetes and Digestive and Kidney Diseases, 327
National Institutes of Health (NIH), 68, 69, 98, 106, 115, 117, 118, 120, 125, 131, 133, 370, 417, 444
National Library of Medicine (NLM), 327
National Research Council (NRC), 121, 323
National Toxicology Program, 9
Natural-based nanocomposites, 451
Natural killer cells, NKs, 21, 31, 36
Natural order, 14
Nature, 14, 19, 21, 27, 42, 74, 93, 107, 241, 264, 284, 306, 341, 365, 440
Nature vs nurture, 93
Nbs (nanobodies), 345
NBTF, 189
NCE, 43, 224, 244
NCI 60, 288
NCTR, 338
NIEHS, 326
ncRNA, non-coding RNA, 75, 77, 79, 81, 101, 103, 442
NDA, new drug application, 116, 350
NDS (nonlinear dynamic systems), 415, 416
Necator americanus, 224
Necropsis, 52
Necroptosis, 52
Negative cooperativity, 260-265
Negative feedback, 5, 22, 30, 325
Neisseria gonorrhoeae, 429
Nervous stomach, 197
Netosis, 52
Network, 1-4, 6, 22, 23, 25, 26, 30, 37, 45, 46, 48, 50, 52, 53-66, 72, 73, 81, 87, 90-92, 98, 105, 109-114, 118, 119, 131, 132, 144, 169, 173, 175, 180, 182, 183, 186, 187, 206, 248, 249, 252, 253, 260, 266, 274, 275, 280, 281, 283, 284, 287, 289, 297-304, 309-323, 328, 338, 352, 359, 364, 368, 373, 374, 379, 380, 389, 412, 418, 425, 429, 438, 448, 449
Network assortativity, 60
Network-centric view, 73
Network dynamics, 3, 61, 281, 311, 374, 379, 380
Network medicine, 66, 280, 290, 297
Network pharmacology, 359
Network proteomics, 61
Network-related drug design, 3
Network theory, 53-66, 338, 389
Neural progenitor cell line (NPC)
Neuroblastoma, 363, 377, 378, 431
Neurodegenerative diseases, 24, 38, 39, 83, 96, 170, 184, 188, 197-201, 203, 246, 266, 313, 371, 393, 413, 424
Neurohormones, 185
Neuroimmune system, 40, 216
Neuroimmunology, 38
Neuroinflammation, 38, 199
NeuroMatrix™, 448
NeuroMend™, 448
Neuromodulators, 24
Neuron, 3, 24, 29, 35, 38, 39, 41, 44, 45, 47, 53, 82, 84, 94, 96, 97, 175, 176, 180, 182-187, 197, 199-201, 208, 212, 214, 272, 371, 376, 420
Neurotransmitters, 19, 30, 45, 96, 97, 179, 185, 187, 197, 271, 287, 290, 400, 401
Neutrophils, 31, 36, 283, 289

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Neurotoxic aggregates, 200
Neurovirology, 38
Neutralism, 54
New Caledonia, 140
New chemical entity (NCE), 43, 224, 244
New Drug Application, 116, 350
New molecular entity
Nexium®, 381, 423
NF-κB, 192, 193, 267, 275, 380
NIAGEN™, 385
Niche-buster drugs, 248
Nicorette®, 381
Nicotinamide adenine dinucleotide oxidized form (NAD^+), 88, 91, 254, 256, 258, 385, 386, 408, 409
Nicotinamide adenine dinucleotide reduced form (NADH), 88, 91, 254, 256, 258, 408, 409
Nicotinamide mononucleotide (NMN), 385
Nicotinamide riboside (NR)
NIH, 68, 69, 98, 106, 115, 117, 118, 120, 125, 131, 133, 370, 417, 444
NIH Blueprint for Neuroscience Research, 131
NIMS (network target-based identification of multicomponent synergy), 300
N-isopropylacrylamide, 335
Nitric oxide (NO), 214, 494
Nitrosophaera, 220
Nivolumab (Opdivo®), 72, 308
Nizatidine, 381
NKCC1 (Na^+-K^+-Cl^-) cotransporter, 371
NLR (NOD-like receptor), 196
nm, nanometers, 255-257, 340-344, 505, 506, 509, 541, 544, 545
nM, nonoMolar, 125, 126, 254, 259, 273, 300, 503
NME, new molecular entity, 43, 244, 245, 250, 285-287, 290, 291, 293-298, 302, 309, 312, 317, 320, 322, 327, 328, 347, 357, 369, 371, 411, 437, 500, 501, 506, 511
N-methyl-D-aspartate (NMDA), 272
NMN, Nicotinamide mononucleotide, 385
NMR, 114, 261, 262, 284, 285, 288, 290, 297, 301, 307, 309, 311, 314-321, 338, 356, 406, 410, 540-555
NMS, Non-motor symptoms (NMS), 200
No observed adverse effect level (NOAEL), 325, 341
Nobel Prize in Physiology or Medicine, 371
Nocebo effect, 26
Nocturnal hypertension, 82
NOD-like receptor, 196
Nodes (in networks), 3, 25, 55-65, 109, 206, 252, 253, 280, 281, 299, 303, 304, 310, 314, 315
NOE (Nuclear Overhauser Enhancement), 551
Nonlinear processes, 21
Nomenclature Committee on Cell Death, 52
Noncoding RNA-activating, 75
No conformational change, negative cooperativity, entropy driven allosteric proteins, 265
Nonlinear dynamics, 23, 416
Noni (*Morinda citrifolia*), 221, 390
Nonioside B, 390
Non-motor symptoms (NMS), 200
Non-nucleoside reverse transcriptase inhibitors, 360
Non-random network, 25, 55
Non-small cell lung cancer (NSCLC), 308
Nonsteroid anti-inflammatory drugs (NSAIDs), 274, 283, 381, 407
Noradrenaline, 30, 272
Norepinephrine, 30, 272
Norgestimate, 274

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

North American Menopausal Society, 115
Norwalk, 215
Notch receptors, 265
Notch intracellular (NotchIC), 265, 266
Nolvadex®, 274
Novartis, 269, 352, 369, 432
Novobiocin, 361
Nrf2/ARE antioxidant system, 324, 361, 384, 386, 391, 407, 408, 411, 413, 415
NSAID, 274, 283, 381, 407
Nuclear erythroid-2 like factor-2 (Nrf2), 324, 361, 384, 386, 391, 407, 408, 411, 413, 415
Nuclear factor kappa beta (NF-κB), 192, 193, 267, 275, 380
Nuclear factor kappa-light-chain-enhancer of activated B cells, 192
Nuclear lamins, 268, 358
Nuclear magnetic resonance (NMR) spectroscopy, 114, 261, 262, 284, 285, 288, 290, 297, 301, 307, 309, 311, 314-321, 338, 356, 406, 410, 540-555
Nuclear receptors (NRs), 272, 338
Nucleoside reverse transcriptase inhibitors, 360
Nucleotide-binding oligomerization domain (NOD) receptors, 41, 42
Nurses, 8, 13, 144-146, 248
Nurse-Family Partnership, 146
Nusinersen, 376
Nutrition, 9, 84, 90-93, 105, 173, 196, 202, 203, 387-390, 418, 429
Obama (President), 106, 133, 137
Obinutuzumab (Gazyvaro®), 360, 428
OCPP (Office of Critical Path Programs), 116
ODDT (Open Drug Discovery Teams), 131
Office of Critical Path Programs, 116
Office of Orphan Products Development, OOPD, 247
Old Friends mechanism, 209, 219
Oligodendrocytes, 38, 288
Omega-3 fatty acid, 407
Omega-3 fats (ω-3 fats), 18, 193, 407
Omega-6 fats (ω-6 fats), 281
OMIM (On-line Mendelian Inheritance in Man), 241, 300
Omnisciome, 105
Oncolytic viruses, 17, 76, 374, 378, 428, 433, 435
Oncosis, 52
Onion network, 60
OOPD, 247
Opdivo®, 72, 308
Omeprazole magnesium, 251, 381
One target–one drug, 359
Open Drug Discovery Teams, 131
OpenEye Scientific Software (chemoinformatic tool), 338
Open reading frame (ORF), 67, 120, 374
Opioid receptors, 271, 337
Opioids, 270, 393
OptiShell™, 347
Oral biofilms, 204, 205
Oral health, 203-205
ORF, 67, 120, 374
Organ chips, organ-on-a-chip, 327, 354, 355, 448
Origin of Species, 12
Orlistat, 403, 421, 529
Orphan drugs, 246, 247
Orphan Drug Act, 246
Orphan Products Clinical Trials Grants Program, 247
Orthosteric ligands, 310
Orudis KT, 381
Osteoblasts, 31-33, 44, 102, 420
Osteocalcin, 31-33
Osteoclasts, 31-33, 44, 102, 420
Osteopontin, 32

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Osteoporosis, 31, 33, 268, 274, 317, 318
OTC drugs, 381, 382
Ovarian follicle, 29
Over the counter (OTC) drugs, 381, 382
Oxalobacter, 221
Oxytocin, 29, 274
Overlapping nodes, 59
P4 medicine, 106, 115, 199
p53 protein, 16, 25, 80, 95, 98, 112, 252, 253, 296, 302, 309, 310, 325, 367, 379
$p66^{Shc}$, 367
Paclitaxel, 342, 360
Pak1, 317
Paleolithic diet, 204
PAMP, 176, 425
Pancreas, 33, 47, 176, 183, 451
Pancreatic β-cells, 33
PANDAS, 199
Paneth cells, 211
PANS, Pediatric Acute-onset Neuropsychiatric Syndrome, 199
Papillibacter, 221
Parabacteroides, 221
Paraplatin®, 276
Parasympathetic nervous system, 186
Parathyroid hormone (PTH), 29
Paravascular transport pathway, 37
Parkinson's disease, 37, 42, 76, 127, 133, 140, 184, 197, 200, 369, 408, 415
Partition function, 260, 261
Party hubs, 58, 111, 314
Pascal, 73, 112
Pathogen-associated molecular patterns (PAMPs), 176, 425
Pathway scoring algorithm, 73, 112
Pattern recognition receptors (PRRs), 183, 425
Pauling-KNF, 260-263
Pawpaw (*Asimina triloba*), 200
PC, Phosphatidylcholine, 193, 195, 340, 415
PCBs, 99
PCDDs, 99
PCPP, 126
PD, Parkinson's disease, 37, 42, 76, 127, 133, 140, 184, 197, 200, 369, 408, 415
PD-1 ligand (PD-L1), 425
PDB, Protein Data Bank, 301, 302, 309, 447, 551, 554
PDC, Pediatric Device Consortia, 247
pDC, plasmacytoid dendritic cells, 425, 426
PDGFR, 110, 269, 271, 449
PDSA (Plan, Do, Study and Analyze), 5
Pediatric Acute-onset Neuropsychiatric Syndrome, 199
Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections, 199
Pediatric Device Consortia (PDC), 247
PEG, polyethylene glycol, 340, 342, 343, 424, 516
Pegaptanib, 278
PEGylated liposomes, 340
Pembrolizumab (Keytruda®), 72, 308, 434
Penicillin, 250
Penicillium citrinum, 253
Pepcid AC, 381
Peptide YY, 177, 185
PER2 gene, 83
Periodontal disease, 189, 203-205
Periodontitis, 203, 205, 223
Peripheral nervous system (PNS), 39, 53, 186
Perivenous space, 37
Peroxisome proliferator-activated receptor (PPAR), 32, 33, 34, 276, 378

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Personal testimonials, 123, 135, 138, 140, 141, 358, 384, 423
Personalized medicine, 4, 52, 67, 112, 116, 118, 298
Peyer's patches, 211
Pfizer, 131, 352, 369
PGE_2, 274
Phagocytes, 52, 53, 342, 426
Phagocytosis, 426
Pharmaceutical Quality for the 21st Century, 351
Pharmacokinetics, 9, 82, 148, 243, 244, 284, 321, 322, 377, 380, 435
Pharmacophore, 285, 315, 336, 337
Phase I, II, II and IV clinical trials, 245, 298, 302, 356-358, 363, 382, 423
Phase II enzymes, 322
Phase I and Phase II metabolism, 322
Phenome-wide association studies (PheWAS), 249
Phenotype, 17, 19, 65, 82, 91, 102, 109, 110, 112, 120, 124, 171, 184, 200, 249, 250, 284, 290, 300, 307, 354, 355, 359, 366, 369-373, 380, 416, 426, 441
Phenotypic screening, 130, 243, 244, 250-253, 359, 369-373, 416, 452
Phenylboronic acid (PBA), 343
Phenyl group, 330, 406, 501, 506, 510, 516, 517, 553
Phoenix Nest, Inc., 131
Phosphatidylcholine (PC), 193, 195, 340, 415
Phosphatidyl inositol 3-kinase (PI3K), 112, 307, 313
Phospholipids, 114, 254, 255, 407
Phosphoproteome, 90
Phosphoproteomics, 105
Phosphorescence, 510
Photoreceptor cells, 45
Physical activity, 5, 97, 108, 186, 211, 368
Physician Quality Reporting System (PQRS), 142
Physiological based pharmacokinetic models (PBPK), 322
Physiome Project, 289
PI3K/Akt, 112, 307, 313
Picornaviridae, 215, 217
Pier luigi Luisi, 1
Pipeline Pilot, 338
Pitolisant, 273
Pituitary gland, 28-31, 33, 175, 176
pKa, 498, 499
PKA, protein kinase A, 318, 319
PKC, 193
Placebo, 26, 125, 210, 211, 219, 224, 367, 368, 386, 422
Planck's constant (h), 505
Plaque, 37, 124, 169, 187, 204, 205, 267, 268, 358, 359
Plaque psoriasis, 275
Plasmids, 68, 85, 86, 277, 375, 444
Plasmodium, 130, 251, 295
Platelet-dependent growth factor (PDGF), 449
Platelet-derived growth factor receptor (PDGFR), 110
Platelets, 18, 29-31, 282, 374, 380
Pleckstrin homology (PH) domain, 313
Pleurotus tuber-regium mushroom, 372
PLK1, 363
Pluripotent stem cells, 51, 108, 170, 278, 298, 329, 355, 375
PMI, 106
PNS, 53
Poliovirus (PV), 374
Pollutants, 21, 208, 440, 502, 503
Polo-like kinase 1 (PLK1), 360
Poly(amidoamine) (PAMAM), 344
Polycaprolactone, 449, 450
Polychlorinated dibenzo-*p*-dioxins, 99
Polychlorinated biphenyls, 99
Polycomb proteins, 88

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Polydextrose, 210
Polyethylene glycol (PEG)
Polymerase chain reaction (PCR), 278, 291
Polymyxin B, 361
Poly(octamethylene maleate (anhydride) citrate), POMaC, 448
Polyol and hexosamine flux, 193
Polypharmacology, 130, 259
Polyploid, 108
Polysaccharides, 173, 189, 201, 212, 448, 538
Polyunsaturated fatty acids (PUFA), 18, 121, 177-179, 220, 281-283, 392
Pomegranates, 190, 384
Poorly liganded iron, 393
Porphyromonas gingivalis, 196, 203
Positive feedback, 21
Post-translational modifications (PTM), 19, 64, 87-93, 101, 268, 304, 311
Power law, 23, 55
PPARα and PPARγ, 32, 33, 34, 276, 378
PPI, protein-protein interactions, 22, 61, 62, 298-309
PPI, proton pump inhibitors, 364, 423, 424
PPI network, 25, 55, 61, 62, 299, 300, 303, 304, 309
PQRS, 142
Precision medicine, 106, 107
Precision Medicine Cohort Initiative Program, 106
Precision Medicine Initiative Predictive biomarkers, 433
Predictive Safety Testing Consortium, 117
Preconception Stress and Resiliency Pathways, 118
Precursor ions, 534, 535
Precursor mi-RNAs, 75, 77
Precursor-mRNA (pre-mRNA), 19, 20, 373, 376, 377
Predictive, preventive, personalized and participatory medicine, 4, 106, 115
Premarin®, 274
pre-miRNA, 75, 77
Premotor cortex
pre-mRNA, 19, 20, 373, 376, 377
pre-mRNA and mRNA-based therapeutics, 376, 377
Pre-operative planning, 451
President Obama, 106, 133, 137
Prevotella, 172, 173, 188, 198, 202, 209, 210, 221
Prilosec®, 251, 381
Primary miRNA, 75
Primary standard, 496, 539
pri-miRNA, 75
Prion, 37, 183, 184
Problem distributors in cellular networks, 253
Problem solvers in cellular networks, 252, 253
Procaine, 336, 337
Process analytical technology (PAT), 351
Processed foods, 219, 389
Product ions, 534, 535
Programmed cell death, 46, 52, 53, 71, 75, 306, 363, 444
Programmable self-replicating manufacturing systems, 344
Programmed cell-death protein 1 (PD1), 308
Prokaryotes, 66, 77, 95, 173, 185, 208, 218
Propaganda, 121
Prophyromonas, 198
Propionibacterium, 172
Propionate, 185, 186, 190, 272, 405, 532
Propranolol, 251
Prostaglandin E_2 (PGE_2), 274
Prostaglandins, 274, 281
Prostate, 65, 120, 145, 190, 272, 274, 281, 414, 427
Prostate specific antigen (PSA), 144

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Protease inhibitors, 252, 360
Proteases, 47, 252, 268, 291, 294, 297, 307, 360, 362
Proteasome, 268, 306, 426
Protectins, 283
Protein, 1-4, 9, 11-25, 29-31, 33-41, 43-52, 54-96, 98-106, 108-113, 124, 129, 169-172, 174, 176, 179-181, 183, 186, 188, 191-194, 196, 200, 204-210, 216-219, 223, 242, 248-250, 252-254, 257, 259-272, 275-281, 283-288, 290-325, 337, 338, 342, 344-346, 353-355, 358-363, 365, 367, 368, 372-377, 379, 381, 385, 412, 413, 424-430, 432, 433, 437-441, 444-452, 518, 535, 537-539, 551, 552, 554, 555
Protein databank (pdb), 301, 302, 309, 447, 551, 554
Protein-DNA interactions, 1, 108
Protein interaction network, 61, 111, 260, 297, 302
Protein kinase A, 318, 319
Protein kinase C (PKC), 193
Protein kinases, 64, 90, 98, 105, 111, 112, 179, 193, 269, 291, 317-319, 337, 367
Protein methyltransferases, 91
Protein–protein interaction networks, 61
Protein–protein interactions (PPIs), 22, 61, 62, 298-309
Protein tyrosine kinases (PTKs), 298, 311
Proteome, 62, 65, 82, 90, 104, 105, 112, 113,263, 322, 359, 535
Proteobacteria, 171, 173, 189
Proteomics, 61, 104-107, 113, 288, 290, 293, 294, 326, 327, 359, 370, 538
Protocatechuic acid, 197
Proton pump, 251, 364, 423, 424
Proton pump inhibitors (PPIs), 364, 423, 424
Protozoa, 167, 223-225, 251
Provenge®, 417
Pyrvinum, 314
PSA, 144
Pseudomonas putrid, 440
Psoriatic arthritis, 275
PSRP, Preconception Stress and Resiliency Pathways, 118
Psychosomatic, 8, 26
Pterostilbene, 384, 386, 390
PTM, Post-translational modifications, 19, 64, 87-93, 101, 268, 304, 311
PubMed, 103
Pulse delay in NMR, 543, 551, 553, 554
Pure Food and Drug Act, 147
PV(Sabin)-Rhinovirus IRES PV Open reading frame (PVSPIRO), 374
Pyroptosis, 52
QbD, Quality-by-design, 351, 352
QMS, 347, 349, 356
QSAR, 244, 291, 295, 297, 338
Qsitefinder, 296
Qsymia®, 421
Quadrupole mass spectrometers, 518, 534
Quality control (QC), 347-349, 353, 354, 356, 406
Quality management system (QMS), 347, 349, 356
Quality Risk Management (QRM), 353
Quantify drug-target interactions, 243, 380
Quantitative analysis, 293, 323, 511, 539, 540, 544
Quantitative structure-activity relationships (QSARs), 244, 291, 295, 297, 338
Quantitative systems pharmacology (QSP), 370
Quercetin, 324, 384
Quorum sensing, 169, 212

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

R Project for Statistical Computing, 338
RAC, Recombinant DNA Advisory Committee, 433, 434
Rac1, 359
Radiation hormesis, 97, 98
Radiofrequency (rf) energy, 314-316, 320, 534, 540-545, 550, 552
Radionuclides, 363
Radioresistance, 98
Raloxifene, 274
Ralstonia, 200
Ramelteon, 272
Random network, 55
Ranitidine, 272, 273, 381
Rapid prototyping, 4, 329, 447, 452
Rare Pediatric Disease Priority Review Voucher Program, 246
Ras-like proteins, Rho, Rab, Rac, Ral, or Rap, 268, 358
RAS protein, 317
RAS (renin-angiotensin system), 8, 24, 365
RCD, Regulated cell death, 52
RDkit (chemoinformatic tool), 338
rDNA (recombinant DNA), 68, 442-444
Reactive oxygen, and nitrogen species (RONS), 188, 192, 193, 196, 306
Reactive oxygen species (ROS), 9, 19, 193, 367, 407, 412
Reactome, 248
Receptor protein kinases, 112
Receptor tyrosine kinases (RTKs), 269, 271
RECIST (Response Evaluation Criteria in Solid Tumours), 434
Recombinant DNA (rDNA), 68, 442-444
Recombinant immunotoxins, 363
Red blood cells, 18, 29, 31, 46, 47, 293, 342
Redox cycles, 81
Redox homeostasis, 83
Reductionist thinking, 1-4, 7-15-20, 35, 66-69, 74, 80, 81, 84-86, 93, 94, 100, 107, 108, 121, 131, 143, 145, 147, 148, 168, 203, 205, 218, 219, 245, 246, 250, 253, 280, 284, 294, 322, 325, 339, 389-391, 414, 417, 418, 421, 428, 428, 436, 438, 502, 506
Red yeast rice, 125, 254, 380, 407, 410
Regenerative medicine, 91, 344, 353, 354, 373, 448, 449, 451
Regulated cell death (RCD), 52
Regulatory networks, 61, 63, 64, 73, 113
Regulatory subnetwork, 63
Regulatory T-cells (Tregs), 212, 429, 432, 433, 436
Relative Process Greenness (RPG), 352
Relaxed state, R state of hemoglobin, 259-262
Remifentanil, 271
Renin, 8, 29, 34, 268, 365
Renin-angiotensin system, 8, 24, 365
Reoviridae, 215
Residue interaction graph (RIG), 310
Resolvins, 283
Respiratory tract infections, 189
Response Evaluation Criteria in Solid Tumours (RECIST), 434
Reproductive system, 29
Resveratrol, 384, 386, 391
Reticuloendothelial system (RES), 342
Retinoblastoma protein (pRb, also called RB1), 112, 263
Retinol, 288
Retrotransposons, 78, 79, 84-87, 93, 168, 170, 215
Reverse transcriptase, 78, 251, 252, 268, 278, 291, 293, 324, 360

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Rheumatoid arthritis, 83, 189, 275, 281, 360
Ribonucleoprotein (RNP), 20, 79, 80
ribosomal RNA (rRNA), 76, 80, 220
Ribozymes, 75, 78, 79, 80, 278
Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), 223
Rich-club network, 59
RIG (residue interaction graph), 310
Rituxan®, 428
RNA, 3, 4, 12, 15, 18-21, 23, 47-49, 54, 55-58, 62, 63, 65, 67, 68, 71, 73-81, 84-88, 91-93, 100-105, 108-110, 112, 168-170, 180, 205, 215-218, 220, 221, 223, 241, 242, 248, 249, 252, 263, 264, 268, 269, 275-280, 288, 290-293, 297, 301, 311, 320, 327, 343, 344, 363, 373-377, 436-438, 441-443, 446, 447, 551, 552
RNA aptamer, 436
RNA-based genes, 74-79
RNA therapeutics, 278
rRNA, 76, 80, 220
RONS, 188, 192, 193, 196, 306
Root cause, 1, 13, 15, 16, 116, 205, 219, 370
ROS, 9, 19, 193, 367, 407, 412
Rosuvastatin (Crestor®), 267
Rozerem®, 272
RNA-based therapeutic, 76, 103, 376, 377
RNA hydrolase, 77, 376
RNA polymerase, 66, 74, 87, 218, 263, 268, 278
RNase, 75, 77, 446
Robustness-related module criticality, 111
Rogaine, 381, 402
Rosaxerone, 95
Roseburia, 200
Rotavirus, 202, 215
RT-PCR, 278
RuBisCO, 223
Rucaparib (Rubraca®), 268
Ruminococcaceae, 221
Ruminococcus, 173, 198, 221
Saccharomyces boulardii, 220
Saccharomycetales, 222
Saccharibacteria, 223
S-adenosylhomocysteine (SAH), 92
S-adenosylmethionine (SAM), 92, 95
Salmeterol, 272
Sanofi, 131
Satiety, 18, 175-177, 186, 391
Satoshi Ōmura, 372
Saturated fats, saturated fatty acyls, saturated fatty acids, 18, 27, 114, 192, 193, 209, 221, 317
Saturation transfer difference NMR (STD-NMR), 316
Saxenda®, 421
Scale free networks, 25
SCFA, 169, 185, 188, 190, 201, 205, 209, 214, 221, 389
SCN, suprachiasmatic nucleus, 82
Second Genome, 387
Secretome, 359
Secukinumab, 275, 428
Select a disease, 243, 245-250, 380
Select a therapeutic target or phenotype, 250
Selected ion monitoring or SIM, 534
Selective estrogen receptor modulator (SERM), 360
Selenium, 94
Self-organization, 2, 287
Self-organized systems, 111
Self-regenerating, 4
Selfish genes, selfish genetic elements, 12, 79, 80, 86, 446
Senathia, 172
Sensipar®, 266, 313, 314
Sentinel Initiative, 117
Sepsis, 83, 198, 251

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Sequential model, with induced fit, 260
Seres Health, 387
Serine/threonine kinases (STK), 269
SERM selective estrogen receptor modulator, 360
Sermo, 132
Serotonin, 41, 180, 181, 184-186, 197, 198, 270, 271, 290, 405
Serratia, 196, 206
Seventh Day Adventists, 188
Severe combined immunodeficiency (SCID), 277
Serevent®, 272
SGN-35, 362, 363
shRNA, 77
Short chain fatty acids (SCFAs), 169, 185, 188, 190, 201, 205, 209, 214, 221, 389
short hairpin RNA (shRNA), 77
Short interspersed nuclear element (SINE), 84
Shotgun sequencing, 69, 105, 167
Sickle cell disease, 445
Signal to noise ratio (S/N), 99, 512, 513, 541, 544
Signal peptidase complex (SPC), 445
Signaling network, 3, 63, 64, 249, 364, 365, 373
Signaling pathways, 32, 38, 59, 63, 64, 90, 115, 121, 127, 176, 193, 198, 263, 271, 279, 291, 311, 323, 364, 428, 450
Silk fibers, 450
Siloranes, 334, 335
Simvastatin®, 251, 267, 404, 531
SINE, Short interspersed nuclear element, 84
Sinecatechins 15%, Verege ®, 43, 411
single strand DNA, 47
single strand RNA, 47
Singulair®, 272
Sipuleucel-T, 427
Sir proteins, 88
siRNA, 77-79, 103, 342-344, 363, 375, 442
SIRT6, 89
SIRTs, 88
Sirtuins, 88
Sitagliptin, 251, 269
Six Sigma, 291
Sleep deprivation, 37, 83
Sleep homeostasis, 81
Sleeping Beauty transposon, 170
Sleep-wake cycles, 81
SM2miR database, 248
Small-activating RNAs (saRNAs), 377
Small GTP-binding proteins Rho, Ras and Rac
Small inhibitory RNAs (siRNAs), 77-79, 103, 342-344, 363, 375, 442
Small-molecule binder of the oncoprotein gankyrin, 373, 379
Small nuclear RNA (snRNA), 20
Small nucleolar RNA, 77
Small ribosomal subunit (30S), 269
Small ubiquitin-like modifier, 90
Smithsonian Institute of American History, 4
SMN, 376
Smoldering inflammation, 108, 188, 189, 196, 197, 199, 203, 204, 267, 283, 306, 368, 412, 413
snRNA, 20
S/N ratio, 99, 512, 513, 541, 544
snoRNA, 77
SNPs, single-nucleotide polymorphisms, 63, 100, 120, 214, 293, 373
Social networks, 73
Social proteome, 112, 113
Somatic nervous system
Somatostatin, 30, 270
SOP, 347, 348, 354, 356, 406, 513-515, 539
Soursop, 140, 200
Soybeans, 190, 446
Spectrophotometry, 504-511

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Spectroscopy, 94, 115, 316, 318, 504-511, 541, 542, 551, 552
Spike-recovery, 502
Spinal cord, 37, 51, 176, 298
Spinal muscular atrophy (SMA), 376
Spinraza™, 376
Spironolactone, 274, 404, 531
ssDNA, 47
ssRNA, 47
Standard Operating Procedure (SOP), 347, 348, 354, 356, 406, 513-515, 539
Staphylococcus, 172, 198, 206, 251, 361
Start codon, 66, 67, 374
Static hubs, 111
Static modules, 111
Statins, 124, 125, 204, 242, 243, 251, 253, 2544, 266, 267, 268, 358, 359, 380, 393, 397, 399, 401, 403, 404, 407-411, 522, 525, 527, 530, 531
Stem cells, 31, 32, 45, 50-52, 79, 90, 91, 105, 108, 170, 278, 286, 298, 327, 329, 354, 355, 371, 375, 378, 420
Stereolithography, 329-336
Stoichiometry, 348, 501, 502, 553
Stomach, 30, 44, 47, 174, 176, 197, 273, 275, 276, 330, 336, 415, 423
Stomatognathic system, 204
Streptococcus, 172, 198, 199, 206, 207, 441
Stroke, 124, 125, 127, 147, 204, 267, 269, 282, 359, 369, 412-414
Structure-activity relationships (SAR), 244, 290, 338, 359
Structure-liability relationships (SLRs), 291
Sub-interactome, 64
Sucrose, 122, 389, 391, 420, 546-549
SUMO, 90
Sub-network, 63, 111, 206, 312
Superfoods, 9, 384, 387-393, 452
Superoxide anion ($O_2^{\cdot-}$), 19
Superoxide dismutase, 127
Suprachiasmatic nucleus (SCN), 82
Survival Motor Neuron (SMN), 376
Sutterela, 198
Sweetened beverages, 419, 420
Swiss Institute of Bioinformatics, 73
Symbiotic relationships, 3, 53, 54, 204, 218, 219
Symbiosis, 54, 168
Sympathetic nervous system, 186, 272
Synapse, 180, 187, 208
Synergistic, 16, 21, 22, 107, 113, 203, 223, 253, 259, 287, 300, 309, 360, 361, 364, 378, 380, 392, 412, 417, 418, 433, 452
Synergy, 21, 22, 200
Synthemax™, 298
Synthetic biology, 435, 436, 437-447
Synthroid®, 274
Syntrophic relationships, 54
Syntrophy, 54
System dynamics modeling, 22
Systematic controllability analysis of biological networks, 303
Systematic evolution of ligands by exponential enrichment (SELEX), 278
Systems-based collaborative approach, 248
Systems biology, 70, 116, 214, 323, 324, 370
Systems technology, 322-336
Systems thinking, 1-7, 13-146, 148, 167-171, 195, 198, 200, 204, 205, 212, 219, 222, 241-452, 502
Systems toxicology, 323
Systems view of allostery and allo-network drugs, 309-314
%T (percent Transmittance), 509, 542

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

T-DM1, 361, 362
T1D, Type-1 diabetes, 182, 184, 436, 437, 451
$T_{1/2}$, 322
Tae Kwon Do, 420
Tagamet HB, 381
Tamoxifen, 274
Tamsulin, 272
Tannerella forsythia, 203
Taq DNA polymerase, 292
Targeting Aging with Metformin (TAME), 368
Targeting interactions between blood platelets and tumor cells, 380
Targeting network dynamics in the tumor microenvironment, 379, 380
Tauro-muricholic acid, 197
TB Accelerator, 132
Tc, 24
TC_{50}, 326
T cells, 36, 38, 39, 71, 72, 202, 212, 277, 374, 426, 427, 429, 430, 432, 433, 436, 441, 444, 445
T cell engineering, 435
TCGA, The Cancer Genome Atlas, 71, 304
TCR, T-cell receptor, 52
Tecentriq®, 72, 308
Tenericutes, 171
Tense state, or T state of hemoglobin, 259-262
Testes, 29
Testosterone, 33, 268, 273, 396, 405, 520, 532
Tetracycline, 361
Tetrahydrofolate, 92, 268
TF, transcription factor, 4, 21, 34, 58, 63, 65, 73, 74, 80, 91, 183, 192, 218, 248, 265, 273, 275, 302, 306, 320, 377, 413, 433, 445
The God Particle, 14
T_H1, 36, 184, 277, 278, 429
T_H2, 36, 184, 277, 278, 429
T_H17 immune cells, 184, 275
T-helper cells (T_H), 35
The Cancer Genome Atlas (TGCA), 71, 304
The Chemistry Development Kit102 (chemoinformatic tool), 338
The Institute for Genomic Research (TIGR), 69
Theory of Everything, 107
Therapeutic targets with formerly unexpected biological roles, 365, 366
THF, tetrahydrofolate, 92
Thimerosal, 134
Think globally while acting locally, 428
Thrombopoietin, 29
Thrombosis, 267, 327, 359, 451
Thymus-derived lymphocytes, T-cells, 36, 38, 39, 71, 72, 202, 212, 277, 374, 426, 427, 429, 430, 432, 433, 436, 441, 444, 445
Tight epithelial junction, 192, 198
Tight junction (TJ), 175, 192
TIL, tumor-infiltrating lymphocytes, 435
Tipping points, 23
Tiprolasant, 273
Tisagenlecleucel-T, 432
TLR, 41, 176, 176, 183, 188, 192, 193, 196, 275, 277, 435
T-lymphocytes, 46, 267, 299, 427, 430
TMA, trimethylamine, 189, 193, 194
TMAO, trimethylamine-*N*-oxide, 189, 193, 194
T_{max}, 322
T-MCA, 197
TMS (tetramethylsilane), 544, 545, 550
TNF-α, 89, 193, 199, 267, 288, 346
TNF-β, 31, 32
Toehold RNA sensors, 443

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Toll-like receptor (TLR), 41, 176, 176, 183, 188, 192, 193, 196, 275, 277, 435
Tooth enamel, 45
Topoisomerase, 88, 268, 276
Topoisomerase inhibitors, 276
Topological Score of Drug Synergy (TSDS), 300
Toreforant, 273
Total quality leadership (TQL), 5
Total quality management (TQM), 5, 6, 13, 142, 145-147, 175, 191, 212, 291, 351, 352, 369, 373, 418, 435, 437, 503
Total quality manufacturing (TQM), 6
Total soluble solids (TSS), 389

ToxCast™, 324
Toxicity Testing in the 21st Century (TT21C), 323, 325
Toxicogenomics, 105, 288, 324, 326
Toxicokinetics, 322
Toxin-Target Database (T3DB), 328
TQL, 5
TQM, 5, 6, 13, 142, 145-147, 175, 191, 212, 291, 351, 352, 369, 373, 418, 435, 437, 503
Traditional Chinese medicine (TCM), 113, 283, 300, 417
Traditional medicine, 42, 98, 107, 113, 243, 284, 370, 405, 415, 417, 424
Trans fats, 27, 169, 192, 214, 391
Transcription of DNA, 4, 18-22, 35, 58, 63-66, 73-84, 86, 91, 183, 192, 218, 248, 265, 273, 275, 302, 306, 320, 377, 413, 433, 445
Transcription factor (TF), 4, 21, 34, 58, 63, 65, 73, 74, 80, 91, 183, 192, 218, 248, 265, 273, 275, 302, 306, 320, 377, 413, 433, 445
Transcription/translation feedback loops, 81
Transient ischemic attack, 359
Transient receptor potential (phytochemical receptors, ion channels), 41
Transcription-translation feedback loop, 82
Transcriptomics and transcriptome, 68, 104, 106, 326, 327
transfer RNA (tRNA), 76, 78, 80, 269
Transgenerational transmission of Holocaust trauma, 93
Translation of mRNA, 19, 66-68, 76, 77-82, 217, 278, 291, 304, 376, 413, 434, 442, 443
Transposons, 78, 86, 84, 85, 87, 170
Transverse relaxation-optimized spectroscopy (TROSY), 316-320
Trastuzumab, 361, 362, 429
Trastuzumab emtansine (Kadcyla®, T-DM1), 361, 362
T_{reg}, regulatory T cells, 212, 429, 432, 433, 436
$T_{reg}1$ cells, type 1 regulatory T cells, 429
Treponema denticola, 203
Triacylglycerides, 27, 41, 114, 204, 221, 222, 267, 378, 389, 407
Tricarboxylic acid cycle, 8, 89, 113
Trichomonads, 220
Trichostatin A, 88
Trichuris suis, 224
Tri-Cyclen®, 274
Triglycerides, 30, 114, 189, 197, 204, 267, 407, 420
Trimethylamine (TMA), 189, 193, 194
Trimethylamine-*N*-oxide, 189, 193, 194
Triplet code, 66, 67, 242
TRMR, trackable multiplex recombineering, 439
tRNA, 76, 78, 80, 269
TROSY, 316-320
TRP, 41

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Tryptophan, 180, 181, 319
TSS, 389
TT21C, 323, 325
TTFL, 82
Tuberculosis, 122, 131, 147, 285
Tumor antigen, 71, 430, 431, 433, 435
Tumor infiltrating T -lymphocytes (TIL), 430
Tumor necrosis factor-α, 89, 193, 199, 267, 288, 346
Tumor necrosis factor-β, 31, 32
Tumor-reactive T cells, 430
Tumor suppressor proteins, 112
Turritopsis nutricola, 28
Two-dimensional NMR, 314, 551, 552
Type 1 collagen, 325
Type-1 diabetes (T1D), 182, 184, 436, 437, 451
Type-2 diabetes, 102, 114, 188, 251, 269, 274, 300, 321, 366
Type-1 nodes, 304
Type-2 nodes, 304
Ubiquination, 306
Ubiquinol, 408-411
Ubiquinone, 268, 358, 408, 409
Ulcerative colitis, 174, 224
Ultraperformance liquid chromatography (UPLC), 124, 293, 511-515
Uncompetitive enzyme inhibition, 258
Unfolded protein response (UPR), 413
Uniprot Database, 90
Universal computer, 344
Universal constructor, 344
University of Cambridge, 248, 296
University of Michigan, 450
University of Wollongong in Australia, 450
University Medical Centre, Utrecht, 248
UPLC-QTOF-MS/MS, 124
US National Research Council, 121, 323
US Pharmacopeia (USP), 356, 513, 539
U.S. Preventative Services Task Force, 115
Using NMR to develop allo-network drugs, 314-321
USPSTF, 115
UVR-6105, 329-333, 335
UV-Vis spectrophotometry, 504-511
Vaccine Injury Compensation Program, VICP, 134
Vagal pathways, 176
Vagus nerve, 175-178, 182, 200
Valvular endothelial cells (VECs), 328
Valvular interstitial cells (VICs), 328
Vancomycin, 199
Vandetanib (Caprelsa®), 372
Variable, diversity and joining regions of antibodies, 15, 75, 86
Vascular cell adhesion molecule-1 (VCAM-1), 267
Vascular cells, 196, 279, 359, 449
Vascular endothelial growth factor A (VEGF), 251, 278, 279, 372, 375, 450
Vascular networks, 328
Vasopressin, 29, 30
VAXXED, 135-141
VCAM-1, 267
V(D)J recombination, 15
Vector, 3, 39, 50, 68, 107, 277, 342, 353, 377, 427, 431
Vedanta Bioscience, 387
Vegan diet, 193-196
Vegetarian diet, 173, 188, 193-196
VEGF, 251, 278, 279, 372, 375, 450
Vemurafinib, 337
Venetoclax, 306, 307
Verify the target, 243, 380
Verrucomicrobia, 173
Vectibix®, 428

EBSCOhost - printed on 2/11/2023 5:23 AM via . All use subject to https://www.ebsco.com/terms-of-use

Vegetables, 27, 96, 123, 192, 197, 200, 220, 306, 324, 356, 384, 389, 391, 392, 422
Veteran's Administration, 117
Viable yellow agouti (*Avy*) locus, 98
Vibrio cholera, 202, 361
VICP, 134, 135
Vioxx®, 283
Virome, 105, 113, 215, 216
Visible Human Project®, VHP, 40
Virtual Physiological Human, 289
Virtual Reality Modeling Language, 113, 114
Viruses, 6, 8, 11, 14-17, 20, 28, 36-39, 43, 46, 47, 50, 55, 57, 76, 77, 84, 85, 87, 105, 113, 130, 147, 167-170, 172, 182, 186, 187, 196, 198, 201, 202, 214-219, 223, 242, 251, 252, 268, 278, 280, 291, 299, 302-304, 339, 342, 346, 353, 373, 374, 377, 378, 411, 424-428, 430, 433, 435, 441-445, 554
Visceral adipose tissue (VAT), 32
Visual cortex, 44, 97
Vitagenes, 96
Vitamins, 9, 29, 33, 34, 69, 94, 125, 126, 173, 177, 180, 211, 273, 274, 360, 381, 382, 384, 391, 393
Vitamin A, 273
Vitamin B_6, 180
Vitamin C, 205, 360
Vitamin D, 29, 34
Vmax, 256, 257
Voice of the patient, 145, 346
VRML, 113, 114
Warburg effect, 89, 307
Warren Buffet, 247
waterLOGSY, 316, 552
Weight loss, 123, 126-128, 147, 197, 209, 215, 393, 396-405, 412, 418, 419-423
Wellcome Trust, 128, 428
West Nile, 445
Whole-exome sequencing (WES), 429
Whole-genome shotgun 454 pyrosequencing analysis, 210
Wikileaks, 136
William Cecil, 372
Winston Churchill, 26
Wnt signaling, 32, 33, 314, 450
Woolly Mammoths, 446
World Wide Web, 55
WS-CAM, whole systems complementary and alternative medicine, 417, 418
Xenical®, 421
X-inactive specific transcript, XIST, 79
X-ray crystallography, 261, 262, 285, 309, 315, 338, 554
X-ray diffraction4, 261
^{90}Y-ibritumomab tiuxetan (Zevalin), 363
Yersinia pestis, 251
Yervoy®, 308
Yoga, 418, 419
Yohimbe plant (*Pausinystalia johimbe*), 126
Zantac®, 272, 273, 381
Zevalin®, 363
Zidovudine, 251
Zika virus, 278, 443, 445
ZINC lead library, 298
Zocor®, 267
Zolpidem (Ambien®), 271

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