Systems Thinking in Medicine and New Drug Discovery

Volume Two

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.

CONTENTS

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

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.

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

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

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

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.

CHAPTER ONE

PERSONALIZED MEDICINE

1.1 P4 medicine: predictive, preventive, personalized and participatory

One of the most important aspects of systems thinking in medicine and new drug development is predictive, preventive, personalized and participatory (P4) medicine [1-3]. It is an outgrowth of stratified medicine, in which people are separated or stratified into separate groups, depending on their genomes, so they can receive the proper therapy [4]. It engages patients and helps them prevent diseases, decide treatments and monitor recovery. Diagnostic tests are changing the paradigms for screening and diagnosing rare conditions. As a result, patients' susceptibilities to diseases can be predicted, along with the likelihood that specific therapies will succeed before starting clinical trials. Interventions will start at an earlier stage in the disease process, often pre-symptomatically, when they are much more cost-effective. Costs of clinical trials will drop while success rates increase when they are done on the correct patient populations. Treatments and potential cures for previously incurable diseases are emerging by separating people and diseases into distinct subgroups. So, people who have the appropriate genome and other –omes or biomarkers that will make them more likely to benefit from the drug being tested can be chosen for clinical trials to test the drug [1]. For example, if a drug or new molecular entity (NME) is being evaluated for treating cancer in patients who have a certain mutation in an oncogene, it would be best if the subjects in clinical trials have that same oncogene. In summary, personalized medicine customizes medicine by using molecular information to understand disease patterns more accurately, to diagnose them better, and to tailor preventive and therapeutic intervention more effectively with fewer side effects [2].

P4 medicine is also known as P4 systems medicine (P4SM) [5] because systems medicine is integral to it [5, 6]. The five pillars of P4SM are cutting-edge technologies, digital infrastructure, personalized data

clouds, new analytical tools and systems biology models. Systems medicine emphasizes prevention and individual participation in one's own health care. It is holistic and quantitative. It includes mathematical approaches in the practice of medicine and new drug development. That is, systems medicine emphasizes prevention and individual participation in one's own health care. It recognizes the important human need for patients and caregivers to be involved in preventing and curing their diseases. At the same time, mathematics is being used to analyze huge datasets from patients, while physicists, chemists, biologists and engineers develop the analytical tools needed to generate the data. All of this is linked through the Internet and used in mobile healthcare applications. So, systems medicine is the application of systems biology to the study of human health and disease [1].

The goal of P4 medicine is to prescribe appropriate, individualized drugs and medical devices for people with different types of nutrition, environment, genes, mRNA, miRNA, epigenetics and/or proteins [2]. Such treatments should be designed for the patient's specific anatomy (size), physiology and environment. Diagnostic devices can monitor vital signs, blood glucose, oxygen or other small molecules. They can monitor brain and heart activities with electroencephalography (EEG) or electrocardiography (ECG or EKG) and do diagnostic imaging. Some can even determine part or all of the genome, epigenome, transcriptome, proteome and metabolome of the patient and/or his or her diseased cells. This approach led to four anticancer drugs being approved by the FDA for use in patients who have specific genetic characteristics that can be identified by a companion diagnostic test [2, 7].

P4 medicine can use –omics data and biomarkers from many people to help everyone work with their physicians to make their own medical decisions [2, 7]. It can also use data from many different tissues or the same tissue at different times in single person. For example, the effects of anticancer therapy on a tumor over time can help physicians and patients predict the prognosis and guide subsequent therapy. Pre-emptive genome-based testing of adults and children in personalized health care is becoming very helpful. Diagnostic tests are now available for over 2000 Mendelian conditions. These tests are changing the paradigms for screening and diagnosing rare conditions. They are identifying patients who will most likely respond to preventive treatments or whose diseases or symptoms may progress differently when compared with others in the general population. Personalized medicine can help identify patients who are more susceptible to certain diseases or disease-related symptoms or are pre-symptomatic. Just as important, personalized medicine engages

patients and helps them prevent diseases, decide treatments and monitor recovery. As healthcare continues to be personalized, the public is expressing their desire to participate actively in healthcare decision-making that is based on analyzing their genomes. So, P4 medicine not only aims to prescribe the right medicines, but also maintain the mental and physical well-being of the patient and caregivers. In the process, systems biology and medicine work together to create a cycle of innovation [2, 7].

Sometimes, a combination of predictive biomarkers is used to make clinical decisions. For example, the effectiveness of anti-PD-1 immunotherapy depends on not just the presence of PD-L1, but also an inflamed T cell microenvironment [2]. The popular media have also joined in this effort. In 1995, scientists were on the Larry King show, asking for volunteers who had a history of prostate cancer to allow their genes to be analyzed. The response was wonderful. Many families were recruited from around the world, and their genes were mapped [2].

Moreover, systems medicine is making the care of people with diseases more cost-effective in both human and financial terms [2]. Treatments and cures for previously uncurable diseases are being developed. This is done, in part, by separating people and diseases into distinct subgroups [1, 2]. Genomic and analyses of other –omics and biomarkers can stratify people into subgroups based on their disease risks, likely reactions to drugs and other clinically relevant factors. For example, breast cancer, which was once classified as a single disease, is now stratified into clinically relevant subgroups based on interactions in genetic, molecular and cellular networks [8]. Stratifications of prostate cancer [9] and Crohn's disease [10] are making diagnoses more effective and interventions more cost-effective because they are based on the underlying causes of the diseases [1]. Surgical decisions are also being made based on disease stratification and individual needs. By focusing on the causes rather than the symptoms of a disease, physicians and patients are doing a better job of preventing diseases from occurring in the first place, or stopping them before they can cause serious damage. Moreover, as we identify and understand the biological networks that are perturbed in diseases, systems medicine will continue to provide a stream of new therapeutic targets for the pharmaceutical industry and biomarkers for patients and physicians [1, 2].

The goal is to develop drugs that will be more effective and have fewer costly side effects, because they will be more personalized [1, 2]. They will target specific strata of the populations of people and types of diseases. It will be cheaper for pharmaceutical companies to do clinical

trials if they are done on the correct patient populations. Interventions (including, but not limited to pharmaceutical interventions) will start at an earlier stage in the disease process, often pre-symptomatically, when they are far more cost-effective. The impacts of interventions will be more accurately monitored, allowing for adjustments to improve outcomes and reduce costs [1, 2].

As part of P4 medicine, almost everybody will have a personal data cloud, which will act as a medical record, with all the health data for each individual - including the genome, blood chemistry, lifestyle data (activity levels, diet and stress), transcriptome and gut microbiome [1, 2]. Actionable information will be given to individuals based on the analysis of data accumulated in their personal data cloud [1]. The data will be collected and analyzed to produce a stream of highly personalized information about each person's health and disease. Furthermore, actionable information can be supplied back to individuals based on the analysis of data accumulated in their personal data cloud. This will be given not just to physicians and other professionals, but also to individuals and those with whom they confide. Finally, systems biology and medicine are working together to create a cycle of innovation. As new biological insight inspires the development of new analytical tools, new tools and technologies produce new data, as new data drives the creation of more analytic tools that advance biological insight [1].

This has been happening ever since the decision was made in 1990 to sequence the human genome in 15 years [2]. Bioinformatic tools were developed and implemented to sort the billions of fragmented sequences into the complete genome (shotgun sequencing). This helped the International Genome Sequencing Consortium and others announce the complete DNA sequence in just 13 years, in 2003. Many government agencies, including the US FDA established collaborations with the Center of Excellence in Bioinformatics and Life Sciences (CBLS); Center for Functional Genomics; Center for Structural Genomics; Office of In Vitro Diagnostics and Radiological Health (OIR); and the Voluntary Genomic Data Submission (VGDS) Program and were quite helpful in solving the human genome. Then, in 2004, the FDA created the Genomics and Targeted Therapy Group. Numerous other groups were formed, including the Personalized Medicine Team in the Center for Biologics Evaluation and Research (CBER) in 2011 and the Division of Systems Biology at the National Center for Toxicological Research (NCTR). Also, the FDA has issued at least 21 guidances that relate to personalized medicines. This includes guidances on pharmacogenomics data submissions, tests, definitions, considerations, applying human factors and cGMP requirements

for combination products. However, reductionist thinking led some to believe that once we knew the entire 'book of life', the role of every gene would be identified and a new age of medicine would appear. Surprisingly, when researchers started knocking out individual genes in mice, they often found that it had no effect. Systems thinking was needed to realize that our genes are just one part of a much more complex whole that comprises the human body and influences health and disease [2, 7].

1.2 Systems primary care medicine

Systems biology started with the goal of "making sense of the genome and its relationship to the whole organism (phenotype) through computational and mathematical modeling" [5]. It integrates holistic data into mathematical models. We are now able to make many measurements and determine several –omics at a higher speed and lower cost that was previously thought. So, very large sets of data are generated, while bioinformatics and computer analysis try to make sense of them. Systems medicine is "the emerging medical application of systems biology to medicine" [5]. It views a human as of a dynamic, integrated complex network and a system of systems. It aims to expand the field of personalized medicine so that it no longer uses reductionist thinking to focus on just genes. Instead, it is holistic and integrates many aspects of biology and biochemistry. It is guided by systems theory. In this process, systems medicine hopes to consider the great complexity of human health and disease, while counteracting fragmentation. Even though this may be relatively new in the field of new drug development and in many medical specialties, it has always (and still does) apply to primary care and family medicine. That is, good, effective primary care physicians (general practice or family medicine) have always tried to communicate well with their patients, whom they know as whole persons with their own life circumstances, close relationships, goals and values. Primary care physicians are especially important because they are the first point of contact for patients seeking help. It is also general, unlike specialties focusing on bodily subsystems or single diseases. The generalist aims to provide the best possible, tailored care for each individual as a whole, complex person who often presents previously uncategorized health problems. They try to help their patients achieve the goals that are meaningful to them. The challenges of primary care medicine are strongly linked to the health problems with which it must deal. Non-communicable, chronic (long-term) and costly health problems are seen quite often. They exhibit multimorbidity and even medically unexplained symptoms. The

primary care physician, as a specially trained human professional, models each patient as a whole person, while integrating a variety of fragmented information to make predictions about what is best for that person at that time. This has been done for quite a long time. Even before science became involved in medicine, traditional healers always considered the socially situated whole person. So, personalized medicine has always had a humanistic nature. However, modern systems medicine has added a technoscientific meaning, which has been called technoscientific holism [5].

This has led some to think that computers will eventually be smarter than physicians – especially when it comes to diagnosing diseases and recommending therapies [5]. They ignore the fact that diseases and therapies can't always be reduced to things that computers can recognize as distinct phenomena. In fact, systems biology considers health and disease to be a continuum. Moreover, different diseases are not distinct, but fluid, interlinked and overlapping phenomena. So, a systems view of disease and health requires using more than just computers [5]. It also needs a human touch as well as some theory and/or philosophy.

1.3 Causality in P4SM

For example, P4SM is consistent with "a systems view where no level is causally or epistemologically privileged" [5]. However, some people feel that there is downward causation or biological relativity [5, 6]. That is, "the whole, with its emergent properties, has some sort of causal or constraining influence over the parts by which it is constituted" [5]. This contradicts reductionist thinking that believes in upward causation. That is, the whole person can supposedly be defined and controlled by the individual parts – especially the genes.

There is an intermediate approach that focuses on organs instead of molecules, metabolites, genomes or cells [6]. It is a middle-out approach that views the human body as a total system of inter-connected cells, tissues, and organs. These are connected by biochemical signals and are controlled by combinations of factors. This has led to an increased interest in physiology that incorporates systems biology. Dynamics and control mechanisms are being illuminated as new, advanced laboratory methods emerge. They are able to recognize the metabolic and regulatory activators as well as the metabolites that are produced by the activities of cells. There is much interest in learning on how different parts interact, communicate, and function in relation to one another across subsystems and ordinary boundaries. Multi-level boundaries in cells and tissues are crossed as

information is passed between different parts. This interest in studying regulatory channels, communication methods and cascading molecular networks is modernizing and improving the more traditional mechanistic approach. This is an interdisciplinary approach that includes dynamic theory design and engineering control models [6].

This approach includes the Human Physiome Project (HPP) [6]. It uses a global network of scientists and physicians who are studying physiology and the ways that organ systems function. The goal of the HPP is to study physiologic problems and create models, while continuing further investigations that should include additional parts of the models as they are discovered. Computational modeling is a key aspect of the HPP. As each team of investigators works on their personal areas of interest, they share their results with others to generate a whole picture. Hopefully, this will lead to computational predictive models for individual patients as well as for entire populations. Another important goal of the HPP is to create a Virtual Human Patient model. It will be a computer graphic of individual physiologic models for each patient [6]. This is building on earlier work by Noble and colleagues that became the Cardiac Physiome Project [11]. Eventually, it is hoped that anyone will be able to visit a physician and obtain a computed Virtual Human Patient model so they will be able to discover their susceptibilities to diseases, track their health and the progress of any diseases that they may have and suggest the best treatments or ways to prevent the diseases from emerging [6].

1.4 Explanation, prediction and control in P4 medicine

In contrast, some systems thinkers think that P4 medicine should still have the goals of explanation, prediction and control. They feel that the whole person and his or her health are quantifiable, predictable and controllable. Supposedly, this can be done by quantifying the interactions of biochemicals in networks. However, bioinformatics, machine learning techniques and artificial intelligence may not be able to interpret large data sets without proper theoretical guidance. Moreover, a virtual human is not a real human and not everybody has the same goals or challenges. There are some human factors that most likely will never be quantified [5].

So, personalized medicine has been defined as, “the ability to customize medicine using molecular information to more accurately understand disease patterns and diagnose disease, as well as to tailor preventive and therapeutic intervention more effectively with fewer side effects” [12]. “It includes not only prescribing medicines, but also maintaining the mental and physical well-being of the patient and care

givers. Pre-emptive genome-based testing of adults and children in personalized healthcare is becoming very helpful, especially when studying diseases with Mendelian inheritance. Diagnostic tests are now available for over 2000 Mendelian conditions. These tests are changing the paradigms for screening and diagnosing rare conditions. Personalized medicine can help identify patients who are more susceptible to certain diseases or disease-related symptoms or are pre-symptomatic. It will identify patients who will respond to preventive treatments differently or whose diseases or symptoms may progress differently when compared with others in the general population. Just as important, personalized medicine engages patients and helps them prevent diseases, decide treatments and monitor recovery. As we continue to personalize healthcare, the public is expressing their desire to participate actively in healthcare decision-making that is based on analyzing their genomes" [12].

1.5 What P4 medicine does

Personalized medicine "tailors medical treatment to the individual characteristics, needs and preferences of each patient" [7]. Actually, it has been used for over 100 years to analyze blood types, to ensure that transfusions don't cause hemolytic reactions. Also, over 50 years ago the genetic basis for the selective toxicities of fava beans and an antimalarial drug (primaquine) was discovered. It is a deficiency in the enzyme glucose-6-phosphate dehydrogenase (G6PD) that is important in metabolism. Then, in 1977, different isozymes of cytochrome P450 2D6 (CYP450 2D6) were found to cause the effects of the anti-hypertensive drug debrisoquine to be exaggerated and last longer in some people than in others. So, genetic differences can cause different pharmacokinetic parameters, such as area under the curve, or AUC. Pharmacogenomics is "the study of how variations of DNA and RNA characteristics affect responses to drugs. It has been a crucial part of personalized medicine for decades" [7].

1.6 Examples of P4 medicine

In chapter 3 of volume 1, some examples of personalized medicine were given. For example, high throughput screening (HTS) is being 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 (iPSCs) 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 [13]. In addition, 3D models are being made from iPSCs that are unique (or personalized) for every patient [14]. Also, organ chips are engineered to mimic and recapitulate important aspects of physiology. They can be used to assess the phenotype of the cells by measuring the expression of genes, as well as the structure, metabolism and function of the cells that are seeded into microenvironments that recapitulate healthy or diseased conditions. When cells are taken from the patient, they can be used to specifically model inherited pathologies at preclinical costs and used on just those who have personalized genetic backgrounds [14]. Moreover, J. Craig Venter has started a new company, Human Longevity Inc™ (HLI) [15]. Its goal is to give "everyone access to the power of data-driven health intelligence". They have already published the results of deep sequencing of 10 545 human genomes [16]. A wholly owned subsidiary of HLI, called The Health Nucleus does extensive testing of clients [17]. They analyze their genomes, gaits and fecal microbiomes. Clients get a prognosis of their present and future state of health. The data gives them information that they can use to modify their diets and behaviors [18]. 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" [18]. There are other companies that are using data-driven –omics data to improve wellness and/or longevity by providing personalized advice on nutrition [19]. They include Arivale [20], Day Two [21] and Google Baseline [22]. There are even some companies that do personalized analyses of microbiomes [19]. They include American Gut [23], Enterome [24], Second Genome [25], Seres Health [26] and DELETE Vedanta Bioscience [27]. 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 are in a conspiracy to keep people sick.

Instead, they are developing and prescribing different drugs and medical devices for people with different genes, mRNA, miRNA, epigenetics and/or proteins, as well as various levels of nutrition, while living and working in different environments [2]. These treatments are designed for the patient's specific anatomy (size), physiology and environment (be it the home, hospital or Intensive Care Unit). Diagnostic devices can monitor vital signs, blood glucose, oxygen and a

variety of small molecules. Some of these devices can perform electroencephalography (EEG) or electrocardiography (ECG or EKG) and do diagnostic imaging. Some can even analyze the genome, epigenome, transcriptome, proteome and metabolome of the patient and/or his or her diseased cells. Also, the patient's blood or tissues can be assayed for different types of enzymes (including CYP isozymes) that catalyze reactions that can metabolize drugs differently and affect their bioavailabilities, or abilities to bind to different receptors. This genetic approach led to the development and rapid approval of tratuzumab, or Herceptin®, for treating and curing 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 "for use in patients who have specific genetic characteristics that can be identified by a companion diagnostic test" [2, 7]. A similar approach was used to find patients with cystic fibrosis (CF) who would respond to a new drug.

Kalydeco® (ivacaftor) was approved for patients with a specific genetic mutation (glycine to aspartic acid on amino acid 551, or G551D) [28]. Kalydeco® was the first drug to address the underlying cause – rather than the symptoms – of CF. Personalized medicine is also important in pediatrics [28]. Genetic tests are available that can be used before conception to detect mutations that are associated with over 150 genetic disorders. After conception, prenatal tests of the mother's blood can diagnose many genetic diseases. Still, many childhood diseases have multiple causes, so they still need personalized medicine. For example, children with type-1 diabetes must monitor their consumption of carbohydrates and monitor their blood glucose concentrations. Unlike adult-onset type-2 diabetes, which can be caused by decades of over-consumption of sugars, fats and calories, type-1 diabetes is not. Still, both types of diabetes require P4 medicine. It must be predictive, preventive, personalized and participatory. Unfortunately, type-1 diabetes is not so predictable or preventable, but type-2 can be prevented in many cases by adjusting one's diet and lifestyle. Both are personalized and participatory as each patient or their caregiver must provide medicine, control their lifestyle and diet, and monitor their blood glucose. There are also *in vitro* fertilization techniques to help couples have a baby. If the mother has a mitochondrial disease, the nucleus from her fertilized egg can be transplanted into the enucleated embryo of another woman. The embryo has functioning mitochondria and can be transplanted back into the mother [28].

One measure of the advance in P4 medicine is that about 2% of the population of the USA uses or has used genetic tests [29]. There are about

1000-1300 tests available for about 2500 conditions and new tests are emerging rapidly. A survey of healthcare claims data for 32 million people in the USA from 2008–2011 found that the cost grew by 14% per year between 2008 and 2011, primarily resulting from increased utilization. They predicted that genetic testing and molecular diagnostics use will continue to grow in the next five years. Moreover, by strengthening the ability to collect and analyze data in P4 medicine we will promote positive changes that benefit patients [29].

Also, scientists are working to develop hand-held devices that will prick the finger and quantify about 2500 organ-specific proteins from all the approximately 50 human organs [30]. The data will be sent by wireless communication to a file server, which will analyze the data and email a report to the physician and patient. Hopefully, this will enable a rapid evaluation of current health. Also, our genomes and metabolomes will enable predictive and personalized medicine. Physicians will learn how to use drugs to re-engineer metabolic networks that have been perturbed by the beginnings of a disease, before symptoms can emerge. Moreover, education will put patients in the position to take more responsibility for charting their own future health choices [30].

Individualized medical devices are being made, too [7]. Three-dimensional (3D) printing was used to make a bioresorbable tracheal splint for an infant who was critically ill. Furthermore, research on induced pluripotent stem cells (iPSCs) will enable people to use their own cells to biosynthesize their own organs when they need a transplant. So, advances in genomics, medical imaging, 3D printing and regenerative medicine, along with increased computational power and the advent of mobile and wireless capabilities, are allowing patients to be treated and monitored in ways that better meet their individual needs [7]. Many surgeons are now able to print 3D models obtained from MRI and/or CAT scans before they perform the surgery. That way, they can see ahead of time what they will face during surgery. At the same time, 3D models can be used by interns to develop and practice surgical techniques before they do their first real surgery.

There are many examples of personalized medical devices [2, 7]. A customized tinnitus masker tailors audio signals to suit each patient's hearing requirements. Pedicle screw spinal systems can be assembled by physicians to fit each patient's unique anatomy based on MRI and/or CT images. There is a software-based EEG analyzer that predicts one's response to various psychotropic drugs. The device predicts the probable response to various medications to help guide clinical decisions. There is also a Zenith Fenestrated AAA Endovascular Graft for patients with

abdominal aortic or aortoiliac aneurysms having morphology suitable for endovascular repair. Each device is tailored to the patient's individual aortic anatomy. Finally, there is an artificial pancreas under clinical investigation that monitors the concentration of glucose in the blood and delivers the proper dose of insulin to diabetics [2, 7].

The FDA and other governments' regulatory agencies evaluate applications for new medical devices and drugs. So, the following goals were described in a recent report from the FDA [7]: "Personalized medicine seeks to reduce the burden of disease by targeting prevention and treatment more effectively. With the help of personalized medicine, the health care 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 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" [7].

1.7 Important collaborations in P4 medicine

The US FDA and other countries' regulatory agencies are collaborating with industry and academia to build an elaborate infrastructure to support personalized medicine. This has been described as the five pillars of systems medicine: cutting-edge technologies, digital infrastructure, personalized data clouds, new analytical tools and systems biology models [1, 2]. Advanced technologies and algorithms can gather and analyze data, while setting up user-friendly databases for further analyses. For example, in 2003 the US NIH started the ENCODE project to identify and define the functional DNA elements that are required for normal genome function. In 2012, about 40 articles were published describing the results that came from an international effort. It is anticipated that the Internet and mobile telecommunication will establish a data cloud in the near future – hopefully for everybody who wants it. It will contain medical records, including the genome, blood chemistry, lifestyle data (activity levels, diet and stress), transcriptome and gut microbiome. Actionable information will be given to individuals based on the analysis of data accumulated in their personal data cloud. Attempts are being made to establish quantitative metrics to help decide when a person is healthy, pre-disposed

to a disease, or in various stages of a disease. The data will also help find biomarkers that will be used to suggest the best therapies for each individual, while monitoring the treatment process, making necessary changes when new conditions emerge [1, 2].

Also, the US National Cancer Institute (NCI), the Moffitt Cancer Center and Stanford University School of Medicine met at the Medicine 2.0 conference in Boston in 2010 [31]. They discussed how sociotechnical frameworks, informatics platforms and health-related policies can be used to encourage data sharing and innovation. This built on the Institute of Medicine's vision for a rapid learning health care system that is encouraging an open source, population-based approach to cancer prevention and control. This learning system is based on a sufficiently advanced digital health infrastructure and "rapid, seamless, secure exchange of useful, standards-based information among authorized individual and institutional senders and recipients" [31, 32]. A learning system that is being used in oncology is called the American Society of Clinical Oncology's Cancer Learning Intelligence Network for Quality (CancerLinQ) system [31, 33]. It was designed to address the growing challenge of managing the huge amounts of data that are emerging from P4 medicine for cancer care. It incorporates data from researchers, providers, and patients so comprehensive clinical algorithms reflecting preferred care at a series of decision nodes for clinical decision support can continually improve [31]. There are also immunology datasets that are available at the National Institute of Allergy and Infectious Diseases ImmPort website [34]. To help encourage open collaborations, the US government passed the America COMPETES Act as part of the Open Government Initiative [28]. This has led to unprecedented amounts of data being available to the public. Two examples include the Data.gov platform, which provides public access to "nearly 450,000 datasets…across 172 federal agencies" [35] and one million microarray measurements of gene expression [36].

In addition to sharing data, public and private entities are collaborating to accelerate innovation [31]. At the same time, the US government's NCI and the Office of the National Coordinator for Health Information Technology (ONC) are working together with the federal Small Business Innovation Research (SBIR) grant program to support the evaluation and dissemination of evidence-based applications for cancer prevention and control [37]. They incorporate crowdfunding to encourage further collaborations and perform market validation of innovations [38].

The FDA is also collaborating with other governments, academia and pharmaceutical companies to develop regulatory standards, reference

libraries, research methods, and tools that can be used to integrate biomarker identification into drug and device development and help make clinical decisions [2, 7]. “The biomarker qualification program aims to provide a framework for scientific development and regulatory acceptance of biomarkers for use in drug development, facilitate integration of qualified biomarkers in the regulatory review process, and encourage the identification of new and emerging biomarkers. There is also a project on microarray and sequencing quality control, a genomic reference library for whole genome sequencing (WGS) platforms and a virtual physiological patient. They are also building a high performance integrated virtual environment (HIVE) for next generation sequencing analysis. Also, high resolution human leukocyte antigen (HLA) typing systems are being developed, as well as molecular tools to facilitate blood group typing. The FDA and other governments’ health care agencies are working with others to design and conduct clinical trials better. They are refining the design of clinical trial and the statistical methods of analysis to address issues such as missing data, multiple endpoints, patient enrichment, and adaptive designs that often arise when developing targeted therapeutics. They are also looking closely at clinical trials of anticancer drugs. This is complicated because many cancers are heterogeneous, each with their own specific genetic makeup. This heterogeneity is one reason why different people with cancer in the same organ respond differently to the same therapies. The I-SPY 2 trial is a collaborative initiative developed under a unique public-private partnership. It includes more than 20 cancer centers. They are trying to better understand heterogeneity and complexity to provide targeted therapies” [7].

It is also essential to have adequate and robust statistical methods to analyze the data that is produced [2, 7]. So, scientists at Booz Allen Hamilton, the FDA supercomputer center, the Genomics Evaluations Team for Safety (GETS) and the Office of Vaccines Research and Review (OVRR) in CBER have been comparing different methods to analyze genomic data to predict patient outcomes and/or prognosis. CDER and CDRH (Center for Devices and Radiological Health) have also been developing new device diagnostics to improve drug safety. They are assessing new device-based algorithms and biomarkers that can distinguish between benign and malignant drug-induced QT prolongation (time between the start of a Q wave and the end of a T wave) in an electrocardiogram [2, 7].

The National Center for Toxicological Research (NCTR) is also studying the biology of cancer [2, 7]. They found that many tumors carry subpopulations of KRAS (Kirsten rat sarcoma oncogene) in mutant cells.

These mutations can lead to acquired resistance to some anticancer drugs. Effective treatments to prevent drug resistance in tumors are being identified by using defined genetic profiles. Also, researchers at CBER are identifying genetic determinants of immunogenicity in patients with Hemophila A. The eventual goal is to predict each patient's risk of immunological response to a given protein therapy before it is used in treatment. Other researchers at CBER are trying to understand better the effects of DNA modifications on the quality of protein products coded by them. By looking at proteins that are involved in blood clotting as models, they demonstrated that while "synonymous" or "silent" mutations do not affect the protein sequence, they may affect protein concentrations as well as protein folding and function. The goal is to determine which mutations are deleterious and which may be safely employed in the design of therapeutic protein products. Hopefully, this will lead to new tools and methods for evaluating the properties of proteins from the gene sequence [2, 7]. "This could have many diverse implications for developing and evaluating safe and effective protein therapeutics, including biosimilar products" [7].

CBER's Office of Vaccines Research and Review (OVRR), together with the Genomics Evaluations Team for Safety (GETS) are also involved in collaborations to identify genetic risk factors that are associated with adverse reactions to vaccines [2, 7]. In addition, the Centers for Disease Control (CDC) and Northern California Health Care (Kaiser Permanente) are trying to see if there are genetic risk factors that predispose children to febrile seizures (caused by high fever) after MMR vaccination. At the same time, the Innovation Center for Biomedical Informatics (ICBI) at Georgetown University is trying to identify genes that are associated with links between vaccines, vaccine components, and several autoimmune diseases. The goal is to help test the hypothesis that autoimmune diseases might occur as adverse reactions to some vaccines [2, 7]. "Pathway models derived from this data may help predict autoimmune reactions to vaccines and other medical products in the future" [7].

Scientists at NCTR are collaborating with the University of Liverpool (in the UK) and the Huashan Hospital (China) to do whole genome sequencing and genetic analyses to identify susceptibilities to carbamazepine-induced hypersensitivity reactions [2, 7]. They are also collaborating with the University of Maryland to identify genetic factors that might interact with certain aspects of lifestyle in the Amish community to see if they contribute to heart disease. The metabolic responses of volunteers were examined after consuming specific diets and drugs that are associated with cardiovascular risk. This included blood

triglyceride concentrations after a high fat meal, blood pressure after consuming much salt (NaCl) with a meal, and platelet aggregation response after taking aspirin or clopidogrel. The DNA from people in the Amish community who showed abnormal responses was sequenced. Genetic association studies are also being done. This work is ongoing. As possible genetic markers are discovered, they are being validated in another group (cohort) of Amish subjects. Identifying genetic factors that interact with drugs or certain diets to increase risks of cardiovascular disease or the efficacy of treatment will lead to patients and their physicians using personalized medicine to improve health [2, 7].

At the same time, the National Cancer Institute (NCI), the National Institute of General Medical Sciences (NIGMS), the University of Maryland and FDA are trying to see if increasing the dose of clopidogrel can increase antiplatelet responses in people who have genetically reduced CYP2C19 metabolism compared to those with normal metabolism [2, 7]. Also, scientists in the Office of Science and Engineering Laboratories at CDRH are using new methods to analyze electrocardiograms to predict which patients will benefit from cardiovascular therapies such as cardiac resynchronization therapy. They can diagnose problems with electrical conduction and quantify scar tissue in the heart. They use different criteria for women and men, since women tend to benefit significantly more than men from cardiac resynchronization therapy. Similarly, the Office of Science and Engineering Laboratories at CDRH is collaborating with George Washington University to develop a microfluidic, high-throughput microchip to test the interaction of tears with contact lenses, care products, and microbes. They aim to provide individual testing results that can guide the prescription of lens materials and hygiene products for patients. So, from the perspective of the FDA and other governments' regulatory agencies, the era of personalized medicine has arrived [2, 7].

So, the FDA has a website with information on personalized nutrition and medicine provided by the Division of Personalized Nutrition and Medicine (DPNM) [2, 39]. The Division has two areas - Biometry and Biology. The main function of Biometry is to develop biometrical methods for all aspects of the FDA's mission, goals and objectives. A subgroup within Biometry analyzes all data from the National Toxicology Program (NTP). The Biology area is studying the broad areas of pharmacogenomics and nutrigenomics - how individuals respond to drugs and nutrients in foods. The overall goals of the DPNM are to develop and implement research strategies that will be able to account for genetic, environmental, and cultural factors that influence the expression of genetic makeups and

produce knowledge for improving personal and public health. These overarching goals will be met with three parallel efforts that develop:

- Integration of omics methodologies to assess an individual's health status and, as importantly, susceptibility to specific chronic conditions influenced by environmental factors including diet
- Means to capture and assess an individual's nutritional, environmental, and activity exposures
- Classification algorithms that integrate the data from omics and environmental assessments that will result in evidence-based and validated biomedical decision making [2, 39].

Genomics is also being used to predict whether a person is more susceptible to a disease [2, 39]. For example, some people have a variation of the BRCA1 gene. They have a higher risk of developing breast, ovarian and possibly even colon cancers. BRCA1 was the first gene found to be an important causative agent in some types of breast cancer. Subsequently, it was found that mutations in the second gene found to be correlated with breast cancer, BRCA2, are associated with breast, pancreatic, gallbladder and stomach cancers. These discoveries affect the second major aspect of pharmacogenomics, targeted therapy. That is, tumors have different genomic variations. Tests that are based on genomics are helping physicians identify cancers that are likely to respond to a particular treatment. The third aspect of pharmacogenomics is testing for drug resistance. For example, the HIV virus genome is always changing, so testing for resistance can help doctors choose the drug that will best match the virus and suppress it [2, 39].

As mentioned in a recent review, "In 2004, the FDA introduced the Critical Path Initiative, with the intent of modernizing drug development by incorporating recent scientific advances, such as genomics and advanced imaging techniques, into the process" [40]. Identifying and quantifying new biomarkers, modernizing clinical trials, bioinformatics, drug manufacturing and collaborations between government, research institutes, academia and industry are all important parts of this initiative. The initiative began because society expects more and better drugs to be developed, but this was not happening. From 1994 to 2004, global spending on research and development of NMEs increased from about $40 billion to $60 billion, while the total number of NMEs approved worldwide decreased from 40 per year to a 20-year low of just over 20 NMEs [2, 40].

Discovering new biomarkers through advanced genomic, proteomic, metabolomic and imaging technologies has a very high priority [2, 40].

"New biomarkers can improve diagnosis, define disease subsets that may differ in response, define individual variability in the drug's molecular target, and provide an early readout of response to therapy. The Biomarker Consortium (http://www.biomarkersconsortium.org) has been formed at the Foundation for NIH, or FNIH, and is funding biomarker trials for PET scanning in non-Hodgkin's lymphoma" [40].

The next part of the Critical Initiative is modernizing clinical trials by establishing better standards for fully automating the trials and managing the data [2, 40]. Bioinformatics is an important part of this. The FDA has the largest set of data on animal testing of NMEs, but it is not in as user-friendly as it could be. So, digitized electrocardiograms are being developed to help scientists evaluate NMEs for adverse cardiac events. Also, bioinformatics is helping to establish quantitative models of disease processes. This includes data on biomarkers, clinical outcomes and the effects of various interventions. The last part of the Critical Initiative is to improve drug manufacturing by incorporating new science and technology, especially modern process control technologies. Process control is used widely in many industries, such as petroleum refining, producing hydroelectric energy and manufacturing electronic devices. Automated analytical instruments, such as GC, GC-MS, ion chromatography, ICP-AES, ICP-MS and LC-MS can continuously monitor parameters such as the composition of petroleum distillates, the chloride content of water and the metal content of electroplating baths. Note that ICP stands for inductively coupled plasma and AES stands for atomic emission spectroscopy. If critical parameters fall outside of control limits, the process is automatically corrected or stopped, until it can be corrected. Similar process control analyses would be useful in drug manufacturing [2, 40].

It is not surprising that there is a big difference between brain cancer and cancer in other tissues [2]. However, even for a given tissue or cell type there can be important differences. So, the National Cancer Institute (NCI) started a $100 million research program to determine the genomes of different cancers [2, 41]. This is being doing in collaboration with the National Human Genome Research Institute to determine the genomes of brain, lung and ovarian cancers [2, 42]. Also, the MD Anderson Cancer Center at the University of Texas worked on a research project called the Biomarker-Integrated Approaches for Targeted Therapy for Lung Cancer Elimination (BATTLE) [2, 41]. They analyzed biopsies of lung cancer cells at different stages of the disease and identified genetic biomarkers. They established the proof-of-principle that molecular-based,

individualized, targeted therapy can be useful in treating lung cancer patients [2, 41].

The NCI Office of Cancer Genomics' Cancer Genome Characterization Initiative (CGCI) supports advanced genomics research on adult and pediatric cancers [2]. They are developing and applying advanced sequencing and other genome-based methods to identify previously undiscovered genetic abnormalities in tumors. The extensive genetic profiles generated by CGCI may inform better cancer diagnosis and treatment [2, 42]. They have completed a pilot lung cancer project. They used next generation transcriptome sequencing, as well as gene expression and epigenome profiling to study the early stages of lung cancer. They discovered some changes in early-stage lung epithelial tumor cells and tested them to learn their roles in cancer development. They also compared changes in the various pathologically-determined epithelial phenotypes (normal, dysplastic, neoplastic, and malignant) to identify alterations that associate with those phenotypes. Alterations that were found to correlate with early-stage phenotypes (dysplastic and neoplastic) probably play important roles in initiating lung cancer. Once this analysis is completed, a new set of regulatory pathways in lung carcinogenesis might be identified. These pathways will be pursued in future studies looking for prospective biomarkers for risk assessment, early diagnosis, and targeted therapies [2, 42].

The CGCI also has a Cancer Genome Anatomy Project (CGAP) that generated much genomics data on cancerous cells that are accessible through the CGAP website (ocg@mail.nih.gov) [2, 42]. Some of this data was put into the SNP500 database. Its goal is to resequence 102 reference samples to find known or newly discovered single nucleotide polymorphisms (SNPs) which are of immediate importance to molecular epidemiology studies of cancer. Together with the Initiative for Chemical Genetics (ICG), the CGCI established Chembank, an interactive database for small molecules. It contains data from hundreds of biomedically relevant small molecule screens that were done on hundreds-of-thousands of compounds. Chembank also provides analytical tools to make data mining easier. They also have a Childhood Cancer Center that has comprehensive information on childhood cancers, including current treatments, clinical trials, prevention, genetics and testing. There is also an initiative to find Cancer Genetic Markers of Susceptibility (CGEMS). It is identifying common inherited genetic variations that are associated with several cancers, including breast and prostate. Data from these genome-wide association studies are available through the Division of Cancer Epidemiology & Genetics website. (http://dceg.cancer.gov/research/how-

we-study/genomic-studies/cgems-summary). The CGCI also validated full open reading frame (ORF) cDNA clones for all the currently defined human genes that code for proteins. Moreover, there is a mammalian cDNA Library from the NIH Mammalian Gene Collection (MGC). It is providing full-length clones for most of the defined human and mouse genes, along with selected clones of cow and rat genes [2, 42].

Finally, the Office of Cancer Genomics (OCG) has an SOP manual [2, 42]. It contains a set of guidelines for investigators participating in OCG projects to characterize tumors on a molecular level. The manual provides templates and protocols for SOPs that apply to all projects, as well as some that apply specifically to individual projects. Investigators must follow the protocols in the SOP when submitting samples and data. The sample and data acquisition process is explained in comprehensive detail to ensure that all materials contributed will be of sufficient quality to be used in the projects [2, 42].

In 2009, researchers started studying the genomes of medulloblastoma, a brain tumor that is found primarily in children, and B-cell non-Hodgkin lymphoma (NHL), a white blood cell cancer [2, 42]. They found many genetic alterations that are potentially important in cancer onset and/or progression that were not detected in previous studies. Additionally, CGCI collaborates with the OCG TARGET initiative to help solve the genetic factors involved in some pediatric cancers that don't respond well to standard therapies. These include acute myeloid leukemia and two rare kidney tumors. Results from some or all of these projects may result in improved survival and quality-of-life for patients afflicted with these diseases. CGCI partners have also begun projects to better characterize cancers from HIV-positive patients, early-stage lung cancer, as well as Burkitt lymphoma. Data is publicly available through the NIH and NCI databases [2, 42].

In December 2012, British Prime Minister David Cameron announced the start of a project to sequence the genomes of 100 000 Britons (100,000 Genome Project or 100kGP) affected with cancer or rare diseases [2, 43]. In July 2013, the Department of Health announced that this initiative would be coordinated by the newly-established, government-owned company Genomics England (London, UK). They are trying to incorporate whole-genome and whole exome sequencing into clinical studies. They also plan to develop strategies on how to use whole-genome sequencing safely and effectively. This will require excellent communication between patients, their caregivers and physicians, due to the large amount of valuable information it can generate [2, 43].

However, it is important to remember that genetics has its limits [2]. Even if genetic testing shows that a person is susceptible to a disease, it does not mean they will get it. At the same time, just because genetic (DNA) testing indicates that a person does not have a genetic defect that makes them susceptible to a disease, that person may still get it. For example, the entire genome of the scientist, J. Craig Venter, was determined. Even though he did not have a genetic defect that would make him more susceptible than others to skin cancer, he still got it. This was most likely because he was exposed to direct sunlight (and UV radiation) when he was a young surfer in California. So, environment is also very important. It can affect the microbiome, virome, epigenetics, proteome, post-translational modifications and mobile genetic elements, which can all affect the phenotypes of diseases. Also, diseased cells have different genetics and epigenetics. Cancers and heart diseases are really many different diseases. So, medicine should be personalized not just for individuals, but also for their particular types of cancers and heart diseases [2].

The Sheik Khalifa Bin Zayed Al Nahyan Institute for Personalized Cancer Therapy at the MD Anderson Center is also involved in personalized medicine [2, 44]. It supports preclinical research and clinical trials. Each patient's tumor biopsy is assayed for abnormal genes and gene products to select therapy with agents that target the product of those particular abnormal genes. This program integrates the results from research into clinical trials. Its goals are to implement personalized cancer therapy and improve patient outcomes. The seminal BATTLE trial in lung cancer treatment demonstrated the practicality of this approach. A series of Phase I/II trials were done. Experimental drugs were chosen based on biomarkers that were detected in the patients' cancers. Several more clinical trials based on genetic and molecular biomarkers in patients' cancers are now underway. They significantly improved response rates in combined targeted therapy in Phase I clinical trials for multiple disease sites. For example, patients treated with a drug targeting the PI3K pathway had a response rate of 35% in those who had mutations compared to the 4 to 11% response rate that is usually observed in phase I trials [2, 44].

Stanford University and the company called Nodality also started looking at leukemia [2, 41]. They prepared fluorescent-labeled antibodies that bind to specific phosphoproteins in important cancer signaling pathways. They used a cell flow method in which cells flowed past a fluorescence detector. Those that had antibodies bound to phosphoproteins fluoresced, so they were detected. This established their concentration, which controlled the extent to which the signaling pathways were

activated in cancer cells [2, 41]. Nodality subsequently described a Single Cell Network Profiling (SCNP) method that can characterize signaling pathways, as well as drug sensitivity and resistance profiles in mast cell leukemia (MCL) bone marrow mast cells [2, 45]. They also measured EGFR pathway signaling and modulation from patients who had non-small cell lung cancer (NSCLC). The epidermal growth factor (EGF) binds to its receptor (EGFR), which is also a tyrosine kinase. It catalyzes the phosphorylation of some of its own tyrosine residues, which activates downstream signaling cascades [2, 45].

Other important studies have been done at the Harvard Partners Center for Genetics and Genomics, which was founded in 2001 with the specific goal of accelerating the realization of personalized medicine [2]. Its name changed in 2008 to the Center for Personalized Genetic Medicine [2, 46]. The change was due to an increasing emphasis on translational medicine. That is, they are trying to translate laboratory results into useful medical treatments and cures. This led to the Personal Genome Project, which was announced by George Church in 2006. It is publishing full genome sequences and medical records of volunteers to enable research into personalized medicine. Also, the Laboratory for Personalized Molecular Medicine (LabPMM) was founded in 2007 to identify specific mutations in genes linked to clinical outcomes in patients with leukemia and lymphoma. Identifying the presence or absence of these mutations is becoming standard for patients with acute myeloid leukemia. There is also a LabPMM that develops patient-specific molecular tests from the DNA in their tumor samples. The ultra-sensitive tests are used by leading cancer treatment centers throughout the world to monitor residual disease and treatment [2, 46].

The Mayo Clinic also has a Center for Individualized Medicine [2, 47]. About 100 000 people in the USA die each year from adverse reactions to medications, while another two million are hospitalized. Hopefully, research at the intersection of pharmacology and genetics (pharmacogenomics) will make it possible to predict people who are most likely to have an adverse reaction to a drug before it is given to them. It may also be able to predict whether a patient will respond well to a particular medicine [2, 47].

There are also Centers for Personalized Medicine at the Roswell Park Cancer Institute (RPCI) [48], Duke [49], Stanford [50], the University of Pennsylvania (Penn) [51], the Sheikh Khalifa Bin Zayed Al Nahyan Institute for Personalized Cancer Therapy [44] and many others [2].

1.8 P4 and evidence based medicine

P4 medicine is also consistent with evidence based medicine that is used to help prescribe appropriate, individualized drugs and medical devices [1, 52]. A patient's blood or tissues can be analyzed for different types of enzymes (isozymes 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 different groups of people. This approach led to the development and rapid approval of tratuzumab, or Herceptin®, for treating and curing patients who have the HER-2 gene that is involved in many cancer signaling pathways. Herceptin targets breast cancer patients who have the type 2 form of receptor for the human epidermal growth factor, or HER2 receptor. Breast cancer can be divided into the following different groups: estrogen receptor positive (ER+), human epidermal growth factor receptor2 (HER2; also called ERBB2 and HER2+) and triple-negative (ER^-, progesterone receptor negative, PR^-, and $HER2^-$) [53]. There are also six independent intrinsic molecular subtypes: normal-like, HER2-enriched (HER2E), luminal (A and B), basal A/basal-like and basal B/claudin-low. However, new methods may discover other subtypes. A statistical analysis called pathway-assisted clustering was used to analyze LC-MS data on plasma proteome samples from breast cancers. It found that cancers can change from one subtype to another. This can be seen by looking for changes in biological and signaling pathways. Similar subtypes share distinct yet similar pathway activation networks, while dissimilar subtypes use different networks. Moreover, the distance or similarity of cancer subtypes based on pathway analysis might illuminate the relationships between breast cancer subtypes [52, 53].

So, instead of just making a medicine that is specific for the DNA that a person is born with, it can be made specific for a type of cancer. This is being done by developing monoclonal antibodies. 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 [2]. Gleevec® is used to treat chronic myelogenous leukemia. More recently, Personalized medicine 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 [1, 52].

So, the following goals were described in a recent report from the FDA [2, 54]. “Personalized medicine seeks to reduce the burden of disease by targeting prevention and treatment more effectively. With the help of personalized medicine, the health care 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 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” [54].

1.9 The Advancing Regulatory Science Initiative

The FDA launched the Advancing Regulatory Science Initiative in 2010 [2, 55]. This initiative 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 [2, 55]. 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 [55].

1.10 The voices of the people

One voice that is heard across all societies is that our children, grandchildren and great grandchildren are of utmost importance. However, the needs of children vary with the different communities in which they live [2, 56]. 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 all 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 [2, 56].

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 [2, 56]. It is also important to recognize 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. So, there is a Nurse-Family Partnership and an Invest in Kids program that provide essential links between communities and healthcare systems. It is also important to be open-minded and flexible. Some communities may want to focus on bullying, while others focus on violent crime and drug abuse. Others may be more interested in obesity by improving diet and behavior (such as exercising). Others may focus their efforts 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. That way, 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 [2, 56]. Good programs can evolve and continuously improve – as in TQM. So, systems thinking and TQM are important in helping our children.

Even the federal government of the USA is getting involved in P4 medicine [57]. In his State of the Union address on January 20, 2015, President Barack Obama said "Tonight, I'm launching a new Precision Medicine Initiative to bring us closer to curing diseases like cancer and diabetes — and to give all of us access to the personalized information we need to keep ourselves and our families healthier." Fortunately, this led to a new era of precision medicine (www.whitehouse.gov/precisionmedicine).

This initiative includes the NIH and focuses initially on cancer. There are already some targeted therapies for treating some types of cancer, included immunotherapy using oncolytic viruses (as described in Chapter 3, volume 1). One goal of the Precision Medicine Initiative is to recruit a cohort of one million people who will have their cell populations, proteins, metabolites, RNA and DNA analyzed. This will be linked with behavioral data and electronic health records. The people who participate in this project will have access to the data on them, but others will not, to protect everyone's privacy. At the same time, the FDA is collaborating with the scientific community to "make sure its oversight of genomic technology supports innovation, while ensuring that the public can be confident that the technology is safe and effective" [57].

1.11 Limitations of P4 medicine

However, there are some limitations to systems and P4 medicine. It can be no more holistic than its computational and mathematical models. It still focuses on specific molecules, making it somewhat reductionist. Some people feel that it has some problems accounting for all aspects of health and disease prevention, as well as what is often called the 'mind' and the 'body' [5, 58-60]. In addition, P4 medicine emphasizes technoscientific innovation, which has often been at odds with humanistic ideals. Computers can tell a person what they should do (such as follow a strict diet), but physicians need to care for patients even when they do what they shouldn't (like drinking sweetened beverages and eating red meat). Over-medicalization is the basis of some criticisms of P4 medicine. Medicalization has been defined as "the process by which aspects of human life come to be defined in medical terms and underlain medical control" [5]. Medicalization and medical control are not the problem – overmedicalization is. Some feel that it threatens the overall balance between benefits and costs (or harm) of over-emphasizing medicine. That is, P4 medicine should include the social, psychological, and behavioral dimensions of illness [5].

In the spirit of TQM, it is also important to listen to the voice of the patient and caregiver. For example, it is doubtful that a computer model will ever be able to predict or explain why a young child's pain and suffering from a minor scratch can be cured instantly by a loving caregiver who puts a colorful bandage on it. In addition, mathematical modeling can't quantify the health effects of things like happiness and love. To do so, there would have to be units in which they can be measured. Many biochemicals can be measured in units of grams per mole, while gene

expression can be quantified by the number or amount of RNA or proteins that are expressed. Happiness and love are essential aspects of human health, but there are no units in which their levels can be expressed. Also, it is possible that some colors or combination of colors can stimulate some healthy emotions. However, there is no way that a placebo can be used for a color (like blue or red). When placebos are given in clinical trials of NMEs, the placebo is a pill (or other dose formulation) that looks just like the actual NME. How can anything look blue or red, but not be blue or red? Moreover, how can anyone be sure that they see the same thing that another sees when they see something that is red or blue? Even more important, some patients may reach the end of their patience with seemingly endless tests and trips to a hospital when they have a terminal illness. They might tell their caregivers that they can't endure any more suffering. So, the next time a caregiver or nurse thinks about sending such a patient to a hospital, the patient might tell them that it might be better to call hospice and prepare for the end in as comfortable a way as possible. That is, P4 medicine may be of limited use in palliative care. Instead of treatment options, the patient may simply need more pain medicine and someone to hold their hand as they slowly die. Indeed, it is an exceptional nurse who can do that. So, remember that systems thinking rejects dogma. One size does not fit all. It is also important to consider the entire human population, and not just those people who can afford to have all the -omics and biomarker tests done on them.

As a result, the concept of P5 medicine has emerged, in which the 5th P stands for population to some and psycho-cognitive aspects of medicine to others, since psychology can't be ignored – especially when caring for patients who have cancer [58-61]. Population science and sociology are concerned with the costs and potential for harm that result from large social, economic and health disparities [58]. They also focus on enhancing population level interventions, such as education, employment, the availability of healthy food and clean water as well as infrastructure. Population science and sociology augment the P4 approach to medicine. It augments prediction by including an ecological model of health that accounts for multilevel determinants of health. It augments prevention by incorporating population screening principles to assess the benefits, harm and costs of preventing diseases. It augments personalization by including the principles of evidence-based medicine that use a formal analytic framework to compare effectiveness. It augments participation by including the public health sector in developing policies, as well as using regulatory science to implement health services research. Population science also focuses on enhancing interventions at the population level

(such as education, employment and infrastructure) as well as individual level interventions to improve health and prevent disease [58].

1.12 Metabolomics

1.12.1 Introduction

Regardless of whether or not patients follow the advice of their physicians – or a computer analysis, it is important to monitor the types and concentrations of metabolites that they produce. That is, in P4 medicine, it is not only important to listen to the outward voices of the patients, but also to pay attention to how they respond to their ever-changing internal and external environments. This is done by analyzing their metabolomics, which includes their metabolites and studying their metabolism [62, 63]. It is useful in diagnosing diseases and is important in epidemiology, epigenetics, hypertension, cancer, toxicology, drug discovery and the understanding of biological processes [63]. Even though metabolomics and metabonomics are often used synonymously, metabonomics has been defined as the "quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [64, 65]. Metabolomics has been defined as "the complete set of metabolites/low-molecular-weight intermediates, which are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism" [66]. Metabolomics has also been called 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 genome wide association studies 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" [62]. It is enabling the personalized phenotyping of metabolites. When combined with genomic and epigenomic data, it is advancing the field of precision medicine [62, 67]. The goal is "to use advanced diagnostic testing to customize an individual's medical treatment according to their specific -omic profiles" [62]. 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) [62]. On the other hand, "pharmacometabolomics aims to identify metabolic traits that offer insights into the intended as well as the unintentional effects of drugs on the human organism" [68]. The FDA simply defined metabolomics and metabonomics as "global metabolic profiling or simply, metabolic profiling" [69]. "Together, genetics, transcriptomics, proteomics and global metabolic profiling comprise the basis of the systems biology approach" [8]. Moreover, the FDA's lab at the NCTR does extensive metabolomic and proteomic research [70-72].

Perhaps one of the most important uses of metabolomics is to help discover new drugs. A recent review article stated that, "Metabolism represents the 'sharp end' of systems biology, because changes in metabolite concentrations are necessarily amplified relative to changes in the transcriptome, proteome and enzyme activities, which can be modulated by drugs" [2, 73]. Systems biology is an integral part of systems medicine [2, 74, 75]. It is also an important part of preventive, personalized and participatory (P4) medicine [74, 75]. Models of human metabolic networks are needed to understand drug metabolism [73]. "One such model, Recon2, can predict the effects of inborn errors of metabolism, identify exometabolites and show how metabolism varies between tissues and cellular compartments. Small molecule transporters for drugs can also transport metabolites, so they are of special importance. Many pharmaceutical compounds enter or leave cells through such transporters. Also, some dietary compounds, such as naringen in grapefruit can be metabolized to produce compounds like naringenin that can alter cytochrome P450 metabolism of statins. So, it is anticipated that the emerging field of systems pharmacology and metabolomics may help to discover and develop new drugs" [73].

In earlier metabolomic studies, quantitation was seldom a priority [62]. Now that governments, pharmaceutical companies, universities and research institutes have gained access to modern instruments like LC-MS, quantitation is being done. Since modern LC-MS instruments are equipped with autosamplers and computers, they can analyze the data from many samples in untargeted, automated metabolomic studies. This also enables 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 [76]. 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 Western and Eastern knowledge is used [76].

1.12.2 Comparison of LC-MS and GC-MS

In mass spectrometry (MS), chemicals (biomarkers and other analytes) are ionized and their ratios of mass to charge (*m/z*) are measured. There are different ways to introduce the sample into the mass spectrometer, or MS. Samples can be carried to an MS in a stream of gas, as is done in gas chromatography coupled to MS (GC-MS), or in a stream of liquid, as in LC-MS. GC-MS is especially useful in breath analysis. Both BC-MS and LC-MS are useful in analyzing blood, urine and a variety of tissues. Tissues with relatively high fat content (like the brain) are often analyzed for the relative amounts of fatty acyls that are covalently attached to fats and lipids. First, the tissues are extracted with a 2:1 (v/v) mixture of chloroform ($CHCl_3$) and methanol (CH_3OH). The $CHCl_3$ phase is saved, since it contains cholesteryl esters, monoacylglycerides (MAGs), diacylglycerides (DAGs), triacylglycerides (TAGs), phospholipids, lysophospholipids, sphingomyelin and other lipids. MAGs, DAGs and TAGs are also known as mono-, di- and triacylglycerols, respectively. Phospholipids include phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Next, the lipids are hydrolyzed, producing free fatty acids (plus glycerol in the case of MAGs, DAGs and TAGs). The free fatty acids are then converted to fatty acid methyl esters (FAMEs) or other volatile derivatives, which are can be separated on a variety of GC columns [79]. Physiological samples can also be derivatized with a solution of methoxamine hydrochloride, which attaches an oxime to oxo groups on reducing sugars (glucose and fructose), followed by trimethylation of hydroxyl, amino and carboxylic acid groups [80, 81]. This converts them to volatile compounds. Another approach is to derivatize fatty acids using pentafluorobenzyl bromide [82].

However, caution must be used when trying to understand the data. Unfortunately, the term fatty acid is often misused when describing the analyses of triacylglycerides and other lipids [83, 84]. For example, the term fatty acid was used two different ways in an article. In the title, it was correctly reported that omega-3 (ω-3) fatty acids can activate the Nrf2 antioxidant pathway, but when analyzing mice brains for changes in brain

lipids during ischemia, the fatty acid content of the brain was reported. Actually, it was actually the concentrations of fatty acyls that were determined. Still, dietary TAGs are hydrolyzed into free fatty acids plus glycerol. It is the free ω-3 fatty acid and not the entire TAG that binds to the Nrf2. On the other hand, it was reported that "omega-3 polyunsaturated fatty acids [sic] (n-3 PUFAs) attenuate ischemic neuronal injury" [85]. It was actually omega-3 fatty acyls that were fed to the mice, in the form of fish oil. In fact, there are no acids of any type in fish oil – just TAGs [83]. So, people with acid reflux can consume fish oil without worrying about ingesting any acids. Fortunately, physicians, nutritionists and most of the medical profession use the correct terms, omega-3 fats and polyunsaturated fats [83].

Communication about lipidomics can be confusing – even in review articles published in very prestigious journals. That is, food chemists and some analytical chemists often use the term fatty acids very differently than other chemists. In freshman chemistry, it is taught that acids have an ionizable hydrogen that produces a proton (H^+) and acidity can be measured using a pH scale. In organic chemistry, a fatty acid has a carboxyl, or –COOH group, while fatty acyls are not chemical compounds, but parts of larger molecules, such as esters. Acids and esters have very different physical-chemical properties, including solubility. However, in analytical chemistry, even though the six classes of lipids were said to include fatty acyls, their structures were shown to contain the –COOH of fatty acids [84]. This could lead some readers to think that free fatty acids are the major form of fats in human cells, which they are not.

The main reason behind this misunderstanding is that the fatty acyl composition of TAGs and phospholipids in foods and biological samples were determined by GC coupled to flame ionization detection (GC-FID) and by GC-MS long before they were determined by LC-MS. Since GC-FID and GC-MS detect volatile derivatives of fatty acids (such as FAMEs), almost all the earlier articles reported fatty acid compositions, not fatty acyls. This is simply human nature. For example, apprentice carpenters are often told that if you give a guy a hammer, pretty soon everything starts looking like a nail. A corollary to that is when you give a guy a GC, pretty soon everything starts looking like a fatty acid.

In total contrast, many people doing metabolic studies use the proper vocabulary. For example, there was an excellent review article on the analysis of biologically active carboxylic acids, based on GC or LC [86]. The proper nomenclature was also used when urine samples of patients with CKD were analyzed without doing a hydrolysis step [81]. This made perfect sense, since lipids like MAGs, DAGs and TAGs are not soluble in

water or urine. Instead, free fatty acids, sugars, amino acids, benzoic acid and oxo acids were derivatized to form volatile compounds containing a trimethylsilyl moiety. The derivatives were analyzed by GC-MS. In that study, other compounds such as carnitine derivatives were identified by LC-MS [81]. In another study, free fatty acids were accurately described as minor constituents, since "the majority of fatty acids exist in the form of esters and amides in lipids" [82]. To determine the total fatty acid composition of blood plasma, saponification by acidification with HCl followed by pentafluorobenzyl bromide derivatization was used prior to GC-MS analysis [82]. Even work coming from very prestigious organizations can be slightly deficient in the description of the ways that samples were prepared. For example, a recent study reported that liver and blood samples were prepared for the analysis of aqueous metabolites by adding "different volumes of resuspension solvent consisting of aqueous 2 mM NH_4OCOCH_3 and 3 mM hexylamine (pH 9.2, adjusted with acetic acid)" [87]. This makes sense since aqueous metabolites are soluble in water. However, supposedly the same resuspension solvent was used to redissolve extracts of liver and blood plasma before analyzing them for lipids – most of which are not soluble in water [87].

In contrast, LC-MS can be used to analyze a variety of physiological samples for intact DAGs, TAGs, phospholipids and free fatty acids without derivatization [88]. In that case, free fatty acids were distinguished from fatty acyls [88]. However, LC-MS can't tell which carbons on a glyceride backbone have specific fatty acyls covalently attached to them. It can only provide the molecular weights of the intact glyceride, such as TAG 52:1, in which the 52 indicates the total number of carbons in the three fatty acyls and the number one indicates that there is only one C=C bond on just one of the fatty acyls [89]. Still, LC-MS can distinguish between free fatty acids like arachidonic acid and fatty acyls, such as the arachidonoyl in lysophosphatidylcholine that was abbreviated as LysoPC(20:4) [90]. In another study, fast switching between positive and negative ion ESI was used to detect nonionic TAGs from cationic and anionic lipids using LC-MS [91].

The principles of MS were described further in the Appendix in volume 1. Regardless of the technique used, the ions that are produced can provide useful information for identifying a compound [83]. The molecular ion can give the molecular mass and formula, if the resolution of the mass spectrometer is good 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} amu. The resolution is 10^{-4} divided by 10^{2}, or 10^{-6}. If masses can be detected with sufficient 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. So, compounds with the molecular formulas C_6H_{12} C_5H_8O and $C_4H_8N_2$ have molecular weights of 84.0939, 84.0575 and 84.0688, respectively. They can be distinguished by high resolution mass spectrometry (HRMS) [83].

However, HRMS can't distinguish isomers that have the same molecular weights or *m/z* values unless one also looks at the fragments that are produced. For example, the mass spectra of isomers in a class of neurotoxic compounds (called acetogenins) that are in tropical fruits in the Annonaceae family (like graviola, *Annona muricata*, and the North American pawpaw, *Asimina triloba*) are different. The structure of one of these neurotoxins, annonacin, is shown in Figure 1. Some isomers of annonacin have one or more of the –OH groups on different parts of the molecule. As a result, they produce different fragments and are retained on a C18 column for different times than annonacin.

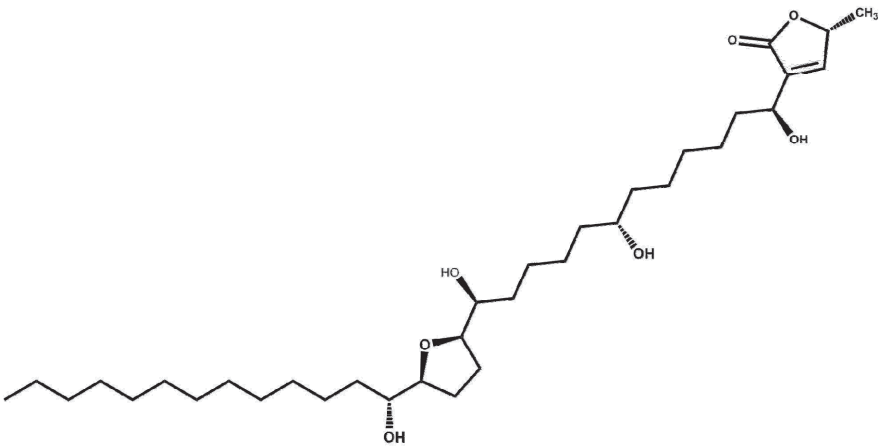

Figure 1. Structure of annonacin $C_{35}H_{64}O_7$, MW 596.87846, IUPAC name: (2S)-2-methyl-4-[(2R,8R,13R)-2,8,13-trihydroxy-13-[(2R,5R)-5-[(1R)-1-hydroxytridecyl]oxolan-2-yl]tridecyl]-2H-furan-5-one.

Many polar and ionic compounds can be separated by LC and detected by MS. Often nonionic compounds, peptides and proteins can be separated from each other on C18 (octadecyl silica) columns using a polar mobile

phase, such as a combination of CH_3OH or acetonitrile with water. Ammonium formate and/or formic acid are added to help produce ions in the MS and control the degree of ionization of weak acids, such as carboxylic acids. C18 columns may be needed if one is trying to separate the naturally occurring neurotoxins annonacin and squamocin from their isomers [92]. Small, ionic compounds, such as amino acids can be separated on ion exchange columns. Many small and highly polar molecules can be separated best using hydrophilic interaction liquid chromatography (HILIC) [93]. A variety of different compounds can be covalently bound to silica to make HILIC columns. This includes amides, saccharides and others. Zwitterionic compounds (ZIC) such as sulfobetaine (ZIC-HILIC) and phosphocholine (ZIC-cHILIC) that contain both positive and negative charges are especially popular in metabolomics and proteomics. The mobile phase should contain at least 2% water. The remaining portion is often acetonitrile. As a result, a layer that is enriched in water is formed on the surface of the HILIC column. This provides a completely different separation mechanism than the one that occurs in reverse phase LC using C18 columns. HILIC columns can be used to separate proteins as well as small molecules, such as acrylamide that can be found in some foods like potato chips. Analytes can be retained by hydrophilic partitioning, surface absorption and electrostatic interactions [95]. HILIC-MS can also be used to separate and detect amphiphilic analytes, such as the acidic lipids PA, PC, PI, sulfatides, ceramides, PE, PS, LPE, LPC and sphingomyelin in kidneys and other biological samples [94]. Alternatively, silica columns and less polar mobile phases can be used in normal phase LC to separate different classes of lipids in lipidomics [95, 96].

LC-MS has been used for both targeted and untargeted (or global) analyses of metabolomes [97]. In targeted analyses, the identity and exact molecular weight of each analyte is known, but in untargeted analyses they are not. Targeted analyses are done most convincingly using LC-HRMS, in which the *m/z* ratio of each analyte is specified by the MRMS computer to within about ±0.005 atomic mass units (amu) and the retention time of the analyte is verified using a primary standard [92]. If the MS is set to detect all masses within a range of 100-1000 amu, millions of ions would have to be monitored to cover all possible analytes. In contrast, if the resolution of the MS is only ±0.5 amu, a manageable number of analytes can be detected - but without the certainty that HRMS can provide. So, targeted metabolomics covers far fewer metabolites than untargeted [97]. However, a new technique called globally optimized targeted (GOT)-MS has emerged. It combines many of the advantages of targeted and

untargeted detection, such as the ability to identify previously unknown metabolites, while providing broad coverage of metabolites and excellent quantitation. It does a global search of precursor and product ions using an LC-triple quadrupole MS. In the initial report, 595 precursor ions and 1890 MRM transitions were obtained from serum in colorectal cancer using both positive and negative ion MS in a range of 60-600 amu (also known as Daltons, Da). The mass resolution was relatively low, so an *m/z* interval of ±0.5 was used with a 10 ms scan time (cycle time 0.6 s) [97].

There are several methods for interpreting MS data, which can contain just the *m/z* values of analytes or the retention times and *m/z* for GC-MS and LC-MS. An excellent review was published recently on MS from a computer scientist's point of view [98]. That is, MS data can be described in different ways. The total ion chromatogram (TIC) shows the sum of all signals (ion intensities) plotted against the retention times (t_r), or length of time in which an analyte is retained on an analytical column, like a C18 column. From this, total ion spectra (TIS) can be obtained that show the *m/z* of all the ions that are produced by one or more compounds with the same t_r. One can also obtain a base peak chromatogram (BPC) that shows the largest peaks for each tr across all *m/z* values. One can also obtain an extracted ion chromatogram (XIC) that shows all the peaks in a chromatogram that have an *m/z* that falls within a small, specified range. Moreover, an isotope trace is the signal produced by a single ion from a single analyte at a particular charge state. An isotopic envelope trace is the group of isotopic traces produced by a single analyte at a particular charge state, usually +1 or -1. To assist in data analysis, there are software packages that reduce the noise, detect spectral features and deconvolute overlapping peaks [98]. Results can be compared to data in mass spectral databases, such as METLIN [99]. There is also an isoMETLIN database for isotopically labeled metabolites [100]. It can search all computed isotopologues derived from METLIN on the basis of *m/z* values and specified isotopes of interest, such as ^{13}C or ^{15}N. Additionally, isoMETLIN contains experimental MS/MS data on hundreds of isotopomers. These data find where the isotopic labels are located within a metabolite [100].

There is also a software (RAMClust) package that can identify spectral features and do spectral matching to annotate MS data [101]. It groups signals from MS data into spectra and annotates their intensities [40]. Features are detected in both MS and idMS/MS data, while relationships between features are determined simultaneously from the MS and idMS/MS data [101]. Another package, called *mzGroupAnalyzer*, automatically extracts features from LC-MS data, determines the structures of

compounds that produced the data and assigns them to the proper metabolic pathway [102]. It can also identify biochemical and chemical transformations in non-targeted metabolomics data [102].

To help identify unknown compounds, one can form chemical adducts of functional groups in what is called chemoselective adduct formation, or CS-tagging [103]. When using isotopically labeled tags, usually the unknown compound can be identified by searching metabolomics databases. There is a program called CheckMol that can find at least 240 different functional groups in molfile databases. However, the method for finding each functional group is unique and hard-coded. To add a new functional group to the list, new code must be written in Pascal and then put it into the proper place in CheckMol. So, another algorithm called Chemically Aware Substructure Search (CASS) was written to find functional groups in existing databases of metabolites [103].

Bioinformatics are also needed to decide which metabolites are the most biologically meaningful and correlate the best with disease diagnosis, pathogenesis or any other aspect of metabolism [104]. Algorithms called XCMS and MZmine have been used to align the retention times from LC-MS data to those in databases. This is needed because the retention times can vary slightly from one instrument to another – especially when using older columns for separating the metabolites. Automated metabolomics can also be done in which MS1 and MS/MS data are acquired simultaneously using an untargeted metabolomics workflow. MS1 data are preprocessed by XCMS. Features are extracted, realigned to correct for differences in retention times and analyzed statistically. The MS/MS data are acquired automatically using data dependent acquisition and are compared to spectral databases. To obtain high-quality spectra, quadrupole TOF MS was recommended. The workflow can save weeks of time needed for mass spectrometry and selection of significant peaks. XCMS can also detect features, correct for differences in retention times, fill gaps in the data, annotate features, predict fragments *in silico* and match spectra to databases. If a data dependent acquisition and MS/MS processing step is included using MetShot (an R package), MS/MS experiments can be automatically generated from a ranked list of interesting precursor features. Such experiments use defined filters that make the MS acquire only the relevant spectra. Finally, data can be uploaded into data clouds where they can be processed [104].

There are also many mass spectral databases that are freely available [105]. This includes the Human Metabolome Database, HMDB, version 3.6 (http://www.hmdb.ca/) [106]. It is a “comprehensive web-accessible electronic database containing detailed information on metabolites found

in the human body". As of August 2015, it contained nearly 42 000 metabolite entries [106]. To help evaluate their usefulness, the Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI; http://msi-workgroups.sourceforge.net) defined four levels of analyte identification. Level 1 requires that at least two orthogonal (unrelated) molecular properties of the putative metabolite be confirmed with an authentic pure compound analyzed under identical analytical conditions. For MS, this is usually retention time and the *m/z* of molecular ions or adducts, such as $[M+H]^+$, $[M+Na]^+$ and $[M+NH_4]^+$. However, it can also include NMR analysis. By contrast, for levels 2 and 3, results only need to be compared with previously published values in the peer-reviewed literature and/or MS database. As a result, only annotations are achieved – not clear identification. Level 4 is for unknown compounds. However, it should be noted that current databases still don't contain experimental data on all known metabolites. However, the Metabolite Standards Synthesis Core (MSSC) initiative by the NIH (http://www.metabolomicsworkbench.org/standards/nominatecompounds.php) is trying to increase and improve their content. Its goal is to generate new compound standards [106].

1.12.3 NMR in metabolomics

NMR measures the absorbance of radio frequency (rf) electromagnetic radiation by organic compounds that are placed in a strong magnetic field [83, 107]. ^1H, ^2H, ^{19}F, ^{13}C and ^{14}N nuclei are magnetically susceptible, so they will absorb rf radiation. Even though NMR spectra can be obtained on solid samples (especially man-made polymers and plastics), much better spectra can be obtained if the solids are dissolved in a solvent, such as water. Since undissolved solids are undesirable, proteins, triglycerides and lipids must be removed from aqueous biological samples by solid phase extraction or other methods. Once the proteins and fats are removed, NMR is especially useful for analyzing blood and urine. Despite being superb for quantitation, ^1H-NMR is not nearly as sensitive as MS, ultraviolet-visible (UV-Vis) and infrared (IR). There is another important difference between NMR and MS, UV-Vis and IR. The locations (chemical shifts) of the peaks (or signals) in an NMR spectrum depend on the concentration, pH and ionic strength of the sample. So, the chemical shifts are different in dilute vs concentrated urine and in blood from different people who have different concentrations of proteins and electrolytes in it. So, NMR spectroscopists look for the chemical shifts that are due to ^1H nuclei in the most abundant metabolite, such as creatinine in

urine. This is in contrast with GC-MS and LC-MS, in which an analyte will be retained on the LC column and reach the MS detector at the same time, regardless of the concentration or ionic strength of the sample. The retention times (t_r) are so repeatable that they can be used together with the mass spectra to verify the identities of the analytes. That is, when doing research under good laboratory practices (GLP), methods based on GC-MS and LC-MS should specify the expected t_r of each analyte of interest, as well as its mass spectrum. On the other hand, a NMR–based GLP method for analyzing an aqueous, physiological sample (especially urine) should not specify that all the peaks (chemical shifts) due to analytes have the same chemical shifts in every sample. Instead, it can specify that the chemical shifts of the peaks due to the most abundant analytes (like creatinine) should be set to a standardized value [83, 107]. If an NMR spectrum is obtained on compounds that are dissolved in a deuterated organic solvent (such as $CDCl_3$), zero ppm is assigned to the chemical shift of tetramethylsilane (TMS). Alternatively, a concentrated buffer, such as 1M phosphate, can be added to keep the pH constant for each urine sample. So, when 640 μL of urine was mixed with 80 μL of 1M phosphate buffer, pH 7.0, the $–CH_3$ and $–CH_2–$ groups in creatinine produced chemical shifts of 3.05 and 4.07 ppm, respectively in the ^1H spectrum [107, 108]. Since TMS is not soluble in water, zero ppm was set using a similar water-soluble compound, 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt (TSP) [108]. Another approach is to use two separate NMR tubes – one that contains the sample and the other that contains the D_2O and standards for setting the chemical shifts [109]. This "prevents isotope exchange between sample metabolites and the deuterated solvent, and is compatible with downstream mass spectrometry" [109]. This was the basis of a high throughput method that used the known chemical shifts for glycerol as a reference in ^1H-NMR spectra of 30 μL samples of urine [109].

For NMR analysis of blood plasma and serum, only enough D_2O to obtain lock is added since the pH is already well buffered [110]. Proteins contain many different hydrogens that can have chemical shifts (peaks) that overlap with those produced by small molecule metabolites. So, the proteins should be precipitated out by adding solvents like acetonitrile or methanol that denature them. Also, fats and lipids are not water-soluble. They can be removed by extracting the physiological sample with 2:1 chloroform:methanol ($CHCl_3$:CH_3OH). Once the interfering proteins, fats and lipids have been removed, the signals or peaks due to small molecule metabolites will become evident, but their exact chemical shifts will be different in samples with different ionic strength and concentrations of

metabolites. Human liver transplant blood will use the added citrate, while heparinized blood will use signals due to glucose to set the chemical shifts. Cerbrospinal fluid (CSF), expressed prostate secretions (EPS), saliva, bronchoalveoloar lavage fluid (BALF) and tissues can be analyzed after adding some D_2O, while bile can be analyzed after adding deuterated methanol. Intact tissue specimens can also be analyzed by high resolution magic angle spinning NMR. Important biomarkers that are seen by NMR include acetate, *N*-acetyl aspartate (NAA), alanine, allantoin, betaine, choline, dimethylglycine (DMG), citrate, creatinine, glucose, glutathione, glycerophosphocholine (GPC), hippurate, ketone bodies, lactate, methionine, methyl amines, phosphocholine, phenylalanine, polyunsaturated fats, succinate, taurine and trimethylamine-*N*-oxide (TMAO) [110]. Acetate was found in the blood, urine and liver in subjects with liver toxicity. Elevated NAA was detected in the brain and cerebrospinal fluid of subjects with cancer or neurodegeneration. There was alanine in tissues of subjects with cancer or brain damage due to ischemia-reperfusion. Alantoin was in rat blood and kidneys. Betaine was in the blood and tissues of subjects with brain damage due to ischemia-reperfusion. Choline was in the tissues of subjects with cancer. DMG, hippurine, creatinine and TMAO were seen in the urine of subjects suffering from kidney disease. Elevated citrate was in the prostate and blood of subjects with prostate cancer. Elevated glucose was seen in the blood, urine and tissues of subjects with drug toxicity, transplants, liver toxicity and ischemia-reperfusion. Glutathione was present in the blood and urine of subjects with damage due to ischemia-reperfusion and drug toxicity. GPC was found in tissues of subjects who had cancer. Ketone bodies were present in the blood of subjects with liver toxicity and dysfunctions. Elevated lactate was seen in the blood, urine and tissues of subjects with drug toxicity, ischemia-reperfusion, transplantation and cancer. Elevated methionine was seen in the blood and tissues of subjects with liver steatosis, transplantation and cancer. Methyl amines were found in the urine and blood of subjects with nephrotoxicity and renal ischemia-reperfusion. PCho was in the tissues of subjects with cancer. Elevated phenylalanine was seen in the blood and urine of subjects with liver toxicity. PUFA were present in the tissues of subjects with oxidative stress and apoptosis. Elevated succinate was seen in the urine, blood and tissues of subjects with kidney and liver toxicity. Taurine was in the urine, blood and tissues of subjects with liver toxicity and osmotic stress [110]. However, the term polyunsaturated fatty acids (PUFAs) is misused sometimes [111]. That is, there are some genuine free PUFAs in the blood and other biological samples, as well as polyunsaturated fatty acyls that are covalently bound to

a glyceride backbone [83]. Genuine PUFAs and some phospholipids can be extracted from blood and other tissues using methanol [112], while triacylglycerides containing polyunsaturated fatty acyls require 2:1 $CHCl_3$:CH_3OH [113].

1.12.4 Receiver operator characteristic (ROC) curves

After doing the spectral analyses and finding putative biomarkers, it is important to do proper quality control [114]. This includes constructing receiver operator characteristic (ROC) curves. That is, very few biomarkers can unequivocally diagnose a disease. There are almost always some false positives and false negatives. Moreover, often there has been very little consistency in how researchers select, assess or report their candidate biomarkers. The simplest, but most naïve way to determine the quality of biomarkers is to indicate the percentage of individuals who are correctly classified as having a disease or not. Unfortunately, this forces the analyst to predetermine the optimal criteria for the classification. There may be a difference between mathematically and ethically optimal boundaries. That is, for the deadly ebola virus disease, it might be essential to identify all people who have the disease, even though there might be some false positives. On the other hand, tests for prostate cancer using PSA as the biomarker produce many false positives for this disease, which progresses very slowly. Such false positives often lead to painful biopsies, radiation and chemotherapeutic treatments and even unnecessary surgeries. So, it is often better to consider the frequency with which a test for biomarkers produces true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN). The sensitivity (Sn) and specificity (Sp) of the method are given by the equations (1) and (2) [114]:

Equation (1): $\mathrm{Sn} = \dfrac{TP}{TP + FN}$

Equation (2): $\mathrm{Sp} = \dfrac{TN}{TN + FP}$

Sensitivity is “the probability of a positive test result given that a subject has an actual positive outcome, and specificity can be considered as the probability of a negative test result given that a subject has an actual negative outcome. Thus, for a given biomarker with a fixed decision

boundary (metabolite concentration or model score) a sensitivity of 0.90 and a specificity of 0.7 indicate that: given a new test subject with unknown clinical outcome, when the resulting test score is above the decision boundary there is a 90 % chance that the subject is correctly classified as a positive outcome; but if the test score is below the decision boundary then there is only a 70 % chance that the subject is correctly classified as a negative outcome" [114].

Different ROC curves can show how changing the classification decision boundary affects the sensitivity and specificity of biomarkers. The analyst plots the sensitivity against the specificity and determines the area under the curve [114]. "A rough guide for assessing the utility of a biomarker based on its AUC is as follows: 0.9–1.0 = excellent; 0.8–0.9 = good; 0.7–0.8 = fair; 0.6–0.7 = poor; 0.5–0.6 = fail" [114].

1.12.5 Acute kidney injury (AKI) metabolomics

To help show how metabolomics is used in medicine, its use in studying kidney diseases will be described next. AKI is due to a sudden and sustained decrease in kidney function that leads to retention of toxic waste products [115]. It is diagnosed by increases in the concentration of serum creatinine, which means that the glomerular filtration rate is lowered. Vascular and tubular defects emerge, along with changes in the expression of several proteins that can be found in the urine and blood. This includes neutrophil gelatinase-associated lipocalin (NGAL), urinary cystatin C, urinary kidney injury molecule (KIM-1), urinary interleukin-18 (IL-18), and glutathione-S-transferase. They are early diagnostic biomarkers. NGAL is released from immune cells and is present at higher concentrations during inflammatory conditions and after heart surgery. The concentration of NGAL in the urine is also useful in evaluating the risk and prognosis of AKI. It can also be used to distinguish between AKI and CKD. At the same time, KIM-1 is a very specific biomarker for kidney injury, since this transmembrane glycoprotein is only expressed in the kidneys. It is upregulated in the proximal tubule cells after ischemic or toxic injury. There are also protein biomarkers that stop the progression of the cell cycle and can be used to predict the risk of AKI. They include insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases-2 (TIMP-2), both of which induce G_1 cell-cycle arrest [115].

LC-MS has been able to find significant changes in the concentrations of several metabolites in blood serum in AKI (formerly known as acute renal failure, or ARF) [116, 117]. The concentrations of creatinine,

acylcarnitines and the amino acids methionine, homocysteine, pyroglutamate, asymmetric dimethylarginine (ADMA) and phenylalanine increased, while arginine, tryptophan and several lysophosphatidyl cholines (LysoPCs) decreased. This included LysoPC(14:0), (16:0), (16:1), (18:0), (18:1) and (18:2). Increases in homocysteine, ADMA and pyroglutamate are also biomarkers of cardiovascular disease, while acylcarnitines are biomarkers of defective oxidation of fatty acids [116, 117].

Inflammation also plays an important role in the pathogenesis of acute kidney injury, or AKI [118]. Dyslipidemia can occur and lead to cardiovascular diseases. However, a diet supplemented with ω-3 fats was able to protect mice from renal ischemic damage and death, while ω-6 fats offered no protection. The supplementary ω-3 fats increased the expression of protectin D1, which led to decreased influx of kidney polymorphonuclear leukocytes and upregulated the expression of heme oxygenase-1 protein and mRNA in injured and uninjured kidneys [118].

Kidney diseases and injuries can also lead to vascular calcification and cardiovascular diseases [119]. Vascular calcification develops in a similar way that bone formation does. First, vascular smooth muscle cells (VSMCs) differentiate into distinct cells that resemble osteoblasts. During kidney failure, the extracellular concentration of phosphate ($H_2PO_4^-$ and HPO_4^{2-}) increases and induces a phenotypic switch of VSMCs into osteoblast-like cells. These differentiated cells secrete bone-associated proteins and release matrix vesicles into the extracellular matrix that can become mineralized. Under healthy physiological conditions, VSMCs release matrix vesicles that are protected from mineralization by inhibitors of calcification. Long-term exposure of VSCMs to inflammation, hormonal changes, oxidative stress and metabolic disorders (including excess phosphate and/or calcium) can lead to vascular calcification. Moreover, disturbances in the rate of bone turnover, or autopoiesis can increase vascular calcification, since bones contain the highest reserve of calcium and phosphorus in the body [119]. So, there is a bone-vascular axis that is important in kidney diseases [120]. That is, changes in mineral (Ca^{2+}) metabolism are associated with "alterations of its hormonal regulation and various forms of bone disease" [120].

AKI has also been caused by consuming aristolochic acid that is present in weight loss supplements containing the Chinese herb Guang Fang Ji [121]. Biomarkers in the urine of rats exposed to aristolochic acid were identified by ultra performance liquid chromatography coupled with quadrupole time-of-flight high definition mass spectrometry (UPLC-QTOF/HDMS) with a resolution of 12 000 [122]. The concentrations of citrate, aconitate, fumarate, glucose, creatinine, *p*-cresyl sulfate, indoxyl

sulfate, hippuric acid, phenylacetylglycine, kynurenic acid, indole-3-carboxylic acid, spermine, uric acid, allantoin, cholic acid and taurine changed significantly in the urine of the rats that suffered from nephrotoxicity [122].

However, UHPLC instruments are more expensive than conventional HPLC, so some hospitals and laboratories don't have them. Instead, core-shell columns that are made using fused core technology can be used with HPLC for metabolomics studies [117]. That is, there is an outer porous layer that is 0.5 μm thick with 90 Å average pore size and 150 m^2/g surface area. It surrounds the 3.4 μm inner particle. Core-shell columns can contain C18, HILIC and other types of packing. They operate at lower back pressures than UHPLC columns. The relatively high surface area makes these columns very useful in separating analytes that have high or low molecular weights in untargeted metabolomics studies [117].

1.12.6 Chronic kidney disease (CKD) metabolomics

The metabolomics analyses of CKD up to 2013 were reviewed [123]. This review described results from not just LC-MS, but also GC-MS, capillary electrophoresis (CE) and NMR. LC-MS was used to show that the concentrations of the following free fatty acids changed in animal studies: arachidonic acid, linoleic acid, palmitic acid, docosahexaenoic acid (DHA), docosapentaenoic acid and eicosapentanoic acid (EPA) all changed during CKD. Moreover, LC-MS found that certain fatty acyls that were parts of lipids were potential biomarkers. Examples included ceramides (18:0/14:0) and (18:0/16:0), PC (16:0/18:2), several LysoPCs, phosphatidylinositol (18:0/20:4), two phosphatidyl serines (PS 18:0/18:0 and PS 18:0/22:5), lysophosphatidyl ethanolamines (LysoPEs 16:0 and 18:2) and monoacylglyceride (24:1). Another lipid, glycerophosphocholine, was also reported to be a biomarker for CKD in animal studies. As expected, creatinine was, too. Several amino acids, carbohydrates, polyols, glucuronides, adrenaline, dopamine, nucelotides, choline, cholic acid, ethanolamine, pantothenic acid, ascorbic acid and trimethylamine-*N*-oxide (TMAO) were also found to be biomarkers [123].

The same review article also described metabolomics studies on people with CKD and end-stage renal disease (ESRD) [123]. The concentrations in the blood serum of lysoPC(16:0), creatinine, phenylalanine and kynurenine were higher in patients with CKD than in healthy subjects. However, the serum concentrations of LPC(16:0), LPC(18:1) and tryptophan were lower in CKD. LC-MS was also used to analyze the plasma of patients with ESRD before and after hemodialysis. Initially, the

concentrations of polar metabolites increased, while some lipids decreased. Dicarboxylic acids (adipate, malonate, methylmalonate, and maleate), biogenic amines, derivatives of nucleotides, phenols, and sphingomyelins were identified as potential biomarkers. There was an increase in relatively low molecular weight triacylglycerols (TAGs) and a decrease in intermediate sized TAGs. In addition, 1-methylinosine was found to be a possible biomarker for estimating the best frequency of hemodialysis [123].

The concentrations of lipids in the blood serum of patients suffering from chronic glomerulonephritis and CKD were also determined in an excellent study [124]. One of the things that made this article so informative and believable is the detailed information provided about the sample preparation. That is, 250 µl of water was added to 210 µl of blood plasma, followed by 1.5 ml CH_3OH with 0.01% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 3 ml $CHCl_3$ and sonication before and after adding the $CHCl_3$. This makes sense, because lipids do dissolve readily in $CHCl_3$ (but not water or a mixture of CH_3OH and water). Internal standards of lysoPC, PC, PE and PS were added, followed by thorough mixing. Finally, another 1.5 mL of water was added, followed by mixing and centrifugation to make two separate phases form. The lower $CHCl_3$ phase was collected, dried to evaporation, redissoved in 250 µl $CHCl_3/CH_3OH$ (2:1, v/v) and diluted with 2.5 ml of hexane/1-prpopanol (3:2, v/v). This was injected on a diol column that was connected to a QTRAP LC/MS/MS. The lipids were separated using a gradient elution made from two solvent mixtures. Solvent A was hexane/1-propanol/formic acid/ammonia solution (79/20/0.6/0.06, v/v/v/v), solvent mixture B was 1-propanol/water/formic acid/ammonia solution (88/10/0.6/0.06, v/v/v/v). The "ammonia solution" was probably aqueous NH_4OH, so the mobile phase probably contained a mixture of formic acid and ammonium formate. This buffer would control the pH of the mobile phase. Nineteen different phospholipids (PIs and PSs) were found to be potential biomarkers [124].

Dyslipidemia occurs often in CKD and can lead to cardiovascular diseases [125]. TAGs increased in blood plasma and the membranes of red blood cells (erythrocytes) of patients who had been on dialysis for 30 months. There were also differences in the fatty acyl content (higher palmitoyl and monounsaturated fatty acyls, with decreased polyunsaturated fatty acyls). Several phospholipids were identified as possible biomarkers. This could have been due in part to increased hydrolysis of PIs to produce the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol [125].

A recent study used LC coupled to a HRMS that could measure masses with an error of <2 ppm [96, 97]. The authors used the term accurate mass instead of high resolution or HRMS, since they are synonymous. Lipids were extracted from low density lipoproteins (LDL) from blood plasma samples taken from 10 patients with normal (healthy) levels of cholesterol, but who had stage 4 or 5 CKD and 10 control subjects. The extraction was done by adding 160 µl of ice cold CH_3OH to 46 µl of serum (containing 25 µg of protein), followed by 320 µl of $CHCl_3$, incubation for 20 min and mixing. Subsequently, 150 µl water was added, followed by another 10 min incubation with mixing. Then, the mixture was centrifuged and the upper (aqueous) phase was removed and re-extracted by adding another 250 µl of ice cold $CHCl_3$:CH_3OH (2:1, v/v) and centrifugation. The upper phase was discarded and the two lower phases (containing $CHCl_3$) were combined and dried under nitrogen. The residue remaining was solubilized in 100 µl $CHCl_3$:CH_3OH (1:1, v/v), diluted further with CH_3OH and injected on a silica column used in the hydrophilic interaction chromatography (HILIC) mode. This was combined with gradient elution using a mixture of isopropyl alcohol (IPA) and acetonitrile with 20 mM ammonium formate to separate 352 different lipids. An Exactive MS (ThermoFisher, Hemel Hempstead, UK) equipped with electrospray and polarity switching was used for detection. The concentrations of total lipids and cholesterol were not changed significantly. The concentrations of TAGs and *N*–acyltaurines increased significantly increased, while PCs, plasmenyl ethanolamines, sulfatides, ceramides, and cholesterol sulfate decreased [97].

There was another article that provided important details on sample preparation before doing a combination of metabolomics and epidemiological analysis [126]. Blood plasma samples were taken from 1434 participants in the Framingham Heart Study (FHS) who did not have CKD when the study started [126]. The FHS is an example of how both reductionist and systems thinking were used and continue to be used in medicine [2, 83]. That is, the reductionist thinking required for quantitative analysis was applied to people living in Framingham, Massachusetts without any preconceived theories (using systems thinking) to look for possible risk factors for cardiovascular diseases, cancer and other illnesses [2, 83]. During the eight-year study on CKD, 123 of the subjects developed the disease [126]. Nine metabolites were found that decreased more than creatinine in those patients. A total of sixteen metabolites were significantly associated with CKD in this FHS. They were xanthosine, citrulline, isocitrate, aconitate, choline, kynurenine, β-aminoisobutric acid, kynurenic acid, TMAO, adenosine, 5-hydroxyindole

acetic acid, quinolinic acid, sucrose, inositol, LysoPC(18:2) and LysoPC(18:1). Note that LysoPC is also abbreviated as LPC. To extract and analyze serum for organic acids, sugars, bile acids and other negatively charged polarmetabolites, 30 µL of plasma were extracted with 120 µl of methanol/water (80:20, v/v) containing an internal standard. After centrifugation, supernatants were injected on an NH2 column and detected using a QTRAP triple quadrupole MS. For lipid analysis, 10 µl of plasma was 190 µl of isopropanol, containing a PC internal standard, followed by centrifugation, injection on a C4 column and detection with the QTRAP. The article concluded by stating that a combination of metabolomics and epidemiology was quite useful in identifying biomarkers [126].

A subsequent article from a different group used both LC-MS and GC-MS in their metabolomics study of CKD [127]. Fortunately, they did provide details of their sample preparation. For GC-MS analysis, they did not try to hydrolyze any of the lipids, but instead formed volatile derivatives of compounds containing hydroxyl groups by methoxamination. For LC-MS analysis, they added 200 µl of ice cold CH_3OH (-20 ^oC) to 50 µl of blood plasma, followed by centrifugation to precipitate out proteins. So, no lipids were solubilized in this process. The supernatant was dried, then re-dissolved in 100 µl water/acetonitrile (95/5, v/v) and analyzed by LC-MS. GC-MS identified gluconic acid, galacturonic acid, lactose, maltose and trihydroxypentaenoic acid as potential biomarkers. LC-MS found many others, including several free fatty acids, such as C22:4 and C22:5. Interestingly, was nothing was mentioned about TMAO [127].

The composition of the intestinal Bacteria changes during CKD [128]. Trimethylamine (TMA) is produced by some of these Bacteria as they metabolize choline, phosphatidylcholine (PC, also known as lecithin) and/or *L*-carnitine [128, 129]. TMA is transported to the liver, where it is converted to TMAO. Dietary sources of choline, PC and *L*-carnitine include lecithin dietary supplements, red meat, liver, egg yolk and dairy products containing relatively high fat content. TMAO is both a renal toxin and a biomarker for CKD. It is also a risk factor in CKD mortality and can also lead to cardiovascular disease [128, 129].

On the other hand, PC is an essential component of human cell membranes and an important dietary source of choline, a precursor of the neurotransmitter, acetylcholine. Its common name is lecithin. PC and choline are important for healthy brains, hearts, skeletal muscles, livers and overall metabolism. For example, Alzheimer's disease patients have lower concentrations of three different PCs in their blood serum than healthy control subjects. A lack of sufficient dietary choline can cause symptoms of subclinical organ dysfunction (fatty liver or muscle damage).

The need for choline is especially high during pregnancy and lactation. If there is insufficient choline stored in the body, the ability to methylate homocysteine to make methionine decreases, so plasma concentrations of homocysteine increase. This can increase the risk for cardiovascular disease, cancer, cognitive decline and bone fractures. So, PC and choline are like many things in life. They must be present at sufficient concentrations to support life, but not be too high to become toxic. Moreover, 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. Still, people who don't eat meat or egg yolks may want to take such supplements. On the other hand, another supplement called citicoline (cytidine diphosphocholine, CDP-choline) may be a better option since it is not metabolized into TMAO and may help brain function as it provides necessary choline.

Others used LC-MS to find that N-α-acetyl-L-arginine, L-kynurenine, N^4-acetylcytidine, N^2,N^2-dimethylguanosine, phenylacetylglutamine, hippuric acid, indoxyl sulfate, N^6-carbamoylthreonyladenosine and an unknown metabolite that produced a positive ion with an *m/z* of 366.1433 were potential biomarkers of CKD [130]. For sample preparation, 450 µl of methanol containing an internal standard was mixed with 50 µl of blood plasma, so lipids were not solubilized [130]. In another article, the same group reported preparing blood serum for LC-MS analysis by mixing 30 µl of plasma were mixed with 270 µl of 90 % CH_3OH. In addition to other previously identified toxins, supposedly the concentration of LysoPE (20:4) was different in the plasma of rats that were in the early stages of CKD [131]. However, LysoPE, like other lipids, has very low solubility in CH_3OH:water (9/1, v/v). Its apparent solubility could be increased by the presence of a variety of natural detergents, including other lipids. So, it is possible that the differences that the authors found in LysoPE (20:4) were due to differences in the amounts of it that were solubilized in the extraction solvent.

A different group did a combined metabolomics and proteomics analysis of urine and a metabolomics analysis of blood plasma from patients with CKD [132]. They did a targeted metabolomics analysis by PITC (phenylisothiocyanate)-derivatization in the presence of isotopically labelled internal standards. They also reported measuring the concentrations of free and total fatty acids as their corresponding methyl ester derivatives (FAMEs) using GC-MS. They did not provide the details on how they hydrolyzed the fatty acyls from the lipids to which they were attached. However, they had to hydrolyze the lipids before the FAMEs

could be formed. They identified 17 plasma metabolites and 17 in urine. Only asymmetric dimethylarginine (ADMA) was found in both plasma and urine. The concentrations of ADMA and some acylcarnitines were higher in patients with advanced CKD, compared to those with only mild CKD. On the other hand, ADMA concentrations were lower in the urine of patients in the later stages of CKD [132].

In a metabolomics study of urine in patients with advanced-stage CKD, a combination of NMR and LC-MS with selected reaction monitoring (SRM) was used [133]. The concentrations of the following metabolites were significantly different between patients with CKD and healthy control subjects: 5-oxoproline, glutamate, guanidoacetate, α-phenylacetylglutamine, taurine, citrate, and TMAO. For LC-MS analysis, urine samples were prepared by adding 50% acetonitrile, which precipitated out the proteins. After recovering the supernatant, analysis was done on an LC coupled to a triple quad MS (LC-(QQQ)-MS) [133]. For NMR analysis, proteins did not have to be removed since they produced very small signals or no detectable signals, due to them being present at relatively low concentrations and each protein having many different types of protons. In contrast TMAO, which has only one type of hydrogen (three identical $–CH_3$ groups), produced only one signal.

1.12.7 Metabolomics of acute kidney injury

Some types of kidney dysfunction are due to acute injuries or damage [117]. Acute kidney injury (AKI) is the term that is used for this. It replaced the term acute renal failure. About two million people die every year from AKI. It can emerge due to inflammation, exposure to environmental toxins, ischemia or obstruction of the urinary tract. Despite advances in therapy, the rates of morbidity and mortality continue to increase. So, many efforts are being made to find biomarkers that can be used for early diagnosis and to help evaluate treatments and prognosis. Even though serum creatinine is easily measured, its concentration can vary due to many factors other than just AKI or CKD. Still, it can be used together with determinations of the concentration of cystatin C in serum and urine as markers of kidney function. In addition, the following serum proteins are upregulated in AKI: kidney injury molecule-1, IL-18, neutrophil gelatinase-associated lipocalin, liver fatty acid-binding protein [117].

To identify and quantify the smaller metabolites in urine as well as blood plasma and serum by LC-MS, the proteins must be removed [117]. This can be done by adding CH_3OH, ethanol, acetonitrile, acetone or

mixtures of these. These solvents denature proteins, causing them to form precipitates that are easily removed by centrifugation and filtration through a 0.45 μm membrane. The filtrate is often diluted into the mobile phase that is used in the LC-MS analysis. To maximize the separation of metabolites (chromatographic peak resolution), silica, C18, bridged ethylsiloxane silica hybrid (BEH) phenyl adsorbent, and/or HILIC columns packed with small (< 2 μm) particles can be used in UPLC. The following compounds have been identified as possible biomarkers in urine for AKI: 2,8-dihydroxyadenine, 2-hetoglutarate, 3-hydroxybutyrate; 5-methyltetrahydrofuran, 3-*O*-methyldihydroxypheylalanine, adenosine, adrenaline, adrenosterone, alanine, allantoin, ATP, citrate, creatine, creatinine, deoxycholic acid, dihydrosphingosine, dimethylamine, dopamine, ethyl-N_2-acetyl-L-argininate, hippurate, hippuric acid, homocysteine, hypoxanthine, isocitric acid, kynurenic acid, lactae, methionine, N-acetylleucine, phenylacetylglycine, phenylalanine, phytosphingosine, proline, puccinate and tryptophan. The following compounds have been identified as possible biomarkers in blood plasma for AKI: 1-methylinosine, 2-deoxyadenosine, 4-ethylphenyl sulfate, adenine, adenosine, adipate, alanine, ceramides, cholic acid, creatinine, dihydrosphingosine, hippurate, hippuric acid, homocysteine, hypoxanthine, indoxyl sulfate, isocitrate, kynurenic acid, kynurenine, L-acetylcarnitine, lactate, leucine, LysoPC, maleate, malonate, methylmalonate, myoinositol, pantothenate, *p*-cresol sulfate, phenyl sulfate, phenylalanine, phytosphingosine, puccinate, taurine, thymidine, tryptophan, xanthosine and xanthurenic acid [117].

People who have cardiopulmonary bypass surgery can be susceptible to AKI [131]. The concentrations of creatinine and the following proteins were determined before surgery, post-operatively and at 24 h after surgery in 93 high risk patients who underwent this surgery: urinary α and π glutathione *S*-transferases (α-GST and π-GST), urinary L-type fatty acid-binding protein (L-FABP), urinary neutrophil gelatinase-associated lipocalin (NGAL), urinary hepcidin and serum cystatin c (CysC). AKI was best predicted by the presence of π-GST, as well as a lower ratio of hepcidin:creatinine after 24 hr, and increased ratios of urinary NGAL:creatinine post-op and serum cystatin C. However, there was most likely a small typographical error in the abstract, which said that it was the hepcidin:creatine ratio and not hepcidin:creatinine ratio that decreased. Throughout the rest of the article, the abbreviation Cr was used, without defining it. However, in the materials and methods section, the method used to quantify urinary and serum creatinine was described, while creatine was not mentioned [131]. Even though creatinine and creatine are

spelled almost the same, they have very different structures (Figure 2) and different biochemical properties [83].

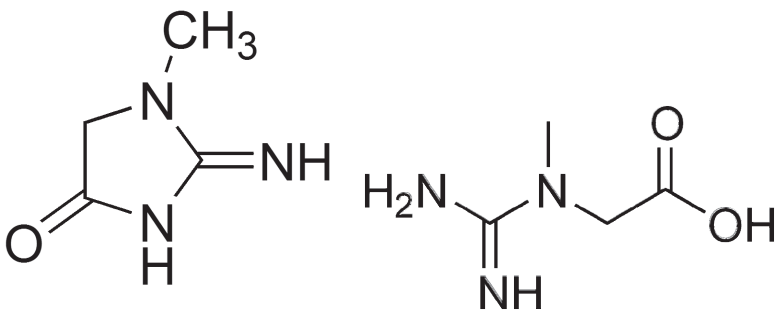

Figure 2. Structures of creatinine (left) and creatine (right).

1.12.8 Metabolomics of neonatal, pediatric and childhood kidney diseases

Kidney disease occurs in children, as well as adults. By the time most healthy neonates reach full term (between 37 and 41 weeks of gestation), they have all the nephrons that they need [132]. However, if their mothers take relatively high doses of steroids such as dexamethasone when they are pregnant fetal nephrogenesis can be impaired. This can lead to fewer nephrons being produced. Similarly, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor antagonists can reduce nephron formation. This is because the RAS (or RAAS) is needed for fetal nephron development. Moreover, maternal use of non-steroid anti-inflammatory drugs (NSAIDs) can lead to undesirable changes in the kidney tubules and fewer nephrons in the neonate [132].

To complicate this, only a few of the drugs that are used in neonatal intensive care units are specifically authorized for neonates [132]. When unlicensed or off-label use is prescribed, it can lead to adverse drug reactions. Moreover, kidney damage often happens in preterm babies. It is a predisposing factor for developing CKD in adulthood.

1.12.9 Metabolomics of kidney cancer

A new genomics-based analysis of almost 900 kidney cancer cases found that there was much molecular diversity within each major histological type that pathologists had previously used to categorize them

[134]. Instead of just three major types of kidney cancer (based on microscopic morphology), nine major subtypes were described. Each subtype had its own distinct alterations in genetics, epigenetics and signaling pathways, as well as patient prognosis. This included changes in gene copy number, DNA hypermethylation and several histone acetyltransferases, as well as the phosphatidylinositol 3-kinase (PI3K), Nrf2-ARE antioxidant and immune checkpoint pathways [134]. The Nrf2-ARE antioxidant pathway is an important antioxidant and anti-cancer mechanism that can be activated by specific dietary phenolic compounds such as (–)-epigallocatechin gallate (EGCG), which is especially abundant in green tea [135, 136]. Dividing kidney cancers into separate subtypes will help identify the most appropriate patients for clinical trials of new molecular entities. It will also help analytical chemists identify biomarkers from metabolomics studies on specific subtypes of kidney cancer. Phosphatidylinositol (18:0/20:4) had previously been identified as a biomarker of CKD [136]. So, it could turn out to be a biomarker for one or more subtypes of kidney cancer. In addition, cardiomyopathy caused by diabetes may be preventable if the Nrf2/ARE system is activated [137].

It should be noted that the Nrf2/ARE pathway is like much of the rest of life. It must be kept in balance. EGCG and other bioactive, natural compounds can activate this pathway and produce important health effects. However, it can lead to many problems when over-activated, including multi-drug resistant cancer [137, 138]. That is, constitutive activation of the Nrf2/ARE pathway can increase insulin resistance, impair lipid accumulation in adipose tissue, and increase hepatic steatosis [137]. Also, Nrf2 deficiency improved glucose tolerance in mice fed a high-fat diet and prevented reductive stress-induced hypertrophic cardiomyopathy [137].

1.12.10 Proteomics

Even though proteomics is often considered to be separate from metabolomics, it is not. As discussed in Chapter 1 of volume 1, proteins, like much of the rest of the human body, are being continuously broken down and remade in autopoiesis. So, the proteome is dynamic, not static and proteins are metabolites [139]. Metabolomics should be more than just accumulating many facts that focus on purely structural and stoichiometric properties of metabolic networks. Even though metabolomics analyses provide stoichiometric models that enable the analysis of the modes of flux, they need to be complemented with information on enzyme kinetics and regulation of metabolism by proteins [139]. In fact, proteomics has

been done longer than genomics [140]. Since 2010, proteomics has gained importance and attracted international attention with the launch of the Human Proteome Project. This has led to an interdisciplinary effort and the coining of two new terms: environtome and social proteome. The environtome 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" [140]. The social proteome is "the subset of the environtome that influences the transition of proteomics technology to innovative applications in society" [140]. The social proteome includes new ways to reimburse physicians and testing labs as well as business innovation models for proteomics diagnostics that depart from the once-a-life-time genetic analyses that have become quite popular. Scientists used the nesting principle for governance of complex systems that was described by Elinor Ostrom, a Nobel Prize winner in Economic Sciences in 2009 [140, 141] to propose here a three-tiered organizational architecture for Big Data science, including proteomics. The proposed nested governance structure contains scientists, ethicists, and scholars in the field of ''ethics-of-ethics''. Its goal is to cultivate a robust social proteome for personalized medicine. Nested governance designs like this ensure that political power in innovation processes is distributed almost evenly and is not concentrated disproportionately in a single overbearing stakeholder or person. Moreover, social and biological proteomes interact synergistically to help realize the full potential of proteomics for personalized medicine in psychiatry in current age of Big Data [140].

It is much more convenient to collect urine than blood from people. So, human urine is a convenient and rich source of biomarkers for proteome analysis [142]. The proper methods for collecting and preparing urine for proteomic analysis have been described [142]. At first, capillary electrophoresis (CE) [143] and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were coupled with MS (CE-MS) to separate proteins based on differences in their molecular sizes and isoelectric points [144]. The CE-MS analysis led to the development of a urinary biomarker model that is specific for CKD [143]. That is, CE-MS was used to analyze urine from 230 patients with CKD and 379 healthy control subjects. This identified 634 peptides that differed significantly between these two cohorts, 273 of which were sequenced. They were combined using support vector machines to produce the 273CKD classifier [143].

In 2D-PAGE, proteins were identified as spots on the gel [143]. These spots were cut from the gels and the proteins were analyzed by MALDI-

TOF-MS. However, 2D-PAGE was not able to separate many proteins that were bound to the cell membrane and MALDI-TOF-MS was ineffective for proteins that were only present at very low abundance. So, nanoscale reversed phase liquid chromatography coupled with nanoelectrospray ionization (nanoESI) emerged as a superior alternative. The combination increased the number of proteins that could be resolved, identified and quantitated in the urine and kidneys. Moreover, reliable protein search algorithms were developed that improved protein identification. Subsequent advances in MS using ion traps (especially Orbitraps) dramatically increased the ability to detect proteins in tissue and biological fluids [143].

So, LC-MS has been very useful in identifying proteins in the urine that are linked to kidney diseases [142]. Proteins were separated on gels, digested (hydrolyzed) and then analyzed by linear ion trap (LTQ)-Fourier transform ion cyclotron resonance trap LTQ-FTICR. A total of 1543 urinary proteins were identified by combining 1281 proteins detected by LTQ-FTICR with 1055 proteins from LTQ-Orbitrap. Peptides produced from hydrolyzed proteins from healthy volunteers by multidimensional LC to identify 1310 of the intact proteins in their urine. Phosphorylated proteins were also profiled in urine. In another study, a LTQ-Orbitrap Velos mass spectrometer was used to find 1452 proteins in unfractionated urine and 617 proteins in glycoproteome [142].

Urine has also been analyzed for exosomes, which are vesicles that originate in multivesicular bodies from every renal epithelial cell type facing the urinary space [145, 146]. They can be isolated from urine by differential centrifugation as the low-density fraction. When analyzed by LC-MS/MS using an LTQ equipped with a nanoelectrospray ion source, 1412 proteins including 14 phosphoproteins were found. The raw data files were searched to try to find amino acid sequences in the NCBI Reference Sequences (RefSeq) human protein database using BIOWORKS software. Phosphopeptides were enriched from the samples using an isolation kit. Fetuin-A and activating transcription factor 3 were found to be biomarkers for AKI. Aquaporin-1 was a biomarker for renal ischemia-reperfusion injury. A scaffolding protein, CD2AP, and microRNA-29c were biomarkers for renal fibrosis. Wilms tumor 1 protein was for patients with focal segmental glomerulosclerosis. Aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin were used to distinguish between IgA nephropathy versus thin basement membrane nephropathy [145, 146].

All this work on the urinary proteome has produced enough data to inspire the development of open-access databases [147]. This includes the

Urinary Protein Biomarker Database (http://122.70.220.102/biomarker/), the HKUPP database (http://www.hkupp.org/), the Urinary exosomes protein database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/index.htm), the MAPU urine dataset (http://www.mapuproteome.com), the Clinical urine proteomic database (http://alexkentsis.net/urineproteomics/) and two urinary peptide biomarker databases, (http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257 and http://122.70.220.102/biomarker/) [147, 148].

However, LC-MS can't do everything. It is not very useful in analyzing urine, renal tissue or any other biological sample for microRNA, more popularly known as miRNA (see Chapter 1, volume 1). That is, it was once thought that DNA can only code for messenger RNA (mRNA), which codes for proteins. We now know that miRNA and other types of RNA are transcribed from DNA and that miRNAs play important roles in regulating the expression of genes into proteins as well as in kidney development, homeostasis and disease [149]. So, much research is also being done on developing methods for discovering miRNAs that can serve as biomarkers for many types of kidney diseases and may even be useful therapeutic targets [143, 149].

1.13 Targeted radiation therapy

Not only prescription drugs, but also radiotherapy is now being targeted precisely [150]. Targeted radionuclide therapy (TRNT) combines a biochemical or nanobody that specifically defines the target and a radionuclide that delivers a cytotoxic payload to kill cancer cells. Its goal is to deliver cytotoxic radiation to cancer cells and cause little or no toxicity to surrounding healthy tissues. Currently, some radionuclides accumulate naturally in tumors. Examples include iodine-131 (^{131}I) for thyroid cancer and strontium-89 (^{89}Sr) and radium-223 (^{223}Ra) for bone metastases. ^{131}I and ^{89}Sr emit β^- particles (electrons), while ^{223}Ra emits alpha particles (α-particles). There are also radionuclides that are conjugated with a peptide that targets tumors. This includes yttrium-90 (^{90}Y) and lutetium-177 (^{177}Lu), each of which can be bound to an octapeptide (octreotide) [150, 151] that is an analogue of somatostatin and binds to two of its receptors (SSTR2 and SSTR5) with high affinity [152]. It is used to treat advanced neuroendocrine tumors [152]. Radiopharmaceuticals are also used as diagnostics and for non-invasive imaging (positron emission tomography, PET) [150]. When radiopharmaceuticals are used for both diagnosis and therapy, they can be called theranostic agents. Even though β^- particles are useful in treating bulky and heterogeneous tumors, α-

particles (which are a helium nucleus that contains two protons and two neutrons) are preferred for treating smaller tumors and small clusters of cancer cells. Targeted alpha therapy (TAT) can deliver highly localized, toxic radiation at a short range (50-100 μm) with a minimum of damage to healthy cells that surround the target, due to the high level of radiation produced and the short lifetime of the α particles. The α-particles can be produced by astatine-211, actinium-225 and bismuth-213, as well as ^{223}Ra. The radionuclides are attached to an antibody that acts as a vehicle to target over-expressed antigens on cancer cells. Examples include epidermal growth factor receptor variant III, human epidermal growth factor receptor 2 (HER2), folate receptor alpha, tenascin-C, CD20, CD33, and prostate-specific membrane antigen. The antibody is often a monoclonal antibody (mAb). However, engineering of mAbs has improved enough so that antibody fragments can be made. They have shorter half-lives in blood serum, so they have much lower liver and bone marrow toxicities. In addition, ligands (like folate), synthetic protein scaffolds (affibodies) and substrate analogs (peptides) can be used as the targeting agent to deliver the toxic radionuclide specifically to the tumor. Research is also being done to use nanobodies to target cancer cells. If successful, they will reduce off-target toxicity and kill just the cancer cells. It is expected that the efficient targeting capacity and fast clearance of nanobodies will make TAT quite effective. In addition, the pharmacokinetic properties of nanobodies are an excellent match with the decay properties of the short-lived α-particle emitting radionuclides astatine-211 and bismuth-213. So, they could become very useful in treating micrometastatic cancers and residual diseases [150].

Another approach to targeted radiation therapy is to use protons instead of high energy X–rays to kill cancer cells and destroy tumors [153]. Protons can be used at higher doses while causing less damage to healthy cells that surround the tumor. That is, they deposit increasing energy at increasing penetration distances that leads to a maximum (the Bragg peak) near the end of the range of the proton beam. That is, if one plots the stopping power of a source of radiation vs the path length, the maximum stopping power is called the Bragg peak. In front of the Bragg peak, the dose of radiation is lower than what is produced by X-rays. Beyond the Bragg peak the dose falls to almost zero. The depth of the Bragg peak can be adjusted by choosing proton beam energies that are appropriate for the depth and volume of the targeted tumor. Hence, much better precision can be achieved compared to conventional radiotherapy. The result is much less toxicity and much faster recovery time. So, proton beam therapy (PBT) has been used to treat pediatric intracranial tumors, ocular tumors,

chordomas and chondrosarcomas in the head and neck, prostate cancer, NSCLS and liver cancer [153-156]. PBT is also known as proton beam radiation, proton radiation and proton teletherapy [156]. PBT was first approved by the US FDA in 1988, but its use has not yet been accepted by everyone in the oncology community. It has been criticized because of the relative lack of clinical trials of effectiveness and its relatively high cost. However, recent advances in PBT therapy and effective advertisements in the media have led to a large increase in its use. That is, there were only two PBT centers in 2003. There were 22 PBT centers in the USA by 2016, with more being planned or already under construction. This is making it easier to conduct clinical trials. So, in 2016, a total of 122 clinical trials were being conducted with 42 052 subjects being enrolled. The most common tumors being investigated in these trials were in the CNS, GI tract, prostate gland and lungs. Placebos are not appropriate in such studies. Instead, PBT and photon therapies (including X-rays) are being compared [156].

1.14 Precision systems medicine that targets cancer stem cells

Precision systems medicine is also being used to target cancer stem cells [157]. This is due, in part, to the realization that cancers are systems-level, network diseases. Cancer cells emerge through a complex set of steps, starting with mutations or rearrangements in DNA that destabilize the networks in the formerly healthy cells. The transformation of malignant tumors occurs in two phases in which an initial increase of network plasticity (flexibility or ability to change) is followed by a decrease of plasticity at late stages of tumor development. The constantly changing intensities of stress factors (hypoxia, inflammation and the either cooperative or hostile interactions of tumor inter-cellular networks) increase the adaptability of cancer cells. At first, nodes in the cellular network become disordered, which disrupts the network, which then causes more nodes to become disordered in a vicious cycle. In contrast, there is a decrease in network plasticity in later stages of carcinogenesis. These changes are due to cancer attractors in the network. Cancer cells must first cross a barrier in the epigenetic landscape that can be lowered by mutations and/or epigenetic changes. This leads to destabilization and a more plastic phenotype. This is followed by cancer cells becoming stabilized while the cancer attractor causes a more rigid, inflexible phenotype. This can lead to the bypass of cellular senescence and to the emergence of cancer stem cells. This initial decrease and subsequent

increase in plasticity does not occur uniformly. Tumors have both early phase (plastic) and late phase (inflexible) cells at the same time. However, it appears that it is difficult or even impossible to define exactly what cancer stem cells are. That is, the properties of cancer stem cells depend on the particular history of stressors that have impinged on a given tumor. Still, cancer stem cells can evolve and produce many inheritable phenotypes [157]. This is consistent with the defining characteristics of cancer stem cells: "the possession of the capacity to self-renew and to repeatedly re-build the heterogeneous lineages of cancer cells that comprise a tumor in new environments" [157]. So, cancer stem cells can adapt to changing environments. Moreover, cancer stem cells can evolve easily due to repeated transitions between plastic (proliferative and dividing symmetrically) and rigid (quiescent, dividing asymmetrically and often more invasive) phenotypes that have plastic and rigid networks, respectively. Thus, cancer stem cells reverse and replay their development many times [157].

So, network models have emerged that try to explain the behavior of cancer stem cells [157]. They are based, in part, on the analyses of gene expression in about 150 different human stem cell lines [157, 158]. Transcriptomes of pluripotent stem cells formed a tight cluster in the cellular network, while those of other stem cells were much more diverse [157]. Pluripotent stem cells shared a common sub-interactome that was enriched in several gene-products, including proteins related to tumorigenesis. Moreover, stem cells contain signaling systems (analogous to circuits) that can alternate between off and on in a way that is regulated in both directions. Many complex model and real world networks undergo abrupt and extensive changes in their topology when they respond to a shortage of resources needed to maintain inter-nodal connections and/or upon environmental stress. These changes are called network topological phase transitions [157]. They cause a large re-organization of network structure, dynamics and function [157, 159]. Since there is much variety in the resources and stressors in tumors and they are magnified further by anti-cancer therapies, the networks of cancer cells respond to this environmental variability with frequent topological phase transitions [157]. In the first stage, dynamic, creative nodes can disrupt existing nodes and edges in the cellular network. In the second phase, some nodes seed rigidity and establish a rigid cluster. Some nodes even promote more rigidity, which leads to a phase transition. Creative nodes (like chaperones and prions) become less dynamic as the core size decreases. A compact core network, separated modules and decreasing water content help accelerate the process of increasing rigidity. High mobility and

invasiveness probably require a more rigid network structure, especially in the cytoskeleton. This allows invasive cancer cells to remain intact when they are exposed to the physical forces needed for migration and metastasis [157].

In addition, cancer stem cells seem to obey the statement made by the German philosopher, Friedrich Nietzsche [157]. That is, "what does not kill us makes us stronger" [160]. That is, conventional chemotherapies can make cancer stem cells stronger [157]. However, some popular anticancer drugs, including metformin, inhibit the mechanisms by which cancer stem cells override cellular senescence. More recently, a new strategy that includes a central hit and network influence are being developed. Central hits attack the network integrity of rapidly proliferating cancer cells selectively. This strategy is most useful when attacking plastic networks. Drugs that influence the cellular network shift the malfunctioning network of a more differentiated cancer cell back to its normal, healthy state. This strategy is most useful when attacking rigid networks. Since cancer stem cells cycle between plastic and rigid states, conventional anticancer drugs that target plastic networks only shift the cycle of cancer stem cells towards the rigid state. So, a multi-target therapy is needed to attack cancer stem cells. Their plastic state can be attacked with central hit drug and their rigid state with a drug that influences the network at the same time. However, efficient targeting of rigid networks (consisting of quiescent cancer stem cells) often requires an indirect approach, in which neighbors of the original therapeutic target are attacked by allo-network drugs [157].

1.15 Cancer prevention

Still, it would be much better to prevent cancer from starting in the first place. An important part of preventing cancer is avoiding toxins and behaviors that can help cause cancer. So, obesity, smoldering inflammation, smoking and exposure to a variety of environmental toxins (including UV rays from the sun) should be avoided or at least minimized. As described in Chapter 2, volume 1, maintaining a healthy gut microbiome by consuming plenty of dietary fiber is also important. At the same time screening and early detection can help prevent many forms of cancer [161]. In addition, several natural bioactive compounds in foods, spices and other botanicals may also help prevent cancer [161-167]. As we will see in Chapter 2 of this volume, this is due in part to the presence of dietary antioxidants that activate the body's natural antioxidant response elements. However, as we will see in Chapter 3, a reductionist approach

should be avoided. That is, the whole diet must be considered and not just individual components. It is also important to consider how foods and spices are prepared and consumed. For example, cinnamon is widely recognized for its health benefits. However, if it is consumed as part of a sweet role or other pastry that is loaded with sucrose (table sugar) as part of the typical American fast food diet, it loses its health benefits. Still, the best-studied compounds that might help to prevent cancer are tamoxifen, aspirin, and NSAIDs [161]. Early studies showed that tamoxifen, a selective estrogen receptor modulator (SERM), reduces the risk of ER-positive breast cancer by as much as 50%. This effect continues long after anticancer therapy ends. In addition, the second-generation SERM raloxifene was found to produce similar preventive effects as tamoxifen in postmenopausal women, but without an increase in the risk of uterine cancer that tamoxifen can cause. These studies led the US FDA to approve raloxifene as an alternative treatment to tamoxifen for reducing the risk of breast cancer women who are at high risk. However, treatment with raloxifene can cause hot flashes and thromboembolic events in some women. In addition, its preventive effects wear off after about three years, after which it retains only about 75% of the effectiveness of tamoxifen for preventing breast cancers. Since there appears to be a trade-off between side effects and effectiveness over the long term, the selection of tamoxifen vs raloxifene as a preventive therapy depends on the characteristics of the patient. Since raloxifene is less likely to cause uterine cancer than tamoxifen, it may be best for postmenopausal women at high risk of breast cancer who still have an intact uterus. However, in postmenopausal women who no longer have a uterus, tamoxifen may be the drug of choice because it is more effective over the long term [161].

In addition, aspirin can reduce the overall incidence of cancer (especially colorectal cancer) and mortality in the general population [161]. However, aspirin can cause excessive internal bleeding that can lead to hospitalization and even death. Moreover, aspirin and other NSAIDs should not be taken by people who have chronic kidney disease. So, it is important for patients and physicians to communicate well with each other, just as customers and manufacturers communicate in TQM.

Since as much as 70% of all cancers may be linked to diet, it is important to learn what types of diets and dietary compounds can prevent cancer [162]. For example, epidemiological data show that most Asian populations have a much lower incidence of colorectal cancer than people in much of the Western world. That is, the incidence of colorectal cancer in India and the United Kingdom (UK) are 4 and 31 per 100 000 people, respectively. This is due, in part, to the higher consumption of curcumin in

India than in the UK. It not only helps to prevent colorectal cancer, but also other kinds of cancer as well as cardiovascular and neurodegenerative diseases, while lowering or preventing smoldering inflammation and helping wounds heal. One way it does this is to target cancer stem cells. It can also exhibit synergistic effects when combined with other dietary compounds (such as piperine in black pepper) and even some anticancer drugs [162]. Many people in India also consume *Withania somnifera* roots (Ashwagandha, Indian winter cherry, Indian ginseng), which has been a part of Ayurvedic medicine for thousands of years [165]. It is used to enhance one's energy and improve performance when exercising. It has important anti-cancer properties (such as inducing apoptosis in cancer cells and preventing angiogenesis) and may even be able to help prevent it [165]. Vegetarians in India also eat no red meat, which can cause cancer. Instead, they eat more fruits and vegetables, which have flavonoids (like quercetin in onions), isoflavonoids (like genstein in soybeans) and neoflavonoids (like calophyllolide in *Calophyllum inophyllum* seeds). They can help to prevent cancer [162]. More important, quercetin can interact synergistically with sulforaphane to prevent the growth of pancreatic cancer cells, due to their ability to attack cancer stem cells. In addition, resveratrol can target pancreatic and glioma cancer stem cells, while preventing smoldering inflammation. Then there is EGCG (which was discussed in Chapter 3, volume 1), which can target many signaling pathways that are important in cancer, thus reducing cancer cell growth, invasion and angiogenesis. There are also bioactive isothiocyanates (especially sulforaphane) in cruciferous vegetables (like broccoli) that lower the incidence of cancer. Its structure is shown in Figure 3. Vitamin A and its precursor, β-carotene, may prevent lung cancer – at least in people who don't smoke or consume tobacco products [162]. Still, it is important to consider how and where foods and beverages that contain these anticancer compounds are prepared and consumed. For example, raw onions have far fewer calories that do deep-fat fried onions. Also, even though red wine is a great source of resveratrol, over-consumption can lead to alcoholism and its attendant adverse consequences. Moreover, there are some societies and religions in which the consumption of alcohol is forbidden. The legal penalties and scorn from one's family and friends would obviate any potential health benefits obtained by drinking red wine. Besides, there are many other bioactive dietary compounds and foods that can exert similar health effects.

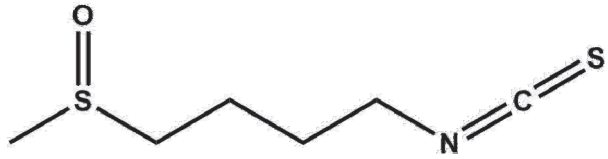

Figure 3. 2D structure of sulforaphane, or 1-Isothiocyanato-4-methylsulfinylbutane.

This includes fisetin, which is found at relatively high concentrations in strawberries [167]. Its structure is shown in Figure 4. It works by affecting several therapeutic targets, including the Nrf2/ARE antioxidant system, as well as the mTOR and NF-κB signaling pathways [167, 168]. It also binds to and disrupts microtubule dynamics and acts as a stabilizing agent with effects that are far superior to the anticancer drug paclitaxel [167]. However, if one adds lots of sugar to one's strawberries and put them into cream before eating them, the health benefits could be obviated – especially if the person is obese. However, remember that the dose is the poison and that we need to listen to the voice of the patient and caregiver. So, if a patient suffers from anorexia, strawberries with lots of cream and sugar could be quite healthy – as long as the patient is willing to eat them.

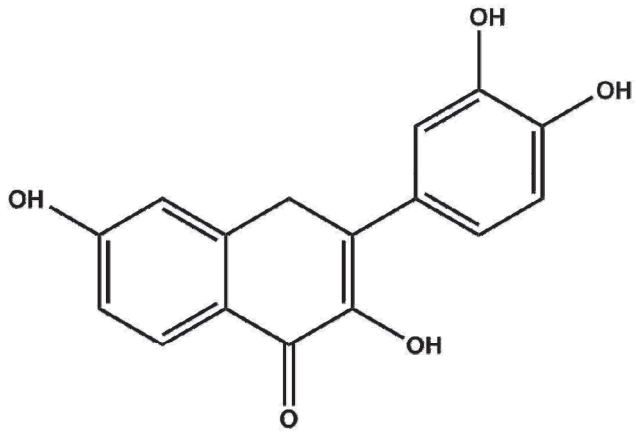

Figure 4. 2D structure of fisetin, or 3,3',4',7-tetrahydroxyflavone.

Cancer may also be prevented and treated by dietary compounds that modulate the immune system [163]. This includes polyunsaturated fats,

dietary fiber, the amino acids arginine, glutamine and tryptophan, as well as vitamins C, D and E. It also includes the minerals zinc and selenium and the botanical, curcumin. The phenolic compounds resveratrol and EGCG are also on the list. Some of these dietary compounds and minerals have many effects. For example, zinc affects mediators of the immune system, as well as enzymes, peptides, and cytokines that affect the proliferation, activation and apoptosis of lymphoid cells. Curcumin in turmeric can modulate the activation of T-cells, B-cells, splenic lymphocytes, cytotoxic T lymphocytes, macrophages, neutrophils, NK cells, and DCs, as well as the secretion of immune cytokines in healthy people. It can also prevent the tumor-induced apoptosis of T-cells and restore progenitor, effector, and circulating T-cells by modulating the JAK/STAT pathway. Polyunsaturated fats, especially DHA and EPA, can also help the immune system. They can act as antioxidants in cell membranes, thus helping to prevent smoldering inflammation that can lead to cancer. They are also precursors in the biosynthesis of resolvins, which also protect against smoldering inflammation. Dietary fiber can also help – not just by encouraging the growth of healthy Bacteria in the gut, but also by producing short chain fatty acids that are anti-inflammatory due to their ability to inhibit TNF-α, IL-8, IL-10 and IL-12 in immune cells [163].

Ginger (*Zingiber officinale*) is an important spice that not only can help to prevent cancer, but also may be able to augment chemotherapy in a multi-targeted, nontoxic approach [166]. It is thought to be an almost universal natural medicine in traditional Chinese and Ayurvedic medicine. The underground stem, or rhizome, has been used for thousands of years to treat many conditions, including nausea. So, many physicians recommend that their patients who are suffering from nausea due to chemotherapy consume ginger. That is, it is antiemetic due to some of its active ingredients being able to act as antagonists for serotonin receptors. It is also anti-inflammatory, as it suppresses the biosynthesis of prostaglandins and leukotrienes. In addition, ginger has many anticancer properties. It can help to prevent skin and colon cancer and may be useful in treating breast, prostate, pancreatic, cervical and ovarian cancer. It is also anti-angiogenic and prevents metastasis. One of its active ingredients, 6-gingerol, inhibits cell proliferation and induces apoptosis in cancer cells. So, many physicians recommend its use in combination with chemotherapy since it acts synergistically with many anticancer drugs [166]. Since it is quite spicy and makes many people feel like their mouth is burning, it is often diluted by consuming it as a component in chewing gum, or by adding it in small quantities to seafood. Still, for people who can tolerate that feeling and enjoy the exotic taste, it can be used as a

natural throat lozenge that will keep one's mouth and throat moist. So, some people who do endurance exercises put a small piece of ginger in their mouth to keep it from becoming too dry as they breathe hard for a long time.

In addition, some researchers are using nanotechnology to deliver natural products and increase their bioavailability [164]. When nanodelivery systems are used to improve chemoprevention, it is called nanochemoprevention [164]. The use of nanostructures to improve the delivery of prescription drugs and to make materials for regenerative medicine was discussed in Chapter 3, volume 1. They are also being used to improve the delivery of bioactive components in natural products [164]. For example, EGCG was encapsulated in polylactic acid-polyvinyl alcohol for sustained release of the EGCG. It has also been encapsulated in gold nanoparticles, bovine serum albumin (BSA) and chitosan. EGCG has also been combined with quercetin in one case and with the phenolic compound herceptin in another. Resveratrol and curcumin have also been encapsulated in nanostructures. A combination of aspirin, curcumin and suforaphene was encapsulated in solid lipid nanoparticles for the prevention of pancreatic cancer. Finally, research is being done on ways to combine natural products with anticancer drugs like cisplatin and 5-fluorouracil to improve their efficacies [164]. So, nanotechnology is finding new uses in delivering natural products and anticancer drugs to prevent and treat cancer.

1.16 Systems medicine 2.0

Systems medicine is also adjusting to changes and improvements in the World Wide Web. The second stage of the World Wide Web emerged from the transition from static web pages to dynamic or user-generated content and the growth of social media. It is called Web 2.0. The term was coined in 2004 as "a set of economic, social, and technology trends that collectively form the basis for the next generation of the Internet, a more mature, distinctive medium characterized by user participation, openness, and network effects" [169, 170]. The main difference between the first generation of the Internet (Web 1.0) and the second (Web 2.0) is interaction [170]. That is, Web 1.0 was mostly unidirectional, while Web 2.0 is bidirectional, in which the user can add information or content to a web page and receive feedback from others who go to the same website or blog. When the technologies of Web 2.0 are used in health care, it becomes Health 2.0 or Medicine 2.0, in which physicians can communicate with their patients, educate them about their conditions and

recommend therapies. Patient participation is also important, so the term patient empowerment 2.0 has emerged [170].

The Web 2.0 has also led to the development of personal learning environments (PLEs) that are centered on students and their needs [171]. At the same time, Web 2.0 social media platforms are promoting dialogs between the research community and the communities they serve [172]. It is not always possible or practical for physicians and patients to interact face-to-face. Social media are producing new channels of communication and providing important support groups for patients and their caregivers. This includes WordPress, which is a "free, open-access blogging tool that allows users to create webpages" [172]. In 2016, about 60 million new posts with 60 million new comments were appearing each month, leading to the creation of about 20 billion pages with about 400 million views. At the same time, Twitter offers microblogs with hundreds of millions of users and Tweets. In addition, the "Center for Clinical and Translational Science's Office for Community Engagement in Research partnered with the Social Media Network at Mayo Clinic to develop and implement a social media communication plan to promote community engagement" [172]. The goal was to support and promote the use of community engagement by researchers. In addition, community representatives increased public support for research on population health. Two social media platforms, Twitter and a WordPress blog, were used to engage researchers and community representatives in online dialogs and include the community in developing educational curricula. Once these platforms became promising, an extensive social media plan with additional applications (eg, Facebook, Storify, and podcasts) was formulated. In addition, more stakeholders (providers, payers, policy makers) are being included. The primary themes of the social media were increasing knowledge about community-engaged research and dissemination of results obtained from the research. Social media platforms spread the news about research results. They also raise the awareness of scientific leaders in community-engaged research, and help to develop a core network of diverse communities that are communicating effectively about health research. The preliminary results were that social media platforms can potentially improve and increase the engagement of community members and other stakeholders in online education [172].

It is important to educate physicians and researchers about the importance of systems thinking, since medical knowledge has and continues to be produced very often using a reductionist paradigm [173]. However, many of them are realizing that the systems medicine paradigm is better and that this takes advantage of increasingly larger datasets. The

data are being shared better. This is being facilitated by the widespread availability of electronic healthcare records. In accordance with the principles of Systems Medicine 2.0, patients are being given easy access to their personal data and records. In the process, as systems medicine is combined with large-scale data sharing, we can understand communicable diseases better. At the same time, personalized medicine is being nurtured and encouraged, while much progress is being made in predicting one's susceptibility to these diseases, which leads to being able to prevent their occurrence in the first place. When such diseases do occur, appropriate treatments and cures are becoming more feasible and widely available. In the process, a synthesis between Medicine 2.0 and systems science has produced Systems Medicine 2.0 [173].

However, systems medicine is not replacing reductionism any more than quantum mechanics replaced Newtonian physics. Both are needed in modern medicine [2, 83] and physics [174]. Instead, systems medicine is augmenting reductionist medicine [173]. As mentioned previously, one important augmentation was the use of precise anticancer agents to treat and cure different subtypes of breast cancer and leukemia. This showed that some diseases that appeared to be the same on first examination turned out to be quite different. This led to the study of the etiome, or the "precise etiological pathways by which genetic and environmental agents cause a disease" [173]. It encourages assigning subdivisions called intermediate pathophenotypes to each category of diagnoses. Even though pathologists may be more interested in the final stages of pathology, many physicians and epidemiologists are more interested in the many factors that caused the original dysfunctions. They are also interested in predicting patients' prognoses, based in part by their health histories. It has been suggested that the etiological phenotype or etiphenotype might be more appealing to clinicians. As such, the determination of the many causes or etiologies of diseases (etignosis) would complement the idea of diagnosis. A second supplement to reductionist medicine is the idea that many patients have more than one disease and that some diseases tend to form clusters in the diseaseome network. For example, heart disease is now viewed as a multi-organ failure, while diabetes increases one's risk of cardiovascular diseases, stroke, chronic kidney disease and neuropathies. Similarly, metabolic syndrome can lead to cardiovascular and neurodegenerative diseases, as well as many types of cancer and autoimmune diseases – especially type-2 diabetes. The third aspect of reductionism is that it is often better to treat complex diseases with several drugs given at the same time [173]. A corollary to this, which was discussed in Chapter 3, volume 1, is that many natural products and

effective prescription drugs have several therapeutic targets – even if they were originally developed based on their effects on just the first target that was discovered.

So, the reductionist paradigm is being modified by network medicine [173]. It requires realizing that complex diseases emerge from perturbations in many genes, RNA and proteins that are linked in several interconnected networks. However, when network medicine only looks at snapshots of the final stages of a disease, it's not very useful. Instead, it is important to look at gradual shifts from a healthy to a diseased state. That is, disease is not simply the opposite of health. They both exist in stages. So, it's important to discover the cascade of events that can lead to a gradual shift from health to disease. That is, network medicine should not just focus on genes, RNAs and proteins, but also include behavior and environment. It should also be concerned with nutrition (from conception through childhood and eventually old age), upbringing (hopefully, but not always in a nurturing family), socioeconomic status, physical activity and exposure to environmental toxins. So, systems medicine should study how genetic and epigenetic factors interact with behavior, psychological and environmental factors to disrupt health and lead to diseases [173].

To do this, a new approach to data collection and analysis is needed [173]. Most risk factors for diseases interact with each other to produce small effects that are nested in many levels. So, data on many patients are required. This will include high throughput technologies that can be used to formulate new hypotheses which can be tested. This is being facilitated by machine learning algorithms on the world's most advanced supercomputer, IBM's Watson. It has data on more than one million cancer patients and the scientific literature on cancer. The datasets that are produced are being examined for 'hot spots' which are loci that show associations between molecular data (-omics), behavioral, environmental, socioeconomic factors and clinical outcomes. This will help to identify etiphenotypical groups and help to find proper preventions and treatments for them. So, the traditional paradigm in which humans generate hypotheses that are then tested on computers is being modified. Now, computers are being used to help generate hypotheses. This requires "seamless collaboration with mathematicians, modelers, and data scientists, so that the right modeling tools can be used and combined to appropriately balance sufficient complexity with practical utility" [173]. It also requires sharing data between researchers and the public, as well as using data from public health records (that have been made anonymous to protect patient privacy) for research. This is leading to the formation of online communities that are creating a new social web. Moreover, Web 2.0

is using crowdsourcing (which has been so successful in Wikipedia) to produce Science 2.0, which is also known as Open Science and Cyberscience 2.0. Online platforms are making data sharing easier as researchers merge and re-analyze each other's data in projects like HapMap and Sage Bionetworks. Some of these projects (like Fight AIDs at Home, PatientsLikeMe and GalaxyZoo) encourage and empower laypeople, patients and their caregivers to help in scientific research. So, Web 2.0 technologies are being used with healthcare records to create Medicine 2.0. In the spirit of TQM, it is narrowing the gaps in communication and collaboration between researchers, physicians, patients and their caregivers. To assist in this, the Joint Initiative on SDO Global Health Informatics Standardization is producing common health informatics standards [173].

Systems Medicine 2.0 is also being assisted by improvements in information technology (IT). Several consortia have been formed that include multi-disciplinary teams that often focus on a particular disease [175]. One example is the Medicine Approach for Heart Failure (SMART) Consortium. It has a heart center, a molecular biology lab with a bioinformatics facility, a proteomics lab and experts who model cell and whole-organs in addition to multi-scale modeling. The IT helps members of the consortium share data and communicate it effectively. It also processes data reliably and reproducibly. The foundation is an In-Memory Database (IMDB). It integrates large biomedical datasets in a single system and enables real-time analysis of data by holding all data in the main memory. It uses platforms that integrate, process and analyze many heterogeneous datasets while supporting the workflow. In addition, the Georgetown Database of Cancer (G-DOC) is a web-based platform that has integrated clinical outcomes and -omics data on cancer. Data are stored in a traditional relational database. GDOC processes data from microarrays and metabolomics studies. It concentrates on the genetic cause and predictions of outcome. This approach is being extended to form a holistic systems medicine approach to studying diseases and helping patients [175].

The SMART consortium follows the Design Thinking (DT) methodology to identify software requirements that are needed to improve the existing research program [175]. DT provides a process framework for constant communication and feedback between the developing team, stakeholders and end users. In the spirit of TQM, they interviewed users (or customers) and consortium members to document and continuously improve the systems medicine research process. Based on the constant user feedback, they extended their prototype and evaluated the new

functionality in workshops and telephone interviews after users tested the software. Feedback was incorporated to plan subsequent phases of development. The design decisions, event-driven processes and applications have been described [175].

Others are using a diffusion-based method for analyzing data [176]. It is based on the concept of diffusion analysis in evaluating gene scores that measure the significance of gene mutations and the likelihood that they will lead to diseases. A diffusion process in statistics is a solution to a stochastic differential equation. Diffusion begins at each gene and then spreads out in the network of protein-protein interactions. Once the diffusion reaches an equilibrium, an algorithm that detects network modules is used to find the network modules that have large distributions in their gene score. Genes in these modules are assumed to be related to the disease being studied, as measured by statistical analyses. A network-based tool called HotNet2 was used to detect driver genes in cancers that have less frequent mutations by searching for significant cancer-related network modules. It's a modification of the original HotNet tool. It is based on heat dispersion. That is, heat disperses from a source and warms up the surroundings. Mutated genes are considered 'hot' and their heat will spread through the network and affect other genes in the network. Genes closer to the hot genes have a higher chance of obtaining more heat from hot genes than genes further away in the network. A corollary to this is that genes near hot genes that have few neighbors have a higher chance of obtaining more heat than genes near hot genes with many neighbors. Instead of using heat diffusion without considering heat directionality (as in HotNet), HotNet2 incorporates the impact of the direction of heat diffusion. The first step of HotNet2 allows nodes to retain some of their own heat in the process of transferring heat to neighboring nodes. In the second step, HotNet2 incorporates information about the direction of heat traveling between nodes for the two genes separately to determine how genes influence each other. The details on how this was incorporated into the Javascript programming language have been described [176].

It is also important to consider the technological and social conditions that are essential parts of the environments in which health data are produced, analyzed, shared and used [177]. This has been called health data ecosystems. The European Federation of Pharmaceutical Industry Associations (EFPIA) commissioned RAND Europe to perform a rapid review of the value of health data. There were two goals. The first was to identify and explain the potential and existing benefits of using health data. The second was to "examine the key drivers of supportive health data ecosystems and their implications for future research, policy or practice"

[177]. It was discovered that healthcare data adds value to research and development, while it improves public health and pharmacovigilance, along with the delivery of healthcare, which improves the overall health system. Data on clinical trials, as well as data reported by patients and clinicians, together with data on behavior and health systems data are improving the health of the whole population. This includes personalized healthcare, which empowers patients and their caregivers to help obtain better healthcare, new and improved diagnostics as well as better treatments. At the same time, health data are helping to design new research projects. For example, magnetic resonance imaging (MRI) data are being transformed into more accurate measurements of body composition that shows the distribution of fat around various organs using a cloud-based, computer-assisted process. When combined with data on Body Mass Index (BMI), the data can describe a person's health status better and be used in research on metabolism. There is also a Structural Genomics Consortium (SGC) that is producing 3D protein structures and entering the results in freely accessible protein databanks. There is another project called Genomics England. It is relating genomics data to data on phenotypes and clinical records to construct a research dataset. At the same time, Genomics England Clinical Interpretation Partnerships are being used as pre-competitive research networks to help with new drug development. Data from clinical studies done by GlaxoSmithKline (GSK) are being shared by the GSK Clinical Study Register. It includes summaries of study protocols, scientific results, clinical studies and complementary information from clinical trials that have been sponsored by GSK. At the same time, timely healthcare data is helping with emergency preparedness. This includes more robust monitoring of infectious diseases and disease outbreaks, while facilitating early warning systems and more proactive strategies for managing the diseases. Large datasets of health records are also making it easier to compare clinical outcomes of patients who have different –omics and behaviors. Finally, electronic health records are helping to establish improved healthcare policies. This can lead to better allocations of resources, while ensuring proper and timely reimbursements so physicians can focus on healthcare instead of how they are going to get paid and maintain their practice [177].

At the same time, Systems Medicine 2.0 now includes bioregulatory systems medicine (BrSM) [178]. It is a systems approach for describing the human body as "a complex biological network of interconnected components" [178]. It is a bidirectional approach that aims to translate scientific discoveries into clinical applications, while providing important medical innovations. BrSM is based on the idea that the complexity of

diseases can be understood better by considering how people's many homeostatic systems interact across all levels of biological organization. It emerged as a response to the limitations of reductionist thinking, which has failed to prevent the rise in healthcare costs and the burden of diseases. BrSM recognizes that small perturbations in a system can lead to much bigger perturbations across the many networks that exist in the human body. It also appreciates the importance of each patient's autoregulatory systems that exist in nested levels of physiological networks. BrSM also realizes that the interconnectivity of networks can be viewed as a global autoregulatory network that is an important indicator of one's health. It is an interdisciplinary approach. The viewpoints of many experts were obtained. They were used to prepare a similarity matrix. Cluster analysis of the matrix identified four major themes: inflammation physiology, biological communication at the microenvironment-scale, biological communication across multi-scale networks, and bioregulatory clinical pharmacology. There were also six intermediate themes: inflammatory network response to perturbation, microenvironment response to inflammation, diagnostics and therapeutic strategy, clinical focus on dysregulation, autoregulation of biological networks, and the patient health-disease continuum. A graph was then constructed in which the horizontal axis represented the biological information and the vertical axis represented the mechanisms of intervention. The experts recognized that the core feature of the BrSM model is that biological systems are complex. A healthy system is one that regulates itself even when confronted with perturbations to the network. This is called autoregulation. An adequate autoregulatory capacity is essential for good human health. These networks are also robust. They can adapt and respond to the inherently dynamic and unstable networks that are influenced by the environment. Robustness is different than homeostasis because it refers to sustained functionality rather than sustained concentrations of metabolites. Metabolic, genetic, epigenetic and protein networks interconnect to create a global biochemical network. The feedback loops that connect and regulate these networks are the foundation of the global autoregulatory network. When one's autoregulatory networks become dysfunctional, diseases emerge. For example, a chronic low level of inflammation (smoldering inflammation) can make these networks dysfunctional and lead to neurodegenerative, autoimmune and cardiovascular diseases, as well as many forms of cancer. Such stressors can propagate throughout networks of interconnected tissues in a ripple-like effect. This propagation helps diseases progress by distorting the flow of information [178].

The concept of modularity is also important in understanding how pathophysiological events tend to organize and how this organization impacts the maintenance of good health [178]. Modules are self-organized units of individual components that are grouped according to rules (like a common function). They allow networks to optimize their dynamics and adapt properly to disturbances. Modularity helps networks control perturbations and the effects of disease on the system by supporting autoregulation. Many diseases are interconnected by shared pathophysiological events. There is a common network that is perturbed in the most chronic diseases. It is called a common disease-state signature. Many diseases also share the same functional modules. This suggests that treatments may be more effective by targeting these biological networks rather than the just single molecules. This overlap of biological networks is particularly useful in understanding the aging process and age-related diseases. So, multitarget drugs that target hubs, bridges, and/or other areas of networks that overlap are often more effective and lead to fewer side effects than those that have just one therapeutic target. Moreover, optimal therapeutic access points may be discovered by utilizing these pathological links between seemingly unrelated diseases. This pathophysiological connectivity may be a better way of developing new drugs in what is known as network pharmacology. That is, networks themselves are becoming the focus of new drug design and discovery [178].

At the same time, inflammation is being understood better. As we will see in Chapter 2, it has been a major topic in medical science for quite some time – mostly due to it leading to many diseases. So, it has been thought as something that should be reduced or suppressed [178]. However, systems thinking and further research has taught us that properly controlled inflammation is also essential for good health. It plays a key role in maintaining tissue homeostasis. BrSM embraces physiological inflammation as an essential part of the autoregulatory capacity of the body. It helps maintain the healthy state of tissues and in restores their proper function when they are affected by disorders or diseases. BrSM recognizes that inflammation can be adequate, insufficient, resolving or excessive – depending on the state of the systems in which it occurs. So, physicians are recognizing that inflammation can be a useful tool when treating patients. It is an essential part of homeostasis and not always pathogenic [178].

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CHAPTER TWO

THE ROLE OF INFLAMMATION

2.1 Previous misconceptions caused by reductionist thinking

Inflammation can be one of the most misunderstood aspects of health when viewed with reductionist thinking. Part of the misunderstanding is based on the assumption that model organisms, such as baker's yeast (*Saccharomyces cerevisiae*)**,** the fruit fly *Drosophila melanogaster*, mice, rats and dogs can tell us what also happens in humans. That is, caloric restriction without starvation has extended the lifespans of these organisms [1]. This even inspired one physician (Roy Walford) to restrict his caloric intake and write a book titled *The One Hundred and Twenty Year Diet: How to Double Your Vital Years* [2]. This idea was based on the assumption that restricting the consumption calories by reducing the consumption of proteins, fats and carbohydrates reduces the total metabolic rate and the production of reactive oxygen and nitrogen species (RONS) and free radicals [3]. This led to the free radical theory of aging, in which it was proposed that the accumulation of damage caused by free radicals causes aging and eventually death. That is, oxidative metabolism of proteins, fats and carbohydrates in the mitochondria produce RONS and free radicals which can oxidize and damage DNA, lipids and proteins and lead to cancer as well as cardiovascular and neurodegenerative diseases [3-5]. This also led the Nobel Prize winning chemist, Linus Pauling to suggest that consuming large amounts (up tp 40 000 mg per day) of the natural antioxidant, vitamin C, could prevent the common cold, the flu and cancer [6-9].

At the same time, endurance athletes (especially those who ran marathons) were encouraged to load up on carbohydrates so they would have enough energy to compete successfully. There is even a phrase in Italian “pasta è basta”, or pasta is all you need to eat. Indeed, carbohydrates can be digested and converted to amino acids, proteins and fats that the body needs. Moreover, many Italians do live long, healthy lives – especially if they consume the Mediterranean diet (that includes

fruits, vegetables, nuts, grains and olive oil, as well as moderate amounts of whole grain pasta and rice, but little or no red meat). In addition, people in almost all countries were encouraged to eat foods that have a high antioxidant capacity, and *in vitro* tests for total antioxidant capacity emerged. They were based on measuring the destruction of oxidized test compounds in direct reactions with the antioxidants in foods. Many dietary supplements arrived on the market. They contained purified antioxidants, such as resveratrol and EGCG that were and still are widely assumed by many to be quite healthy at any dose. Even today, many advertisements for popular 'superfoods', beverages and dietary supplements highlight the huge antioxidant activities of their products. One advertisement pictures the antioxidants in their product as being powerful ninja warriors, armed with swords so they can kill the evil free radicals that can cause heart disease and cancer. This assumes that dietary antioxidants exert their health effects by reacting directly with RONS and free radicals, destroying them. They were very popular years ago and led to one 'superfruit', açaí, being identified as having the highest antioxidant activity of any food [10]. So, the U.S. Department of Agriculture (USDA) listed the *in vitro* antioxidant capacities of many foods and spices. Unfortunately, Doctor Roy Walford died from amyotrophic lateral sclerosis (ALS, commonly known as Lou Gehrig's disease) at the age of 79. Linus Pauling died from prostate cancer at the age of 93.

Moreover, as scientists and physicians learned more about human nutrition, they realized that the caloric restriction that worked for other organisms did not work for humans. This required changing the paradigm from reductionist thinking to systems thinking. As described in Chapter 1, volume 1, reductionist thinking assumed that there is a linear relationship between the dose and the physiological effect of potential toxins. It also assumes that if a small amount of an antioxidant that is present in food (like EGCG in green tea), then much larger amounts in a dietary supplement would be even healthier – especially if they were 'natural'. This was an important part of the free radical theory of aging. It led many to deny the existence of hormesis. In contrast, systems thinking does recognize the nonlinear and cyclic nature of life, while accepting the concept of hormesis – especially when biochemical mechanisms are discovered that explain the health benefits of low doses of something like selenium and the toxicity of high doses. This has now been shown to apply to mitochondria, as well as the proteasome and endoplasmic reticulum (ER) in a process called mitohormesis. This is the basis for the Proteasome, Endoplasmic Reticulum and Mitochondria (PERM) hypothesis [3, 11]. It proposes that mitochondria, proteasomes and ER

regulate cellular survival decisions when cells are under oxidative stress. They coordinate and modulate apoptosis and autophagy. It includes the idea that RONS are important signaling substances that enable cells to trigger their survival mechanisms. That is, hormesis recognizes the toxic effects of moderate levels (or doses) of RONS are needed for good health. Still, at higher doses, RONS and free radicals can cause increasing levels of oxidative damage that can have seriously consequences, including senescence, cardiovascular diseases, muscle weakness and death [12].

In addition, the antioxidant paradox became apparent [13]. Dietary antioxidants have low bioavailabilities. Also, giving large doses of dietary antioxidant supplements to human subjects has seldom had any preventative or therapeutic effects. Most antioxidants (especially phenolic compounds) don't work by reacting directly with RONS and free radicals. So, the USDA removed the list of antioxidant capacities from their website years ago.

At the same time, it is important to use systems thinking and realize that inflammation can be not just a cause or a symptom of many diseases, but it is also essential for good health [5]. When pathogenic microorganisms invade the body, immune cells kill them by causing oxidative damage and inflammation. Although there may be some collateral damage to the surrounding healthy cells and tissues, this does get repaired. However, when the immune system is overactive, it can incorrectly identify environmental chemicals, foods and even one's own cells as foreign and mount a potentially fatal autoimmune response or allergic reaction. So, inflammation, like so much else in the body, must be carefully controlled. Inflammation occurs when parts of the body become red, warm, swollen, and damaged. This can happen when the immune system responds to foreign materials, irritation, bone or nerve damage, infection by microorganisms, and ischemia (lack of blood flow), followed by reperfusion. Inflammation is caused by the production of RONS and free radicals, as well as histamine, pro-inflammatory cytokines, prostaglandins and eicosanoids. Prostaglandins, eicosanoids, and leukotrienes are made from arachidonic acid, as described in Chapter 3, volume 1. Prostaglandins were first discovered in the prostate gland. They all have 20 carbons, so they are eicosanoids, as are leukotrienes. Prostaglandins have a five-carbon ring and are derived from arachidonic acid. COX catalyzes their synthesis. They are found in almost all tissues and organs. There are nine known prostaglandin receptors. They are can be ligated (or bound) to G-protein coupled receptors. One type of prostaglandin receptor regulates inflammation. Prostaglandins have short half-lives, so they exert their effects locally, as either paracrine (locally active) or autocrine (active

on the same cell that makes them). They can produce fever and dilate blood vessels. Leukotrienes are also 20-carbon derivatives of arachidonic acid, but their synthesis is catalyzed by 5-lipoxygenase (5-LOX). As the name implies, leukotrienes have three C=C double bonds and are made by leukocytes (white blood cells). They help regulate blood flow. One of them, leukotriene 4, or LBT-4, attracts white blood cells to the site of an infection, and it increases the permeability of blood vessels [5].

Inflammation can be caused (or induced) by infection, tissue injury or by tissue stress and malfunction [5]. Inducers interact with sensors, such as toll-like receptor 4 (TLR4), immunoglobulin E (IgE), a subcellular particle called the NALP3 inflammasome or a protein called the Hageman factor. The sensors then initiate the production of several inflammatory mediators. These include tumor necrosis factor alpha (TNF-α), IL-6 (interleukin-6), prostaglandin E_2 (PGE_2), vasoactive amines, IL-1β and bradykinin. Tocilizumab (Acterma®) is a monoclonal antibody that binds to the IL-6 receptor and is approved for treating rheumatoid arthritis. So, inflammatory mediators affect the cells and tissues, and are also called effectors. Target cells include endothelial cells, hepatocytes, leukocytes, the hypothalamus and smooth muscle cells. So, Bacteria can produce lipopolysaccharides, which interact with TLR4, which initiates the production of TNF-α, IL-6 and PGE_2. This affects endothelial cells, hepatocytes, leukocytes, the hypothalamus and other cells. Bacterial inducers include pathogen-associated molecular patterns (PAMPs) and virulence factors. Host cells have receptors for PAMPs, which trigger inflammation [5].

Microbes stimulate the innate immune system and its TLRs and nucleotide-binding oligomerization domain (also known as NOD-like receptors) [5]. The cytoplasmic receptor NOD2 senses peptidoglycan fragments and activates nuclear factor κB (NF-κB) signaling, which leads to inflammatory immune responses. Dysregulation of NOD2 signaling can lead to inflammatory diseases, such as Crohn's disease and Blau syndrome. Macrophages and mast cells that reside in the affected tissue produce inflammatory modulators. These include cytokines, chemokines, vasoactive amines (histamine and serotonin), eicosanoids and products of proteolytic cascades. So, there is an interconnected network of genes in NF-kB signaling [16]. At the same time, allergens are sensed by IgE, which produces vasoactive amines (such as histamine), which activate endothelial cells and smooth muscle cells. Another class of inducers, crystals of monosodium urate and calcium pyrophosphate dehydrate are sensed by NALP3, which produces IL-1β, which activates endothelial cells, hepatocytes, leukocytes, the hypothalamus and other cells. These

crystals are formed in the diseases called gout and pseudogout. The fourth type of inducer, the protein called collagen, is sensed by the Hageman factor, which is mediated by bradykinin, which activates endothelial cells and smooth muscle cells. The Hageman factor (also known as factor XII) becomes activated by contact with collagen and other components of the extracellular matrix that are produced when the vascular endothelium is damaged in stressed or malfunctioning tissues [5].

There are also some lipids, called lipoxins, resolvins and protectins that are anti-inflammatory [5]. A low dose of aspirin (81 mg) stimulates the production of lipoxins. Higher doses have no effect on lipoxin synthesis. Anti-inflammatory lipids are released in the later stages of inflammation and restore the normal, healthy condition. The successful initial inflammatory response eliminates infectious agents. This is followed by a resolution and repair phase, which is mediated by macrophages that are already in the tissue and macrophages that are drawn to the damaged site. They switch the lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, which inhibit the recruitment of neutrophils and promote the recruitment of monocytes. They work together with resolvins, protectins, transforming growth factor β and other growth factors to remove dead cells and start remodeling tissues [5].

2.2 Chemistry of free radicals and RONS

If an atom or molecule has one or more unpaired electrons and can exist on its own, it is a free radical [14]. In contrast, the electrons in most atoms and molecules are in pairs that tend to be located in atomic or molecular orbitals. One electron in the pair has a spin quantum number of +1/2, while the other is -1/2. Free radicals are produced by homolytic, heterolytic, or redox reactions. However, not all reactive oxygen species (ROS) are free radicals. Some like hydrogen peroxide (H_2O_2) have reactive oxygen atoms. Similarly, reactive nitrogen species (RNS) can be either free radicals or simply molecules that have a reactive nitrogen. Note that some people use the term reactive oxygen and nitrogen species (RONS) instead of ROS and RNS.

The most abundant free radicals that are produced by cells are superperoxide ($O_2^{\bullet-}$) and nitric oxide (NO, also written as NO•). Superoxide is produced through either incomplete reduction of O_2 in electron transport systems or as a specific product of enzyme-catalyzed reactions. NO is produced by reactions that are catalyzed by nitric oxide synthases (NOS). Both superoxide and NO also react to form other ROS and RNS. For example, superoxide dismutase catalyzes the formation of

hydrogen peroxide (H_2O_2) and subsequently hydroxyl radicals in the presence of transition metals. NO undergoes reactions that produce peroxynitrite ($ONOO^-$) and subsequently other RNS [14].

H_2O_2 can produce free radicals, such as the hydroxyl radical (•OH) [14]. H_2O_2 is stable and permeable to cell membranes. It can be formed by the dismutation of superoxide, as shown in eaction (1):

Reaction (1): $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

The cytotoxicity of H_2O_2 is due primarily to its production of the hydroxyl radical when it reacts with Fe^{2+} or Cu^{2+} in the Fenton reaction, as shown in reaction (2):

Reaction (2): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$

This is part of the Haber-Weiss reaction, in which iron or copper are kept in their reduced state (Fe^{2+} and Cu^+) by superoxide and subsequently produce the hydroxyl radical [14]. The hydroxyl radical is highly reactive and damaging. It can oxidize lipids and lead to atherosclerosis and heart disease, as stated in the iron hypothesis [14, 15].

Hydroxyl radicals oxidize and destroy molecules close to where they are produced in the cell, because they are so reactive and not permeable to cell membranes [14]. They are probably the most damaging ROS in cells [14].

Nitric oxide (NO) is produced in reactions catalyzed by neuronal NOS (NOS1), inducible NOS (iNOS, or NOS2) and epithelial NOS (NOS3) [14]. The NOS3 form occurs under inflammatory conditions. NO also binds to the Fe^{3+} ion in guanyl cyclase, which activates it and produces cyclic GMP (cGMP). NO also reacts rapidly with superoxide to produce peroxynitrate, as shown in reaction (3).

Reaction (3): $O_2^{\bullet-} + NO\bullet \rightarrow ONOO^-$

Peroxynitrate and its protonated form (ONOOH) are strong oxidizing agents that can deplete thiol (SH) groups, damage DNA and add a nitrate to proteins [14].

Hypochlorite (OCl^-) is another important ROS [14]. Its acid form (HOCl, hypochlorous acid) is produced by a reaction between H_2O_2 and a chloride ion (Cl^-) that is catalyzed by myeloperoxidase, as shown in reaction (4):

Reaction (4): $H_2O_2 + Cl^- \rightarrow HOCl + OH^-$

Hypochlorite is mostly formed by neutrophils. It can damage important biochemicals by oxidizing thiols (SH), lipids, ascorbate (vitamin C) and NADPH. When acidified as HOCl, it can also cross cell membranes and cause both fragmentation and aggregation of proteins [14].

In addition, free radicals can start chain reactions in some biochemicals, such as polyunsaturated fatty acids (PUFA) and polyunsaturated fatty acyls that are a part of fats and lipids [14]. The hydroxyl radical (•OH) starts the peroxidation by removing a hydrogen atom from a methylene carbon ($-CH_2-$) side chain, to make a carbon-centered, lipid radical, plus water. The carbon-centered, lipid radical can undergo a rearrangement to make it more stable. Two lipid radicals can cross-link to make a conjugated diene. More often, the lipid radical will donate an electron to O_2, forming a peroxyl radical (designated LOO^-, where L is the PUFA) and propagating a chain reaction. The chain reaction is terminated by making a cyclic peroxide or cyclic endoperoxide and other products, including malondialdehyde. Lipid peroxidation can damage cell membranes, making them more rigid, while making proteins lose their proper function [14].

2.3 How antioxidants really work

Even though vitamins A and C as well as CoQ10 can react directly with RONS and destroy them, other antioxidants seldom do this. Moreover, many RONS act as biochemical messengers in normal, healthy metabolism [3, 13, 16, 17]. RONS have nonlinear, hormetic effects. At low concentrations, they can have definite health effects. As mentioned in Chapter 1, volume 1, exercise is an example of hormesis. When we exercise, RONS are produced and there is some temporary inflammation. Vigorous exercise increases blood flow, oxygen consumption and the production of RONS, while modulating the growth factor signaling cascade and increasing the availability and function of neurotransmitters. That is, exercise induces oxidative stress [15]. Regular exercise causes adaptive changes in cellular antioxidants. Redox balance regulates the generation of force in muscles. Maximum force is produced when the redox state is balanced. That is, there should be a proper balance between the concentrations and activities of pro-oxidants (RONS) and antioxidants. RONS are required to promote the healthy responses of muscles to exercise. Free radicals contribute to muscle fatigue and a temporary increase in their size (a phenomenon called ‘pumping up’). Contracting

muscles release superoxide radicals into the interstitial space. However, regular exercise increases the activities of endogenous enzymes that function as antioxidants in cardiac and skeletal muscles. This increases with the intensity and duration of exercise. At the same time, vitamin E can protect membranes in cardiac and skeletal muscle cells from oxidative damage. In addition, endurance exercises protect cardiac myocytes so that they can resist damage caused by ischemia-reperfusion that can occur in strokes. This cardioprotection is due to an increase in the activity of superoxide dismutase [16].

The discovery that NO is the signaling biochemical that causes vasodilation led to a paradigm shift [16]. It is a RON that is harmful in some cases, but can also promote the biosynthesis of more mitochondria (mitochondrial biogenesis) [18]. That is, not only do cells undergo autopoiesis, so do mitochondria [12]. It was once thought that the only role of mitochondria was to produce energy in the form of ATP. This is still recognized as a major role of mitochondria in healthy cells. However, it is now known that mitochondria also play important roles in apoptosis, autophagy, the production of RONS and free radicals, as well as handling Ca^{2+}. Moreover, dysfunctional mitochondria that produce less ATP exist in aging skeletal muscle, the heart and adipose tissues. This contributes to cardiovascular diseases and the age- and obesity-related decrease in muscle mass, as well as a decline in the function of skeletal muscles, which is known as sarcopenia. Even though there are fewer mitochondria in white adipocytes, they are still essential in their function. They are needed for the secretion of adipokines – the hormones that are produced by adipose tissue (AT). Moreover, mitochondrial dysfunction in AT can lead to insulin resistance and cardiac dysfunction. So, maintaining mitochondrial function in AT is important in increasing one's lifespan and healthspan (the number of years that one remains healthy). On the other hand, obesity (especially excess abdominal fat) accelerates the physiological decline that occurs during aging [12].

The structure of the myocardium (muscle tissue in the heart) changes as one ages [12]. So, it's important to maintain as many healthy mitochondria as possible. This requires maintaining proper mitochondrial structure. Since the heart needs much energy, a decrease in mitochondrial function contributes to age-related myocardial dysfunction. Cardiomyocytes have two different types of mitochondria: the subsarcolemmal mitochondria (SSM), which are located beneath the plasma membrane and the interfibrillar mitochondria (IFM), which are between the myofibrils. IFM have more ADP-stimulated respiration, while SSM produce proteins faster than IFM. SSM in aged rodents maintain their respiratory capacity,

while IFMs consume less O_2. This leads to a decrease in the activity of the protein complexes III and IV in the electron transport chain (ETC). Also, mitochondrial biogenesis tends to be impaired during aging. This is due to lower expression of important regulators of mitochondrial biogenesis, such as peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha, or PGC-1α). In addition, the lipid called cardiolipin (structure shown in Figure 1) is an important part of the structure of cristae (the partial partitions in a mitochondrion formed by infolding of the inner membrane). As a result, cardiolipin affects the activities of the protein complexes in the ETC. Aged mitochondria have less cardiolipin and remodeled cardiolipin. So, consuming acetyl-L-carnitine (a natural component of mitochondrial membranes) can increase the amount of cardiolipin and the amount of ADP-stimulated respiration [12].

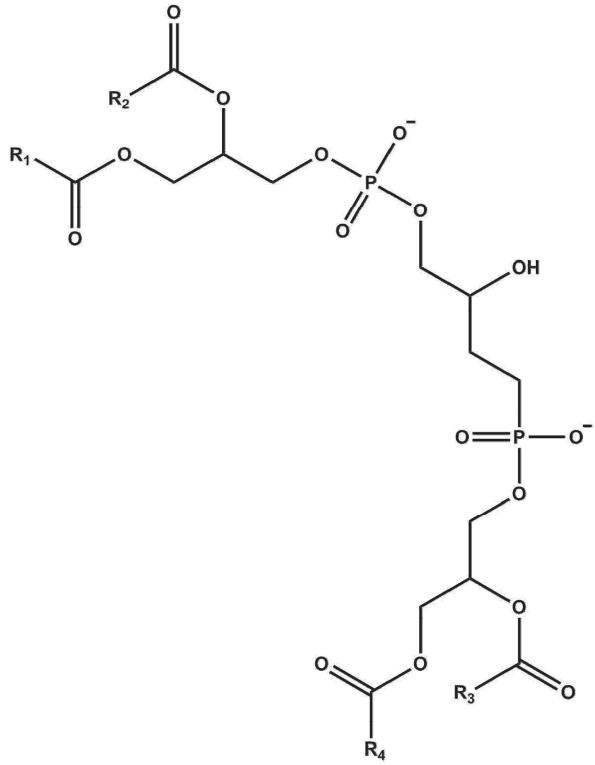


Figure 1. 2D structure of cardiolipin.

During aging, the activities of protein complexes in the ETC in cardiomyocytes tend to decrease [12]. As a result, oxidative phosphorylation decreases, as does the generation of RONS and free radicals. This leads to leakage of electrons from the ETC. They can react with O_2 to produce the superoxide anion, which can be reduced to hydoxyl radicals and H_2O_2. The ETC is not the only part of mitochondria that produce RONS and free radicals. There are also monoamine oxidases (MAOs) that transfer electrons from amines to O_2 to produce H_2O_2. MAOs also produce a protein called $p66^{Shc}$. Under normal physiological conditions, $p66^{Shc}$ is found primarily in the cytosol, but during stress it moves to the mitochondria, where it helps make H_2O_2. An increase in the concentration of $p66^{Shc}$ can contribute to the increase in RONS and free radicals in aged cardiac SSM. Excess RONS and free radicals damage lipids and proteins, which leads to cardiac dysfunction and death. In addition, mitochondrial DNA is not protected by histones and is located near the site where RONS are made. So, it is quite susceptible to oxidative damage. Mutations in mitochondrial DNA in the heart decrease mitochondrial replication, as well as the mitochondrial mass and the mitochondrial antioxidant system. This can lead to heart failure. So, the free radical theory of aging is still considered to be valid by many, since increased formation of RONS is associated with a decrease in lifespan [12]. Still, it is important to remember that some RONS are needed for proper health.

So, mitochondria are very dynamic and undergo morphological changes, including opening the mitochondrial permeability transport pore (MPTP) during stress, as well as a well-regulated turnover during healthy conditions [12]. However, giant mitochondria with disorganized cristae emerge during aging and after excess endurance training in a degenerative response. So, mitochondria undergo both fission and fusion. This occurs to a lesser extent in myocytes than other tissues, though. Mitochondrial fission and fusion contribute to the segregation of damaged mitochondria and their removal. Damaged mitochondria are separated by fission and removed by mitophagy (destruction of mitochondria). Note that autophagy is the degradation of cytosolic components by the lysosome, while mitophagy is a type of autophagy in which dysfunctional mitochondria are sequestered into autophagosomes and delivered to lysosomes. It is important for cells to maintain proper quality control of mitochondrial fission and fusion to ensure proper function. Mitochondrial fission precedes mitophagy, which can be triggered by RONS, a loss of the mitochondrial membrane potential and opening of the MPTP. One of the regulators of mitophagy is the protein called Parkin, which is

dysfunctional in some people who have Parkinson's disease. In addition, the efficiency of autophagy and mitophagy tends to decrease in the aging heart [12].

There are also age-related changes in skeletal muscle mitochondria [12]. This leads to sarcopenia and a large decrease in muscle strength. The age-related changes in skeletal muscle mitochondria are similar to those that happen in the heart. Like the heart, skeletal muscles have both SSM and IFM. SSMs produce more RONS and free radicals while being fragmented and degraded more than IFMs. On the other hand, IFMs are more susceptible to apoptotic stimuli and opening of the MPTP. Still, SSMs and IFMs are interconnected. As most people age, the mitochondria in their skeletal muscles are less efficient and have decreased capacity to produce energy. There is a decrease in mitochondrial DNA (mtDNA), as well as ATP production and O_2 consumption. A larger proportion of mitochondria in the elderly become depolarized and dysfunctional. The decline in mitochondrial function is also due to less physical activity, but this can be prevented by exercise, even though exercise causes a temporary increase in RONS and free radicals. Mitochondrial dysfunction leads to apoptosis in skeletal muscles. Aged skeletal muscles accumulate dysfunctional mitochondria and have impaired mitophagy. This leads to the accumulation of non-degradable lipofuscin, a fine yellow-brown pigment granules composed of lipid-containing residues of lysosomal digestion. It is considered to be one of the aging pigments that appear in aging liver, kidney, heart muscle, retina, adrenals, nerve cells, and ganglion cells. At the same time, mitochondrial fusion is inhibited. Giant mitochondria accumulate due to hyper-fusion. In addition, fewer new mitochondria are produced, leading to a decrease in the mitochondrial content of sedentary elderly people [12].

So, exercise and physical activity are two of the most effective ways of slowing down the loss of muscle strength and mass [12]. It increases mitochondrial turnover. It was shown that five months of endurance training induces systemic mitochondrial biogenesis, prevents mtDNA depletion, increases mitochondrial oxidative capacity and prevents dysfunction in skeletal muscle. So, endurance exercises can attenuate or even prevent mitochondrial dysfunction in aging skeletal muscles, even though it produces RONS and free radicals. The RONS and free radicals play important roles in major signaling pathways that regulate skeletal muscle quality control and dynamics of mitochondria. So, consuming too many antioxidants can impair the health benefits of exercise. It should be noted that this refers to high doses of vitamins A and E and not to the comparatively low doses of phenolic compounds that are present in many

colorful fruits and vegetables. It is also interesting to note that endurance training can increase one's antioxidant response, which leads to lower concentrations of RONS and free radicals. Still, even though the beneficial effects of endurance training compared to strength training is well-established in younger people, it may not apply to people over 80 years of age when mitochondrial content is no longer as adaptable [12].

Mitochondrial dysfunction also occurs in obese people and people who have type-2 diabetes [12]. In addition, a premature aging phenotype can be seen in the liver, heart, AT and skeletal muscles. There is also some metabolic inflexibility. So, reduced mitochondrial capacity may be a cause and not just a consequence of insulin resistance [12]. However, it is also possible that feedback between organs that have reduced mitochondrial capacity and the pancreas can set up a vicious cycle in which cause and effect become almost indistinguishable, which is a hallmark of systems thinking (see Chapter 1, volume 1). Moreover, "Sarcopenia and obesity both pose a health risk for elderly people, but in combination, they synergistically increase the risk for negative health outcomes" [12, 18].

There are some therapeutic strategies that target the regulation of mitochondrial biogenesis, dynamics and improve the ability of the respiratory chain in mitochondria to scavenge toxins [12]. For example, benzafibrate (which is approved for lowering LDL cholesterol) increases mitochondrial biogenesis and oxidative phosphorylation. In addition, metformin as well as dietary sources of resveratrol and quercetin can increase the biogenesis of mitochondria [12]. However, as we will see in a subsequent section of this chapter, dietary supplements that contain large doses of purified resveratrol and quercetin (as well as many other phenolic compounds) activate the Nrf2/ARE antioxidant response system that is over-activated in multidrug resistant cancers.

Mitochondria in AT are also important in maintaining good health [12]. It has been known for some time that AT is important in energy storage and the expenditure of energy. As mentioned in Chapter 1, volume 1, AT is also an important endocrine organ that secretes adipokines that have essential interactions with heart and skeletal muscle. Impaired mitochondrial function in adipocytes is often associated with reduced oxidative metabolism of fatty acids. This can lead to an increase in cytosolic free fatty acids that can cause deterioration in the function of other organs. At the same time, adipocytes have a limited capacity to grow in size. When they reach their limit (which happens in obesity), lipids are deposited ectopically in not just skeletal muscles and cardiac myocyes, but also in liver and pancreatic beta cells. This leads to lipotoxicity as fatty acids enter into harmful metabolic pathways such as ceramide

biosynthesis, which causes apoptosis of lipid-loaded cells. However, lipotoxicity and lipoapoptosis can be prevented by treatment with PPARγ agonists (like rosiglitazone) and leptin, as well as by limiting caloric consumption [12].

AT also affects one's lifespan and metabolism throughout the body [12]. Obesity not only decreases the efficiency and performance of white adipose tissue, but also accelerates aging. At the same time, mitochondrial dysfunction in AT can lead to insulin resistance, cardiac dysfunction and hypertension. However, there is not just white adipose tissue, but also thermogenic brown adipose tissue (BAT). It is present during early postnatal development, but absent or present only in small amounts in adults. Brown adipocytes help to maintain the balance between energy storage and energy expenditure. BAT has a high oxidative capacity due to the many mitochondria in it. White adipose tissue can undergo browning during exposure to the cold (which causes one to shiver, or shake uncontrollably), PPARγ agonsists, leptin, natriuretic peptides, or beta-adrenergic stimulation. So, prescription drugs that increase the browning of white adipose tissue may increase energy expenditure and limit weight gain, thus helping to prevent obesity and slow down the aging process. However, browning of AT also occurs in some cancer patients. So, prescription drugs that accelerate this process can contribute to cachexia, a muscle wasting disease that occurs in many form of cancer [12].

In addition, *N*-acetylcysteine (NAC) can improve the performance of respiratory muscles [16]. NAC improves human exercise performance during submaximal exercise (60-80% of maximal oxygen consumption, or VO_2 max), but not during high intensity exercise (≥90% VO_2 max). The increase in RONS that occurs during exercise alters the expression of genes in muscles and contributes to adaptations in skeletal muscles caused by exercise. Relatively high doses of Vitamin C can be detrimental to one's performance in high intensity exercises, but not endurance exercises [16, 19].

Aerobic exercise increases the number and volume of mitochondria in skeletal muscles, which increases one's oxidative capacity [19]. It also increases the energy demand and Ca^{2+} turnover. This is detected by 5' adenosine monophosphate activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), Ca^{2+}/calmodulin-dependent kinases (CaMKs), sirtuins and mitogen-activated protein kinases (MAPKs). They activate the transcription factors PGC-1α, Nrf2 and mitochondrial transcription factor A (mtTFA), which regulate mitochondrial biogenesis. Note that mTOR is also known as mechanistic target of rapamycin. Antioxidants interfere with this increase in mitochondrial biogenesis. They

(vitamins C and E) can also prevent the enhanced insulin sensitivity that exercise and RONS can cause. In addition, antioxidant supplements can increase muscle damage and the concentrations of creatinine kinase and heat-shock proteins that occur during such damage. Antioxidant supplements can also decrease the activation of PGC-1α that is caused by exercise. This transcription factor is a key regulator of skeletal muscle adaptation to exercise. When antioxidant supplements are taken prior to exercise, they can prevent the increase in blood pressure and flow-mediated dilation that occur during exercise. So, RONS can act as intracellular signaling molecules to promote adaptations that help muscle cells to tolerate future stress better [19].

Exercise causes thermal, metabolic, hypoxic, and mechanical stress [20]. In the long term, though, regular exercise protects neurons in every part of the brain in which this has been studied. Vigorous exercise increases blood flow and oxygen consumption, while modulating the growth factor signaling cascade and increasing the availability and function of neurotransmitters. Also, exercise induces increased generation of not just RONS 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 RONS-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 RONS cause oxidative damage, while moderate amounts induce an adaptive response to oxidative challenge [20]. This has been called the oxygen paradox, in which adaptations against RONS and O_2 toxicities are due to oxidative changes in the cells that are affected [21]. Subtoxic doses of oxidants can cause changes rather fast in antioxidant enzymes that become more active when oxidized. Also, oxidative damage that causes mutations in DNA produced mutated species that were better able to adapt to changes in the O_2 concentration in the atmosphere [21].

So, dose is not the only important independent variable in hormesis. Time is too [21]. That is, the behavior and properties of the important, natural antioxidant glutathione (GSH) system vary with time. At first, cells respond to oxidative stress by direct enzyme modification. The concentrations and amounts of GSH increase and GSH-dependent protective enzymes are activated. In the first few seconds or minutes, microsomal glutathione-S-transferases protect cells by catalyzing the scavenging of RONS by GSH. This short-term adaptation is due to the modification of a redox-sensitive group on the enzyme that increases its activity. Also, short-term adaptations target GSH by regulating gamma-glutamylcysteine synthetase (γGCS), which catalyzes the rate-limiting step

in the biosynthesis of GSH. Third, the supply of cofactors that enzymes need can be modified. RONS can oxidize the SH group in GSH to make glutathione disulfide (GSSG). GSSG must be reduced back to GSH for cells to be protected against oxidative damage. This reaction is catalyzed by glutathione reductase (GR), in a reaction that converts NADPH to $NADP^+$. To prevent depletion of NADPH, an adaptive mechanism evolved that can stimulate the production of NADPH. During oxidative stress, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inactivated by oxidation. Since GAPDH is required for glycolsis, cellular metabolism is redirected into the pentose phosphate pathway, which produces NADPH. After several hours, a hormetic response is seen at the transcriptional level by up-regulating Nrf2-mediated expression of enzymes involved in GSH synthesis. In the long run, adaptations occur at the epigenetic and genomic levels. That is, after hours or days, RONS up-regulate endogenous antioxidant enzymes. They oxidize the SH groups on Keap1, which is bound to Nrf2. This increases the nuclear translocation of Nrf2 and increases the transcription of antioxidant response elements (ARE). This in turn leads to increased biosynthesis of GSH and protection against oxidative damage. Also, low O_2 tension induces another long-term effect through the hypoxia-inducible factor 1-α (HIF1α)-pathway. During normoxic conditions, prolyl oxidase enzymes catalyze the hydroxylation of HIF1α. Subsequently, an E3 ligase (Von Hippau Lindau protein) binds to HIF1α, which is ubiquitinylated and hydrolyzed by proteasomes. However, during hypoxia or low O_2 tension, there is not enough O_2 to keep the central iron of prolyl hydroxylase oxidized. HIF1α is no longer hydroxylated, so the O_2 increases in the long-term. In contrast, if there is too much oxidative stress, *S*-sulfonated glutathione is not attached to GAPDH. Instead, GAPDH is *S*-sulfonated. This changes the protein-protein interactions of GAPDH. Instead of being bound to its inhibitor, it binds to seven in absentia homologs (Siah1), which have a nuclear localization signal [21].

2.3.1 Poorly liganded iron and the Fenton reaction

Many dietary antioxidants are phenolic compounds that can bind to Fe^{2+} and Cu^+, preventing these metal ions from undergoing the Fenton reaction, which produces the deadly hydroxyl radical (•OH) [5, 22]. That is, differences in the amounts of stored iron can explain why men are more prone to heart disease than women. Iron stores in men rise after adolescence, but remain low in women. It only begins to rise after the age of about 45 in women. The maximum sex difference in heart disease is also at age 45. Moreover, medicines like aspirin that cause gastrointestinal

bleeding may protect against heart disease by decreasing iron stores. This may also partly explain the protective effect of NSAIDs on Alzheimer's disease. That is, a systems biology analysis showed that poorly liganded iron and copper (free iron and copper) can help cause not just Alzheimer's, but also Parkinson's and Huntington's diseases. Free iron and copper can also lead to diseases caused by prions, bacteriocides and chemical toxicity [22]. That is, there is a continuous autocatalytic production of hydroxyl radicals involving poorly liganded iron, leading to cell death via apoptosis. That is, once Fe^{3+} is produced in the Fenton reaction it can react with superoxide anions, $O_2^{\cdot-}$, (also produced by mitochondria) to regenerate Fe^{2+} [5, 22].

Vitamin C (ascorbic acid or ascorbate at physiological pH) can also reduce Fe^{3+} to Fe^{2+} [5, 22]. The hydroxyl radical is quite reactive and can damage many cellular components – not just lipids. It can also release Fe^{2+} from Fe-S catalytic centers in proteins like ferritin, thus producing more free Fe^{2+} that can react with more H_2O_2 to produce more hydroxyl radicals in an autocatalytic reaction. In a related reaction, the superoxide anion can react with nitric oxide (NO) to make the highly reactive peroxynitrite anion, $ONOO^-$, which can react with tyrosine or cysteine in proteins (to make nitrosylated proteins), with DNA (to make 8-hydroxy-20-deoxyguanosine) and with fatty acyls to make nitrosylated fats. So, to prevent the formation of excess hydroxyl radicals and superoxide anions, it is important to keep Fe^{2+} fully liganded [5, 22].

That is, iron has up to six individual chelation sites, arranged octahedrally, and many ligands will bind to only some of them [5, 22]. For example, many dietary phenolic compounds can chelate iron and copper. However, partial chelation by ascorbate will transform vitamin C into a pro-oxidant (promoting the production of hydroxyl radicals) and not an antioxidant, which is the healthy form of it. So, the binding of incompletely liganded iron (usually bivalent) to inappropriate cellular structures can cause catalytic activities that kill cells. This can lead to not just atherosclerosis, but also stroke, age-related macular degeneration, prion diseases, sepsis, septic shock, viral infections and neurodegenerative diseases. It may also contribute to microbial, plant, animal and chemical toxicities [5, 22].

2.3.2 The Nrf2/ARE system

In contrast, some dietary antioxidants can activate the Nrf2/ARE antioxidant signaling system that was introduced in Chapter 3, volume 1. Foods and beverages that have high concentrations of antioxidants (such as phenolic compounds) can help prevent cardiovascular diseases and

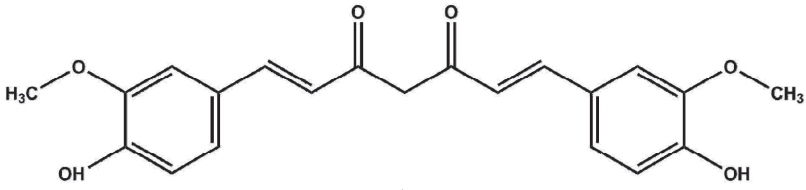
many types of cancer [5, 23, 24]. In the past, it was thought that dietary antioxidants exerted their health benefits by reacting directly with RONS and free radicals, thus destroying them [25]. This led to the development and use of several *in vitro* assays to measure antioxidant capacities [26]. So, many foods and beverages (such as green tea) have been called 'super-foods', due to their high *in vitro* antioxidant capacities [27]. However, there is now much evidence to refute the hypothesis that dietary antioxidants act *in vivo* by reacting directly with RONS and free radicals [28]. Instead, several specific compounds exert their health effects by activating the endogenous antioxidant response elements (AREs) that are present in cells [29]. This should not be confused with androgen response elements, which are quite different. They respond to the hormone, androgen and don't involve Nrf2. In many cases, transcription of AREs is activated by the nuclear erythroid-2 like factor-2 (Nrf2), which is a transcription factor [30-32]. So, the Nrf2 signaling system is often called the Nrf2-ARE or Nrf2/ARE signaling system. It controls the expression of AREs by binding to their promoter regions [33]. Some of the dietary compounds that have been shown to activate the Nrf2/ARE signaling system are listed in Table 1. The structures of five of them that are phenolic compounds with similar structures are shown in Figure 2.

Table 1. Partial list of dietary compounds that have been shown to activate the Nrf2/ARE signaling system [23].

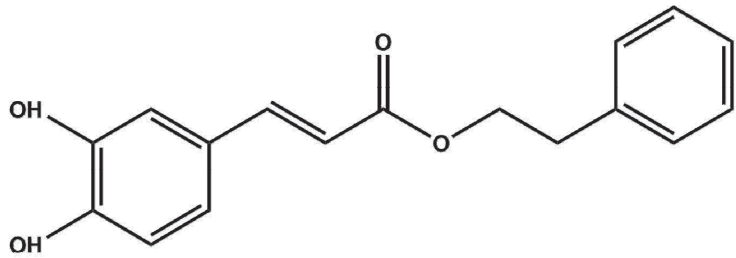
Compound	**Dietary Sources**[a]	**References**
Epigallocatechin-3-gallate (EGCG)	Green tea	[34]
Curcumin	Turmeric	[35]
Carnosol	Rosemary	[35]
Zerumbone	Ginger	[35]
Caffeic acid phenethyl ester (CAPE)	Honeybee propolis and many plants	[35]
Ethyl ferulate	Many plants, including eggplant	[35]
Sulforaphane	Broccoli and other cruciferous vegetables	[35]
Resveratrol	Red wine, Itadori tea	[36]
Quercetin	Many foods, including capers	[37]
Cyanidin & cyanidin-3-*O*-glucoside	Pomegranates, grapes, blueberries, açaí	[38]

Compound	Dietary Sources[a]	References
Catechin	Many foods, including cocoa and tea	[39]
Epicatechin	Many foods, including cocoa and tea	[40]
Kaempferol	Many foods, including green tea and berries	[40]
Naringenin-7-O-glucoside	Many foods, including tomatoes	[40]
Procyanidin B2	Many foods, including cocoa and grape juice	[40]
Genistein	Soybeans	[40]
Butein and phloretin	Fruits, vegetables, nuts, tea, coffee, red wine	[40]
Xanthohumol	Comon hops (*Humulus lupulus*)	[40]
Luteolin	Many foods, including celery and broccoli	[41]
Tangeretin	Tangerines and other citrus fruits	[41]
Ellagic acid	Pomegranates	[42]
Oleanolic acid	Many plants, including olive leaves	[43]
Ganodermanondiol	Lingzhi mushrooms	[44]
Echinatin	Licorice	[45]
Chlorogenic acid	Green coffee extract, coffee	[46]
N-methylpyridinium	Coffee	[46]
Ursolic acid	Apple peels and many other foods and spices	[47]
Hydroxytyrosol	Olive oil and olive leaves	[48]
Rosmarinic acid	Rosemary	[49]
Protocatechuic acid	Raspberries and many other foods	[50]
Phloroglucinol aldehyde	Metabolite of anthocyanins	[51]
p-coumaric acid	Many foods, including peanut and tomatoes	[52]
Ferulic acid	Many herbs used in traditional Chinese medicine	[53]
Isoorientin	Açaí, *Sasa borealis*	[16]
Ascorbic acid	Vitamin C, citrus fruits	[54]

a – Only some of the main dietary sources are listed



A



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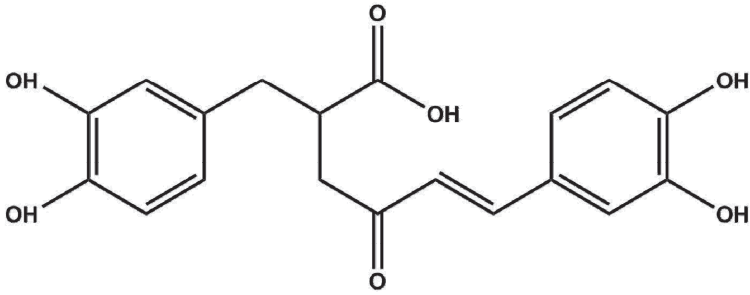

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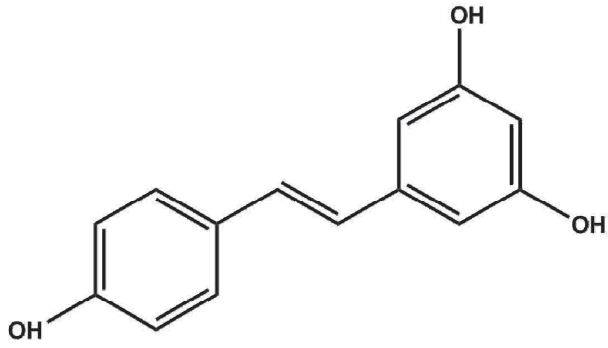

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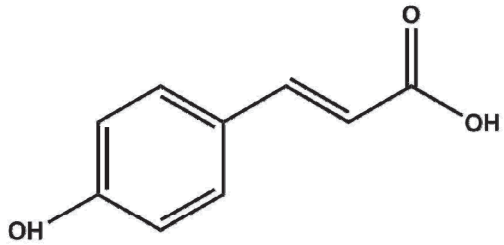

E

Figure 2. Structures of curcumin (A), Caffeic acid phenethyl ester or CAPE (B), rosamarinic acid (C), resveratrol (D) and *p*-coumaric acid (E).

Some of these compounds deserve more elaboration. For example, cyanidin and cyanidin-3-*O*-glucoside are anthocyanins that have been reported to activate the Nrf2 system [38]. However, it was not the intact anthocyanin, but a metabolite (phloroglucinol aldehyde) produced by gut Bacteria that activated the Nrf2/ARE system in one study [51]. Protocatechuic acid, cyanidin-3-*O*-glucoside, syringic acid, vanillic acid and gallic acid did not activate the Nrf2/ARE system by themselves. Their metabolites (produced by gut Bacteria) did. So, one's ability to activate the Nrf2/ARE system by consuming dietary anthocyanins and other phenolic compounds may depend on having a healthy gut microbiome [55].

There are also many compounds from Asian, African and American fruits, vegetables and natural remedies that activate the Nrf/ARE system [16, 37]. This includes extracts of *Withania somnifera* (Ashwagandha), *Sutherlandia frutescens* (Sutherlandia) and *Euterpe oleracea* (açaí) [37]. There is also at least one dietary supplement that appears to activate the Nrf2/ARE signaling system. It is called Protandim® and contains Ashwagandha, bacopa extract, green tea extract, silymarin, and curcumin

[56]. They appear to act synergistically [57]. However, one should be careful in controlling the doses of compounds and/or supplements that activate the Nrf2/ARE system. It has potentially deadly properties when over-activated [31, 32, 63-70]. The Nrf2/ARE system is over-activated in some forms of multidrug-resistant cancer and cardiovascular diseases [31, 32, 63-70]. It should also be noted that ellagitannins in pomegranates, walnuts, strawberries and other fruits can indirectly activate the ARE genes and exert many health effects [70, 71]. They are converted to urolithin A by select Bacteria in the gut. The structure of urolithin A is shown in Figure 3.

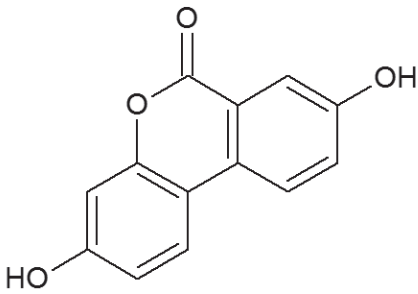

Figure 3. Structure of Urolithin A.

However, the ability to produce urolithin A can depend on the status of the gut microbiome [72]. It was produced by gut Bacteria in normal rats, but not in rats that had ulcerative colitis. Moreover, urolithin A increased the concentrations of probiotic bifidobacteria and lactobacilli, as well as probiotic strains of *Clostridium*. This prevented the colonization and invasion of colonic tissue by pathogenic enterobacteria [72]. However, the relative concentrations of different species of Bacteria in the gut and intestines are different in people who have excess abdominal fat and have metabolic syndrome [73]. The gut microbiome also tends to be healthier in vegetarians and vegans [74, 75]. So, ellagic acid and ellagitannins may or may not be metabolized to urolithin A very efficiently, depending on the status of the gut microbiome [76]. So, it is noteworthy that urolithin A is commercially available and that it has multiple health effects [77]. There is also a prescription drug for treating multiple sclerosis (dimethyl fumarate) that acts by activating Nrf2 [78].

There are also some compounds that can inhibit the Nrf2/ARE signaling system, instead of activating it [79-87]. Some of them are listed

in Table 1. It should be noted that three of them, EGCG, ascorbic acid and luteolin, are listed as both activators (Table 1) and inhibitors (Table 2). This is because they were tested at different concentrations. For example, it took >200 μM EGCG to inhibit the Nrf2/ARE system in human lung adenocarcinoma A549 cells *in vitro* [82]. It is almost impossible for concentrations of EGCG to ever be so high *in vivo*. That is, the maximum concentration of EGCG that was found in the blood plasma of human subjects in a pharmacokinetic study was 77.9±22.2 ng/ml, or about 0.17 μM [88]. Even when EGCG is inserted into nanoparticles, its maximum concentration in blood plasma was 704 ng/ml, or 1.5 μM [89]. Similarly, 0.83 μM luteolin was shown to activate the Nrf2/ARE system in hepatocytes that had been exposed to the carcinogenic dioxin, TCDD (2,3,7,8-tetrachlorodibenzodioxin), at a concentration of 0.2 nM [90]. On the other hand, 1 μM luteolin inhibited the Nrf2/ARE system in hepatocytes *in vitro* [80]. So, the effect of luteolin may depend on the type of cell to which it is administered. Similarly, the effect of ascorbic acid seems to depend on the type of cells to which it is administered. It activated the Nrf2/ARE pathway in rat RAW 264.7 macrophages when present at concentrations of 10 to 300 μM and increased the survival of endotoxemic mice at a dose of 300 mg/kg, *i.p.* [54]. On the other hand, 125 μM (0.125 mM) ascorbic acid restored the sensitivity of leukemia cells to the anti-cancer drug imatinib by inhibiting the Nrf2/ARE system [82]. In another study, 1000 μM ascorbic acid antagonized the activation of Nrf2/ARE caused by administering resveratrol to hepatocytes *in vitro* [91]. It specifically antagonized the endogenous antioxidant enzyme, heme oxygenase-1 (HO-1), which is an ARE that is activated by resveratrol. However, resveratrol still exerted its antioxidant effects by activating the antioxidant enzyme called paroxonase, which is not activated by Nrf2 [91]. It should be noted that relatively high concentrations of ascorbic acid in blood plasma can be obtained by consuming high doses of vitamin C as a dietary supplement. That is, when the daily oral dose was increased from 250 to 2500 mg, the concentration in plasma increased from 68 to 85 μM [21]. Moreover, liposomal and intravenous doses of ascorbic acid can produce plasma concentrations up to 400 and 15 000 μM, respectively [93].

Table 2. Partial list of dietary compounds that have been shown to inhibit the Nrf2/ARE signaling system.

Compound	Dietary Sources[a]	References
Ferulic acid	Many plant seeds and cell walls, including *Ferula foetida*	[54, 79]
Luteolin	Many foods, including celery and broccoli	[30, 80]
EGCG	Green tea and green tea extract	[30, 81]
Ascorbic acid	Vitamin C and citrus fruits	[30, 82]
Apigenin	Fruits, vegetables	[30, 83]
All-*trans*-retinoic acid	From β-carotene	[30, 84]
Brusatol	*Brucea javanica*	[30, 85]
Trigonelline	Fenugreek seeds	[30, 86]
Ochratoxin A	*Aspergillus*, *Penicillum*	[30, 87]

It is important to maintain a healthy balance in the amount of RONS and the redox state of cells [64]. So, the activity of the Nrf2-ARE antioxidant system must be turned on only when it is needed. Its activity is limited by the binding of an inhibitor protein called Keap1 [30]. So, it is sometimes called the Keap1-Nrf2-ARE signaling system. Under conditions of low oxidative stress, Nrf2 is bound to Keap1, which is anchored to actin in the cytoskeleton in the cytosol [30]. This complex makes the Nrf2 protein accessible to reaction with the ubiquitous protein called ubiquitin [94]. Ubiquination causes many transcription factors (including Nrf2) to be broken down (hydrolyzed) in subcellular organelles called proteasomes when DNA transcription should not be activated [94]. However, this breakdown of Nrf2 can be prevented by breaking the bonds between it and Keap1 [30, 94]. 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. These natural antioxidant elements include the enzymes superoxide dismutase (SOD), thioredoxin, thioredoxin reductase, sulfiredoxin, NAD(P)H:quinone oxidoreductase-1, HO-1, glutathione reductase, glutaredoxin, glutamate cysteine ligase, glutathione S-transferase, UDP-glucuronyl transferase, thioredoxin reductase, peroxiredoxin sulfotransferase and γ-glutamate cysteine ligase catalytic subunit [40, 95, 96]. In addition, the expression of over 500 genes is modulated by the

Nrf2/ARE pathway [40]. This includes phase I and II detoxfication enzymes, transport proteins, proteasome subunits, chaperones, growth factors and their receptors, as well as some other transcription factors [40].

This is an example of a biological regulatory process that enables metabolism to adapt to changes and the needs of the entire organism [40]. Such regulatory processes require a signal and a sensor to switch-on the adaptive process, a transducer, a modulator of sensitivity, an effector, and a way to switch the signal off. It is also important that such processes communicate (or crosstalk) with other signaling systems [40]. It should be noted that biochemists use the term crosstalk very differently than engineers. In electronics, crosstalk is when a signal transmitted by one circuit (such as a radio frequency transmitter) causes an undesirable effect (such as noise) in the other circuit (such as a radio frequency receiver). In biochemistry, crosstalk (communication) between signaling pathways is not just advantageous, but absolutely necessary to support life.

Keap1 is the redox sensor of the Keap1-Nrf2 system [23, 29, 30]. The reactive sulfhydryls in the cysteine residues of Keap1 can sense oxidative stress. Once it is released from the cytosolic complex with Keap1, Nrf2 becomes phosphorylated at Ser40, so it can enter the nucleus. Its activity can be decreased or enhanced by activating or inhibiting its export out of the nucleus. If Nrf2 becomes phosphorylated again – this time at Tyr568 – it can be exported out of the nucleus. There are nuclear export signals in the leucine zipper domain and transactivation domain of Nrf2. They can be blocked by binding to the musculo-aponeurotic fibrosarcoma protein [23, 29, 30].

So, Nrf2 undergoes translocational oscillations from cytoplasm to nucleus [97]. The frequency modulations in the oscillations are linked to the activation of cytoprotective responses caused by oxidative stress. So, the Nrf2 transcription factor functions like a wireless sensor. Under basal conditions, Nrf2 activity is repressed by binding to Keap1, which enables its degradation. This was initially interpreted as meaning that activators (like those listed in Table 1) stabilize Nrf2 and prevent its proteolysis, while increasing the concentration of Nrf2 protein in the cytoplasm. This leads to Nrf2 being translocated into the cell nucleus, where it activates the transcription of AREs. However, the activator concentration that produces half-maximal transcriptional response does not increase the intercellular Nrf2 protein concentration. Instead, translocational oscillations of Nrf2 increase in frequency, decrease in amplitude and activate ARE-linked genes. Nrf2 is then inactivated by acetylation, expelled from the nucleus and reactivated by reactions catalyzed by deacetylase and phosphatase enzymes in the cytoplasm. So, Nrf2 function is driven by its reactivation.

The oscillatory translocation of Nrf2 in and out of the nucleus provides for repeated sensing of the cytoplasmic stress status and direct conveyance of this to the site of ARE-linked gene transcriptional control. The frequency of stress surveillance is intensified when the host cell is challenged by the positive coupling of increased translocational frequency of Nrf2 to increased transactivational activity, the system samples the cytoplasmic stress status more frequently when homoeostasis is challenged. This increases the cell's ability to sense the stress status and trigger an increase in the ARE response when there is abnormal cell dysfunction and damage. This enables a balanced and well-regulated response to metabolic and environmental challenges and a quick return to homoeostasis. Moreover, inactivation of Nrf2 in the nucleus prevents abnormal accumulation of active Nrf2 and loss of transcriptional fidelity. This is analogous to engineering sensor in which dynamic resolution depends on a high refresh rate. This is because the sensor reassesses key aspects of the environment at an appropriately high rate. That is, Nrf2 goes through cycles of sense, response, inactivate and reset. This mechanism implies that there are several receptors for Nrf2 activation that may bind to low doses of stimulatory ligands [97].

So, oxidative stress and other primary signals are sensed by Keap1 [23, 29, 30]. They are transduced into the expression of AREs, modulated by phosphorylation of Ser40 and Tyr568 and turned off by nuclear export and subsequent destruction of Nrf2 in the proteasome. This is done by ubiquination. Several proteins are required, including a Cullin-3 based ligase (Cul3) that targets the Nrf2 protein in the Keap1-Nrf2 complex. The effectors of the primary signals are the target genes that code for AREs. The signals can be turned off by not just nuclear export, but also by other mechanisms. There are also Keap1 proteins in the nucleus. They can bind to Nrf2 and target it for degradation in nuclear proteasomes. The actin cytoskeleton must be polymerized for it to bind to the Keap1-Nrf2 complex. Cellular oxidants can activate the enzyme phosphatidylinositol 3-kinase (PI3K), which depolymerizes the actin. Re-polymerization allows Nrf2 to be exported from the nucleus. Moreover, actin can be covalently modified by the attachment of glutathione. This leads to the de-polymerization of actin. This can be prevented by Grx, which is a small redox enzyme that uses glutathione as a cofactor. The Keap1-Nrf2 signaling system also activates the transcription of DNA coding for proteins like Cul3, Rbx1 (ring box protein 1) and Keap1 that are cytosolic inhibitors of this system. Finally, there are many enzymes that can eliminate the system's signals or prevent them from being formed in the first place [23, 29, 30].

The Keap1-Nrf2 signaling system is also affected by crosstalk with other signaling systems [5, 94]. As mentioned previously, Nrf2 can be phosphorylated and dephosphorylated. This links it with protein kinases and phosphatases. In addition, it is affected by crosstalk with MAPK, casein kinase 2, the protein kinase R-like endoplasmic reticulum kinase, protein kinase C, PI3K and its partner, Akt [94]. That is, PI3K catalyzes the biosynthesis of phosphatidylinositol (3,4,5)-trisphosphate or PtdIns(3,4,5)-P_3, which activates Akt, also known as protein kinase B. It was named Akt because it was first found in a retrovirus called Akt8 [5]. In addition, the tumor suppressor protein p53 has antioxidant functions that include activating the transcription of the gene coding for Nrf2 and the proper maintenance of mitochondria function, which limits the production of RONS [98].

However, p53 and Nrf2 have many different effects on different types of cells and under different physiological conditions. For example, p53 activates ferroptosis, which Nrf2 inhibits [99]. Nrf2 is affected not only by the p53 protein, but also by several other signal transduction systems [100]. This includes the AMP-activated kinase (AMPK), which is a central hub in the network that controls cellular energy homeostasis. It decreases anabolism and increases catabolism, improves endothelial function, reduces inflammation, and improves redox balance. Moreover, AMPK works and communicates with (crosstalks) with the Nrf2 system to protect cells from damage caused by unfolded proteins. This causes an unfolded protein response (UPR) that counteracts ER stress. AMPK and Nrf2 interact to support the UPR and prevent cardiovascular diseases. Tight cooperation between AMPK and Nrf2 controls cellular redox, energy and protein homeostasis [93].

Normal vascular function is important for cardiovascular health. This requires continual turnover of proteins, which is done in proteasomes [101]. Protein turnover is needed to help regulate signaling cascades by controlling the concentrations of transcription factors. It also allows damaged proteins to be replaced, thus preventing cellular oxidative damage. Proteasomal dysfunction in aging and atherosclerosis may cause vascular dysfunction. This can prevent proteasomes from removing oxidized proteins, producing large protein aggregates. They are extensively cross-linked and can be further modified by advanced glycation end products, lipid peroxides or ubiquitin, preventing protein unfolding and consequently degradation by the proteasome. Moreover, protein aggregates can inhibit proteasomal activity directly [101].

Nrf2 also interacts with a protein deglycase called DJ-1 (also known as Parkinson disease protein 7) [102]. It protects neurons from oxidative

stress and aggregation of the protein α-synuclein, which can lead to Parkinson's disease. DJ-1 also acts as a natural antioxidant by activating the Nrf2/ARE system. It does this by binding to Keap1, preventing it from inhibiting Nrf2 [102].

Nrf2 also interacts and crosstalks with the Notch signaling pathway [103]. The Notch signaling pathway influences the cell cycle as well as cellular differentiation, survival, proliferation and apoptosis. It transduces primary signals at the cell membrane of target cells. It goes into the nucleus to activate the expression of several genes. The exact responses depend on the type of cells and their needs. The Notch pathway exerts pleiotropic effects in each tissue that expresses the Notch protein. Thus, Notch-signaling networks regulate various events in embryonic and postnatal development. Like the Nrf2 signaling system, Notch is conserved from worms (*Caenorhabditis elegans*) to humans. They can be regulated by reciprocal transcription. That is, Notch1 targets the expression of the gene coding for Nrf2 and Nrf2 targets Notch expression. Nrf2–Notch crosstalk protects against endogenous and exogenous stressors by activating the expression of defense systems. This leads to cytoprotection, while maintaining cellular homeostasis and tissue organization. These effects may vary between different tissues and within specific regions, such as the niche where adult tissue stem cells or progenitor cells reside [103].

Even though the Keap1-Nrf2-ARE signaling system exists in so many animals, the level of its activity is quite variable [104]. It is much more active in the relatively long-lived naked mole-rat (*Heterocephalus glaber*) than in other rodents with shorter lifespans. Moreover, species that live longer are more resistant to both chronic and unpredictable stressors. They are also more resistant to age-related diseases, including cardiovascular diseases. However, it is not the concentration of Nrf2 itself that controls its total cellular activity. Instead, it is the concentrations of Keap1 and the β-transducin repeat containing protein (βTrCP), both of which target cytosolic Nrf2 for proteolytic destruction. So, it was suggested that βTrCP could be a good therapeutic target. It is conserved in mice, mole-rats and humans. It could be a better target than Keap1, since low concentrations of it produce fewer harmful side effects than those caused by low levels of Keap1 [104].

However, the βTrCP protein does not act in isolation [105]. As mentioned previously, phosphorylation of serine residues in Nrf2 enable it to dissociate from the complex with Keap1 and enter the nucleus. There are several protein kinases that can catalyze this phosphorylation. They include protein kinase C, RNA-like endoplasmic reticulum kinase, casein

kinase 2, the SRC (sarcoma) family of protein kinases and glycogen synthase kinase-3 (GSK-3). In addition, the PI3K-Akt signaling system induces the expression of one of the genes coding for an ARE, HO-1. The PI3K-Akt signaling system also enables Nrf2 to sustain cell proliferation by reprograming glucose and glutamine metabolism. It does this by first targeting glycogen synthase kinase-3 (GSK-3). GSK-3 catalyzes the phosphorylation of the SRC-related kinase, FYN. This tyrosine kinase is translocated to the nucleus, where it catalyzes the phosphorylation of Nrf2 at Tyr568. This targets the phosphorylated Nrf2 for nuclear export and degradation in the cytosol. The β-TrCP protein recognizes phosphorylated Nrf2 and targets it for ubiquination and proteolysis. So, Keap1 and β-TrCP have been described as limiter and regulator valves, respectively. They control the movement of Nrf2 in and out of the nucleus of the cell. Under normal redox homeostasis and the absence of stimulation by a growth factor, they both act to limit the flow of Nrf2 into the nucleus. Under normal redox homeostasis, but in the presence of signaling by a growth factor, the Keap1 'valve' stays closed while the β-TrCP 'valve' opens to release a small percentage of the Nrf2 for entry into the nucleus. During both redox imbalance and receptor signaling, the Keap1 and β-TrCP 'valves' open the flow of Nrf2 into the nucleus. This combination is unlikely under normal physiological conditions, but could be caused by pharmaceutical intervention. That is, drugs might be developed that could reduce the concentration of Nrf2 by targeting the GSK-3/β-TrCP system [105].

2.4 The role of the Keap1-Nrf2-ARE signaling system in preventing cardiovascular diseases

The Keap1-Nrf2-ARE signaling system can prevent cardiovascular disease (CVD) by preventing smoldering inflammation. It does this by activating the natural antioxidant systems in cells. Smoldering inflammation is a chronic, relatively low level of inflammation that is caused by an excess of RONS and free radicals [5]. The heart requires much energy that is produced mostly by mitochondrial oxidative phosphorylation [29]. It consumes more energy than any other organ. Even when resting, it uses about 8–15 ml O_2 min/100 g heart. This can increase to as much as 70 ml min/100 g heart when exercising vigorously. Every day the adult heart beats about 100 000 times, pumping approximately 10 tons of blood throughout the body, and recycling around 6 kg of ATP. The RONS and reactive nitrogen compounds produced as by-products can cause inflammation if they are not destroyed effectively [29].

So, inflammation plays an important role in atherosclerosis and the emergence of CVD [5]. Atherosclerosis is the main process underlying CVD. It starts when endothelial cells that line the intima are activated by saturated fatty acids and/or cholesterol. It leads to the expression of adhesion proteins on leukocytes, making them bind to the endothelium. Once they are bound, they can migrate through the endothelium to the intima where they can attract monocytes that can change into lipid-laden foam cells. This process is often enhanced in people who have type-2 diabetes. After immune cells and inflammatory mediators interact, atheroma (degeneration arterial walls) can emerge and rupture-prone atherosclerotic plaques are made. Pro-inflammatory signaling pathways are also involved in thrombosis, the late stage of atherosclerosis. It is responsible for most of the clinical complications of CVD. So, CVD can be a major consequence of obesity-induced inflammation and type-2 diabetes [5].

Inflammation is more important than cholesterol concentrations in causing CVD [5]. About half of all heart attacks and strokes occur in people with normal or even low concentrations of cholesterol in their blood. Normal, healthy endothelial cells (ECs) on the innermost surface of arterial walls are able to resist adhesion by leukocytes [106]. When a person smokes tobacco, consumes too much saturated fat, is hyperglycemic or resistant to insulin, and has metabolic syndrome with high blood pressure, adhesion molecules are expressed by ECs [5]. This enables leukocytes to attach to the arterial wall. VCAM-1 then binds to monocytes and T lymphocytes, which are found in early atherosclerotic plaques. This can be prevented by laminar blood flow, which activates some anti-atherosclerotic mechanisms. This includes the expression of the natural anti-oxidant, superoxide dismutase and an increase in nitric oxide (NO) synthase [5]. There is also an increase in the concentration of NO, which causes vasodilation and limits the expression of the gene coding for VCAM-1 [5, 107].

So, it is important to note that the Keap1-Nrf2-ARE system can sense shear stress in ECs and protect against vascular dysfunction and atherosclerosis [106]. Nrf2 is highly sensitive to laminar fluid shear stress, which interacts with the epithelium to maintain vascular homeostasis. This is done by linking biomechanical forces with signal transduction to maintain a balance in the redox state. ECs respond to changes in shear stress to modulate redox signaling. This leads to changes in the expression of AREs, the inflammatory phenotype and cell alignment as well as structural remodeling of blood vessels. Also, when ECs are exposed to oscillatory disturbed shear forces, the expression of histone deacetylases

(HDACs) is induced. One of the HDACs catalyzes the deacetylation of Nrf2, thus decreasing its activity. There is another important epigenetic mechanism that affects Nrf2 activity. Redox-sensitive microRNAs (miRNAs) can modulate the concentrations of Nrf2 and some of the regulators of Nrf2 signaling. The expression of these miRNAs is different in laminar compared to oscillatory fluid shear stress. Thus, there are several mechanisms by which Nrf2 can react to fluid shear stress and help prevent CVD [106].

Another way that Nrf2 helps to prevent CVD is by protecting mitochondria from oxidative stress [108-111]. Cardiomyocytes have more mitochondria than any other type of cell [110]. However, they produce H_2O_2 as a byproduct of oxidative phosphorylation. As mentioned before, the Keap1-Nrf2-ARE signaling system activates the production of natural antioxidants. This includes glutathione, thioredoxin, and NADPH [109]. Nrf2 also upregulates the transcription of genes that code for uncoupling protein 3 (UCP3). Nrf2 influences mitochondrial biogenesis by maintaining the concentrations of nuclear respiratory factor 1 and peroxisome proliferator-activated receptor γ coactivator 1α, as well as by promoting purine nucleotide biosynthesis. When some of the mitochondria become irreparably damaged, Nrf2 stimulates mitophagy [111].

In healthy cells, mitochondria exist in elaborate networks and provide cells with ATP by oxidative phosphorylation of nutrients through a series of protein complexes. In this process, protons (H^+) and electrons are separated [112]. Electron transport is coupled to the active transport of H^+ across the inner mitochondrial membrane. This is accompanied by a proton gradient that helps make ATP. An electron transport chain is coupled to this proton motive force. However, this process is not completely coupled. Some protons leak through the inner mitochondrial membrane in a process that is mostly controlled by five uncoupling proteins, UCP1 – UCP5 [112-114]. This decreases the membrane potential and helps to limit the production of excess RONS by mitochondrial complexes I and III [113]. UCP4 and UCP5 are primarily located in neurons [114]. UCP1 is an important adaptor of thermogenesis in brown adipose tissue in mammals [112-114]. UCP2 is expressed in white adipose tissue, liver, and cardiac and skeletal muscle, while UCP3 is mostly expressed in brown adipose tissue and skeletal muscle, and at lower levels in cardiomyocytes (cardiac muscle cells) [113]. The expression of UCP2 increases as the concentration of RONS increases, subsequently producing a negative feedback that limits the production of RONS [111]. Both UCP2 and UCP3 help control the production of RONS and the oxidative damage that they can produce in the heart if not properly controlled [111]. Nrf2

can protect mitochondria and prevent myocyte death by activating the transcription of UCP2 and UCP3 [111, 113].

Nrf2 also activates HO-1, which prevents apoptosis in cardiomyocytes [108]. That is, when HO-1 is over-expressed, it can produce carbon monoxide (CO), which stimulates SOD and mitochondrial H_2O_2 production. This activates protein kinase B (more frequently known as Akt), which deactivates glycogen synthase kinase 3-β, which allows more Nrf2 to be released from Keap1 and be translocated to the cell nucleus. The accumulation of nuclear Nrf2 opposes apoptosis and necrosis caused by the anti-cancer drug, doxorubicin. Even though higher concentrations of CO are toxic, lower concentrations can be healthy. That is, CO can bind to the reduced *a3* heme of cytochrome *c* oxidase and increase H_2O_2 production. Despite its toxicity at higher concentrations, H_2O_2 is important in signal transduction and production of more mitochondria [108].

The Keap1-Nrf2-ARE signaling system supports the activity of another subcellular organelle – the endoplasmic reticulum, or ER [115]. In CVD and other diseases, unhealthy changes in cells can lead to ER dysfunction and an abnormal accumulation of unfolded proteins. That is, for proteins to function properly, they must fold into a specific structure and not exist as unfolded, random coils that are not very soluble in the cytosol or cell membranes. The folding process is partly controlled by the ER. However, when the protein-folding capacity of the ER is overwhelmed, ER stress and cardiac hypertrophy ensue. Moreover, a reduction in blood flow caused by atherosclerotic coronary artery disease and hypoxia can induce ER stress. Transmembrane sensors in the ER detect the accumulation of unfolded proteins. They activate transcriptional and translational pathways that deal with unfolded and misfolded proteins. When the UPR fails to control the concentrations of unfolded and misfolded proteins in the ER, apoptosis is induced. Interventions against ER stress as well as activation of the Keap1-Nrf2-ARE system reduce myocardial infarct size and cardiac hypertrophy in the transition to heart failure. Finally, activation of the Keap1-Nrf2-ARE system may be important in ischemic preconditioning, in which the heart is subjected to one or more episodes of nonlethal myocardial ischemia-reperfusion before coronary artery occlusion can occur [115].

It should also be noted that statins are administered to patients experiencing myocardial infarctions or CVD [106]. They were developed based on their ability to inhibit the rate limiting step in cholesterol synthesis, catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase. Since being introduced to the clinic, many other healthy effects of statins have been discovered. This includes stabilizing plaque, maintaining

endothelial function, anti-inflammatory actions and antioxidant capabilities. Recently, it has been shown that statins can also activate the Keap1-Nrf2-ARE signaling system [106].

The Keap1-Nrf2-ARE signaling system is also important in maintaining the renewal of cardiomyocytes [29]. That is, for many decades, the heart was thought to be a post-mitotic organ. It is now known that some cardiac remodeling can occur – especially during aging. The adult heart contains cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells and extracellular matrix proteins. Proper heart function depends on healthy cardiomyocytes and the sarcomeres in them that are formed by contractile proteins. Even though the number of cardiomyocytes virtually does not change in adulthood, the amount of sarcomeres does. The change is variable and can be modified as an adaptive response to stress conditions. This can lead to many biochemical and functional changes, such as alterations in Ca^{2+} handling, signaling cascades and energy metabolism. However, cardiomyocytes become more susceptible to oxidative stress during the aging process, resulting in necrotic and apoptotic cell death. The decrease in the number and proper function of cardiomyocytes leads to age-related changes in hearts that are also associated with augmented remodeling processes. This causes an increased heart size, a change from elliptical to spheroid shape, left ventricle wall thickening and increased systolic pressure. Cardiac remodeling in the elderly is often accompanied by interstitial and perivascular fibrosis, thickening of coronary vessels and increased calcification in the myocardium. The loss of functional cells can decrease the regenerative activity of the heart from 1% per year at 20 years of age to 0.4% per year at 75 years. A decrease in the number of sinoatrial node cells is also associated with aortic stenosis development in 2% of aged adults. This can lead to attenuated diastolic function and cardiac output, decreased maximum stroke volume, lower circulating blood volume and increased arterial stiffness. However, Nrf2 can help protect against CVD. On the other hand, age-related deregulation of the Keap1-Nrf2-ARE signaling system causes an increase in oxidative stress in cardiomyocytes and the vascular system. So, specific compounds (Table 1) that activate this signaling system may help prevent CVD, type-2 diabetes, renal failure and neurodegenerative diseases [29].

However, like so many things in life, the Keap1-Nrf2-ARE signaling system must be balanced. That is, even though it may be quite healthy to activate it to a limited extent by consuming dietary antioxidants at the concentrations that they occur in foods, it should not be over-activated [29, 31, 58, 65, 66, 117-119]. For example, some RONS are needed to help

control normal insulin signal transduction and glucose-stimulated insulin secretion in pancreatic β-cells. So, persistent activation of the Keap1-Nrf2-ARE system can prevent the required RONS signaling. Some of the detrimental effects of overactive Keap1-Nrf2-ARE signaling include worsening insulin resistance, impairing lipid accumulation in adipose tissue, and increasing hepatic steatosis in leptin-deficient mice. In addition, some oxidative modification of proteins is needed for proper ubiquitination and protein degradation. If the Keap1-Nrf2-ARE system is over-activated, it can decrease necessary protein oxidation, chronic reducing stress, deubiquitination and downstream protein degradation pathways. This can cause cardiac hypertrophy and remodeling [117]. Reduction stress can occur when there is an imbalance between oxidants and antioxidants, in favor of the latter [65]. It was originally defined as an excess of NADH, but now is known to include other reducing agents, such as NADPH and reduced glutathione. An excess of biochemical reducing agents can lead to damaged lipid membranes, deposition of triacylglycerides (triglycerides), mitochondrial dysfunction, cytotoxicity, cardiac ischemic injury and an increased risk of Alzheimer's disease [65].

Finally, multi-drug resistant cancers often have an overactive Keap1-Nrf2-ARE signaling system [117]. So, even though the lower concentrations of dietary antioxidants that are present in green tea as well as many fruits and vegetables may help prevent CVD and cancer, the much higher concentrations and doses in dietary supplements may help cause CVD and multi-drug resistant cancer.

2.5 Role of the Nrf2/ARE antioxidant system in cancer and neurodegenerative diseases

The Nrf2/ARE antioxidant system is also important in cancer since it prevents oxidative damage and therefore many types of cancer [120, 121]. This is one way that phytochemicals in many fruits and vegetables help to prevent cancer. The hormone melatonin also stimulates the expression of phase II enzymes, which detoxify carcinogens [121]. It also stimulates the Nrf2/ARE system, which helps prevent prostate, ovarian and breast cancers [122-127]. Like many natural products and several highly effective prescription drugs, melatonin has various therapeutic targets. It inhibits the initiation, progression and metastasis of many types of cancer [122]. At the same time, it has potent antioxidant and anti-apoptotic effects on healthy cells [126]. Conversely, melatonin has pro-oxidant as well as anti-proliferative, anti-angiogenic and immunomodulatory properties in many hormone-dependent cancers. It helps regulate the expression of the

estrogen receptor. It also affects the activities of protein kinases, calcium/calmodulin signaling, the cellular redox state, cytoskeletal reorganization and function, as well as fatty acid metabolism. It also suppresses some types of intracellular signal transduction, while activating others [126]. For example, it activates the SIRT1/PGC-1α pathway, which stimulates biosynthesis of more mitochondria [128]. So, melatonin is a remarkable hormone that is able to exert just the right effects on the right targets to help maintain good health.

Not only melatonin, but also proteasome inhibitors like bortezomib (Velcade®, FDA-approved for treating people who have multiple myeloma) suppress the degradation of Nrf2 and enhance its translocation into the nucleus [128]. Proteasome inhibitors upregulate the body's natural antioxidant and detoxifying enzymes by stimulating the transcription of the genes that code for them. This includes superoxide dismutase (SOD), glutathione peroxidase (GP), hemoxygenase 1 (HO-1), and NADPH:quinone oxidoreductase (NQO1). Melatonin modulates the activities of three separate substrates (Nrf2, NF-κB and Bcl2/Bax) when it inhibits the proteasome [128].

However, as in the heart, the Nrf2/ARE system can have deadly effects when it is over-activated in cancer cells [129-134]. That is, the effects of an activated Nrf2/ARE system depend on context, or where it occurs, and on the degree to which it is activated [129]. Its effects depend on the stage of the cancer, or the extent to which it has developed. Fully malignant cells are autonomous. They are very different from dysplastic (but not yet fully neoplastic) cells in a premalignant lesion. Premalignant cells are under much greater control from inflammatory cells and other stromal cells in their microenvironment. Moreover, they don't yet have enough DNA damage to make them autonomous. So, increasing Nrf2 activity, which would decrease both inflammatory and further oxidative or mutagenic stress, appears to be beneficial during premalignant states and may help limit further carcinogenesis. So, higher Nrf2 activity can be anti-carcinogenic in the early stages of tumorigenesis, when the human body is still trying to control premalignant carcinogenesis. However, it can become pro-carcinogenic when it makes fully malignant cancer cells become resistant to treatment [129]. The prognosis of patients with tumors that have very an active Nrf2/ARE system is poor because it increases cancer cell proliferation and promotes chemoresistance and radioresistance [130]. Also, Nrf2 regulates the expression of the multidrug resistant protein-3 (MRP3) in both human bronchial epithelial and non-small cell lung cancer (NSCLC). This protein, when combined with upregulated detoxification enzymes like glutathione S-transferases (GSTs), can lead to

the increased hydrophilicity of the cell membrane. This makes it easier for the cancer cells to excrete many anticancer drugs, including chlorambucil, cisplatin, etoposide, and doxorubicin [130].

Nrf2 also reprograms metabolic pathways towards anabolism, while augmenting purine synthesis and influencing the pentose phosphate pathway [131]. That is, Nrf2 redirects glucose and glutamine into anabolic pathways. Under physiological conditions, Nrf2 signaling is turned on by the presence of oxidative stressors in the cellular microenvironment, but is rapidly deactivated. However, under pathological conditions the tight regulation of Nrf2 changes. There is less responsiveness to cell stressors. At the same time, when Nrf2 is over-activated mammalian cells under adverse conditions have a survival advantage. This applies to cancers of liver, lung, colorectal, pancreas, prostate, gall bladder and ovaries [131].

The Nrf2 protein targets at least 18 genes in ovarian cancer cells, including one that codes for the ATP-binding cassette (ABC) transporter, ABCF2 [133]. ABC proteins are in a superfamily that uses the hydrolysis of ATP to power the transport of foreign chemicals, including anticancer drugs. There is a promoter region near the *ABCF2* gene that is an ARE. So, Nrf2 activates the *ABCF2* gene by targeting this promoter. The ABCF2 protein plays an essential role in the development of cisplatin resistance in ovarian cancer. So, targeting ABCF2 may be a new strategy to prevent such resistance and make cisplatin treatments more effective and long-lasting [133].

Since oxidative damage is also an important cause of neurodegenerative diseases, the Nrf2/ARE antioxidant system plays an important role in preventing them [37]. So, not only do many fruits, vegetables and spices have phytochemicals in them that prevent neurodegenerative diseases by activating the Nrf2/ARE system [37], but so does melatonin [135-137]. In addition, antidepressants activate the Nrf2/ARE system [138].

Melatonin can also help as an adjunct to other treatments for posttraumatic stress disorder (PTSD), in which many patients lose proper control of their circadian rhythm – especially when they have recurrent nightmares [136]. This adversely affects their neuroendocrine immune systems. It affects their sleep, cognition, memory, metabolism, pain, neuroimmunomodulation, stress endocrinology and physiology, as well as circadian gene expression, oxidative stress and epigenetics. Adding melatonin to the treatment of patients with PTSD can help them get their badly needed restful sleep [136].

2.6 Smoldering inflammation

There is something called para-inflammation, low grade or smoldering inflammation [5, 139]. It is also known as metabolic inflammation or meta-inflammation [140] and chronic inflammation [141]. It is a relatively low level of inflammation that is caused by obesity and/or periodontal disease. It can lead to diseases such as type-2 diabetes, many forms of cancer, asthma, and atherosclerosis [5, 139-141]. However, most of the experiments that showed this were done on mice which were kept in the cold, below their thermoneutral zone [142]. So, it was important that a recent study showed that thermoneutral housing strongly affects the development of metabolic inflammation, insulin resistance, and atherosclerosis. Mice housed at these more comfortable, warmer conditions tend to develop metabolic inflammation in adipose tissue and in the vasculature at an accelerated rate. However, this increased inflammatory response contributes to the progression of atherosclerosis but not insulin resistance. Also, obesity can cause smoldering inflammation in white adipose tissue (WAT) by recruiting macrophages. "These findings not only suggest that metabolic inflammation can be uncoupled from obesity-associated insulin resistance, but also point to how thermal stress might limit our ability to faithfully model human diseases in mice" [142].

So, studies done on human subjects often are more informative. A recent study showed a clear link and probable cause and effect relationship between inflammation and cancer [143]. An anti-inflammatory drug, canakinumab, not only reduced the risk of cardiovascular complications in heart attack victims, but also reduced the risk of cancer – including lung cancer. The incidence of cancer in patients who received the highest dose of canakinumab decreased by 67%. It targets IL-1β and the NLRP3 pathway in inflammation, which is in inflammasomes [5]. Note that NLRP3 stands for NOD-like receptor family pyrin domain containing 3. Inflammasomes regulate early adipose tissue inflammation. Also, inflammasomes are pattern-recognition receptors that assemble into larger structures that control the maturation and secretion of proinflammatory interleukins such as IL-1β. NLRP3 releases the cysteine protease caspase-1, which catalyzes the conversion of procytokines into their mature active forms. The expression of NLRP3 increases in obesity. Several endogenous stress signals, including glucose, palmitate, cholesterol crystals, islet amyloid peptides and RONS, can induce inflammasome activity [5].

In many cases, these diseases of inflammation can be prevented by avoiding periodontal disease (see Chapter 2, volume 1), obesity, *trans* fats

and saturated fats that are in the typical fast food diet that many people in the USA consume. Instead, unsaturated fats are much better [5]. Lipoxins are made from arachidonic acid, but the resolvins and protectins are made from omega-3 fats, such as alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fats can be taken as dietary supplements such as fish oil. They are also present in fatty seafood, such as salmon and scallops. Eskimos eat lots of fatty fish, so they have a very low incidence of arthritis and heart disease [5].

Inflammation is important in all stages of heart disease, from its beginning, development and final, tragic stages [5, 144]. Proper diet and cardiovascular exercise can limit smoldering inflammation and help prevent heart disease. Lipoxin A4 is important in asthma, a prevalent disease of chronic inflammation [5, 145]. It helps regulate the activation of natural killer (NK) cells and type 2 innate lymphoid cells (ILC2s) [5, 146]. ILCs express high levels of cytokines that are important in the pathogenesis of the inflammatory response. There are also type 1 and type 3 ILCs (ILC1 and ILC3). ILC1s include NK cells and interferon-γ (IFN-γ)–producing ILC1s. Group 3 ILCs produce IL-17A and/or IL-22. All ILCs express the IL-2Rα (CD25) and IL-7Rα (CD127) subunits, as well as the common γ chain (CD132), which is the other subunit for the receptors for IL-2 and IL-7. ILC2s also express the receptors for IL-33 (ST2) and IL-25 (IL-17RB). ILC2s cells secrete high levels of interleukin 5 (IL-5) and IL-13 in response to the epithelial-derived cytokines IL-25 and IL-33 as well as thymic stromal lymphopoietin. IL-5 and IL-13 are crucial for causing the allergic response. Both have been targets of monoclonal antibodies used in human trials for treating asthma [5, 146]. IL-5 is the most potent growth, differentiation, and survival factor for eosinophils and is important in eosinophil chemotaxis. IL-13 has a role as a central mediator in asthma pathogenesis. ILC2s secrete IL-13 in response to prostaglandin D_2 (PGD_2) by activating a GPCR. They also express the receptor for lipoxin A_4. NK cells are highly activated in severe asthma. They down-modulate the airway inflammatory response by inducing apoptosis in eosinophils. On the other hand, ILC2s promote airway inflammation by secreting interleukin-13 (IL-13). Despite their opposite functions, both NK cells and ILC2s express the receptor for lipoxin A4. In fact, lipoxin A4 increased the ability of NK cells to induce eosinophil apoptosis and decreased IL-13 production by ILC2 cells. Lipoxins are anti-inflammatory for neutrophils and eosinophils and may help 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. So, lipoxin D_4 receptor agonists and/or synthetic lipoxin A_4 analogues might be good therapeutic agents for asthma [5, 145, 146].

Inflammation plays an important role in all stages of atherosclerosis and cardiovascular disease [5, 144]. Atherosclerosis is the main process underlying macrovascular disease. It starts when endothelial cells that line the intima are activated. This can be induced by saturated free fatty acids or cholesterol, but it leads to the expression of leukocyte adhesion molecules. So, leukocytes bind, then migrate through the endothelium to the intima where they can attract monocytes which ultimately transform into lipid-laden foam cells. This process can be enhanced in people who have type-2 diabetes. After immune cells and inflammatory mediators interact, the process can continue to atheroma and make rupture-prone atherosclerotic plaques. Inflammatory pathways are also involved in thrombosis, the late stage of atherosclerosis. It is responsible for most of the clinical complications of macrovascular disease [5, 144]. So, macrovascular disease can be a major consequence of obesity-induced inflammation and type-2 diabetes [5, 147]. Inflammation may be more important than cholesterol levels in causing heart disease. Fifty percent of all heart attacks and strokes occur in people with normal or even low levels of cholesterol [148]. Normal, healthy endothelial cells (ECs) on the innermost surface of arterial walls resist adhesion by leukocytes [5, 144]. Smoking, consuming large amounts of saturated fats, obesity, high blood pressure, hyperglycemia and insulin resistance lead to the expression of adhesion molecules (such as vascular cell adhesion molecule-1, or VCAM-1) on ECs. This enables leukocytes to attach to the arterial wall. VCAM-1 binds to monocytes and T lymphocytes, which are found in early atherosclerotic plaques. Oxidized lipids, NF-κB and TNF-α can also stimulate VCAM-1 expression. They can be prevented by laminar blood flow, which has some anti-atherosclerotic effects. This includes expression of the natural anti-oxidant, superoxide dismutase and an increase in nitric oxide (NO) synthetase. These cause an increase in the concentration of NO, which causes vasodilation and limits VCAM-1 gene expression [5, 144].

However, when blood flow is disturbed, lesions can form, leading to the binding of monocytes to the arterial endothelium [5, 144]. Monocytes subsequently enter the vessel walls, causing a fatty streak to start developing. This is the first stage of atherosclerosis. Next, monocytes mature into macrophages. They express scavenger receptors and engulf modified lipoproteins. Cholesteryl esters accumulate in the cytoplasm while macrophages change into foam cells, which have an increased concentration of lipids. Next, T-lymphocytes of the adaptive immune

system enter the arterial cell walls and cause more inflammation. They are attracted to the arterial cells by chemokines that are induced by interferon-γ [144].

The next stage is progression to the formation of plaque, which can begin early in life, when a person is still in their early twenties [5, 144]. Several cytokines, including IL-1β, IL-1α, IL-6, IL-18, TNF-α and TNF-β have important roles in this process, as are the macrophage colony stimulating factor (MCSF) and the monocyte chemoattractant protein-1, MCP-1. Eventually the plaques rupture, leading to thrombosis. In healthy arteries, there is a fibrous cap that contains collagen to make them strong and resilient. The cap keeps blood from reaching the lipid core. When the cap ruptures, a thrombus or blood clot forms. Inflammation interferes with the integrity of the collagen matrix by blocking the creation of new collagen fibers and by destroying existing collagen. T-lymphocytes participate in this pro-inflammatory process. They produce CD40, a cell surface protein, and IL-1. They increase the production of enzymes in macrophages that catalyze the destruction of collagen. The CD40 ligand also stimulates macrophage production of tissue factor VII, which initiates the coagulation cascade [5, 144].

Frequently, these harmful events can be prevented by lowering the risk factors by not smoking, engaging in regular strenuous cardiovascular exercise and by consuming a healthy diet, with fewer simple carbohydrates, no red meat and more fresh fruits and vegetables [5, 144]. Since this is not possible for many people, physicians often monitor an important biomarker for generalized inflammation - the high-sensitivity C-reactive protein, or hsCRP. It binds to phosphatidylcholine that is present on the exterior surface of dead or dying cells and activates the complement system of the innate immune system [5, 144]. In healthy postmenopausal women, it is a more accurate predictor of risk for a heart attack than total cholesterol or LDL [5, 148].

Obesity is also linked to smoldering inflammation in adipose and liver tissues [5, 149]. This can lead to insulin resistance and type-2 diabetes through the pro-inflammatory nuclear factor-κB (NF-κB), which is activated by the phosphorylation of the regulatory protein IκB by IκB kinases (IKKs). One of these kinases, IKKε, is elevated in adipose tissue in people who consume a high fat diet. In an attempt to identify a small molecule inhibitor of IKKε, a library of 150 000 compounds was screened and one of them (amlexanox) bound with high affinity to the IKKε receptor. This prescription drug is approved for treating asthma allergic rhinitis and aphthous ulcers. When it was given to mice on a high fat diet, it “prevented weight gain, improved insulin sensitivity, attenuated hepatic

steatosis, reduced adipose tissue inflammation and promoted energy expenditure in adipose tissue through increased thermogenesis" [5, 149].

Another group studied the receptor activator of NF-κB ligand (RANKL), a potent activator of NF-κB that is involved in bone homeostasis [5, 150]. They showed that binding of RANKL to its receptor in the liver activates NF-κB and promotes pro-inflammatory cytokine expression in the liver. On the other hand, blocking RANKL signaling improved insulin resistance in the liver and prevented diabetes [5, 150]. Moreover, RANKL antagonists such as denosumab have been proven to be safe in patients and are currently used to treat osteoporosis. So, they may be effective in breaking the link between inflammation and diabetes [5, 149].

Inflammation is also important in neurodegenerative diseases. Omega-3 fats are very important anti-inflammatory nutrients that are important in the brain, where they play an important role in cognitive function and behavior. The typical American (USA) fast food diet contains little or no omega-3 fats [151]. Instead it contains high levels of *trans* fats and saturated fats, which increase the risk of heart disease and may be partly responsible for the poorer academic performance of children in the USA, compared to other countries that don't eat so much unhealthy food.

Inflammation is also an important factor in Alzheimer's disease [5, 152, 153]. This was shown recently in a continuation of the Framingham study [5, 152]. The goal of the Framingham study was (and still is) to monitor the health of thousands of people for decades, and for several generations. Even though the original goal was to see if there were some factors or lifestyles that increase one's susceptibility to cardiovascular diseases, it has also looked at Alzheimer's disease and dementia. The health of thousands of people has been monitored. It was found that people who had higher concentrations of pro-inflammatory cytokines in their blood were more likely to get Alzheimer's disease [5, 152]. It has also been shown that people who take anti-inflammatory NSAIDs earlier in life have a lower incidence of Alzheimer's disease [5, 153]. However, once a person gets Alzheimer's disease, NSAIDs are no longer effective [5].

Inflammation is also an important factor in stroke, which can be caused by the temporary lack of blood flow to the brain (ischemia), followed by re-perfusion. Ischemia-reperfusion causes glia and astrocytes to become activated and produce RONS, which cause oxidative damage and inflammation. Dietary antioxidants can help prevent this [154].

RONS, free radicals and inflammation are also involved in cancer [5, 155, 156]. In the 19th century, doctors observed that tumors often arose at sites of chronic inflammation [5, 157]. In the 20th and 21st centuries,

epidemiological studies found that smoldering inflammation is an important risk factor in many types of cancer. At the same time NSAIDs have been shown to help prevent cancer [5, 157]. Damage to DNA by RONS is one of the widely accepted causes of cancer [5, 155]. Most mutations caused by RONS convert deoxyguanosine into deoxythymidine (G → T). Also 8-hydroxydeoxyguanosine can accumulate when one is exposed to nitrosamines and polynuclear aromatic hydrocarbons produced by smoking tobacco. Some viruses, such as hepatitis B and C, can also cause chronic inflammation and liver cancer. Moreover, G → T mutations can be caused by aflatoxins, which are known human carcinogens. Some types of cancer cells also produce RONS of their own. It can be induced by oncogenes, such as *RAS* [5, 155]. Also, pro-inflammatory cytokines, such as IL-1β, IL-6, IL-23 and TNF-α are produced after genes are activated by transcription factors, such as transcription factor nuclear factor NF-κB and STAT3, or signal transducer and activator of transcription activator 3 [5, 157]. NF-κB is a coordinator of innate immunity and inflammation and is a tumor promoter. It activates the transcription of genes that code for pro-inflammatory cytokines, adhesion molecules, enzymes (such as COX and inducible nitric oxide synthase, iNOS) and angiogenic factors [5, 157].

It is also important to consider tumor-associated macrophages, or TAMs [5, 157]. They infiltrate most, if not all, tumors. They usually have the M2 phenotype and promote tumor growth and angiogenesis, as well as remodel tissues and suppress adaptive immunity. In the process, vascular endothelial growth factor (VEGF) stimulates angiogenesis and attracts monocytes into both the primary and the metastatic tumor. Then the monocytes secrete more pro-angiogenic factors, which stimulate angiogenesis further. Chemokine receptors and their ligands also direct the migration of tumor cells during inflammation. When healthy cells are transformed into cancer cells, they start to express chemokine receptors that lead to their migration and survival at their metastatic site [5, 157].

Also, microRNAs (miRNAs) in the bloodstream play important roles in endothelial cell migration and angiogenesis [5, 158]. When their concentrations change, it can lead to cancer. So, they may be good biomarkers to detect early signs of it. Some of the miRNAs in tumor cells are packaged into microvesicles and delivered to endothelial cells. One of them, miR-9, reduces the activity of the JAK-STAT pathway, which is one of the major oncogenic signaling pathways that is activated in a several types of cancer. JAK is an abbreviation for Janus Kinase, a family of nonreceptor tyrosine kinases that transducer cytokine-mediates signals by the JAK-STAT pathway. STAT proteins play a crucial role in tumor cell

proliferation, survival and invasion, and help form a unique tumor microenvironment. Also, in endothelial cells, JAK1 is a proangiogenic protein and a target of miR-17-92. Even though it is probably difficult to target miRNAs, it may be possible to target the JAK-STAT pathway. So, several inhibitors of this pathway for treating other diseases are in later phases of clinical trials since they have already been shown to have acceptable toxicological profiles [5, 158].

In addition, the adaptive immune system surveys the body and eliminates newly produced tumors, in a process called tumor editing [5, 155]. However, if this fails and the tumor gets large enough to be detected in a clinical setting, the adaptive immune system can be suppressed by cytokines such as IL-10. Regulatory T cells also infiltrate tumors. This suppresses both the innate and adaptive immune systems [5, 155].

Sex steroid hormones can also stimulate tumors [5, 155]. For example, prostate cancer is stimulated by androgens and IL-1β. The inflammatory cytokine IL-1β is made by macrophages in the tumor microenvironment. It converts androgen receptors from being inhibitors to becoming stimulators of prostate cancer growth. Also, females are less susceptible to cancer of the liver since it is not a target of sex hormones [5, 155].

So, inflammation can be healthy or unhealthy. It kills invading Bacteria, as part of a healthy immune response. It is unhealthy when it is unresolved in smoldering inflammation. This occurs during obesity and chronic infections and can be caused by periodontal disease. Omega-3 fats and polyunsaturated fats help protect against smoldering inflammation because they are converted into resolvins and protectins, which resolve the smoldering inflammation and protect against further inflammation. So, omega-3 fats and polyunsaturated fats can help prevent diseases of inflammation, including arthritis, cancer, diabetes, heart disease, stroke, Alzheimer's disease, hormonal diseases, osteoporosis, inflammatory bowel disease, pelvic inflammatory disease, and many other diseases.

2.7 The complement system and toll-like receptors in health and disease

As described in the previous section, inflammation can lead to a variety of deadly diseases – most notably cardiovascular diseases. Atherosclerosis is the main process underlying them. It's a leading cause of morbidity and mortality throughout the world [159]. Unfortunately, many patients are asymptomatic at first. Atherosclerosis is not recognized until an acute thrombotic event like a myocardial infarction (MI), stroke or sudden death occurs. Moreover, the prevalence of atherosclerotic disease

and its related costs are increasing, not only in industrialized countries, but also developing ones [159].

As described in section 5.5.6.1, cholesterol and inflammation are both important factors in atherogenesis and atherosclerosis. In addition, lipoproteins that are trapped and retained by matrix proteoglycans in the intimal layer of the arterial can be oxidized [159]. This triggers an innate immune response. The bidirectional interaction between inflammation and lipids leads to an accumulation of lipid-filled macrophages in the intima. They eventually form a lipid core in lipid-filled cells, and trigger the apoptosis and necrosis. Subsequently, cell debris and cholesterol crystals accumulate. The lesion retains a stable phenotype when it is provided with local cytokines. They stimulate smooth muscle cell proliferation and the synthesis of extracellular matrix proteins. In addition, a central lipid core and a thick surrounding layer of smooth muscle cells form with fibrous connective tissue to make a fibrous cap [159].

At the same time, atherosclerosis is a dynamic process [159]. Stable lesions can be transformed into unstable lesions that are more susceptible to being ruptured. Unstable plaques not only have a large lipid core and a thin fibrous cap, they have an imbalance between pro- and anti-inflammatory mediators. This leads to more infiltration of T cells and activation of macrophages, as well as higher rates of apoptosis and increased expression of pro-inflammatory cytokines, chemokines and proteolytic enzymes in unstable plaques. Several types of immune cells are involved in atherosclerotic inflammation. Overexpression of cytokines (IFN-γ and TNF) that are produced by T helper 1 (T_H1) cells can lead to the production of advanced and unstable plaques. So, excess T_H1 activity can destabilize plaques. Fortunately, regulatory T cells (T_{reg}) can protect against atherosclerosis by suppressing T_H1 cells and through anti-inflammatory effects. Recently, B cells have also been shown to be involved in atherosclerosis. B2 cells can help cause atherosclerosis, while B1 cells help attenuate the atherosclerotic process by secreting IL-10 [159].

Moreover, macrophages (prototypical cells in the innate immune system) play a key role in lipid accumulation and inflammation during the initiation of atherosclerosis [159]. There are inflammatory (M1) and resolving (M2) macrophages. Lipopolysaccharides and IFN-γ that are released from T_H1 cells promote the polarization of M1 macrophages by activating TLR4. Cytokines (IL-4 and IL13) that are released from T_H2 cells promote M2 polarization of macrophages. There are four types of M2 macrophages: M2a, M2b, M2c and M2d. The M2a macrophages are wound-healing macrophages. M2b macrophages are induced when they

are exposed to immune complexes and TLR ligands or IL-1 receptor agonists. They produce both inflammatory (e.g., IL-6 and TNF) and anti-inflammatory cytokines (IL-10). M2c macrophages are induced by IL-10 and glucocorticoids. M2b and M2c macrophages are regulatory macrophages. Finally, M2d macrophages are induced by a combination of agonists of TLRs and the adenosine A2A receptor. They have high concentrations of IL-10 and VEGF and play a role in angiogenesis. There are also Mhem macrophages that are present in regions of hemorrhage, and M4 macrophages that express matrix metalloproteinases that can destabilize carotid plaques. M1 polarization is induced when TLR2 and TLR4 are activated. T_H2 related cytokines (but not TLR activation) are important for M2 macrophage polarization. In addition to TLRs, complement activation can lead to M1 polarization since mice who don't have enough of the C3 complement protein had fewer M1 macrophages and more M2 macrophages [159].

That is, there are three pathways through which the complement system can be activated [159]. They all converge on the hydrolysis of C3 to produce C3a and C3b. Activated C3 also produces an enzyme, C5 convertase, which catalyzes the conversion of the C5 protein to C5a and C5b. C3a and C5a are anaphyltoxins that lead to the production of inflammatory mediators and anaphylaxis. The complement system is part of our innate defense against infections. There are more than 40 membrane-bound and soluble proteins in the system. The water-soluble proteins are mostly secreted by liver cells, monocytes and macrophages. However, the traditional view of the complement system as being mostly a host defense system against microbes has changed. We now know that it's a surveillance system that can be activated quickly by sensing any danger to the host. It contributes to maintaining tissue homeostasis and promotes tissue regeneration and repair. On the other hand, undesired or uncontrolled activation of the system can induce tissue damage and organ dysfunction in the host [159, 160].

This is another example of how yesterday's solutions can become today's problem. That is, the same things that enable the innate immune system to fight bacterial infections and eliminate cellular debris also enable it to trigger inflammatory and autoimmune diseases [160]. The harmful effects are made worse by advanced age, several genetic factors and autoimmune diseases. Also, the complement system can be activated inappropriately in some patients who have biomaterials inserted into them or have organ transplants. In some cases, the innate immune system can attack host cells and contribute to smoldering inflammation. When the complement system is activated in tumor microenvironments, it can lead

to the recruitment and/or activation of myeloid-derived suppressor cells. When over-activated, the complement system can induce systemic inflammatory response syndrome that can lead to hyper-inflammatory conditions and events, including sepsis. In addition, as we age, our body's ability to completely remove cellular debris deteriorates. As a result, debris accumulates and the innate immune system is continuously activated. This can lead to diseases caused by smoldering inflammation, such as age-related macular degeneration. Moreover, the kidneys are especially susceptible to damage caused by the complement system. However, recent research has identified some tipping points between healthy physiological and unhealthy pathological activities of the innate immune system. These are emerging as possible new therapeutic targets. This includes the C1 esterase inhibitor (C1-INH) which catalyzes the inactivation of C1r, C1s and MASP-1/2 proteins that are involved in the classical and lectin pathways for activating the complement system. It also includes the therapeutic monoclonal antibody eculizamab that binds to the C5 protein, preventing it from being activated and blocking the production of C5a. It was first introduced as a treatment for paroxysmal nocturnal hemoglobinuria and was subsequently approved for treating atypical hemolytic uremic syndrome. Other NMEs are being developed that target the complement system and are undergoing clinical trials. For example, mirococept (APT070) is being tested for its ability to provide localized tissue protection during kidney transplantation. It contains a recombinant fragment of complement receptor 1 linked to a membrane-tethering moiety (an important part of a molecule). When perfused after organ collection, mirococept lines the endothelium of the donor organ to restrict complement-mediated damage that occurs after transplantation. Convertase-mediated amplification can also be impaired by inhibiting factor D, which is a bottle-neck in the formation of C3 convertase which is important in the complement system [160]. Patients with age-related macular degeneration (AMD) are being enrolled in a phase III trial of the anti-FD antibody, lampalizumab [160, 161]. Many more antibodies, biologics and small molecule NMEs that target the complement system are also being developed [162]. Antibodies have the advantage of targeting protein-protein interactions. Some (like TT30) target regulators of the complement system. Others target mediators of natural immune evasion or unrelated protein scaffolds. There are also some (like Zimura) that target oligonucleotide-based ligands such as aptamers or peptides (such as analogs of compstatin). New drugs that target the complement system are also being developed to treat neurodegenerative diseases as well as injuries and infections in which the complement system is over-activated [162].

So, several parts of the innate immune system (including the complement system and TLRs) are being targeted in new drug development for preventing and treating atherosclerosis [159]. Oxidized lipoproteins in the arterial wall are potentially dangerous stressors. The innate immune system helps to eliminate them. Several pattern-recognition receptors (PRRs) are used in this first line defense. The innate immune response not only has rapid pro-inflammatory effects, but also triggers adaptive immunity, resolves inflammation and repairs tissues. However, chronic exposure to stressors in the arterial wall can lead to a loss of immune homeostasis. TLRs and the complement system are key regulators of this homeostasis. The complement system affects both B and T cells. However, recent research indicates that there is much bidirectional communication (crosstalk) between TLRs and the complement system. So, there is a complex interaction between these pathways in atherogenesis. This is leading to new therapeutic strategies for repairing and stabilizing atherosclerotic lesions [159].

So, the complement regulator C1-INH (Berinert®, ZYLEDIG®) is approved for treating hereditary angioedema, a condition with life-threatening recurrent swellings due to low concentrations of the C1-INH esterase inhibitor [159, 163, 164]. “This leads to the inhibition of the classical complement cascade, the contact system, the blood clotting cascade and the fibrinolytic system. The result is the prevention of local inflammation by complement and bradykinin” [164]. In June, 2017, the US FDA approved a version of this drug (Haegarda®) for subcutaneous administration [165].

In conclusion, inflammation is like many things in life. It must be carefully controlled to maintain a healthy, well-balanced life.

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CHAPTER THREE

THE ROLE OF NUTRITION AND LIFESTYLE

3.1 Nutrition

For many people, maintaining proper nutrition is one of the easiest and most important things that they can do to maintain good health. There is even a popular phrase, "You are what you eat". As discussed in Chapter 2, volume 1, our diet greatly affects the composition of microbes in our gut, which plays a major role in shaping our feelings and behavior. However, for many other people, it is almost impossible to eat healthy. Childhood trauma can cause some people to become anorexic. Unfortunately, there are also some adolescents who become anorexic as they try to look like professional models. Similarly, there are a variety of addictive drugs that suppress one's appetite. Moreover, opiates (including those that are obtained by prescription) can cause constipation, which can also suppress one's appetite. Similarly, there are anticancer agents that can cause nausea. At the same time, there are hundreds of millions of people (especially in the USA and its territories) who consume the typical fast food diet that consists of huge quantities of saturated fats, calories and beverages that are loaded with simple sugars (sucrose, glucose and/or fructose) [1]. Unfortunately, many people find such foods to be delicious. There is even a large industry that synthesizes food additives that aim to make the fast food tastier [1].

Fortunately, the fast food diet has not become popular in all western countries. For example, when the first McDonald's restaurant opened in Rome, Italy, there was a large demonstration against it [2]. The demonstrators started a Slow Food organization (website: http://www.slowfood.com/), dedicated to the very Italian process of spending plenty of time preparing the food, enjoying lively discussions, slowly eating the food and singing songs after the meal is over. Similarly, many European and Latin American tourists who come to the USA are unpleasantly surprised by how fast they are supposed to eat their meals in restaurants. That is, our Mexican, Central and South American friends reject the fast foods of the USA. When instant soups were introduced into South

American markets, they failed miserably, because cooks were certain that the only way to prepare a healthy meal was to do it slowly. Moreover, it is much better to eat slowly and chew, rather than gulp one's food. It takes about a half hour for ingested food to affect our brains and make us feel satiated. When one eats a large meal in just a few minutes it's very easy to overeat. Instead, people can eat much more than if they eat slowly. Another very common problem in the USA is sugar-sweetened soda pop and fruit juices, which have been shown to stimulate the appetite. In fact, many people in the USA have been brainwashed into thinking that the only way to enjoy a movie is to have a large soda and box of buttered popcorn. The soda (or any sugar-sweetened beverage) will cause a rapid increase in blood glucose levels, which can eventually lead to type-2 diabetes. Even though these sweetened beverages contain the 'natural' fruit sugar (fructose), they are very unhealthy. The fructose is rapidly converted to glucose, which does much damage [2].

Instead, it is much better to drink water [2]. Unfortunately, billions of people don't have adequate access to clean water. Still, in parts of the world that have plenty of clean water, it is important to drink at least eight 8-ounze glasses of water every day to maintain good health. Caffeinated beverages, sweetened fruit juices and soft drinks are often considered to be dehydrating, even though usually they are not [3]. The USDA recommends different amounts of water for people of different age and sex, such as 3.7 L/day for adult men and 2.7 L/day for women. Even though sweetened beverages may not be dehydrating, they are a major contributor to obesity in the USA and other countries.

The USDA also has guidelines and recommendations for how much of each food group one should consume to have a healthy diet [2, 4], as well as a database that lists the nutrient levels in many foods [5]. They recommend eating 6-11 servings from the bread, cereal, rice and pasta group, 3-5 servings of vegetables, 2-4 servings of fruits, 2-3 servings from the milk, yogurt, and cheese group and 2-3 servings from the meat, poultry, fish, dry beans, eggs and nuts group [4]. They claim that milk products are important because they provide protein, vitamins and minerals (especially calcium). They recommend choosing lean meat and poultry without the skin as well as eating fish, beans and peas often. They also recommend using fats, oils and sweets sparingly and limiting saturated fat to less than 10% of calories, or about 1/3 of total fat intake. They recommend preparing meats in a low-fat way by trimming away all the visible fat and then broiling, roasting or boiling instead of frying them. They recommend going easy on egg yolks and making larger portions by using egg whites. They also point out that nuts and seeds are high in fat, so

they should be eaten in moderation. They also stated that some health authorities recommend limiting dietary cholesterol to less than or equal to 300 mg per day and salt (NaCl) to no more than 240 mg per day [4].

The USDA recommendations are based on the consumption of 2000 Calories per day diet, suitable for a sedentary man 51 – 70 years old, or a sedentary woman 19 – 30 years old [2, 4]. Of course, the number of Calories and servings of food should be higher for active people. The recommended daily Caloric intake for active women aged 19 – 30 is 2400 Calories and for active men 19 – 30 years old it is 3000 Calories. Note that there are two different definitions of Calories and calories. In a physics class, one calorie (lower case) is defined as the amount of energy needed to raise the temperature of one gram of deionized water in the liquid phase by 1 ^{o}C. Actually, calories and kilocalories are not SI (metric) units and are somewhat imprecise. That is, the exact amount of energy needed to raise the temperature of liquid water by 1 ^{o}C depends on the starting temperature of the water. Still, the difference in the amount of energy needed to heat liquid water from 1 ^{o}C to 2 ^{o}C is very close to the amount of energy needed to heat liquid water from 10 ^{o}C to 11 ^{o}C, so the original definition of a calorie is close enough to be used for most practical purposes. One kilocalorie is the amount of energy needed to increase the temperature of 1 kg (1000 g) of water 1 ^{o}C. However, labels on containers of foods and beverages are based on a different definition. They use the commonly accepted definition that one Calorie (upper case) in food is the same as one kilocalorie as defined by physicists. So, theoretically, the 100 Calories in a small apple have enough stored energy (as carbohydrates) to increase the temperature of 100 kg of liquid water 1^{o}C, if it is burned up completely in a bomb calorimeter [2].

However, it should be noted that the caloric content of food is not based on an artificial experiment, in which a small sample of food is burned up completely in a bomb calorimeter in the presence of much O_2 [2]. This type of calorimeter contains a thermometer that measures the increase in temperature that is caused by complete combustion. However, not all calories are equal when considering the effect on health and obesity. It is more important to consider the effect of different foods on the glucose concentration in the blood, or the glycemic index [2].

To measure the glycemic index, a measured amount of food or beverage (usually 50 g) was given to ten different people, and the changes in amount of glucose in the blood were measured after two hours [2]. The change in glucose concentration vs time, for 120 min was plotted. The area under the curve was the glycemic index and the average for ten subjects was used. Two different benchmarks can be used. Usually, glucose is used

and is assigned a glycemic index of 100. A 100 Calorie candy bar has a higher glycemic index that a 100 Calorie apple because it contains fructose instead of complex carbohydrates that are in apples. In addition, apples have dietary fiber. So, they are much healthier and less likely to lead to obesity than candy. Foods such as most fruits and vegetables have relatively low glycemic indices of 55 or less. Whole wheat products, brown rice and table sugar have intermediate glycemic indices of 55 – 69. Corn flakes, white rice, baked potato, watermelon, white bread, cereal and candy have high glycemic indices of over 69. This is all based on glucose having a glycemic index of 100. So, the caloric content of food is based on the ingredients. Protein and carbohydrates are assigned the value of 4 Cal/g and fat 9 Cal/g, regardless of the source. Bomb calorimetry is not used in the assignment of caloric content [2].

The USDA recommendations have their limits, though [2]. There is no distinction made between different types of meat or fish. It is now known that there is a big difference between healthy unsaturated fats (in fish) and unhealthy saturated fats (in meat). Unsaturated, polyunsaturated and omega-3 fats are especially important for the developing brains of babies and infants. In adults, they help prevent smoldering inflammation and diseases that it can cause. On the other hand, saturated fats and *trans* fats that are found in meat and partly hydrogenated oils can lead to obesity and smoldering inflammation [2].

In addition, the recommendation of maintaining a low-fat diet many scientists have realized for some time that this is wrong [6]. That is, "Mainstream nutritional science has demonized dietary fat, yet 50 years and hundreds of millions of dollars of research have failed to prove that eating a low-fat diet will help you live longer" [6]. This was written in 2001. In 1984, the president of the American Heart Association (AHA) told *Time* magazine that if everyone went along with the recommendation to have a low-fat diet, "we will have [atherosclerosis] conquered" by the year 2000. Moreover, the Surgeon General's Office itself had just published its 700-page landmark "Report on Nutrition and Health," which claimed that fat is the single most unwholesome part of the American (USA) diet. In 2001, it was written that "During the past 30 years, the concept of eating healthy in America has become synonymous with avoiding dietary fat. The creation and marketing of reduced-fat food products has become big business; over 15,000 have appeared on supermarket shelves. Indeed, an entire research industry has arisen to create palatable nonfat fat substitutes, and the food industry now spends billions of dollars yearly selling the less-fat-is-good-health message" [6].

The idea that a low-fat diet was healthy was based on the discovery that the saturated fats in meat and dairy products elevate the concentration of cholesterol in the blood [6]. This was shown to be an over-simplification in "the single most dramatic diet-heart trial ever conducted: the Lyon Diet Heart Study, led by Michel de Lorgeril of the French National Institute of Health and Medical Research (INSERM)" [6] and published in February 1999 in the peer-reviewed journal *Circulation* [7]. The investigators randomized 605 survivors of heart attacks into two groups. All the participants were taking cholesterol-lowering drugs. They counseled one group to eat an AHA "prudent diet," very similar to that recommended for everyone in the USA. They counseled the others to eat a Mediterranean-type diet, with more bread, cereals, legumes, beans, vegetables, fruits and fish but less meat. The total amount and types of fat consumed by the two groups was quite different. However, the concentrations of HDL, LDL and total cholesterol in the two groups were almost identical. However, after four years the Mediterranean diet group had only 14 cardiac deaths and nonfatal heart attacks compared to 44 for the "American" diet group. The authors concluded that the "protective effects [of the Mediterranean diet] were not related to serum concentrations of total, LDL or HDL cholesterol" [6, 7]. So, as we will see in section 6.1.1, the effects of dietary fats on health are more complicated that what was realized almost two decades ago and what is being told to the public by advertisers of "fat-free" and "low-fat" foods.

Also, there is no USDA recommendation regarding organic foods, or genetically modified (GM) crops (more often known as GMOs) [2]. More importantly, the dangers of eating mass-produced meat in the USA are not indicated. The advantages of vegetarianism are not described. The advantages of eating or drinking soy, rice or almond 'milk' instead of cow's milk or other dairy products, are not mentioned – even though they contain more calcium (Ca^{2+}) than milk. Finally, the importance of antioxidants is not stressed [2].

It is better to eat many types of fish than to eat red meat [2]. Fish and vegetable oils are better than butter, margarine, or solid fat from pork or beef. Cold water oily fish, such as salmon, anchovies, herring and mackerel, are good sources of omega-3 fats. Some of the omega-3 fats, such as alpha linoleic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential and may prevent heart disease. However, farm-raised Atlantic salmon has been found to have relatively high levels of toxic pollutants, called PCBs and dioxins [2, 8].

At the same time, the USDA does not distinguish between mass-produced, industrialized meats and meat from animals that are grown on

the free range [2]. That is, cattle, pigs, hogs and even poultry are mass produced in industrialized Confined Animal Feed Operations (CAFOs) [9]. Over 1000 chickens are kept in a room that is the same size as a small bathroom [2, 4]. Also, meat packing plants often employ undocumented workers who have no rights and very little training [1, 2]. They work in close quarters and use very sharp knives in their work. Most of them are missing one or more fingers, due to accidentally cutting themselves. The missing fingers most likely end up in the meat. At the same time, CAFO animals are fed antibiotics, with the hope of preventing bacterial infection. Unfortunately, it doesn't work very well. Most of the animals produced by CAFOs contain Bacteria. To survive, these Bacteria must be resistant to antibiotics. So, the major source of antibiotic resistant Bacteria is not people who have been given too many antibiotics, but animals from CAFOs [1, 2]. Antibiotics tend to give animals (and people) diarrhea. So, CAFO animals, especially cattle, spend their entire lives knee deep in their own fecal material [1, 2, 9]. So, beef is often contaminated with *E. coli* from fecal material. The fecal material (especially from hogs) is a major source of environmental pollution. CAFO animals produce much more fecal material than all the humans on our planet! [1, 2, 9].

So, if you must eat beef, pork, turkey or chicken in the USA, it is better to eat those that are grown on the free range [1, 2, 9]. That is, the animals should be allowed to roam freely, eat (and defecate) in the open range, or pastures. When they defecate, the fecal material enters the soil instead of our rivers and drinking water (as in CAFOs). Sometimes meat produced in this way is labeled organic, meaning the producer has spent some extra money to have his or her organization inspected. However, in her book, *Animal, Vegetable, Miracle*, Barbara Kingsolver told us that it is just as good (and usually better) if you buy your meat and other foods from local producers who you know, even if it isn't certified as organic [9]. That is, organic foods that must travel hundreds or thousands of miles to reach you are much worse for the environment than locally produced foods. That is, it takes much fossil fuel (which produce the greenhouse gas, CO_2) to transport foods over a long distance. This has a terrible impact on the environment. In contrast, free range meat will not have growth hormones, antibiotics, fecal material, or human flesh in it. Similarly, there are advantages to eating locally produced grains, cereals, fruits, and vegetables [1, 2, 9].

It is also much better for the environment to be a vegetarian. The international meat industry generates about 18% of the world's greenhouse gas emissions (more than transportation) and switching to vegetarianism can reduce your carbon footprint by up to 1.5 tons of CO_2 per year [10]. In

an article in *Scientific American*, it was reported that beef production generates greenhouse gases that contribute to 13 times more global warming than chickens and 57 times as much as potatoes [11]. Still, in her book, *Animal, Vegetable, Miracle*, Barbara Kingsolver pointed out that it is almost impossible to be a complete vegetarian, since there are almost always a few pieces of small insects that are parts of the foods we eat [9]. She points out that Hindus are often strict vegans and think that they eat no animal products at all. However, when Hindus began immigrating to England in the early 1900s, many of them became anemic because of a lack of protein. The English farmers used different farming methods that kept the number of insects much lower on their fruits and vegetables than in the Indian subcontinent, so the protein intake of the immigrants decreased, causing anemia [9]. I also remember my son's biology teacher telling his class that if you sleep in the basement of your house and snore, you probably swallow an occasional spider or two.

So, it is important to use systems thinking when deciding what to eat. That is, our healthcare system can't work effectively if it is embedded in badly dysfunctional systems. No amount of systems thinking in medicine or new drug discovery can be effective if global climate change and antibiotic-resistant Bacteria grow unchecked. At the same time, one should remember that no single food (or 'superfood') can make up for bad eating habits. So, it doesn't really help to drink beverages that only have one calorie because they contain artificial sweeteners when one is consuming a large hamburger with fried potatoes and a sweet pastry for dessert. At the same time, some dietary supplements like iron may be very good for some people (like pregnant women), but extremely bad for others (like men over 50 years of age). Other examples will be described later in this chapter.

It is also important to realize that some of the same type of misleading advertising that is done to sell some dietary supplements is used to help sell many foods and beverages. One of the best examples of this is the concept of 'natural foods'. Many foods in grocery stores and health food stores are labeled "all natural" [12]. So-called 'natural foods' have grown from a small niche market to a multi-billion industry. It has also been estimated that 97% of all households in the USA buy 'natural' foods. Surveys have shown that many people think that 'natural' means organic. However, the US FDA does not have a legal definition of the term 'natural', even though the terms organic, low fat, low sodium and high fiber are defined. Instead, the FDA has an informal policy that considers 'natural' to mean that "nothing artificial or synthetic (including colors regardless of source) is included in, or has been added to, the product that would not normally be expected to be there" [12, 13]. Since the FDA and

other governments' regulatory agencies are reluctant to try to stop food retailers from labeling their products as being 'natural', the Center for Science in the Public Interest filed class action lawsuits to try to stop this practice [12]. However, to the best of my knowledge, they have not been successful, since stores are still full of products labeled as being 'natural' and advertisers continue to use this term – even on products that contain high fructose corn syrup or are genetically modified organisms (GMOs).

Just as one can understand systems thinking better by understanding its opposite (reductionist thinking), 'natural' foods might be better understood by considering its opposite – unnatural. Some people might think that anything made or produced by humans is unnatural. By this definition, organic foods that are grown by people on well-regulated farms would be unnatural. Moreover, this definition of unnatural assumes that humans are not a part of nature, but are always enemies of nature. This is certainly not compatible with systems thinking and is probably not compatible with reductionist thinking or even science itself. Unfortunately, it is a big part of misleading advertisements and thinking that is based on greed and superstition.

However, this does not mean that processed foods that often contain large quantities of salt and sugar are healthy. Still, even the term 'processed foods' is more useful in advertising than in science or law. For example, native Amazonians process completely organic açaí. They process it by washing the berries and sometimes by freeze-drying them. So, it might be said that they are processed, even though they can be quite healthy – especially for undernourished natives and endurance athletes who need to consume plenty of calories and healthy fruits without having to consume very much fructose (which is present at much higher concentrations in most other fruits).

So, it is important to remember that one should look at a person's entire diet, state of health, lifestyle and personal attitudes when trying to decide if a certain food or dietary supplement is healthy. For example, açaí has been advertised in the USA as being capable of making people lose weight fast. There is even a You Tube video that shows amazing photos of women who supposedly lost a lot of weight and body fat by eating açaí for just a few weeks [14]. This might have some truth if you consider people who consume the wide variety of products that are fraudulently labeled as containing açaí [15]. These adulterated products are often just dilute grape or raspberry juices that contain almost no calories, because they contain no açaí. That is, açaí is an oleaginous fruit, like olives. It produces an oil and not a juice when it is squeezed. Since most people in the USA think of fruits like oranges, apples and grapes that do produce a juice when

squeezed, they think the same is true for açaí. So, they might really believe that foods labeled as containing 100% açaí juice with no water added are genuine. They are not. Water is often added to açaí in Brazil to make light açaí. Still, it is a slurry that contains many solids. It is not like orange, apple or grape juices [15]. So, if someone in the USA consumes the typical fast food diet and tries to lose weight by adding genuine açaí (like Sambazon açaí) to their diet, they will be disappointed and may even gain weight.

A person's state of health and lifestyle can also be crucial in deciding what is healthy and what is not. For example, people who have cancer should have very different diets that those who are obese but don't have cancer. So, the European Society for Clinical Nutrition and Metabolism (ESPEN) [16, 17], the U.S. National Cancer Institute (NCI) [18] and the American Institute for Cancer Research [19] have published evidence-based guidelines and recommendations for nutritional care in patients who have cancer. The ESPEN guidelines were commissioned and financially supported by ESPEN and by the European Partnership for Action Against Cancer (EPAAC), an initiative by the European Union (EU) [19]. It was estimated that the deaths of 10-20% of patients with cancer can be attributed to malnutrition rather than to the malignancy itself [17, 20]. This is because malnutrition and a decrease in muscle mass are frequent in cancer patients [16]. Both of these have terrible effects on clinical outcome. They can be due to not eating enough, by decreased or complete termination of physical activity and dysfunctions in catabolism. So, as in TQM, a quality control process should be established at each institution that treats cancer patients. It should ensure that patients are properly screened and monitored for malnutrition, so it can be treated when it emerges. In all patients (with the possible exception of those receiving palliative care), energy and substrate requirements should be met by offering interventions that include counseling and nutritional intervention. As in TQM, it is essential that healthcare professionals communicate regularly with patients and their caregivers. Nutritional care should also be accompanied by physical activities and exercise. This is because the loss of skeletal muscle - with or without loss of fat - leads to physical impairment, post-operative complications, chemotherapy toxicity, and mortality. In addition, systemic inflammation often occurs in patients who have cancer [16].

In patients with cancer, systemic inflammation inhibits proper utilization of nutrients and promotes catabolism, or the breakdown of complex, larger biochemical (like protein) to form smaller, simpler metabolites (like amino acids) [17]. In cancer, this is a destructive form of

metabolism, since it leads to muscle breakdown. Updated nutritional strategies now suggest considering nutrition with anti-catabolic and inflammation-suppressing ingredients. To reduce catabolism and systemic inflammation, the ESPEN suggested that it might be good for cancer patients to take anti-catabolic and inflammation-suppressing agents. Studies have indicated that oral nutritional supplements that contain essential amino acids or high-dose leucine may improve protein synthesis in skeletal muscles. Fish oil, a good source of long chain omega-3 fats, is also suggested for improving appetite, while increasing lean body mass and body weight in patients with advanced cancer and at risk of malnutrition [17]. However, they were not specific about what types of inflammation-suppressing agents should be taken. As discussed in Chapter 2 of this chapter, vitamins A and C as well as CoQ10 can react directly with RONS and destroy them. On the other hand, many other dietary antioxidants exert their effects by activating the Keap1-Nrf2-ARE signaling system, which is often over-activated in multi-drug resistant cancers. So, it might be better for cancer patients who are suffering from anorexia, malnutrition and/or a loss of skeletal muscle to take vitamins A and C, as well as CoQ10 instead of other dietary antioxidants.

It is also important to increase immunosurveillance and the effectiveness of the immune system through nutrition [21]. So, new formulas containing defined quantities of essential amino acids, ω-3 fats and nucleotides have been developed to help support the immune system. This is called immunonutrition or pharmaconutrition, since they can act like pharmaceutical agents, as opposed to standard nutrients. The complex inflammatory, immune, and oxidative stresses that occur during the postoperative period have led some people to see if nutrients given at relatively high doses may improve patients' recoveries and prognoses. Researchers have looked at a combination of arginine, omega-3 fats, glutamine and/or nucleotides. Arginine is normally a non-essential amino acid that becomes essential during critical illnesses. It is crucial for proper lymphocyte function. It's also a precursor of nitric oxide and hydroxyproline, both of which play a key role in repairing connective tissue. A deficiency of arginine can cause the adaptive immune response to not respond properly, due to abnormalities in T-cell receptors. Omega-3 fats are anti-inflammatory. They decrease the synthesis of pro-inflammatory eicosanoids, while reducing interactions between leukocytes and platelet-adhesive endothelial cells. They also inhibit the expression of inflammatory genes and stimulate glutathione production, which can decrease oxidative injury. At the same time, glutamine is a major fuel source for macrophages, lymphocytes, and enterocytes. Glutamine is also

involved in intracellular signaling. It increases the expression of heat shock protein, prevents apoptosis and decreases inflammation. Glutamine supplementation is especially important when catabolism is activated, because glutamine reserves can become depleted rapidly. A deficiency of glutamine can lead to impaired immunologic function and the breakdown of the intestinal epithelial border function. Nucleotides are also important in the development of the immune system, as well as tissue growth and differentiation. This is especially true for tissues with rapid turnover such as skin, intestinal mucosa, cells of the bone marrow and lymphocytes. Nucleotides participate in the maturation, activation, and proliferation of lymphocytes. They stimulate the phagocytic activities of macrophages. They modulate the delayed hypersensitivity response, response against tumor and grafts, immunoglobulin production and the response to infection [21].

It is also extremely important to keep in mind the social systems in which a person lives. For example, pork may simply be unhealthy for people living in most countries, but it can be deadly if one tries to import it into countries that follow Islamic laws. Similarly, a person would be ill-advised to try to bring beef into those parts of India where it is illegal. Even if there are no laws or social taboos, it is still important to consider one's state of health when trying to decide what foods might be healthy. For example, strawberries can be quite healthy due to their high content of vitamin C, dietary fiber and the antioxidant fisetin. However, if sucrose (table sugar) and cream are added to them, it would make them quite unhealthy – especially for people who consume a fast food diet.

It should also be remembered that there are many poor people, especially women and children, who suffer from hunger, malnutrition and starvation [2]. Long before people lived long enough to die of cardiovascular and neurodegenerative diseases or cancer, they struggled to find enough food. Humans were hunters and gatherers, long before they became farmers. Civilizations arose near rivers, when people learned to cultivate wheat, barley, millet, rice and squash. Calendars emerged to help farmers know when rivers (like the Nile) would flood, when crops should be planted and when they should be harvested. Eventually, people learned to make metal tools, which helped them plow and cultivate fields and irrigate crops. In time, a scientific revolution emerged during the Renaissance and Age of Enlightenment. This encouraged experiments in plant breeding to increase crop production. It also led to crop rotation, or rotating the planting of grains and legumes, to conserve nutrients in the soil. Other developments included adding limestone to soil, and many inventions, such as a seed drill, cotton gin, and the mechanical reaper. By

the late 1800s, steam power began to replace animal power on the farm. The demand for food for urban workers and raw materials for industry brought about major changes in world trade. Large agribusinesses emerged in capitalist economies, but central planning emerged in Communist countries. Mass starvation was used as a means of control by Stalin in the Soviet Union and by Mao Zedong in the People's Republic of China. This caused the death by starvation of tens of millions of people, especially in Ukraine and rural China. Fortunately, this did not happen in the USA and other non-Communist countries. Large agribusiness has not caused starvation, even though it has displaced most small farmers in the USA [2].

In 1968, the director of US Agency for International Development (USAID) coined the term Green Revolution to describe the worldwide transformation of agriculture that led to significant increases in food production between the 1940s and 1960s [2]. The Green Revolution started in 1943 with the establishment of the Office of Special Studies. It was a collaboration between the Rockefeller Foundation and the Mexican president, Manuel Avila Camacho. They aimed to develop high-yield varieties of maize and wheat. The Mexican government invested heavily in rural infrastructure and new varieties of seeds were used. Mexico became self-sufficient in wheat production by 1951 and has been exporting it ever since. The Green Revolution spread to India, where the Ford Foundation was involved. The Minister of Steel and Mines, C. Subramaniam worked with the International Maize and Wheat Improvement Center (CIMMYT) to improve plant breeding, irrigation, development and financing of agrichemicals [2].

By the late 1970s, rice yields increased by 30 percent. Next, the Rockefeller and Ford Foundations jointly established the International Rice Research Institute (IRRI) in the Philippines in 1960 [2]. High-yield varieties spread throughout the Philippines, followed by Indonesia, Pakistan, Sri Lanka, and many other countries throughout Latin America, Asia and North Africa. Another organization (USAID) helped subsidize the development of rural infrastructure and shipping fertilizer. Subsequently, the Consulting Group on International Agricultural Research (CGIAR) in 1971 responded to some earlier criticism of the Green Revolution by developing a more holistic view of agriculture [2]. So, not only do non-profit foundations and governmental organizations collaborate now to help human health, they have done so for many decades.

3.1.1 Fats

The role of fats in human health is more complicated than first thought by scientists and physicians [6, 7]. This is consistent with systems thinking which realizes that today's problems often come from yesterday's solutions and over-simplified dogma should be avoided. That is, most people in the USA in the 1940s and 1950s felt that red meat helped win World War II by providing badly needed protein to soldiers, sailors and Marines. Moreover, the U.S. government told civilians that food will win the war, as meatless Mondays were proclaimed. So, when the war was over, the American (USA) public became even more enamored with meat – especially beef and pork. It was considered almost un-manly and unpatriotic not to eat meat. Very few people even knew about the important differences between saturated and unsaturated fats. There are also important differences between *trans* and *cis* fats. The structures of saturated, *trans* and *cis* unsaturated fats that have 18 carbons are shown in Figures 1-3. Note that the International Union of pure and Applied Chemists (IUPAC) uses the letter *Z* for *cis*, where Z comes from the German word, zusammen (together), while *E* is used for *trans*, where E comes from the German word entgegen (opposite). That is, the two hydrogens in the carbon-carbon double bond are either on the same side (*Z*) or opposite side (*E*) of the double bond. Note that the structures of the saturated and *trans* fat are almost linear. They can pack together rather tightly in cell membranes. As a result, the melting points of these two are higher than the unsaturated *cis* fat, which has a non-linear structure that disrupts the tight packing of the other fats. This makes the membranes more fluid and flexible.

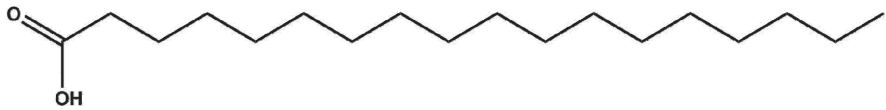

Figure 1. 2D structure of stearic acid, IUPAC name octadecanoic acid. Note that only the hydrogens on the –OH of the carboxylic acid group (carbon number 1) are shown. All other carbons have two attached hydrogens, as $–CH_2–$.

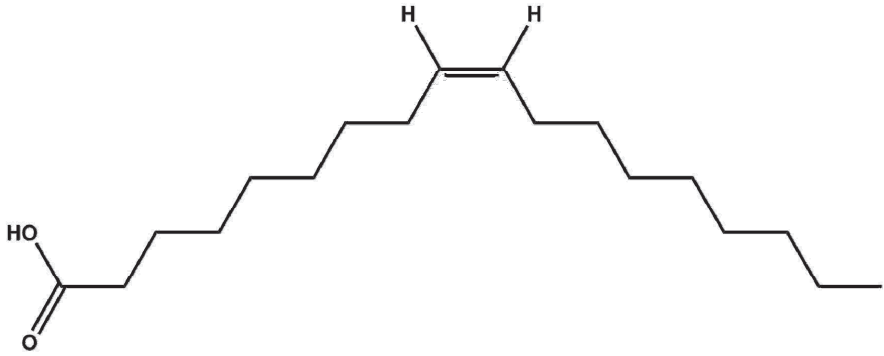

Figure 2. 2D structure of oleic acid, IUPAC name (9*Z*)-octadec-9-enoic acid. Note that only the hydrogens on carbons 9 and 10 and the –OH of the carboxylic acid group are shown. All other carbons have two attached hydrogens, as $–CH_2–$.

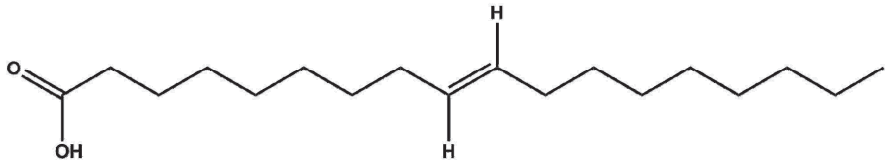

Figure 3. 2D structure of elaidic acid, IUPAC name (9*E*)-octadec-9-enoic acid. Note that only the hydrogens on carbons 9 and 10 and the –OH of the carboxylic acid group are shown. All other carbons have two attached hydrogens, as $–CH_2–$.

As the American (USA) public became more obese and the Framingham Heart Study showed that high concentrations of cholesterol in the blood can cause heart attacks, fat became thought of as being bad. At the same time, reductionist thinking led people to believe the over-simplification that if excess body fat was unhealthy, then people should reduce their consumption of dietary fat and cholesterol. So, margarine and partly hydrogenated vegetable oils were thought to be healthier than butter, while few Americans (except those of Italian, Greek or other Mediterranean descent) had even heard of olive oil. Being able to afford butter was a sign of affluence. At the same time, the word ‘fat’ was used as a derogatory term to insult people who were obese. People (especially in the military) were taught that other people became fat because they didn’t have enough self-discipline. They were fat because they ate too much and didn’t exercise enough. In fact, fat is a type of molecule and nutrient, not a description of a person. Obese people are not fat, they are

obese. This is not due to a personality flaw or a lack of self-discipline. Often it is due to bad advice.

That is, a low-fat diet can be deadly [22]. This was shown in the Prospective Urban Rural Epidemiology (PURE) of 125 287 participants from 18 countries in North America, South America, Europe, Africa, and Asia [22, 23]. That is, participants who cut back on fats had far shorter lives than those enjoying plenty of fats, even if they came from butter, cheese and meats. Even though the British National Health Service (NHS) warns people not to eat too much saturated fat, the group that ate little saturated fat had a 13% higher incidence of death. The group that consumed the highest amount of total fat had a 23% lower incidence of death. More importantly, the group that ate the highest amount of carbohydrates had a 28% higher incidence of death. The authors of the Canadian study stated that consuming a proper balance of fats and carbohydrates was best for the health of the population. That is, about 35% of all of one's calories should come from fats. They concluded that "reducing saturated fatty acid intake and replacing it with carbohydrate has an adverse effect on blood lipids. Substituting saturated fatty acids with unsaturated fats might improve some risk markers, but might worsen others. Simulations suggest that ApoB-to-ApoA1 ratio probably provides the best overall indication of the effect of saturated fatty acids on cardiovascular disease risk among the markers tested. Focusing on a single lipid marker such as LDL cholesterol alone does not capture the net clinical effects of nutrients on cardiovascular risk" [23]. Note that ApoB is involved in lipid metabolism and is the main constituent of very low density lipoproteins (VLDL) and LDLs, which are unhealthy when their concentrations in blood are too high. ApoA1 is a component of HDLs, which are healthy when their concentrations in blood are relatively high.

However, this does not mean that vegetarians should become carnivores. First, industrial-scale production of beef, pork, turkey and chickens in CAFOs is terrible for the environment. Moreover, they are a main source of antibiotic-resistant Bacteria. Second, there is no dietary fiber in meat. At the same time, consuming fresh fruits and vegetables makes people more satiated, so they won't have the appetite to over-consume other foods. In contrast, sweetened beverages can stimulate the appetite and lead to over-consumption of other foods in the typical 'American' (USA) fast food diet.

So, it is noteworthy that long-term consumption of animal flesh increased the risk of hypertension in three prospective cohort studies of people who did not have high blood pressure when the study began [24]. This is important because red and processed meats are widely known to be

bad for the heart and brain as well as leading to many forms of cancer, especially colorectal cancer. At the same time, seafood is believed to be healthy (unless it is fried) and poultry is controversial (even if it is not fried). However, the study was based on participants' responses to a food frequency questionnaire that only asked how much of each type of food was eaten [24]. So, it was based in part on reductionist thinking. It is very important to consider exactly what types of seafood were consumed, how the foods were cooked, what else people ate, how much they exercised, how stressful their lives were, how happy they were and to what extent they were exposed to ubiquitous environmental obesogens. All of these are extremely important factors that could have confounded the study.

That is, not all seafood is the same. Oily seafood (such as salmon, scallops and mackerel) are high in omega-3 fats, so they might help prevent heart attack, stroke, dementia, cancer and even attention deficit hyperactivity disorder [2]. However, some types of seafood tend to have relatively high levels of toxic pollutants, such as heavy metals (like mercury) and dioxins (which were used in Agent Orange in the Vietnam War). So, some government scientists recommend not eating farm raised Atlantic salmon and eating no more than two servings a week of wild salmon [25]. Please note that most grocery stores sell 'fresh' salmon at a higher price than frozen salmon, even though the 'fresh' salmon was raised on farms thousands of miles away and could be several days old, while the frozen salmon was caught wild and has much less contamination from pollutants like dioxins [2, 25]. So, when the USDA recommends meat or fish, there is no distinction between farm raised and wild salmon. However, there are websites that list seafood that should or should not be eaten [26-28]. Also, the National Resources Defense Council has a website that warns pregnant women about the dangers to their babies of eating too much seafood that is contaminated with mercury [29].

In 2004, the FDA and Environmental Protection Agency (EPA) advised pregnant and nursing mothers and their young children to limit their consumption of fish that are low in mercury to no more than 12 ounces (about two portions) per week [30]. This was challenged by another group of experts, the Maternal Nutrition Group and the National Healthy Mothers, Healthy Babies, who recommended that such women and children should eat at least 12 ounces of fish per week. They recommended fish like salmon, tuna, sardines and mackerel [31]. "The Group found that eating fish is the optimal way to gain the benefits of long-chain omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Seafood is the richest dietary source of DHA and EPA in Americans' diets. The Group also recognized that selenium, an

essential mineral found in certain ocean fish, accumulates and appears to protect against the toxicity from trace amounts of mercury" [31]. However, please note that fish like salmon actually contains omega-3 fatty acyls and not omega-3 fatty acids, but the fatty acyls in triglycerides are converted to free fatty acids when the triglycerides are digested.

However, it is possible to consume protein, omega-3 fats and polyunsaturated fats without eating seafood [2]. Beans, nuts and soy products are good substitutes for meat and seafood as sources of protein. DHA and EPA can also be obtained as dietary supplements, derived from seaweed. This avoids the problems with mercury or dioxin contamination, and it avoids possible allergies that some people have to fish or fish oils. Also, it is important to know that the healthy polyunsaturated fats can react with light and oxygen in the air and turn rancid. Thus, olive oil and flaxseed oil should be kept in dark bottles. So, there is much more to know about the details of a healthy diet, and not all the foods in the so-called meat and meat substitute group are the same [2].

Soybeans and soy products are in the meat, fish, nuts and bean food group and are especially healthy to people who aren't allergic to them [2]. They can provide the necessary protein that meat, fish, nuts and beans provide, while offering other unique benefits. Soy does not have any cholesterol, antibiotics, growth hormones or blood, unlike mass-produced, industrialized cow's milk. Moreover, soy proteins may be much better than animal protein as they can help to prevent cancer. Soy also has isoflavone antioxidants, which also prevent cancer and heart disease. It should be noted that isoflavones are phytoestrogens. That is, they are plant-derived compounds that can bind to isolated estrogen receptors *in vitro*, similar to tamoxifen, which is used to treat breast cancer [32]. Unfortunately, this has caused some people to mistakenly think that soy formula should not be given to infants [33]. Countless millions of babies and infants around the world are given soy formula as their major source (or only source) of food [2]. Many of them are allergic to cow's milk and many of their parents refuse to eat meat or dairy products for religious reasons. Still, almost all these babies grow up to have healthy children and grandchildren [2].

3.1.2 Protein

Not only are not all fats the same, neither are all proteins. As a system thinker, one realizes that the proteins are not consumed in isolation, but as part of a whole food. So, it is not so much the protein that is in mass-produced (CAFO) beef and pork, it is the saturated fats that are unhealthy.

Moreover, proteins tend to make one feel satiated sooner than carbohydrates [34]. For example, whey proteins in dairy products stimulate the secretion of the satiety hormone cholecystokinin (CCK). The effects of these and other proteins add to those of opioid peptides that are also in dairy products and other sources of protein because opioid proteins slow the transit time of food. The whey protein α-lactalbumin also improves memory and has an anxiolytic (calming) effect. In addition, phosphorylated peptides from casein (in dairy products) help to prevent tooth decay (caries) [34].

High protein meals also decrease the concentrations of ghrelin (which stimulates appetite) and lower the concentration of PYY and GLP-1, which also stimulate satiety [35]. In addition, functional MRI (magnetic resonance imaging) analyses have shown that high protein meals tend to stimulate the parts of the brain that help to control food motivation, reward, and cravings as well as executive function [36]. Proteins could be especially important for the elderly, who suffer a loss in muscle mass and functional capacity [37]. Even though the U.S. Institute of Medicine's Recommended Daily Allowance (RDA) for protein is 0.8 g per kg of body weight per day for all adults, regardless of age, the elderly may need more. Some scientists have recommended increasing the RDA for all adults. It also appears as if high protein diets may be more effective than standard protein diets in losing weight when caloric intake is limited. Some researchers have also proposed a 'Catabolic Crisis' model to help explain muscle loss in which a catabolic insult (such as inactivity, injury or malnutrition) can lead to anabolic resistance and an aging phenotype with decreasing muscle mass. So, prevention and treatment that target older (>65 years of age) people should also be done on middle aged people (40-65 years) [37].

Fish, marine algae, milk, other dairy products eggs, nuts, legumes (beans) and meat are the main sources of dietary proteins in many cultures [38-49]. Several cereal grains (barley, buckwheat, kamut, maize, oats, rice, rye, spelt and wheat), legumes (beans, chickpeas, mung beans, peas and soybeans), cruciferous vegetables (broccoli, canola and rapeseed), oil seeds (from sunflowers, sesame and walnuts), as well as garlic, sweet potatoes, cocoa, flaxseed, grapes and spinach contain bioactive peptides [48]. When fish proteins are hydrolyzed, they produce peptides that have anti-inflammatory and healing properties due to them inducing proliferation and migration in intestinal epithelial cells [38]. Egg whites contain many bioactive peptides, including some tripeptides that have anti-inflammatory, antioxidant and anti-hypertensive properties. That is, they can help prevent hypertension (high blood pressure) by inhibiting the

angiotensin converting enzyme (ACE). In addition, hydrolysates of soy proteins have anti-inflammatory properties. Moreover, a fermented soybean product (Chungkookjang) that is popular in Korea exerts anti-inflammatory effects in breast cancer cells by downregulating cytokines and activating TGF-β signaling. One of the peptides that can be obtained from soybeans, lunasin, has widespread anti-inflammatory effects including suppressing NF-κB activity, reducing cytokine expression and reducing the concentration of cyclooxygenase-2 (COX-2). It also has antioxidant and anticancer properties [38]. Soybeans can be fermented with Bacteria (*Bacillus subtilis* and lactic acid Bacteria) and fungi (Mucor, Aspergillus and Rhizopus species) [46]. They have anti-hypertensive, anti-obesity, hypocholesterolemic, immunomodulatory, antimicrobial, antioxidant, anti-diabetic and anticancer activities [46, 47]. In addition, soy milk, an aqueous extract of soybeans, is a good source of bioactive peptides [47]. So, in Japan, soybeans and food products obtained from them are included in the set of "Foods for Specified Health Use" [48]. There is also a class of bioactive peptides that called cyclotides that come from plants. They have been found in wheat, maize, and rice. They can suppress the immune system, so they are being used as scaffolds to develop new drugs to treat autoimmune diseases [48].

Rapeseed (a bright-yellow flowering member of the family Brassicaceae), is another important source of bioactive peptides [45]. Some of these peptides can inhibit renin and ACE. So, they can prevent hypertension. In addition, peptides from hemp seeds, flaxseeds, yellow seaweeds (*Palmaria palmate*), peas and peanuts can inhibit renin and/or ACE [45]. Note that rapeseed oil is similar to canola oil. That is, canola was created by plant cross-breeding of rapeseed plants to produce varieties that have much less erucic acid (which is inedible or even toxic at high doses) and glucosinolates, which are very pungent.

Bioactive peptides are also in the milk of cows, goats, sheep, buffalos and camels [40]. They have anti-microbial, immunomodulatory, antioxidant and inhibitory effects on many enzymes. They can also help prevent thrombosis and antagonize the effects of many toxins. They can help prevent cancer, osteoporosis and high blood pressure [40].

Human breast milk is an especially good source of bioactive peptides and proteins [50-52]. However, some of the bioactive peptides are cryptic and are sometimes called cryptides [48]. They are not released from the proteins in which they exist until they are digested [48, 53]. Bioinformatics are being used to help discover such peptides [53]. It takes information that is in databases like BIOPEP to identify cryptic peptides that are food proteins. The universal protein knowledge base (UniProtKB)

is used, along with SwissProt and TrEMBL. Just because the amino acid sequence of a cryptic peptide is found, that doesn't mean that it can be obtained by hydrolysis of the protein (i.e. proteolysis). So, bioinformatics techniques are used to simulate the specificities of protease enzymes. This includes the enzyme action tools of BIOPEP, ExPASy PeptideCutter (http://web.expasy.org/peptide_cutter) and PoPS (http://pops.csse.monash.edu.au). The peptides that are predicted by *in silico* proteolysis are matched with bioactive peptides in databases for predetermined bioactivities [53].

Some sources of bioactive proteins and peptides are especially good. For example, soy protein can help reduce the concentration of cholesterol in the blood by upregulating the activity of the LDL receptor [54]. Since soy protein isolate also contains isoflavones, it has positive effects on some indices of bone metabolism. This includes increasing the concentration and activity of insulin-like growth factor-1 (IGF-1) [54]. In addition, fermented soy products (like soy sauce) contain bioactive peptides that have anti-hypertensive, antimicrobial, antioxidant, anti-diabetic and anticancer activities [46]. Diets that are rich in soy protein also help to prevent many types of cancer [55, 56]. The microalgae that are commonly known as Spirulina (actually *Arthrospira platensis*) are also an excellent source of protein and other bioactive ingredients [57]. It is about 60% protein with all the essential amino acids. It also has more β-carotene than any other whole food. It is an excellent source of gamma linoleic acid, phycocyanin, many B vitamins (but not vitamin B_{12}), minerals, trace elements, chlorophyll and enzymes, including superoxide dismutase, an important antioxidant. Spirulina has almost twice the concentration of Ca^{2+} as cow's milk, seven times the protein as tofu, 31 times as much β-carotene as carrots and 51 times as much iron as spinach. It can protect the kidneys and liver, while also preventing anemia. It has important anticancer activities and benefits for diabetics and their cardiovascular system. It can remove heavy metals from the body and help control allergic rhinitis. It can also build immunity and help provide resistance to viral infections. In 1974, the United Nations wrote that Spirulina is one of the best foods for the future [57]. It also contains bioactive peptides that have antimicrobial, antiallergic, antihypertensive, antitumor and immunomodulatory properties [58]. The bioactive peptides can also promote cardiovascular health [59]. In addition, protein from okra, beans, chickpeas and other legumes are quite healthy [60-62]. However, people who have advanced stages of chronic kidney disease can suffer from proteinuria, or excess protein in the urine. Such patients will most likely be advised by their nephrologists to limit their protein intake.

3.1.3 Carbohydrates

Like fats and proteins, not all carbohydrates are the same. Still, they have important similarities. Like fats, they are stored energy [63]. Plants and photosynthetic algae make them from water and CO_2 during photosynthesis. They have the general formula $(CH_2O)_n$, thus the name carbohydrates (or hydrated carbon). Monosaccharides like glucose and fructose are readily soluble in water. After being absorbed in the gut, they are transported through the bloodstream and into the tissues. Fructose is converted to glucose, which can be stored as glycogen (a branched biopolymer with glycogen subunits or –mers). Alternatively, glucose can be broken down in glycolysis to produce acetyl-CoA, which enters the tricarboxylic acid cycle to produce CO_2 and energy in the form of ATP. Unlike plants, animals have a limited ability to make other polymers of glucose, but mammals can make dimers, such as lactose and some oligosaccharides that are in breast milk. The main dietary sources of complex carbohydrates are wheat and rice. Other sources include sorghum, millet, barley, rye and oats. Sugar cane and sugar beets are main sources of sucrose, which many people consider to be quite tasty [63]. However, once a person eliminates glucose, fructose and sucrose from his or her diet, foods and beverages that contain them may no longer taste very good. For example, many people outside the USA consider typical American (USA) soda pop to be way too sweet to be palatable.

Carbohydrates have been classified into different groups [63]. Oligo- and polymeric carbohydrates can be classified based on their degree of polymerization, type of linkage (α vs β) and the monomers that make them up. The prefixes α and β can also apply to monomers. The structures of α-D-glucose and β-D-glucose are shown in Figures 4 and 5. Note that glucose (and other simple sugars like fructose and galactose) can exist as a six-member ring (pyranose) or a five-member ring (furanose).

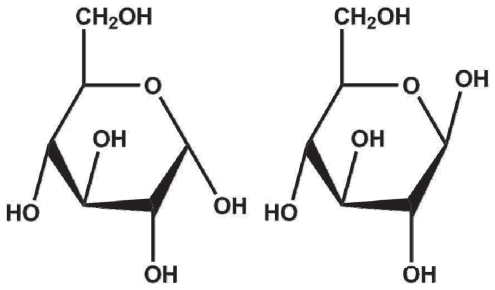

Figure 4. Structure of α-D-glucopyranose (left) and β-D-glucopyranose (right).

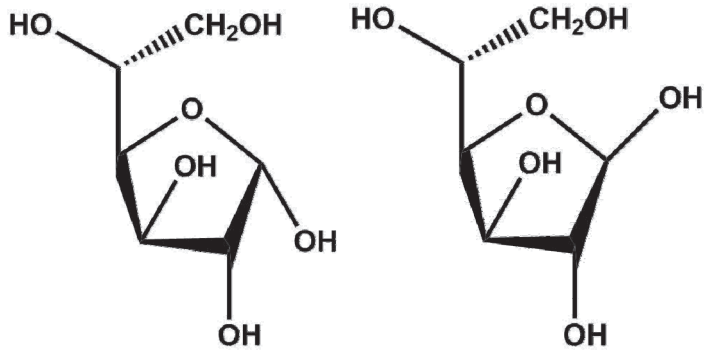

Figure 5. Structure of α-D-glucofuranose (left) and β-D-glucofuranose (right).

So, the three main dietary monosaccharides are glucose (Glc), fructose (Fru) and galactose (Gal) [63]. Honey and either cooked or dried fruits are main sources of free glucose and fructose. Fresh fruits and some vegetables contain lower concentrations of glucose and fructose. Corn syrup is produced by the hydrolysis of corn starch. It contains lots of glucose. High-fructose corn syrup contains both fructose and glucose. Unfortunately, both corn and high-fructose corn syrups are used in the food industry. Even more unfortunate, advertisers state accurately that fructose is a natural sugar and many consumers assume that this makes it healthier than glucose or sucrose. It is not. Fructose is the sweetest of all the common food carbohydrates and is used to sweeten carbonated beverages [63].

The main disaccharides are sucrose (αGlc(1→2)β-Fru) and lactose (β-Gal(1→4)β-Glu) [63]. Oligosaccharides have monosaccharides that are covalently linked by glycosidic bonds. Organic and polymer chemists would say that disaccharides have a degree of polymerization (DP) of two. Somewhat arbitrarily, oligosaccharides are defined as those that have a DP from 2-10. However, nutritionists and many food chemists consider disaccharides (especially sucrose) to be simple sugars. Human breast milk contains some important oligosaccharides. Polysaccharides can have a DP as high as 10^5. Polysaccharides can be either α-glucans (starch) or non-α-glucans. Cereals, potatoes, cassava, legumes and bananas are examples of foods that contain starch. It is made up of two different biopolymers, amylose (DP about 10^3) and amylopectin (DP 10^4 to 10^5). In animals and humans, glucose is stored as glycogen in the liver and skeletal muscles. Non-starch polysaccharides are in plant cell walls and are the main

component of dietary fiber. The most abundant is cellulose, which is packed close together in insoluble microfibrils. Hemicelluloses are a diverse group of polysaccharides that contain a mixture of hexoses and pentoses (5 and 6 carbons, respectively) and have a DP of 150-250. Pectins are also in plant cell walls. They are mostly biopolymers of β-1,4-D-galacturonic acid, with smaller amounts of rhamnose, galactose and arabinose as side chains. Some of the uronic acids are converted to esters as they are methylated or acetylated. There are also calcium (Ca^{2+}) and magnesium (Mg^{2+}) salts of uronic acid in pectins. Algae also contain non-starch polysaccharides. This includes carrageenan, agar and alginate. Carrageenan and agar are highly sulfonated. Carrageenan can react with milk products, so it is often used in dairy products and chocolate [63].

The labels on foods and beverages must indicate the total protein fat, protein and carbohydrates. Total fat can be determined by extracting foods or beverages with hexane. Triacylglycerides are the primary type of fat in most fatty foods and beverages (like whole-fat milk and cream). They are esters of glycerol plus three fatty acids. To determine the fatty acyl (or fatty acid) content of dietary fats, first the hexane used to extract them is evaporated off. Then the fatty acids are hydrolyzed (broken off) off the triacylglycerides. The fatty acids are converted to volatile fatty acid methyl esters (FAMEs) by reacting them with boron trifluoride methanol (BF_3-methanol). The FAMEs are separated by gas chromatography and can be detected by flame ionization. Total protein is measured by determining the total nitrogen content of the food or beverage using the Kjeldahl method [64]. The sample is heated and digested in sulfuric acid, which decomposes the proteins and converts the nitrogen in them to ammonium sulfate. Potassium sulfate is added to increase the boiling point of the mixture. The solution is distilled in the presence of a known amount of excess sodium hydroxide, which converts the ammonium salts to ammonia (NH_3). The amount of excess, unreacted sodium hydroxide is quantified by titration with an acid. The number of moles of sodium hydroxide that reacted with the ammonium salts is calculated. Since the average nitrogen content of proteins is about 16%, the total protein concentration is often calculated by dividing the total nitrogen content by 0.16 or multiplying by 6.25 (which is 1/0.16). However, the actual nitrogen content of proteins can vary from about 13-19%. So, the conversion factor could range from 5.26 to 7.69. So, some people use different factors that depend on the source of the protein, such as 5.18 for nuts and seeds and 6.38 for milk. Carbohydrates are calculated by difference. That is, the percent protein plus fat is subtracted from 100 to get the total carbohydrates [64].

It should be noted that the Kjeldahl method actually measures the total nitrogen concentration and not the total protein. For unadulterated foods, the Kjeldahl method is sufficient and easily automated, so it is cost-effective. However, in 2007 and 2008 pet foods and infant formulas were found to be adulterated with the deadly poison, melamine. It was added to increase the apparent protein concentration. It does that quite effectively, because it is 66% nitrogen. Its structure is shown in Figure 6. So, the FDA and other governments' regulatory agencies routinely analyze infant formulas and other foods for melamine, to make sure that it is not present and to remove any product from the market that is found to contain it.

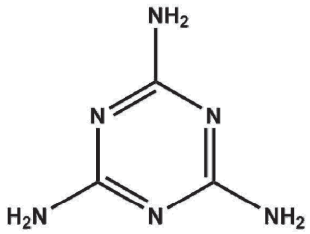

Figure 6. 2D structure of melamine, or 1,3,5-triazine-2,4,6-triamine; $C_3H_6N_6$, MW 126.12 g/mol.

The carbohydrate content of wine and fruit juices as well as most fruits and vegetables is much more than the total fats and proteins. So, the total concentration of soluble carbohydrates (excluding insoluble dietary fiber) is often measured by determining the total soluble solids, or TSS [65]. That is, wine and fruit juices contain simple sugars (mostly fructose with some glucose and sucrose) that are optically active and refract light. So, the amount of light refracted is measured with a refractometer and the percent TSS are calculated and expressed as degrees Brix (or ^{o}Bx), in which one degree Brix would be produced by a 1% aqueous solution of sucrose. That is, this method measures the concentration of carbohydrates that are soluble in water. However, there is a class of carbohydrates that is not soluble in water. They were called fatty acid glycosides when they were identified in the Polynesian fruit called noni (*Morinda citrifolia*). The structure of one of them is shown in Figure 7. They might be more properly called fatty acyl glycosides. They are fats that are not soluble in hexane or chloroform and carbohydrates that are not soluble in water. So, they would not be detected by standard analytical methods. Instead, they can be detected in methanolic extracts of several fruits by using NMR [65]. The fatty acyl glycosides that have been found in noni are beneficial

to the immune system [66, 67]. They may also have antibacterial, anticancer, analgesic, hypotensive, anti-inflammatory, immune-enhancing, anti-inflammatory and cancer chemopreventive properties [68, 69].

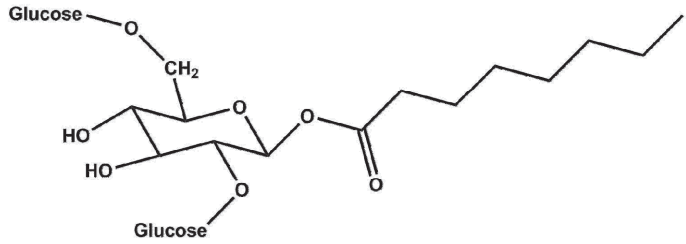

Figure 7. Structure of 2,6-di-*O*-(*α*-D-glucopyranosyl)-1-*O*-octanoyl-*α*-D-glucopyranose, also known as nonioside B; $C_{26}H_{46}O_{17}$, MW 630.273505, $[M+Na]^+$ *m/z* = 653.26327.

Fatty acyl glycosides are not the only type of carbohydrates that can play critical roles in the neuroendocrine immune system and overall human health [2, 70-73]. There are glycoproteins that are widespread and have many healthy effects on the neuroendocrine immune system [2, 70]. As mentioned in Chapter 1 of volume 1, the outer membranes of human eukaryotic cells have human leukocyte antigens, HLAs, that are unique in everybody. 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. Cancer cells, though, often have different patterns of glycosylation. At the same time, class II HLAs present extracellular protein antigens to helper T cells, activating them and triggering an immune response. The cluster of differentiation (CD) proteins CD4 and CD8 are membrane-bound glycoproteins on T cells. There are two main types of thymus cells, or T cells. They are $CD4^+$ cells, which oversee the immune response and $CD8^+$ cells which do most of the actual killing. There are also interferons, which are glycoproteins that are released in response to viruses, Bacteria, parasites and tumor cells. There are also some important glycoprotein hormones. This includes thyroid-stimulating hormone (TSH). It stimulates the production of thyroxine (T_4), which is converted into triiodothyronine (T_3), which stimulates the metabolism of almost every tissue in the body. Erythropoietin (EPO) is an essential glycoprotein hormone that controls

the production of red blood cells. Thrombopoietin is a glycoprotein hormone that regulates the production of blood platelets in the bone marrow. Follicle stimulating hormone (FSH) is a glycoprotein that regulates growth, development, sexual maturation and reproductive processes. Glycoproteins are also important in intercellular communication. For example, GPCRs are glycoproteins. Glycoproteins are also important in cancer cells. The permeability glycoprotein, P-glycoprotein, pumps anti-cancer drugs out of cells. For this reason, it is also known as the multidrug resistance protein 1 or MDR1 [2, 70].

In addition, glycosylation is the most abundant form of posttranslational modification of proteins [2, 70-73]. Glycosylation affects many biological systems, including intercellular communication, the immune response and inflammation [70-73]. The genes that code for proteins that catalyze glycosylation are often called glycogenes [72]. They form a network with microRNAs (miRNAs) that help regulate glycosylation [72, 73]. The human glycome, or collection of all the glycan structures in human cells, is orders of magnitude larger than the human genome [2].

Carbohydrates can also be a part of deadly natural toxins [2, 70]. For example, lipopolysaccharides (LPSs) are on the cell walls of gram negative Bacteria such as *Salmonella typhimurium* and various species of *Campylobacter* that can cause food poisoning. *Campylobacter* species can activate the immune system through proteins that are glycosylayed with N-acetyl α-D-glucosamine (α-GlcNAc) [70, 74]. However, even food and beverages that are not contaminated with pathogenic bacteria can also be quite harmful to human health if they contain simple carbohydrates such as glucose, fructose or sucrose.

That is, over-consumption of simple carbohydrates and foods with a high glycemic index can lead to hyperglycemia, smoldering inflammation, metabolic syndrome, an increase in abdominal fat and type-2 diabetes, as well as dysregulated immune responses [2, 70, 75, 76]. When over-consumed, glucose and its metabolites (such as methyl glyoxal) can react with proteins and body tissues [2, 70]. The products of these reactions are advanced glycation end products (AGEs) as well as RONS that are quite toxic. One important example is glycated hemoglobin, usually known as HbA_{1c}, the concentration of which is elevated in diabetes. At the same time, a chronic excess of glucose can change glucose metabolism. Some of it is converted into fructose 6-phosphate, which enters the hexosamine pathway. This subsequently produces *N*-acetylglucosamine, which can react with serine and tyrosine residues in protein kinases that are important in signal transduction. Also, some of the excess glucose can be converted to sorbitol, which enters the polyol pathway in a reaction catalyzed by

aldol reductase. In the process, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) is converted to nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$). This decreases the amount of NADPH that is available to synthesize reduced glutathione, as well as nitric oxide, *myo*-inositol and taurine. Nitric oxide (NO) is a vasodilator in blood vessels. In healthy endothelial cells, insulin activates endothelial nitric oxide synthase (eNOS). In diabetes, endothelial insulin resistance impairs the activation of eNOS, reduces endothelium-dependent vasodilation, and eventually promotes atherosclerosis. Moreover, when the endothelium is inflamed due to activation by NFκB, cellular signaling pathways controlled by insulin can become dysfunctional. This decreases the bioactivity of NO and increases the release of cytokines. Also, glutathione is needed to destroy RONS. The elevated concentrations of RONS cause oxidative damage and smoldering inflammation. They can react with NO to make especially reactive peroxynitrate ($ONOO^-$), which reacts with some proteins, altering their function [2, 70]. The formation of AGEs and an informative figure that shows the biochemical pathways of AGE formation can be found in a review article [77].

N-(carboxymethyl)lysine (CML) is also an important AGE [2, 70]. It accumulates in adipose tissue and fatty liver while dysregulating the production of cytokines. CML is an important ligand for the receptor for AGE (RAGE). By binding to its cognate receptor, AGEs not only cause insulin resistance, but also pancreatic β-cell dysfunction and apoptosis. AGEs can also oxidize lipids, damage mitochondria and cause apoptosis in cardiomyocytes. They can also cause diabetic cardiomyopathy [2, 70].

AGEs can cause smoldering inflammation, which can lead to insulin resistance, atherosclerosis and the vascular complications of diabetes [2, 70, 78]. Although AGEs mostly occur as metabolic products of excess glucose consumption, there are dietary sources of them, too. They are produced by the Maillard reaction during the heating and browning of foods and beverages. Consumption of glycated proteins can encourage the growth of several harmful Bacteria in the gut. This decreases the production of anti-inflammatory short chain fatty acids by helpful Bacteria and helps luminal endotoxins enter into the blood. In addition, AGEs change the structure and function of extracellular proteins such as collagen. This impairs wound healing, while changing cellular adhesion and movement and dysregulating intercellular communication. At the same time, intracellular AGEs can react with mitochondrial proteins, leading to altered transport of electrolytes, and increasing the production of RONS. This, in turn, leads to mitochondrial dysfunction. Moreover,

AGEs can catalyze the formation of RONS, leading to oxidative stress and inflammation at sites where the AGEs accumulate [2, 70, 78].

Third, when AGEs bind their cognate receptors like RAGE they trigger the production of a cascade of pro-inflammatory proteins [2, 70, 78]. This includes NF-κB, TNF-α and monocyte chemoattractant protein 1 (MCP-1). Also, the pro-inflammatory enzyme, NADPH oxidase, is activated while RONS are produced and NF-κB activity is sustained. The activated NF-κB transcription factor stimulates the transcription of genes coding for the pro-inflammatory cytokines IL-6, TNF-α, C-reactive protein, chemokines, pro-coagulants (like thrombin), vascular endothelial growth factor and adhesion proteins. NF-κB also upregulates the expression of RAGEs, which increases the amount of inflammation. Moreover, RAGE is an important part of the innate immune system since it can interact with microbial products (pathogen-associated molecular pattern, PAMP, molecules) as well as endogenous substances that are released after tissues become damaged and inflamed. Finally, RAGE is also involved with Toll-like receptors that help to coordinate and regulate inflammatory responses [2, 70, 78].

So, AGEs are important modulators of the immune system. However, our bodies have ways to defend against them [2, 70, 78]. This includes the glyoxylase system, which catalyzes the detoxification of several dicarbonyl AGEs and activates the Keap1-Nrf2-ARE signaling system [2, 70, 78].

Overconsuming glucose, fructose and/or sucrose can also impair the neuroendocrine immune system by producing abdominal fat and adipocytes (fat cells) [2]. They release the following pro-inflammatory proteins: IL-1, IL-6 and TNF-α [70, 76]. These proteins can lead to smoldering inflammation while being false alarms to the neuroendocrine immune system. In time, the responsiveness of the neuroendocrine immune system decreases, delaying its response to infections. Also, obese people tend to have fewer white blood cells that are needed to fight infection and the cells they do have are less phagocytic. Overconsumption of simple sugars can reduce white cell phagocytosis. In contrast, the natural glycoside stevioside (from the South American stevia plant, *Stevia rebaudiana*) may be anti-inflammatory and improve phagocytosis. Also, dietary fiber can reduce inflammation by feeding endogenous *Bifidobacterium* and *Bacteriodes* [70, 76]. These bacterial species help the immune system protect against the development of inflammatory diseases [2].

In contrast, a lack of sufficient dietary carbohydrates can be very harmful in people who are suffering from malnutrition, starvation,

anorexia or bulimia, and limit the performance of extreme endurance athletes [70, 76, 79, 80]. In addition, when sugars are attached to phenolic compounds to make flavonoid glycosides, they produce anti-inflammatory compounds like hesperidin (Figure 8) that activate Keap1-Nrf2-ARE signaling system. On the other hand, the glycosylated compound aloin (Figure 9) from *Aloe vera* is not generally recognized as safe for oral consumption, even though it has been sold as a laxative [70, 81].

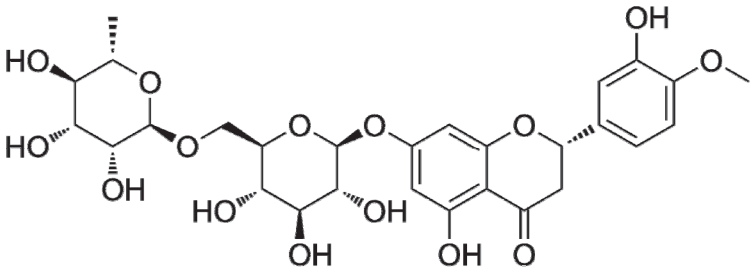

Figure 8. 2D **s**tructure of hesperidin, a flavonone glycoside in citrus fruits.

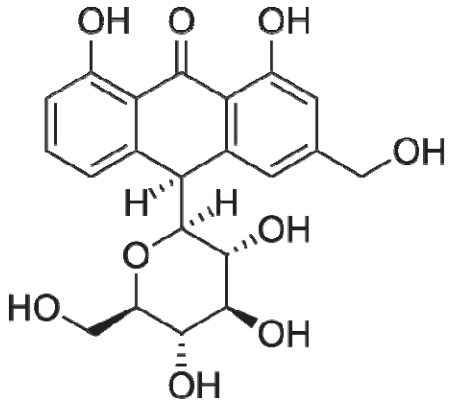

Figure 9. 2D **s**tructure of aloin, a toxic anthraquinone glycoside.

There are also some dietary water-soluble polysaccharides that affect the neuroendocrine immune system [70]. The International Union of Pure and Applied Chemistry (IUPAC) considers polysaccharides and glycans to be synonyms and defined them as “compounds consisting of a large number of monosaccharides linked glycosidically” [82]. However, the glycans are also referred as the carbohydrate portion of a glycolipid,

glycoprotein or proteoglycan (a core protein with one or more covalently bound glycosaminoglycan chains) [70]. The glycosaminoglycans chondroitin sulfate, heparan sulfate and keratan sulfate are the main components of the extracellular matrix [70]. Chondroitin sulfate, which is in many popular dietary supplements, has immunomodulatory and anti-inflammatory effects [70, 83]. The structure of one unit in a chondroitin sulfate chain is shown in Figure 10. It impairs the production of pro-inflammatory cytokines and enzymes in chondrocytes, which are the cells in healthy cartilage that produce and maintain the cartilaginous matrix [83]. It was also more effective than placebo in reducing pain, joint swelling and effusion while improving articulate function and in preventing the joint space in the knee from narrowing in patients with osteoarthritis [70, 83], an autoimmune disease [84].

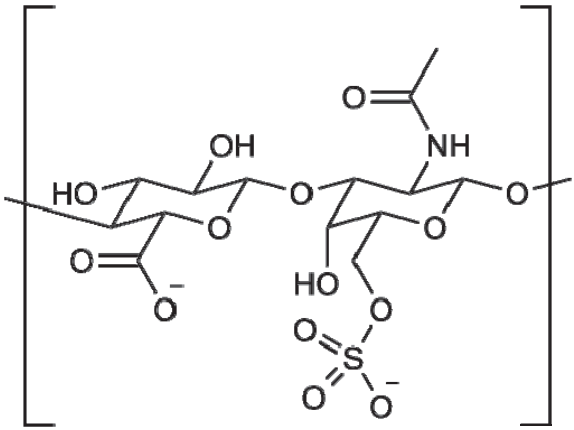

Figure 10. 2D structure of one unit in a chondroitin sulfate chain.

Glucosamine may also be a useful addition to cyclosporine A in treating psoriasis, due to its immunomodulatory effects in autoimmune diseases induced by T-cell inflammation [70, 85]. In a mouse model of psoriasis, 300 mg/kg glucosamine plus a low dose (10 or 30 mg/kg) of cyclosporine prevented the development of skin lesions, while lowering the concentrations of cytokines produced by the helper T-cells, T_H1 and T_H17, as well as increasing $CD4^+$ and $CD25^+$ regulatory T-cells [85]. Glucosamine has also been shown to be able to decrease inflammation in the lungs that is caused by cigarette smoke by inhibiting inflammatory signals produced by RONS [86]. That is, cigarette smoking activates NADPH oxidase, which produces RONS that activate the AMPK

signaling pathway as well as NFκB signal transducer and activator of transcription proteins 3 (STAT 3) and IL-8. Glucosamine decreased these effects [86]. Finally, glucosamine and chemically modified glucosamine can be made into nanoparticles to improve the delivery of drugs and/or genes to therapeutic targets [70, 87].

There are also many immunomodulatory water-soluble polysaccharides and polysaccharide-protein complexes in traditional medicines, edible mushrooms, many types of seaweed, oats, soybeans, ginseng, turmeric, licorice and several fruits [70, 88-106]. They enhance and/or activate immune responses in macrophages and the complement system [70, 88]. They increase the cytotoxic activity of macrophages against cancer cells and microorganisms, while activating phagocytic activity and increasing the concentrations of RONS and NO. Water-soluble polysaccharides also increase the secretion of cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-8, IL-12, IFN-γ and IFN-β. So, they have immunomodulatory, anti-tumor activity, wound-healing and other therapeutic effects [70, 88].

For example, polysaccharides isolated from blue-green algae, *Spirulina platensis*, have anti-tumor and anti-metastatic activities [70, 88]. Many other polysaccharides from several sources have anti-cancer activities as well [89]. Dietary polysaccharides also have healthy effects on the gut microbiome, which can improve one's nutrition, immune response, resistance to pathogens intestinal epithelial development and energy metabolism [89]. They may also improve communication between the gut microbiome and the host's brain [90]. That is, the gut microbiome and brain communicate with each other through endocrine, immune, neural and metabolic pathways. So, improvements in the gut microbiome can improve cognitive function [70, 90].

In addition, at least 131 drugs based on carbohydrates are listed in the United States, European, Japanese and Chinese Pharmacopoeias and 18 are marketed [70, 91]. This includes pentosan polysulfate sodium, or Elmiron® that the FDA has approved for interstitial cystitis. There is also Icodextrin, a glucan with a molecular weight of about 16 kiloDaltons (16 kDa) that is a colloid osmotic agent obtained from corn starch. It is used in peritoneal dialysis and for treating chronic kidney failure. There are several other carbohydrate-based drugs in development as well [70, 91].

Soluble polysaccharides can bind to pattern recognition receptors, such as the TLRs that link innate and acquired immunity [70, 92]. There are also scavenger receptors (SRs) such as a β-D-glucan receptor called Dectin-1. Scavenger receptors recognize and scavenge modified LDLs. Dectin-1 is a mannose receptor and complement receptor type 3 that are on the surfaces of macrophages and many types of dendritic cells. There are

some soluble innate immune receptors in tissue fluids that are also circulating in the bloodstream. This includes C-reactive protein, mannan-binding lectin, lipopolysaccharide-binding protein and proteins in the alternative and classical complement pathways. When immunostimulatory polysaccharides bind to their cognate receptors, signaling pathways are activated, causing genes to be transcribed. For example, when fucoidin binds to SRs on macrophages, two protein tyrosine kinase signaling pathways are activated. On the other hand, the binding of soluble (β1→3)-D-glucans to CR3 stimulates immune function through overlapping signaling pathways. This includes the focal adhesion kinase, spleen tyrosine kinase, phosphatidylinositol-3 kinase, protein kinase B (also known as Akt), p38 mitogen activated protein kinase, phospholipase C and protein kinase C (PKC). The binding of (β1→4)-D-mannans to TLR4 activates the extracellular signal-related kinase (ERK) 1/2, c-Jun N-terminal kinase JNK 1/2 and p38 mitogen-activated protein kinase (MAPK). When polysaccharides activate transcription factors, genes coding for mediators of inflammation are transcribed [70, 92].

To obtain many water-soluble polysaccharides from plants and their fruits, ethanol is added to the aqueous extracts until it reaches a concentration of about 75% by volume [70, 93-103]. This makes the polysaccharides insoluble, so they can be isolated by filtration. They have molecular weights ranging from 10^3 to over 10^6 Da. For example, a standardized extract from the roots of *Echinacea augustifolia* that contain 8 mg of a soluble polysaccharide with an average molecular weight of about $2x10^3$ Da upregulated the mRNAs coding for IL-2 and IL-8, while the mRNAs for the pro-inflammatory cytokines TNF-α and IL6 were downregulated in monocytes in healthy people [70, 101]. A different polysaccharide from the Asian and Brazilian fruit litchi (*Litchi chinesis*) with a molecular weight of about $4.72x10^4$ promoted the proliferation of splenocytes in mice, while enhancing the toxicity of NK cells [95]. Two soluble polysaccharides with average molecular weights of $8x10^4$ and $6x10^5$ were also isolated from black currant (*Ribes nigrum* L.) fruit juice [97]. They worked synergistically to stimulate the activity of macrophages [97]. Soluble polysaccharides from edible algae (including seaweed) can interfere with the migration of leukocytes to sites of inflammation, while others inhibit iNOS [70, 104].

At the same time, a protein-polysaccharide complex (PSK, Krestin), from the mushroom *Coriolus versicolor* is quite popular in Japan [70, 105]. A different peptide-polysaccharide from *C. versicolor* is popular in China due to its anti-cancer and immunomodulatory properties. Each of them can cause the concentrations of several cytokines to increase. These

cytokines subsequently induce gene expression. PSK (Krestin) is a β-glycan-protein complex containing 25-38% protein. The polysaccharopeptide isolated in China has an average molecular weight of about 10^5 Da and has a structure that is similar to that of PSK. These and several other polysaccharide-protein complexes have anticancer activities [70, 105].

Mushrooms are popular folk medicines that have attracted considerable attention because of their efficient antitumor activities [106]. They contain polysaccharides, including (1→3)-β-D-glucans. They modulate the neuroendocrine immune system and have anticancer properties. They stimulate T cells and other immune cells. These polysaccharides can trigger several cellular responses, such as the expression of cytokines and NO. Many of these polysaccharides bind to polypeptides and proteins to form complexes that have strong antitumor activities [106].

So, a variety of soluble, immunomodulatory polysaccharides and polysaccharide-protein complexes have been obtained from many different sources [70]. However, an extensive analysis of the most widely consumed fruits and vegetables to see if they have immunomodulatory polysaccharides in them has not been done. Furthermore, individual polysaccharides have not been quantified, since there are no primary standards. That is, sufficient quantities of purified standards that can be used to calibrate an analytical method are not available. So, it is only possible to determine the concentration of total ethanol-insoluble, but aqueous-soluble polysaccharides by weighing the filtered precipitate obtained after adding enough ethanol to an aqueous extract of a food or other sample to reach 75% ethanol [70].

There are also many galactolipids in vegetables that may have several health benefits [70, 107]. Acylated steryl glucosides (ASGs), monogalactosyldiacylglycerols (MGDGs), steryl glucosides (SGs), ceramide monohexosides (CMHs) and digalactosyldiacylglycerols (DGDGs) have been found in a variety of foods [70, 108]. MGDG and DGDG are present in a variety of legumes, leafy vegetables, stem vegetables, root vegetables and fruits [107]. Different spinach samples had anywhere from 546 to 38 880 mg/kg fresh weight MGDG. Sweet potato leaves had the most DGDG, with 22 640 mg/kg fresh weight [107].

There are at least three patents or patent applications for products that contain galactolipids [70, 107]. One describes a product obtained from spinach leaves that contains MGDGs, DGDGs and sulfoquinovosyl diacylglycerols. It can inhibit DNA synthase and cancer cell growth, so it has anticancer activity [70, 107]. The structure of sulfoquinovosyl dipalmitoylglycerol is shown in Figure 11.

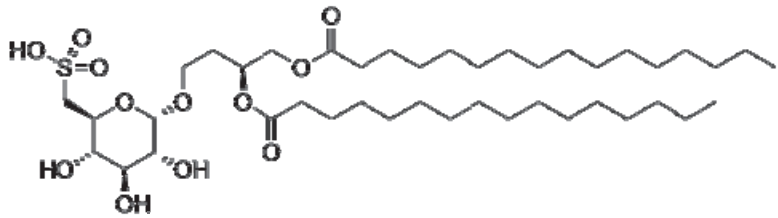

Figure 11. Structure of sulfoquinovosyl dipalmitoylglycerol.

Another patent describes a product containing MGDGs that was isolated from the fruits of dog rose (*Rosa canina*) [70, 108]. It is used to treat inflammatory conditions and acts by preventing chemotaxis and the oxidative burst response of leukocytes. A third patent describes a way to prepare anti-inflammatory MGDGs and DGDGs from a several other plants [70, 108].

Galactosylceramides (GalCers) are also important bioactive galactolipids [109-111]. They are also known as galactocerebrosides. Ceramides are esters of sphingosine and a fatty acid. The generalized structure of ceramides is shown in Figure 12.

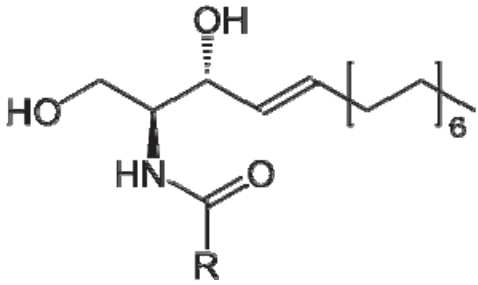

Figure 12. Generalized 2D structure of ceramides, where R is a fatty acyl.

Monoglycosylated ceramides are present in the cell walls and cell membranes of just about all cells [70, 109]. GalCers and their metabolites regulate nerve cells, protein kinase C (PKC) and the function of hormone receptors. One important metabolite, ceramide, induces the dephosphorylation and inhibition of the protein kinases Akt, PKC and MAPK [70, 109]. This makes them antiproliferative and anti-apoptotic [70, 111]. Also, some of the Bacteria that cause food poisoning activate

the immune system through proteins that have *N*-acetyl-α-D-glucosamine attached to them [70, 74].

Until recently, it was thought that glycosphingolipids in mammalian cells only had sugars with β-anomeric carbons [70, 112, 113]. However, it was recently reported that the natural ligands for natural killer T cells (NKTs) are α-linked glycosylceramides [70, 113]. Six different GalCers were isolated from the marine sponge *Agelas mauritianus* and found to have potent anti-tumor activities [109]. This led to the development of a synthetic α-GalCer for treating autoimmune diseases [114, 115]. It has potential uses not only in treating autoimmune diseases and allergies, but also cancer [116]. It activates invariant NKTs (iNKTs) by binding to CD1 receptors on antigen-presenting cells. This triggers the secretion of cytokines. This synthetic α-GalCer suppresses the stimulation of eosinopoiesis by inducing apoptosis [116]. Also, targeted delivery of α-GalCer to CD8a+ dendritic cells optimizes the antitumor responses of type I iNKTs [117]. It is also effective when used in combination with a lipopolysaccharide (LPS) in a mouse model of lung metastasis [118]. They acted synergistically to enhance the immune responses that were specific to tumor antigens and suppressed tumor growth [70, 118]. So, there are several galactolipids and glycolipids that affect the immune system and human health [70].

3.1.4 Vitamins and minerals

Vitamins are organic compounds that human eukaryotic cells can't make for themselves. They must be ingested in food, beverages and/or dietary supplements to prevent syndromes such as scurvy, beriberi and pellagra [119]. People who have inadequate vitamin intake due to consuming the typical American (USA) fast food diet or other factors might be well-advised to take vitamin supplements. The Food and Nutrition Board, National Academy of Sciences and National Research Council of the USA have listed recommended daily allowances (RDA) of protein, vitamins and minerals [120], as shown in Tables 1 and 2.

Table 1. Food and Nutrition Board, National Academy of Sciences and National Research Council of the USA Recommended Daily Dietary Allowances for Vitamins [120].

Group	Vit. A µg[1]	Vit. D µg[2]	Vit.E mg[3]	Vit. K µg	Vit.C mg	Thia-min mg	Ribo-flavin mg	Niacin mg[4]	Vit. B_6 µg	Folate µg	Vit.B_{12} µg
Infants 0-0.5 yr	375	7.5	3	5	30	0.3	0.4	5	0.3	25	0.3
Infants0.5-1 yr	375	10	4	10	35	0.4	0.5	6	0.6	35	0.5
Children1-3 yr	400	10	6	15	40	0.7	0.8	9	1.0	50	0.7
Children4-6 yr	500	10	7	20	45	0.9	1.1	12	1.1	75	1.0
Children7-10 yr	700	10	7	30	45	1.0	1.2	13	1.4	100	1.4
Males 11-14 yr	1000	10	10	45	50	1.3	1.5	17	1.7	150	1.7
Males 15-18 yr	1000	10	10	65	60	1.5	1.8	20	2.0	200	2.0
Males 19-24 yr	1000	10	10	70	60	1.5	1.7	19	2.0	200	2.0
Males 25-50 yr	1000	5	5	80	60	1.5	1.7	19	2.0	200	2.0
Males>50 yr	1000	5	5	80	60	1.2	1.4	15	2.0	200	2.0
Females11-14 yr	800	10	10	45	50	1.1	1.3	15	1.4	150	2.0
Females15-18 yr	800	10	10	55	60	1.1	1.3	15	1.5	180	2.0
Females19-24 yr	800	10	10	60	60	1.1	1.3	15	1.6	180	1.6
Females25-50 yr	800	5	5	65	60	1.1	1.3	15	1.6	180	1.6
Females>50 yr	800	5	5	65	60	1.0	1.2	13	1.6	180	1.6
Pregnant	800	10	10	65	70	1.5	1.6	17	2.2	400	2.2

Lactating0-6 mo	1300	10	12	65	95	1.6	1.8	20	2.1	280	2.6
Lactating7-12 mo	1200	10	11	65	90	1.6	1.7	20	2.1	260	2.6

1 Retinol equivalents = 1 μg retinol or 6 μg of β-carotene

2 As cholecalciferol, 10 μg of cholecalciferol = 400 IU (International units) of vitamin D

3 α-tocopherol equivalents

4 Niacin equivalents = 1 mg niacin or 60 mg of dietary tryptophan

Table 2. Food and Nutrition Board, National Academy of Sciences and National Research Council of the USA Recommended Daily Dietary Allowances for Protein and Minerals [120].

Group	Protein (g)	Ca (mg)	P (mg)	Mg (mg)	Fe (mg)	Zn (mg)	I (µg)	Se (µg)
Infants 0-0.5 yr	13	400	300	40	6	5	40	10
Infants0.5-1 yr	14	600	500	60	10	5	50	15
Children1-3 yr	16	800	800	80	10	10	70	20
Children4-6 yr	24	800	800	120	10	10	90	20
Children7-10 yr	28	800	800	170	10	10	120	30
Males 11-14 yr	45	1200	1200	270	12	15	150	40
Males 15-18 yr	59	1200	1200	400	12	15	150	50
Males 19-24 yr	58	1200	1200	350	10	15	150	70
Males 25-50 yr	63	800	800	350	10	15	150	70
Males>50 yr	63	800	800	350	10	15	150	70
Females11-14 yr	46	1200	1200	280	15	12	150	45
Females15-18 yr	44	1200	1200	300	15	12	150	50
Females19-24 yr	46	1200	1200	280	15	12	150	55
Females25-50 yr	50	800	800	280	15	12	150	55
Females>50 yr	50	800	800	280	10	12	150	55
Pregnant	50	1200	1200	300	30	15	175	65
Lactating0-6 mo	65	1200	1200	355	15	19	200	75
Lactating7-12 mo	62	1200	1200	340	15	16	200	75

Note that there is an important difference between the RDA for protein and the recommendations of the U.S. Institute of Medicine (IOM), which include one's body weight in the recommendations [120]. The IOM recommended a daily protein intake of 0.8 g/kg/day [120]. This might seem to be helpful if one considers that a tall, 100 kg man with a healthy body weight and little abdominal fat will most likely need more protein than a much shorter man weighing 60 kg who also has a healthy body weight and little abdominal fat. However, it's a dangerous over-simplification if one compares the dietary requirements of a shorter 100 kg man who is obese and has much abdominal fat. This is especially true if such a man (or woman) became obese by eating lots of red meat and assumes that they should continue doing so. That is, one should use systems thinking and consider not just the total amount of protein, but also the other things that are in the source of the protein, how it is prepared and what other foods and beverages one consumes. So, seafood that is fried in lard will be much worse than seafood that is baked, broiled or poached in a mixture of water and olive oil. However, even seafood that is cooked in a healthy way (or even uncooked in Sushi) can be of little help if it is served with fried potatoes and a sweetened beverage that is topped off with a sweet roll (even if it has cinnamon in it).

However, the recommended dietary allowance for protein can be misunderstood in other ways [121]. The RDA was determined based on the results of as many studies as possible that estimated the amount of protein needed to "avoid a progressive loss of lean body mass as reflected by nitrogen balance" [121]. The Food and Nutrition Board of the IOM admitted the limits of this approach that did not consider any relevant physiological endpoint and the data came almost exclusively from men who were college-aged. It is quite likely that the elderly need more protein than the young [121]. It is also important to remember that the RDA is defined as "an estimate of the *minimum* daily average dietary intake level that meets the nutrient requirements of nearly all (97 to 98 percent) healthy individuals" [120]. Moreover, the energy requirements of a man who weighs 70 kg is much higher (3067 kcal/day) than a woman who weighs 57 kg and is over 18 years of age (2403 kcal/day) [121]. Note that the units of energy used (kcal) would be readily recognized by physicists, but many nutritionists might prefer Calories (where 1 Calorie = 1000 calories = 1 kcal). However, the total of the RDAs for carbohydrates, fats and protein for people of the same weight would supply only 970 and 886 kcal/day for men and women, respectively [120]. So, the Food and Nutrition Board of the IOM included an Acceptable Minimum Distribution Range (AMDR), as well as tolerable upper intake level in their recommendations [120,

121]. The AMDR reflects optimal intake of nutrients, instead of just the minimum requirements [121]. The daily requirement for energy for a sedentary 19-year old man who weighs 76 kg and is 1.76 m tall is about 37.8 kcal/kg/day. So, the RDA of 0.8 kcal.kg/day would be less than 10% of the energy intake. This is much less than the 35% that is recommended by the AMDR. This led to a widespread misinterpretation of the recommendations. The historically familiar term 'recommended daily allowance' was used, but it was defined in a way that many misinterpreted. That is, the word 'recommended' implied that the RDA is not a requirement, but just a suggestion. That is, the word 'requirement' implies that a minimal amount is needed. Also, the term 'allowance' might imply to many people that it is the permissible amount and not a minimum amount. The AMDR also concluded that there is no upper limit of the amount of protein that one should consume and that the percentage of total energy obtained from protein should be 10-35%. This might reflect the lack of definitive research on the subject, but it also implies an uncertainty in the amount of fat and carbohydrates that one should consume. Still, there is much evidence to support the idea that the optimal level of protein intake should be higher than the minimum recommended. This is important for maintaining proper muscle mass, strength and function as well as for supporting bone health, maintaining energy balance. It is also important for maintaining proper cardiovascular function and wound healing. So, it was recommended that the term 'minimal daily requirement' should replace 'recommended dietary allowance'. Still, it is the RDA that is recognized by most people and has the greatest influence on dietary practice [121].

So, the AMDR has published Dietary Reference Intakes (DRIs) for total digestible carbohydrates; dietary fiber; total fat; ω-6 polyunsaturated fats (like linoleic acid, structure shown in Figure 13); ω-3 fatty acids (like α-linolenic acid, structure shown in Figure 14); saturated, *trans* fats and cholesterol; as well as protein and amino acids, including the indispensable amino acids, histidine, isoleucine, leucine, lysine, methionine plus cysteine, phenylalanine plus tyrosine, threonine, tryptophan and valine [122]. They also published DRIs, adequate intakes and upper limits for vitamins and briefly described their functions [123]. It includes some vitamins and nutrients (biotin, choline, pantothenic acid and riboflavin) that are not listed in Tables 1 or 2. Note that the upper limits of biotin, pantothenic acid, riboflavin, thiamin, vitamin B_{12} and vitamin K have not been determined for any age group, while upper limits for choline, folate, niacin, vitamin B_6 and vitamin E have been determined and are listed for all age groups except infants. Upper limits for vitamins A and D are given

for all age groups, including infants. The DRIs, adequate intakes (AIs) and upper limits of vitamins A, C and D are shown in Table 3.

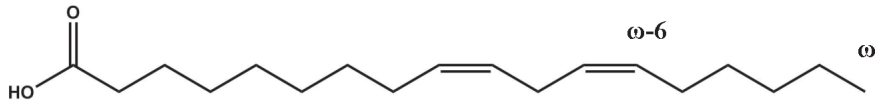

Figure 13. 2D structure of linoleic acid, an ω-6 polyunsaturated fat. IUPAC name: (9*Z*,12*Z*)-9,12-Octadecadienoic acid.

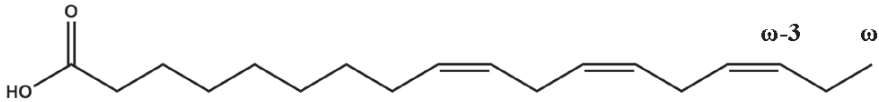

Figure 14. 2D structure of α-linolenic acid, an ω-3 fat.

Table 3. Dietary Reference Intakes (DRIs), Adequate Intakes (AIs) and Upper Limits (ULs) of vitamins A, C and D.

Group	Vit. A DRI (µg/d)	Vit. A AI	Vit. A UL	Vit. C DRI (mg/d)	Vit. C AI	Vit. C UL	Vit. D DRI (µg/d)[a]	Vit. D AI (IU/d)[a]	Vit. D UL (IU/d)[a]
Infants 0-6 mo	ND	400	600	ND	40	ND	ND	400	1000
Infants7-12 mo	ND	500	600	ND	50	ND	ND	400	1500
Children1-3 yr	210	300	600	13	15	400	10	600	2500
Children4-8 yr	275	400	900	22	25	650	10	600	3000
Males9-13 yr	445	600	1700	39	45	1200	10	600	4000
Males 14-18 yr	630	900	2800	63	75	1800	10	600	4000
Males 19-30 yr	625	900	3000	75	90	2000	10	600	4000
Males 31-50 yr	625	900	3000	75	90	2000	10	600	4000
Males 51-70 yr	625	900	3000	75	90	2000	10	600	4000
Males>70 yr	625	900	3000	75	90	2000	10	800	4000
Females9-13 yr	420	600	1700	39	45	1200	10	600	4000
Females14-18 yr	485	700	2800	56	65	1800	10	600	4000

Females19-30 yr	500	700	3000	60	75	2000	10	600	4000
Females31-50 yr	500	700	3000	60	75	2000	10	600	4000
Females51-70 yr	500	700	3000	60	75	2000	10	600	4000
Females >70 yrs	500	700	3000	60	75	2000	10	800	4000
Pregnancy≤18 yrs	530	750	2800	66	80	1800	10	600	4000
Pregnancy19-30 yrs	550	770	3000	70	85	2000	10	600	4000
Pregnancy 31-50 yr	550	770	3000	70	85	2000	10	600	4000
Lactation≤18 yrs	885	1200	2800	96	115	1800	10	600	4000
Lactation19-30 yrs	900	1300	3000	100	120	2000	10	600	4000
Lactation31-50 yr	900	1300	3000	100	120	2000	10	600	4000

a One IU of vitamin D is equal to 0.025 μg, so IU * 0.025 = μg

Some of the most important vitamins, like folic acid, are added to enriched breads, cereals, flours, corn meals, pastas, rice, and other grain products. At physiological pH, folic acid exists as folate, but dietary supplements and enriched foods have folic acid in them. A lack of folic acid causes birth defects, such as spina bifida, which leaves the victim severely disabled. Folic acid is also found in multi-vitamin supplements, and these are recommended for pregnant women. However, not all pregnancies are planned and not everyone can afford to buy multivitamins. So, not just the US FDA, but also the governments of many other nations fortify foods with folic acid, also known as folate and vitamin B_9. This is because the concentration of folate in the blood plasma of pregnant women affects DNA methylation and folate is essential for fetal development [124]. So, even before conception and certainly after pregnancy and delivery, women are advised to take dietary supplements that contain folate and eat foods that are enriched in folate [124]. So, if a nurse, physician, pharmacist or employee of a pharmaceutical company or the FDA is ever told by an uninformed person that they are all in a conspiracy to keep people from having access to lifesaving dietary supplements, just tell them about folic acid. We are all working together to make sure that everyone has access to it. Many of us also strongly encourage almost everyone to take dietary supplements that contain vitamin D.

This is because vitamin D has been shown to have many important health effects beyond the classical effects on mineral homeostasis and bone metabolism [125]. Actually, there are several forms of vitamin D. Vitamin D3 (cholecalciferol) is a prohormone that is converted to 7-dehydrocholesterol in the skin when it is exposed to UV radiation [126]. It is biologically inactive, so it's metabolized to calcifediol, or 25-hydroxyvitamin D3 (25-D) in the liver and then to 1α,25-dihydroxyvitamin D3 (1,25-D) in the kidneys before function [126]. That is, the inactive 25-D (25-OD) form of vitamin D is converted to the active 1,25-D (1,25(OH)2D) form by a reaction in the mitochondria of kidneys that is catalyzed by 1-alpha hydroxylase, also known as CYP27B1 [125]. The structures of vitamin D3, 25-D and 1,25-D (1,25(OH)2D) are shown in Figures 15 and 16. The active, hormonal form of vitamin D3 acts through a nuclear receptor to induce its many effects, including calcium absorption, phosphate absorption in the intestine, Ca^{2+} mobilization in bones and Ca^{2+} reabsorption in the kidney [126]. This has been known for quite some time [125]. However, more recently, CYP27B1 has been also found in the parathyroid glands, adrenal medulla, pancreas, skin, nodes, cerebellum and monocytes. Monocytes express both CYP27B1 and the

nuclear vitamin D receptor and use the conversion of 25OHD to 1,25(OH)2D to promote innate antibacterial responses to infection, mainly through the induction of the antibacterial cathelicidin in the lysosomes of macrophages and polymorphonuclear leukocytes. So, 1,25(OH)D helps the innate immune system respond properly to infections. Also, a deficiency of 25-D can lead to bone fractures, infection, cancer and autoimmune diseases. This deficiency also increases risk of cardiovascular disease, mortality and progression of aortic calcification. It also increases the incidence of other cardiovascular risk factors, including hypertension, insulin resistance, diabetes, and dyslipidemia. In contrast, dietary supplements containing cholecalciferol have anti-inflammatory properties. So, adult patients suffering from chronic kidney disease and are on dialysis patients may want to take 200 000 IU monthly for three months, followed by 80 000 IU monthly for the next three months. In addition, vitamin D has also been shown to inhibit the iron regulatory hormone hepcidin in human cells and in healthy volunteers. So, vitamin D also plays an important role in regulating iron metabolism [125].

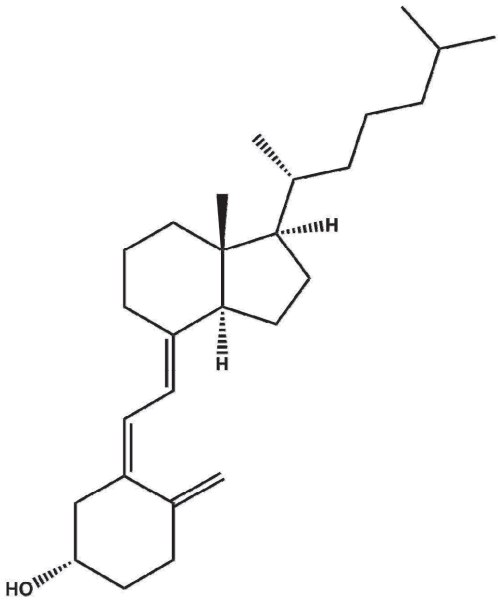

Figure 15. 2D structure of vitamin D3, also known as cholecalciferol.

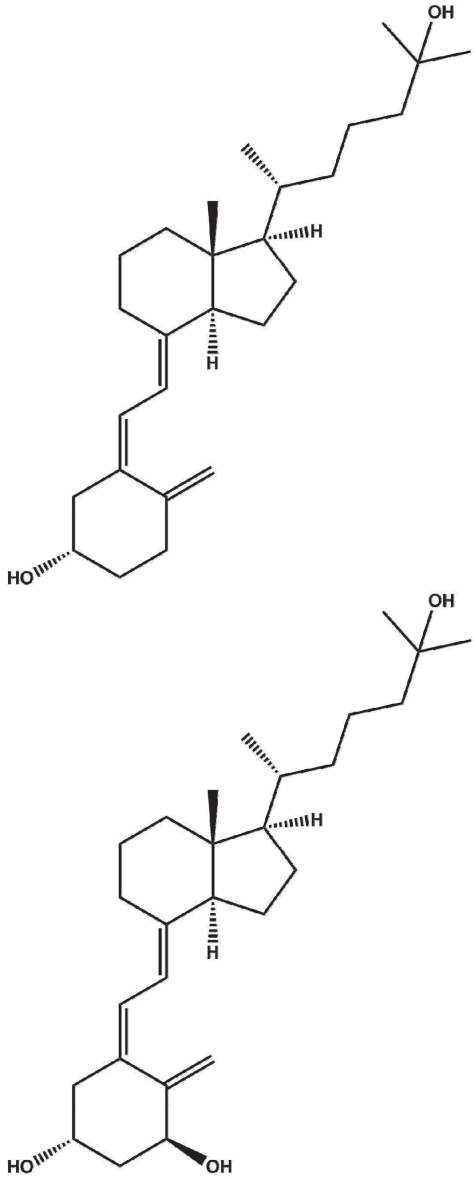

Figure 16. 2D structure of 25-hydroxyvitamin D3, also known as calcifidiol and 25-D (left) and 1α,25-dihydroxyvitamin D3, also known as calcitriol and 1,25(OH)2D (right).

Even though vitamin D can help prevent cardiovascular diseases when it is consumed in sufficient amounts [127], it can increase the risk when consumed excessively [128]. That is, vitamin D has a hormetic or U-shaped (nonlinear) dose-response curve. There is high toxicity at zero dose and inadequate doses. Toxicity gradually decreases to almost zero at adequate doses, but starts to rise nonlinearly at higher, toxic doses. The physiological effects are best evaluated based on the concentration of 25OD that circulates in the blood stream. The IOM classified circulating 25OD concentrations of 50 – 125 nmol/L as adequate, 30 - 49.99 as inadequate and >125 nmol/L as potentially harmful [128, 129]. The potentially harmful effects have been known for quite some time [128]. Excess vitamin D can lead to soft tissue calcification in infants, as well as infantile hypercalcemia with supravalvular aortic stenosis, mental retardation and craniofacial malformation. On the other hand, it can prevent cardiovascular disease, as demonstrated by epidemiological data. In other words, mortality in Australia due to coronary heart disease and stroke was lowest during the winter and highest during the summer, when people were exposed to more UVB sunlight. Moreover, mortality was lower in northern latitudes in the USA, where there is less exposure to UVB sunlight. There have been some randomized controlled clinical trials, in which vitamin D was given at doses from 200 to 5000 IU per day (5 to 125 μg per day). The results were mixed. About half of the studies showed some improvements in lipid parameters, inflammatory markers, endothelial function and cardiac function. With the exception of one subject who was HIV positive, none of the studies found an increased risk or adverse side effects when compared to the subjects who received placebo. There were also some studies in which doses ranged from 20 000 IU weekly to 300 000 IU monthly that had mixed results. Four out of 13 studies reported benefits, while none of them reported adverse side effects. There are more studies being planned in the USA, Australia, New Zealand and Europe [128].

This includes the Biochemical Efficacy and Safety Trial of vitamin D (BEST-D trial) [130]. Its goal is to determine effects of daily dietary supplementation with 100 μg or 50 μg vitamin D3 or placebo, when administered for 12 months, in 305 ambulant community-dwelling older people living in Oxfordshire, England. The primary analyses will compare mean plasma concentrations of 25(OH)D over 12 months, as well as the proportion of participants with a concentration >90 nmol/L who receive the 50 or 100 μg doses. “Secondary analyses will compare the two active doses (both separately and when combined) with placebo. Additional end-points include biochemical assessments of safety, blood pressure, arterial

stiffness, falls, fractures, heel and wrist bone density, grip strength and physical performance and echocardiographic assessments of cardiac function in a random sample of participants" [130].

So, caution is advised when thinking about taking relatively large boluses of vitamin D [131]. This could be very important, since vitamin D is becoming quite popular. Sales in the USA alone increased from $40 million dollars in 2001 to $600 million in 2011. One of the most popular doses that is recommended by some is a bolus of 50 000 IU of ergocalciferol (D2), taken once weekly. This has become popular due to its ease of use and fast results. However, there have some cases of vitamin D toxicity and related complications caused by prescriber error, prescriber and pharmacy communication error and long term consumption of high dose vitamin D. For example, an 82-year old woman who presented with nausea and vomiting had hypercalcemia (14.1 mg/dl or 141 μg/mL). Unfortunately, the patient had been taking 50 000 IU of ergocalciferol twice daily by accident. Her prescription was inaccurately written for 50 IU twice daily. The prescribing physician may have intended it to be 500 IU twice daily, but the script was interpreted by the pharmacist as 50 000 IU twice daily. Her total 25-hydroxyvitamin D concentration in the blood at the time of diagnosis was 338 ng/ml (0.338 μg/mL). It took five months before toxicity was seen. It is especially concerning that vitamin D can be purchased easily over the counter and people can take as many pills containing 1000 IU of vitamin D as they want, whenever they want [131]. This could be especially dangerous to people who don't see a physician regularly because of a lack of health insurance or a general distrust of the medical community.

In contrast, patients who are only at the beginning stages of chronic kidney disease may benefit from taking vitamin D [132]. Many of them have low concentrations of 25(OH)D in their blood and this is linked with risks for morbidity and mortality. Moreover, patients with chronic kidney disease tend to produce less 25(OH)D. This can lead to skeletal disorders, such as osteoblast or osteoclast cell defects, an imbalance in bone turnover and deterioration of bone quality, as well as non-skeletal disorders, including metabolic syndrome, hypertension, immune dysfunction, hyperlipidemia, diabetes and anemia. A pharmacological dose of 1,25(OH)2D dose can cause hypercalcemia and hyperphosphatemia as well as a dynamic bone disorder, which increases vascular calcification. Conversely, supplementation with native vitamin D reduces the risk of hypercalcemia and hyperphosphatemia, which may play a role in managing bone and cardio–renal health and ultimately reducing mortality in patients. However, a combination of native vitamin D and active

vitamin D can enhance therapy benefits of secondary hyperparathyroidism because of 1α-hydroxylase activity in parathyroid gland. This is important because many patients with chronic kidney disease die from mineral and bone disorders that are related to cardiovascular events and infectious diseases. Secondary hyperparathyroidism and hypocalcemia are two other complications that can occur [132].

So, vitamin D can help prevent morbidity and mortality caused by chronic kidney disease, as well as respiratory tract infections [133], Parkinson's disease [134], infectious diseases [135] and cancer [136] by inhibiting angiogenesis [137]. Vitamin D supplementation can also help improve musculoskeletal health and immunity, while decreasing autoimmunity [138]. Vitamin D increases the activity of the innate immune system, while restraining the adaptive immune system [138]. This leads to improved outcomes in autoimmune diseases and may even lower the risk of getting an autoimmune disease in the first place. Also, vitamin D is more important during pregnancy than at any other time in life. That is because the mother is the sole source of vitamin D in the developing fetus [138].

Vitamin D also helps skeletal muscles [139] and the performance of athletes [140]. Skeletal muscles are a direct target of vitamin D [139]. It stimulates myogeneis, which is the formation of muscle tissue. It is especially important in developing fetuses. Vitamin D exerts its effects by increasing the expression of pro-angiogenic factors and decreasing the expression of two inhibitors of angiogenesis and myogenesis (fibroblast growth factor-2, FGF-2, and tissue inhibitor of metalloproteinases, TIMPs). That is, well-regulated angiogenesis is good for muscles because it is essential for developing new capillaries and tissue repair. So, angiogenesis is another example of how systems thinking is important. There must be a balance between pro- and anti-angiogenic factors for wound healing and other physiological processes [139]. In addition, vitamin D, like many other natural products, has different effects on angiogenesis, depending on the cellular environment in which it is located. That is, vitamin D stimulates angiogenesis in skeletal muscles, but inhibits lymphangiogenesis [141]. Since cancer metastasis requires the formation of new blood vessels (angiogenesis) and usually starts in the lymph nodes, inhibiting angiogenesis there can prevent metastasis. Vitamin D also inhibits angiogenesis in prostate and colorectal cancer [142, 143].

Vitamin D is also important in helping muscles recover after exercise [140]. So, vitamin D helps athletes recover faster after exercising, while increasing the force and power that skeletal muscles produce and

increasing the production of testosterone. Moreover, it interacts with vitamin K to increase athletic performance [140].

So, it's important to be able to recognize vitamin D deficiencies [144]. Physical symptoms include symmetric low back pain, proximal muscle weakness, muscle aches and throbbing bone pain that can be exacerbated with pressure over the sternum or tibia. The biochemical symptom of a deficiency is when the concentration of 25(OH)D in blood serum is less than 20 ng/mL (50 nmol/L). Insufficiency is when the concentration is 20 to 30 ng/mL. The American Academy of Pediatrics recommends that infants and children receive at least 400 IU per day from diet plus supplements to prevent vitamin D deficiency. For adults, a daily intake of at least 700 to 800 IU can reduce the risk of falling and breaking one's bones. Adults that are deficient in vitamin D might want to take 50 000 IU per week of ergocalciferol (vitamin D2). After the concentration of vitamin D in the blood increases to an acceptable range, 800 to 1000 IU of cholecalciferol (vitamin D3) should be consumed daily from the diet and/or supplements [144].

Still, some children and youths need special management of their vitamin D deficiency [145]. So, the National Institute for Health and Care Excellence recommends that all pregnant and breastfeeding women as well as children under five years of age take vitamin D and other vitamins (including folic acid) [145].

Still, the idea that healthy people should take vitamin D supplements is controversial to some scientists [146]. Many say yes, especially when they consider the fact that the Scientific Advisory Committee on Nutrition recommended that people take 10 μg of vitamin D daily to protect musculoskeletal health in people aged 4 years or older. This could be especially important since people are advised to stay out of the sun or put on sunscreen if they do go out in the sun. Others say no, because the concentrations of vitamin D in the blood that are needed to prevent insufficiency are poorly defined and based on somewhat arbitrary decisions by clinical societies and international bodies that don't always agree with each other [146]. For example, The IOM recommends 20 ng/ml based on bone health studies [147]. This is much lower than the 30 ng/ml that the US Endocrine Society recommends. To achieve concentrations of the latter in the blood, about 4000 IU of vitamin D would be needed each day. However, the safety of this dose should be examined in further controlled trials. For example, consuming 4000 IU of vitamin D increases the risk of upper respiratory tract infections in asthma patients. Systems thinking also recognizes that one dose does not fits everyone. Instead, the amount of vitamin D that should be consumed is affected by diseases that

a patient may have [147]. It is also felt that the recommendations for widespread supplementation "will inevitably lead to overdose in some" [146]. Moreover, several randomized trials showed that patients with high concentrations of vitamin D in their blood or are taking large doses of vitamin D (above 800 IU) had an unexpected increased risk of falls and fractures [146]. Also, since some supplements contain vitamin D plus calcium, it's important to note that excess dietary calcium can increase the risk of myocardial infarction and adverse cardiovascular events [146, 148].

So, excess vitamin D can be toxic [149]. There are some things that can make a person more susceptible to its toxicity. This includes a high intake of calcium supplements, chronic kidney disease, low concentrations of estrogen, the existence of sarcoidosis (the growth of tiny collections of inflammatory cells in different parts of your body - most commonly the lungs, lymph nodes, eyes and skin [150]) or other vitamin D-hypersensitivity syndromes that may be caused by an overproduction of 1,25(OH)2D [149].

There is also controversy over whether or not it is advisable to take multivitamins and/or multimineral dietary supplements. Multivitamin/multimineral supplements have been defined by the U.S. NIH as, "any supplement containing 3 or more vitamins and minerals but no herbs, hormones or drugs, with each component at a dose less than the tolerable upper level determined by the Food and Nutrition Board" [151]. However, an NIH fact sheet expanded this definition to include supplements that "contain vitamins and minerals at levels substantially higher than the recommended values and may also include other nutritional and herbal ingredients" and those that are supposed "to enhance performance or improve immune function, or for weight control, are often composed of vitamins and minerals in combination with herbal or specialty ingredients such as coenzyme Q10, probiotics, and glucosamine. These may also include nutrients at levels substantially above recommended levels" [152]. Some scientists and nutritionists feel that multivitamin/multimineral supplements "can be safe for long-term use (>10 y)" [153]. However, a meta-analysis of previous clinical trials concluded that they had no effect on the risk of mortality [154]. However, the Physician's Health Study II found that daily multivitamin supplements "modestly but significantly reduced the risk of total cancer" [155]. However, they "did not reduce major cardiovascular events, MI, stroke, and CVD mortality after more than a decade of treatment and follow-up" [156]. In contrast, another study found that dietary supplements containing several minerals and vitamins reduced the risk of cardiovascular disease mortality in women who took them for over three years [157]. There was

also a French study, The Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX), found that 7.5 years of supplementation with low doses of antioxidant supplements "lowered total cancer incidence and all cause mortality in men but not in women" [158]. It was suggested that this could be due to the lower concentrations of some antioxidants (especially β-carotene) in men before the study was started [158]. However, there is a Choosing Wisely® initiative that is being led by the American Board of Internal Medicine Foundation that aims to improve the quality of healthcare and encourage discussions on "avoiding wasteful or unnecessary medical tests, procedures, and treatments" [159]. Their first recommendation is "don't take a multivitamin, vitamin E, or beta carotene to prevent cardiovascular disease or cancer" [159]. They also recommend "don't routinely perform prostate-specific antigen-based screening for prostate cancer" [159]. Even though the first recommendation may be valid, many people do continue to self-medicate by taking multivitamin/mineral supplements. The second recommendation may be scientifically valid, but might be poor legal advice. That is, until physicians stop being sued for malpractice by patients who develop prostate cancer but did not have their blood analyzed for prostate-specific antigen (PSA), they might be well-advised to continue ordering the test [159]. So, physicians should not only listen to the voices of the patient and their caregivers, it's also important that they listen to legal advice. If not, they may find that some of their patients whose lives they have saved many times with proper therapy will not be at all grateful. Instead, they will use reductionist thinking and solve the blame instead of solving the problem. In the process, the cost of malpractice insurance will continue to be exorbitant, which will help keep the cost of healthcare high. This does not mean that physicians should be immune to malpractice lawsuits that are based on solid scientific evidence. It just means that until the legal system catches up with medical science, tests for PSA will continue to be ordered.

Systems thinking can also help in the evaluation of whether or not a person should take multivitamin/multimineral dietary supplements. The iron and copper in multivitamin/multimineral supplements can react with naturally produced hydrogen peroxide to produce the very dangerous, toxic hydroxyl radical. This is part of the iron hypothesis that states that poorly liganded iron can cause atherosclerosis and heart disease, as well as many other diseases. This is especially problematic for men, because they don't lose iron through menstruation. This hypothesis also explains why women tend to outlive men. Fortunately, there are multivitamins for men over 50 years of age that don't contain iron. They do contain copper,

though. So, it is possible that some of the clinical studies that found no effect on lifespan or morbidity were due to the fact that many of the men who took the supplements were over 50 and took the ones that contain iron. If so, then it is possible that some of the men in the study did suffer from increased morbidity and mortality. At the same time, the men over 50 who did not take supplements that contained iron may have had less morbidity and mortality. If both assumptions were true, the effects on the overall population would have balanced out and the conclusion would have been that multivitamin/multimineral supplements had no effect.

Still, copper may have some important health benefits, especially for people who consume many fresh fruits and vegetables that contain antioxidants that also are able to act as ligands and form tight complexes with copper. Scientists at the Lawrence Berkeley Lab in California showed that copper regulates lipolysis by altering the activity of phosphodiesterase 3B (PDE3B), which catalyzes the degradation (hydrolysis) of cAMP [160, 161]. That is, copper is essential for the hydrolysis of fats, so energy can be produced. However, one of the authors warned against taking dietary supplements that contain copper because it can cause imbalances in other nutrients, especially zinc. Still, the Food and Nutrition Board estimated that only about 25% of the people in the USA get enough copper from their diets (700 μg per day) [160]. Some of the best sources of copper that are not in meat are spirulina (6.8 mg in a 112 g serving), sesame seeds (5.9 mg in a 144 g serving) and dry cocoa powder (3.3 mg per 86 g serving) [162]. There are also several types of meat that have even more, but eating meat can have many adverse health effects, especially when it is mass-produced in CAFOs.

3.1.5 Mother's milk – the only true superfood

Even though the superfood cult may want us to believe that there are many superfoods that are good for everybody, the only genuine superfood is human breast milk. The WHO [163], American Academy of Pediatrics [164] and U.S. Department of Health and Human Services [165] have recommended breastfeeding for at least the first six months of life and including it in a mixed diet for at least until the infant is two years old. Human milk contains bioactive peptides and proteins [51]. It also contains small molecules, growth factors, hormones, lipids and carbohydrates [166, 167]. Breast milk is vital in protecting infants from neonatal sepsis and for the promotion of infant growth and development [166]. Some of the small molecules include urea, creatinine, nucleotides and free amino acids. Nucleotides are essential nutrients during early life. They can change

enzymatic activities and help mediate metabolism. Furthermore, nucleotides are known to be beneficial for the development, maturation and repair of the gastrointestinal tract (GIT), as well as the development of the microbiota, and immune function. Immunoglobulins are among the most important proteins in breast milk. They are present at relatively high concentrations early in lactation. The most predominant class of immunoglobulins are secretory IgA (SIgA), followed by secretory IgG (SIgG). They provide immunological protection to the infant, while his or her own immune system matures. They target the infectious agents encountered by the mother during the perinatal period and then target the same infectious agents that are most likely to be encountered by the infant. SIgA is the primary protective agent of breast milk. In colostrum, SIgA concentrations are around 12 mg/ml while mature milk contains only about 1 mg/ml. Thus, the colostrum provides an important protective role. Breast milk also contains SIgA antibodies against bacterial adhesion sites such as pili. Since SIgAs are relatively resistant to proteolysis, they can provide protection against pathogens in the GIT. That is, breast milk contains antibodies against *Vibrio cholerae*, *Campylobacter*, *Shigella*, *Giardia lamblia* and respiratory tract infections [166].

Human breast milk also contains growth factors [167]. This includes epidermal growth factor (EGF), which is essential for the maturation and healing of the intestinal mucosa. It stimulates enterocytes to increase the biosynthesis of proteins and DNA, while also increasing cell division and the absorption of both water and glucose. EGF also inhibits apoptosis and corrects harmful changes in intestinal and liver tight junction proteins that are induced by the pro-inflammatory cytokine, TNF-α. EGF concentrations are highest in early milk and decrease over lactation. Brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) in human milk are essential for proper development of the baby's enteric nervous system. BDNF enhances peristalsis (the involuntary constriction and relaxation of the muscles of the intestine that creates wave-like movements that push its contents forward), which is often impaired in the guts of premature infants. GDNF increases the survival and outgrowth of neurons. There are also IGF-1 and IGF-2, as well as IGF binding proteins and IGF-specific proteases in human milk. IGF-1 increases tissue growth. Vascular endothelial growth factor (VEGF) regulates angiogenesis. Erythropoietin (EPO) stimulates the production of red blood cells, so it prevents anemia. Hormones that regulate growth, calcitonin and somatostatin, are also in human milk. Adiponectin, leptin, resistin and ghrelin in breast milk also regulate metabolism and suppress inflammation. Human milk also contains

macrophages, T cells, stem cells and lymphocytes that stimulate an infant's immunity. There are also cytokines and chemokines that help cells communicate with each other and influence the activity of the infant's immune system. The most abundant cytokines in human milk are in the TGF-β family. They regulate inflammation and wound repair, while helping to prevent allergies. Granulocyte-colony stimulating factor (G-CSF) helps a baby's intestines develop by increasing the depth of villi (small, finger-like projections), the depth of crypts and the proliferation of cells. There are also some pro-inflammatory cytokines that are present at low concentrations in breast milk. They recruit neutrophils and enhance intestinal development. IFNγ enhances the T_H1 immune response, while suppressing the T_H2 allergic response. Complexes between milk SIgAs and antigens are taken up and processed by intestinal dendritic cells in babies. This enables them to recognize antigens in a non-inflammatory environment. There are also innate, multi-functional proteins that protect babies from infection. The most abundant is lactoferrin. It binds iron in Bacteria, viruses and fungi, which kills or inactivates them. A form of lactoferrin produced by recombinant DNA is in clinical trials in Italy to prevent infections in pre-term infants. Another glycoprotein in human milk, lactadherin, prevents infection by rotaviruses, which can kill newborn and very young babies by causing diarrhea. Lactadherin also promotes healing in inflamed intestines. Then there is the multifunctional protein, bile salt stimulating lipase (BSSL). It protects infants from viral infection, including Norwalk and HIV. There are also mucins that protect infants from HIV and rotavirus [167].

Milk is often classified into colostrum, transitional milk and mature milk [166]. However, these are not distinct classes of milk, but refer to the gradual changes in the content of milk throughout lactation. Colostrum, the first milk produced, is quite different from mature milk. It contains high concentrations of whey protein, while caseins are almost undetectable. The average content of protein in breast milk gradually decreases from the second month to the seventh month, after which the concentration of protein levels off. Colostrum contains low concentrations of both lactose and fat compared to mature milk. Lactose production is highest in the fourth to seventh month, after which it decreases, while a gradual increase in the concentration of lipids occurs [166].

Lipids are the largest source of energy in breast milk. About 98% of the lipids are triacylglycerides [166]. The rest are mono- and diacylglycerides, free fatty acids and cholesterol. There are over 200 fatty acyls in human breast milk, with oleoyl being the most abundant. There are also short-chain fatty acids (SCFA) in breast milk. They are also

essential for proper maturation of the gastrointestinal tract. There are also sphingomyelins that are especially important for forming myelin sheaths in the CNS. They improve the neurobehavioral development of infants who have a low body weight at birth. Breast milk lipids can also inactivate various pathogens, including Group B *Streptococcus* (GBS). Breast milk also contains over 400 different proteins which provide nutrition and have antimicrobial as well as immunomodulatory activities, while stimulating the absorption of nutrients. The antibodies found in breast milk are there due to antigenic stimulation of maternal mucosa-associated lymphoid tissue (MALT) and the bronchial tree. So, they target the infectious agents encountered by the mother during the perinatal period as well as the infectious agents most likely to be encountered by the infant [166].

There are also many complex carbohydrates in milk, as well as the disaccharide, lactose [166]. Human milk has a higher concentration of lactose than any other species, due to to the high energy demands of the human brain. There are also human milk oligosaccharides (HMO) that babies can't digest. Instead, they nourish the gastrointestinal microbiota. HMOs encourage the growth of beneficial Bacteria, such as *Bifidobacterium infantis* in the baby's GIT. This protects the baby from being colonized by pathogenic Bacteria. So, HMOs help prevent neonatal diarrhea and respiratory tract infections [166].

The concentrations of the nutrients vary considerably during a single feeding as well as the age of the baby, so that his or her needs are met as well as possible [166]. Table 4 shows the range of concentrations of nutrients in human milk from 30 Italian mothers from when their babies were one to six months old [168].

Table 4. Ranges in average concentrations of components of human milk from Italian mothers who were nursing their babies from ages one to six months, adapted from reference [168].

Component	Range in average concentrations
Energy (kcal/100 mL)	62.4-68.3
Carbohydrates (g/L)	7.28-8.05
Lactose (g/L)	72.4-80.3
Glucose (g/L)	0.24-0.26
Galactose (g/L)	0.09-0.13
Protein (g/100 mL)	0.96-1.38
Non-protein nitrogen (d/dL)	0.17-0.23
Fat (g/100 mL)	2.71-3.20
Saturated fats[a]	36.8-39.0

Component	Range in average concentrations
PUFA[a]	15.2-16.3
Linoleic acid[a]	12.8-14.0
Arachidonic acid[a]	0.51-0.52
α-linolenic acid[a]	0.61-0.69
Eicosapentaenoic acid[a]	0.10-0.12
Docosahexaenoic acid[a]	0.24-0.30
n-3 LC PUFA[a, b]	0.48-0.56
n-6 LC PUFA[a, b]	1.11-1.22
Total n-3 LC PUFA[a]	1.07-1.23
Total n-6 LC PUFA[a]	14.1-15.1
n-3/n-6 ratio	0.08-0.09

a – Percent of fatty acyls in milk lipids
b – LC PUFAs are long chain polyunsaturated fatty acyls

In comparison, the average protein concentration in breast milk from 79 women in Idaho and Washington state (in the northwest part of the USA) was 10.1 mg/mL (equal to 1.01 g/100 mL) [169]. The average concentrations of lactose and total lipids were 64.1 mg/mL (64.1 g/L) and 4.6 g/100 mL, respectively. So, this sample of women had breast milk with about the same protein concentration, but less lactose and more fat (also known as lipids) than the Italian cohort summarized in Table 4. Also, the most abundant fatty acyl in the triacylglycerides was oleoyl in both the mothers from the northwest part of the USA, and those with the Italian mothers (Table 4). It was also found that most of the cells in breast milk are leukocytes that can help protect the mother and her baby from infection. In addition, breast milk contains a diverse community of Bacteria at concentrations ranging from 10^3 to 10^6 cells/mL. The most abundant Bacteria in the breast milk of the American (USA) women were *Streptococcus* (26.7%) and *Staphylococcus* (18.7%) species, but 14 other were also identified. The immune and bacterial cells are closely linked. That is, the concentration of milk protein was inversely related to the relative abundance of eosinophils and *Novosphingobium* species. Lactose was negatively associated with macrophage/secretory epithelial (MSE) cells, neutrophils and somatic cell count. The concentration of lipids (fat) in the milk was positively correlated with MSE and somatic cell count. The relative amount of behenic acid (C22:0) covalently attached to mono-, di- and triacylglycerides (as behenoyl) was positively associated with abundance of neutrophils and negatively correlated with MSE. Arachidonoyl (C20:4n6) was positively correlated with eosinophils, while α-linolenoyl was positively associated with the concentration of lymphocytes. Both

somatic cell count and neutrophil concentrations were inversely correlated with the abundance of six types of Bacteria. On the other hand, MSE was positively correlated with the abundance of five types of Bacteria. The concentration of the HMO lacto-*N*-tetrose was negatively correlated with MSE and somatic cell count. The concentrations of total HMO and the HMO 2'-fucosyllactose were positively associated with the relative abundance of *Streptococcus* species. The concentration of the HMO 3'-fucosyllactose was inversely associated with the relative abundance of *Ralstonia* and *Novosphingobium* species. There are other downstream effects of the Bacteria, immune cells and HMOs on lactation. For example, macrophages play an important role in the development of the mammary gland during puberty. However, not only the signaling molecule colony-stimulating factor 1 from macrophages, but also signals from other immune cells whose abundance is affected by HMOs help maintain the alveolar system during lactation. Moreover, HMOs can prevent the binding of pathogenic Bacteria to glycans that are on the surfaces of host cells and alter the immunomodulatory responses of peripheral blood mononuclear cells. These interactions help maintain homeostasis in the lactating mammary gland [169].

The bioactive molecules in breast milk guide the development of the baby's neuroendocrine immune system and gut microbiome [166]. As mentioned in Chapter 2, volume 1, 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 with 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. 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. HMOs are especially important in enduring proper development of the baby's microbiome. There is a gradual increase in the concentration of fat the beginning, known as fore milk, to the end of a feeding, hind milk, while lactose shows an inverse correlation to the change in fat content. There is a diurnal variation in the concentration of

milk fat, with a peak at midmorning and a low overnight, varying from about 5 g/100 mL to about 3 g/100 mL. The fatty acyl profile of breast milk is affected in part by the maternal diet. This is especially true the monounsaturated omega-6 and omega-3 fatty acyls. Dietary fats are transferred rapidly to breast milk, so within 2 to 3 days, breastmilk changes to where it mimics that of dietary fat [166].

As mentioned in Chapter 2, volume 1, mother's milk also contains healthy Bacteria that colonize her baby's gut during breastfeeding and establish the baby's healthy microbiome. Human milk contains about 400 different species of Bacteria [170]. They establish colonies of helpful Bacteria (including *Staphylococcus* and *Streptococcus* species) that help prevent the baby's gut from being colonized by other, pathogenic Bacteria and help make flu (influenza) vaccines more effective. In addition, mother's milk contains pioneering colonizers such as *Bifidobacterium longum* that carry several gene clusters that enable babies to metabolize HMOs. This allows infants to digest breast milk [171]. These HMOs also help prevent infection by pathogenic Bacteria [172]. HMOs are different than the oligosaccharides that are in other mammals. This is one reason why breastfeeding is better than formula. HMOs also act as prebiotics to stimulate the growth of healthy Bacteria in a baby's gut. They also prevent the adhesion of harmful Bacteria and act as receptor decoys that keep pathogenic Bacteria from colonizing mucosal surfaces. They are especially useful in preventing the growth of *Streptococcus agalactiae*, more often known as Group B Streptococcus (GBS). It's a common cause of neonatal sepsis and meningitis. HMOs also help keep GBS from forming biofilms that would protect them from the infant's developing immune system [172].

Not just the HMOs, but also other parts of the milk glycobiome (milk glycans) influence microbiota development and the overall health of the gut [173]. They protect against infectious diseases and act as prebiotics, selecting for the growth of beneficial intestinal Bacteria. The prebiotic effect helps to prevent diseases such as necrotizing enterocolitis, a common and devastating disease of preterm infants. The neonatal intestinal mucosa, luminal nutrients and microbiota ensure proper homeostasis in the developing gut. So, establishing a health-promoting gut microbiome early in life is crucial [173].

The extent of the beneficial effects of breastfeeding depend partly on the diet of the mother [174]. Even though maternal nutrition has little or no effect on many of the components of her milk, her intake of fatty acyls (dietary source of fatty acids) does. Maternal consumption of PUFAs (especially DHA) can help her baby's brain develop properly. That is, a

baby's brain grows fast, starting at about 350 g at birth and increasing to 925 at year one. In addition, there is extensive arboration (tree-like growth and connections) in the dendrites and axons of neurons. So, maternal consumption of fish and seafood like salmon and scallops that contain high levels of DHA will help her breastfed baby's brain develop properly [174].

Vitamin D is another important component of human breast milk [175]. Since parents are advised to keep their babies and infants from being exposed to very much sunlight, the only sources of vitamin D are breast milk, infant formula and dietary supplements. Human breast milk does not have enough vitamin D in it to meet the minimum daily requirements of infants. In a clinical trial, infants who only consumed breast milk received <20% of the daily dose recommended by the IOM. The concentrations of foremilk and hindmilk were determined. As babies begin nursing, the foremilk has a relatively low concentration of fat, which quenches his or her thirst. After a few minutes of nursing (hindmilk), the fat concentration increases so that it can provide needed Calories for growth. In addition, the average concentration of vitamin D_3 in the hindmilk of women in the clinical study was higher than the foremilk. Moreover, the babies and infants of mothers in the study who took vitamin D supplements had higher concentrations of 25(OH)D in their blood than those who did not. However, most of the mothers in the study were following the advice of the Danish National Board of Health and gave their babies and infants a daily supplement containing 400 IU of vitamin D. So, most of the infants did have sufficient vitamin D_3 circulating in their blood, regardless of the vitamin D_3 content of their mother's breast milk [175].

The amount of IgG antibodies in a mother's breast milk can also affect their baby's and infant's susceptibilities to bronchiolitis, which is a common respiratory disease that mostly affects children under the age of two years [176]. The IgG antibodies can be absorbed by breastfeeding babies and help increase their resistance to bronchiolitis. It is often caused by respiratory syncytial virus and presents with clinical symptoms of wheezing, tachypnea (abnormally fast breathing) and coughing. So, it is significant that mothers of infants who had bronchiolitis had lower concentrations of IgG in their breast milk [176].

The concentration of branched chain fatty acyls (BCFA) in mother's breast milk is also affected by her diet [177]. It should be noted that reference [177] used the term fatty acid instead of fatty acyl. Certainly, the mono-, di- and triacylglycerides are sources of fatty acids (and glycerol) once they are digested, but breast milk contains very low concentrations of

free fatty acids. Regardless of the nomenclature used, BCFAs help promote the formation of a healthy gut microbiome in infants because they favor the growth of healthy Bacteria like *Staphylococcus aureus* and *Brevibacterium* species that have them in their cell membranes [177].

The body mass index (BMI) of a mother is another factor that can influence the composition of her breast milk [178]. A higher BMI was associated with an increase in the concentrations of insulin and leptin in obese mothers. There were important sex differences. That is, the insulin concentration was 229% higher in obese mothers who were nursing female infants than in mothers who had normal weights and 179% higher in obese mothers who were nursing male infants. Both overweight and obese mothers produced breast milk that had a higher concentration of leptin than mothers who had a normal, healthy BMI. That is, overweight and obese mothers had 96.5% and 315.1% higher leptin levels than normal weight mothers, respectively. The concentrations of leptin also decreased by 33.7% as the breastfed babies aged from one to six months. So, the breast milk of mothers with the highest BMI tended to be obesogenic, leading to exaggerated growth and abnormalities in the percent fat in their babies. However, the authors stated that their findings, "should not be construed to imply that obese mothers should not breastfeed; on the contrary, our findings provide for a greater understanding in the role maternal obesity, and other known obesogens play on HBM composition" [178]. Even though the authors did not mention it, even breast milk from obese mothers provides healthy Bacteria and many important nutrients that infant formula can't provide. In addition, breastfeeding helps mothers and their babies bond and build a close, loving relationship that can last a lifetime and subsequently be passed on to the next generation.

So, breastfeeding is being recognized as having a lifelong effect on both the mother and her children [179]. It can protect against childhood infections and malocclusion (abnormal alignment of the upper and lower teeth). It can also increase the child's intelligence, while reducing the risk of obesity and diabetes. Breastfeeding can also lower the risk of breast and ovarian cancers as well as diabetes in the mothers. It also tends to increase the length of time that it takes a woman to become pregnant again. Recent epidemiological evidence has shown that breastfeeding does have benefits for both the mother and her children, regardless of their socio-economic status. It was estimated that "scaling up of breastfeeding to a near universal level could prevent 823 000 annual deaths in children younger than 5 years and 20 000 annual deaths from breast cancer" [179].

In addition, breastfeeding is a smart investment in people and economies [180]. It tends to reduce infant morbidity and mortality, increase

Intelligence Quotient (IQ) score, improve achievement school and increase the earnings of the parents. It also contributes to equity by giving all breastfed children a nutritional and psychological head start. So, breastfeeding can help reduce poverty and help to achieve the goals of the World Bank and Global Sustainable Development Goals to end extreme poverty and increase shared prosperity by 2030. In fact, both Bangladesh and Brazil have shown that comprehensive strategies can increase the amount of breastfeeding in society [180]. Similar efforts by the La Leche League (where leche is the Spanish word for milk) in 85 countries are increasing the number of women who breastfeed and the number of employers who support their breastfeeding employees in their efforts [181].

However, not all mothers are able to breastfeed their babies, through no fault of their own. They can still give their babies plenty of nutritious infant formula, along with love and affection. The babies can (and usually do) grow up to be fine, healthy adults. So, it's important to use systems thinking and realize that it would be bad for the mothers and their babies, as well as society as a whole to try to make a woman feel like she is being a bad mother if she can't breastfeed her babies.

3.2 Role of Lifestyle

There are other aspects of lifestyle besides diet that can affect human health. For example, untreated depression is a major risk factor for developing Alzheimer's disease [182]. In addition, mental stress and unhappiness are bad for one's health and could limit the effectiveness of some habits that are beneficial to happy people. Consistent with this, a recent study showed that the Mediterranean diet only reduced the risk of heart disease in people who had an income of at least $46 000 per year [183]. Having a higher education (which tends to increase one's earning potential) also helped. The authors suggested that this was due to wealthier and better educated people eating more organic fruits and vegetables, which have higher concentrations of antioxidants and lower concentrations of the toxic metal cadmium, as well as fewer or no pesticide residues, when compared to non-organic fruits and vegetables [183]. However, it's also possible that people with limited incomes are more likely to worry about how they are going to feed their children or pay their bills. That could be at least as important as any other factor.

So, people are doing scientific research on ways to increase happiness [184]. In addition, a clinical trial is being planned, in which a comprehensive intervention program will be used for three months,

followed by a three-month follow up [185]. The trial will be called ENHANCE, or Enduring Happiness and Continued Self-Enhancement. The goal is to recruit 160 participants and enroll them into two different versions of this study. One will compare in-person (n=30) with wait-list controls (n=30). The second will compare online (n=50) to with wait-list controls (n=50). The primary outcome will be both self-reported and objective assessments of health as well as psychological mediators (psychological needs) and moderators (personality) of treatment outcomes [185]. This is consistent with systems thinking because happiness is a feeling that is not quantifiable and can't be reduced to a linear combination of parameters. It is also consistent with TQM, which emphasizes listening to the voices of the customers who often express their feelings. So, researchers have shown that the level of one's chronic happiness is controlled by three factors: genetics, circumstantial factors and activities (and habits) that increase happiness [184]. It has been estimated that about 50% of the variance in the happiness of a population depends on genetics, 10% on circumstances and 40% on intentional activities and habits. When considering adaptation and dynamic processes, the third factor (activities and habits) offers the best opportunities for increasing one's happiness. That is, genetics may tend to influence one's set point or range of happiness. However, some circumstantial factors, like the death of one's child or spouse will almost always cause sadness. Indeed, it is important to allow time for grieving and not just simply start taking antidepressants because of social pressures to always appear happy. Since genetics and depressing circumstances are beyond one's control, they have been a cause for pessimism about one's ability to become happier [184]. However, as discussed in Chapter 1, volume 1, nature (genetics) can't be easily separated from nurture (environment). That is, children who are raised by their biological parents receive not just their DNA, but also varying degrees of psychological and physical support (or nurture) from the same people. In addition, other relatives, friends and society in general can be quite nurturing or, during wars, quite devastating.

There are also personality traits that affect one's tendency to be happy [184]. Two of them, neuroticism and extraversion, are especially important. Another source of pessimism is that any gains that may increase happiness are only temporary, because people can adapt rapidly to any changes that occur in their lives. This is known as a hedonic treadmill. That is, new circumstances or events can make people happier at first, but then they tend to take those changes for granted and return to their former set point of happiness. For example, lottery winners are usually no happier than they were before they won the lottery about a year after they win all

their money and many paralysis victims are not nearly as unhappy as one might assume [184, 186]. However, this pessimism is rejected by those who realize that some interventions (such as meditation, prayer, volunteer work and counseling) can improve one's short- and long-term happiness [184]. Moreover, practicing certain virtues such as gratitude, forgiveness and thoughtful self-reflection can improve one's happiness. There are also cognitive and behavioral strategies that can improve happiness. This includes focusing one one's goals in life and take a positive, optimistic attitude about life, while realizing that many things are not as important as they may have seemed at one time [184]. Indeed, the power of positive thinking has been recognized for quite some time [187].

Another reason for optimism is that older people tend to be happier than younger people [184]. There is a socioemotional selectivity theory that proposes that older people tend to learn to structure their lives and pursue those goals that maximize positive emotions [184, 188]. This is consistent with the idea that people can learn to increase their well-being sustainably [188]. One can also be optimistic because genes don't control destiny [184]. They influence happiness indirectly by influencing the kinds of experiences and environments one has or tries to have. So, the unwanted effects of genes can be overcome by actively avoiding situations that detract from well-being or by avoiding being tempted to engage in harmful behaviors. It may also be important to match one's healthy behaviors with one's personality. For example, a natural extrovert might want to have more regular contact with people. An introvert might want to read a good book instead [184]. Mindfulness, or focusing on the present (while forgetting about the past and not worrying about the future), can also be quite useful – especially when coupled with meditation [189].

A corollary to this is that loneliness is quite unhealthy [190]. It has adverse effects on the cardiovascular and neuroendocrine immune system. However, it's the subjective experience of loneliness that's harmful, not just the number of social contacts a person has [190]. So, monks who choose to live an isolated lifestyle for religious reasons can be quite healthy, but a politician who loses an important election can struggle to avoid depression – even though he or she may have millions of social contacts. Still, previous studies have shown that people who feel lonely upregulate several genes that encode signaling molecules that promote inflammation and downregulate genes that encode anti-inflammatory signaling molecules [190]. They also tend to downregulate genes that help defend against pathogenic viruses. However, there is hope. Even though loneliness and depression can appear to be contagious, counseling, therapy and a beneficial change in one's life (like finally finding a good job or

meeting new friends) can help. Moreover, it's not loneliness that is inherited – it's one's susceptibility to feeling pain when alone that is partly inherited. Still, the heritability of loneliness is comparable to that of depression, but less than that of hypertension and dyslipidemia [190]. However, feeling lonely and being temporarily depressed are natural, healthy reactions to severe losses, such as the suffering and death of a loved one. Many psychiatrists and counselors would agree that a certain amount of grieving is essential in overcoming such losses. So, it would be wrong to think that it's always best to treat loneliness and depression with antidepressants alone. Counseling and spending time with others who feel your loss can be quite helpful – as can prayer, yoga, meditation and endurance exercises. Also, it can be quite helpful to read inspiring or informative books that lead one to exercise more, be kind to others, eat healthier and/or focus on the positive aspects of one's life and accept the things that are beyond our control. Of course, it also helps to have the wisdom to know what one can and can not control.

Unfortunately, one thing that can't be controlled is childhood trauma, which can have lasting harmful effects on one's health [191]. It increases the risk of physical and mental illnesses, due to dysregulation of the neuroendocrine immune system - even after one reaches adulthood. Several studies have shown that childhood trauma can lead to increased concentrations of C-reactive protein and other inflammatory compounds, depending on the type of trauma. That is, childhood trauma can act synergistically with physical illnesses and adult psychological trauma to cause an increase in adverse effects [191]. Fortunately, common sense and life experiences support the idea that happy, positive childhood experiences have lasting healthy effects on one's health. Moreover, the positive parenting skills that one learns from good, loving parents can be used when children become parents themselves.

If a person knows his or her physical limits and exercises the proper amount, it can improve one's state of mind (or brain state) [192]. If one doesn't exercise so much that he or she hurts one's self, it can shift the brain state toward excitation in local circuits in the cerebral cortex and increase the non-oxidative consumption of carbohydrates. Instead, carbohydrates can be converted into neurotransmitters, such as glutamate and gamma amino butyric acid (GABA). In addition, electroencephalographic (EEG) power will increase across all delta, theta, alpha, and beta frequencies, and over all brain regions that have been studied. These effects can carry over so that the concentrations of glutamate will increase when resting. In contrast, the concentration of glutamate and GABA decrease when a person is depressed. So, exercise

can help relieve and even cure depression in some people [192]. It can also help make a person smarter and happier and even increase the number of neurons in his or her hippocampus [193].

As mentioned in Chapter 1, volume 1, exercise has hormetic effects on the human body. This includes hormetic effects on both cognition and mood [193]. Many of these effects are linked to neurogenesis in the adult hippocampus. A combination of physical activity and cognition has been essential for human survival since ancient times. Our ancient ancestors had to process information as they exerted themselves while searching for food, hunting, running away from potential predators, searching for sexual partners or simply when playing and dancing. The ways that our brains process information changes with the level of physical activity. Moreover, exercise and physical activity change the morphology and function of the brain. Our adaptive brains exhibit plasticity as we respond to different levels of activity and neural processing. Many things change in existing cells. This includes the metabolism of brain cells, gene expression, the sizes of brain cells and processes, as well as the properties of the blood-brain barrier, synaptic plasticity and the number of neural dendrites and synapses. In addition, new neurons emerge in the hippocampus. Exercise also increases blood flow and O_2 consumption as it modulates the signaling cascades triggered by growth factors. It also increases the availability of neurotransmitters and improves their function [193]. In addition, exercise can help to counteract aging-related memory impairment [194].

However, exercise also causes thermal and metabolic stress [193]. This can include both hypoxia and oxidative stress, as well as mechanical stress. That is, exercise increases the production of RONS (oxidative stress) and lactate when skeletal muscles become sore from being over-worked (hypoxic stress). When the lactate threshold is exceeded, brain mitochondria can become dysfunctional and the concentration of BDNF decreases. There is also an inverted U-shaped hormetic effect on cognition. Exercise not only increases RONS, but also induces the activities of antioxidant enzymes. The exact shape of the curve depends on the duration of the exercise and one's level of physical fitness. That is, there may be little or no effect on cognition when a very fit person performs simple physical activities and it will take more exercise to reach the lactate threshold. In contrast, if a person tries to exercise too hard, he or she can injure his or her self very quickly and suffer from long-term injuries. In addition, high intensity exercise often leads to decreased working and declarative memory that is resolved after one recovers from the exercise. Motivation is also a factor. Few people would run a marathon

if they didn't have peer pressure or support, along with a competition for which they were preparing. On the other hand, few people become concerned about their health or cognition when they exert themselves while trying to save others (especially their children) from a serious physical threat. Still, dehydration from too much exercise can impair information processing, cognition and one's ability to make appropriate decisions (like stop running when you feel totally exhausted and dehydrated). Moreover, one might question the decision-making abilities of professional athletes who continue to compete in severe contact sports that cause concussions and debilitating injuries to the back and knees even after they have made more money than what most people could spend in a lifetime. Of course, this could be due to social pressures more than just exercise, although the two are hard to separate. In contrast, a lack of physical activity can lead to severe health problems, while regular, moderate exercise is one of the best things for a person's health [193].

However, some have questioned the validity of previous studies that appeared to show that a lack of physical activity is unhealthy, because subjects in those studies self-reported their level of physical activity [195]. Moreover, the effects of both the total amount of sedentary behavior and its accrual in prolonged, uninterrupted bouts were not determined. So, it was quite noteworthy when a recent article appeared that described a study that was done on 7985 subjects, in which the length of sedentary behavior was monitored with an accelerometer that was mounted on their hips. It was found that "Both the total volume of sedentary time and its accrual in prolonged, uninterrupted bouts are associated with all-cause mortality" [195]. So, many now realize that "sitting is the new smoking" – even for runners and other people who exercise [196, 197].

However, it would be reductionist to think that exercise alone can keep a person healthy or prevent all diseases. A healthy diet, a positive attitude and strong, loving relationships and some simple good luck are also important. That is, good things (like a long healthspan and lifespan) do happen to people who lead very unhealthy lifestyles. Unfortunately, bad things (like cancer) also happen to people who lead very healthy lifestyles, albeit in a badly polluted environment. So, it's important for even very healthy people to have yearly physicals and do monthly self-examinations to try to feel for any lumps that might emerge. For women, it's important to examine yourself "several days after your period ends, when your breasts are least likely to be swollen and tender. If you are no longer having periods, choose a day that's easy to remember, such as the first or last day of the month" [198]. Of course, men can also get breast cancer and testicular cancer, so we should do regular self-exams, too.

Unfortunately, many people don't trust the medical profession and don't have yearly physicals or get mammograms done even after they are over 44 years old. Even if a woman doesn't have a family history of breast cancer, she can still get it. Remember that we live in an environment that is much more polluted than our parents or grandparents did and obesity is a growing problem. Pollutants that can lead to obesity will be discussed in Chapter 4. So, it's important to repeat again that physicians and pharmaceutical companies are not in a conspiracy to keep us sick. Instead, there is an open collaboration to keep us healthy and find cancer at its earliest stages, while it can usually be cured (especially for all types of breast cancer except triple negative breast cancer).

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CHAPTER FOUR

THE EFFECTS OF A TOXIC ENVIRONMENT

4.1 Introduction

As mentioned at the end of Chapter 3, no drug or preventative measure can be very successful in a highly polluted, unhealthy environment. Even though all chemicals (including clean water) can be toxic if taken at too high of a dose, many chemicals in the environmental are toxic at very low doses. Still others are quite healthy at low doses. Fluoride (F^-) at a concentration of 0.7 – 1.2 μg/mL in tap water is an excellent example [1]. It increased the incidence of bone tumors (osteosarcomas) in male rats given high doses of F^- for two years [2]. However, the presence of 0.7 to 1.2 ppm (μg/mL) has been associated with a decrease in cavities (dental caries) [3-7]. Fluorine is the most electronegative element in the Periodic Table. It can easily exchange with hydroxide (OH^-) in hydroxyapatite in teeth [8-10]. The electrostatic attraction between F^- and Ca^{2+} in hydroxyapatite is stronger than the attraction between OH^- and Ca^{2+}. So, fluoridated hydroxyapatite is more crystalline and stable than the form that only contains OH^- [8, 11]. This makes it is less soluble in acid, which protects the teeth from Bacteria that cause dental caries. Also, F^- influences the chemical exchange reactions that occur between the minerals in teeth and the surrounding fluid in bacterial plaques [12]. Moreover, it can induce apatite formation from calcium and phosphate [13]. These days, F^- is also added to polymeric dental sealants and given to children to prevent caries [1, 14].

This is an important issue for health care professionals who are often asked their opinions on such 'controversial' subjects [1]. Although physicians may feel that they are usually not qualified to give dental advice, they should remind their patients to floss daily and get regular dental examinations and treatment, when a dentist recommends it. Chronic gum infections can lead to smoldering inflammation in the heart, brain and the rest of the body. This can increase one's susceptibility to cardiovascular disease, stroke, neurodegenerative diseases and many forms of cancer. Flossing can prevent periodontal disease and cavities. So, it has been added to drinking water in most parts of the USA and many other

countries for over a half century. Before 1960, almost all children in the USA developed several cavities. It was terrifying to go to the dentist and endure the pain of drilling out the decayed part of the tooth. So, many people learned to fear and avoid dentists. This led to an epidemic of periodontal disease and chronic infections. In contrast, people born after 1960 who drank fluoridated water seldom had any cavities. They learned to trust dentists and their advice. Many of them learned to floss regularly and didn't suffer from dental caries and periodontal disease. So, a strong case can be made for the idea that fluoride in drinking water at concentrations between 0.7 and 1.2 mg/L will prevent cancer by preventing chronic infections. Unfortunately, some popular books, websites and magazine articles have been published that claim the opposite – that fluoride causes cancer [15-17]. As long as you don't steal some sodium fluoride from a chemistry lab and eat relatively large amounts of it, F^- will not cause kill you or cancer. So, it's important to use systems thinking and look for hidden connections. If you drink fluoridated water, it will indirectly prevent cancer. Unfortunately, dentists are starting to see cavities in children whose parents only let them drink bottled water that has no F^- added to it [1]. If they grow up fearing dentists, they may avoid getting regular dental care as adults – especially since most insurance policies don't cover it.

Still, the U.S. government and many others have been and still are very interested in identifying environmental toxins [1]. In the USA, the NIH has a research institute called the National Institute of Environmental Health Sciences that aims "to discover how the environment affects people in order to promote healthier lives" [18]. It is also called the National Toxicology Program (NTP) and the Environmental Toxicology Program (ETP) [1]. The NIEHS evaluates the possible toxicities of chemicals that are of special interest to the public. The decision on which ones to study is based on the level of public exposure to the chemicals and their expected toxicity. For example, many people take laxatives, so the NIEHS tested the toxicity of the former main ingredient in laxatives, phenolphthalein, and found it to be carcinogenic in rodents at very high doses. The NIEHS also studied the toxicity of PCBs and dioxins, because they were known to be very toxic even at low doses and they are almost everywhere in the environment. Some of the most dangerous chemicals are persistent organic pollutants (POP), such as dioxins and PCBs. These compounds are extremely stable, so they persist in the environment. They have extremely low solubility in pure water, but can dissolve in water that is polluted with detergents. They are not biodegradable, so they accumulate in the fatty tissues of animals and humans who consume them and are biomagnified.

That is, when animals eat algae or plants that have dioxins, or when people were exposed to Agent Orange in Vietnam, dioxins and PCBs accumulate in their fatty tissues and the concentrations increases (magnifies) in the animal or human. When predators eat contaminated prey, the PCBs and dioxins are biomagnified further. For this reason, PCB and dioxin concentrations in salmon can be dangerously high, especially in farm-raised salmon that have been fed fish food that is contaminated. There are many other persistent POPs. To deal with them, the Stockholm Convention on POPs was signed by 131 nations in 2004. Its aim is to eliminate the world's most persistent and bioaccumulative toxic substances [1].

However, there is some controversy about what the NIEHS has done in the past [1]. When they tested toxicity, they made some assumptions that toxicologists often made. They assumed that when they gave rodents or other test animals very high doses of a chemical, it provided useful information. Although this might have seemed unwise from a scientific point of view, it may have been the only way to test a compound ethically before a liver on a chip became widely available. The idea was that there is a small fraction of the human population that might be highly susceptible to a chemical. It might have been scientifically sound to give the chemical to seven billion rodents, but this was obviously impractical. Instead, the NIEHS assumed that if they gave extremely high doses of a chemical to a few rodents, that this would help determine whether the chemical was toxic to a small fraction of the human population. It also assumed that chemicals that are toxic to rodents will also be toxic to humans. This can be wrong when considering whether a chemical can cause cancer. Rats and mice are naturally more susceptible to cancer than humans. Let's borrow some nomenclature from analytical chemistry and use it to illustrate the point. When an analytical chemist injects increasing concentrations of a chemical on an instrument like an LC-MS, she or he hopes that there is a nearly linear calibration curve. This is analogous to a dose-response curve. Similar to a dose-response curve, analytical chemists also inject nearly pure deionized water or some other solvent that contains none of the test compound (or analyte). This is called a blank. Analytical chemists expect that the injection of the blank (or placebo in toxicology) will produce zero response in the detector, such as an MS. This does not happen with rodents (or people) when they are given a placebo. Some of them still get cancer. In addition, rats and mice are much more susceptible to cancer than humans. Instead of getting no toxic response to placebo, many rodents die of cancer from 'natural' causes [1]. For example, a certain strain of rats, called F344, was used in 2-year carcinogenicity

studies by the NIEHS. Almost 90% of the male rats developed testicular cancer, 50% developed leukemia, and 30% developed cancer of the pituitary and 30% developed thyroid cancer [19]. That is, tumors were not uncommon - even in rodents that were in the control group, and sometimes people get cancer, even if they live very healthy lifestyles with almost no exposure to environmental toxins [1, 19].

Also, the NIEHS almost always tested one compound at a time [1]. This was consistent with their elementary science education that taught them that the proper way to do an experiment was to change only one variable at a time. For many years, the NIEHS tested only one compound at a time. So, when they decided to test the toxicity of a botanical called goldenseal (*Hydrastis canadensis*), they didn't test the entire herb, but instead they tested two of the ingredients, berberine and hydrastine. They each tested each one separately. However, much scientific evidence emerged to support the idea that consuming an extract or a purified ingredient will not have the same physiological results as the entire food or botanical. As a result, some dietary supplements, such as grape seed extract, were tested as the entire extract, instead of testing individual compounds purified from an extract [1]. However, as discussed in Chapter 3 of volume 1, systems toxicology has become much more popular and effective in predicting toxicities because it uses a liver on a chip to test the toxicities of not just individual compounds, but also combinations of them.

Still, regardless of the methods used to establish that a substance is toxic, it is obvious that there are many environmental toxins [1]. When combined with daily stress, imbalances can occur. When our neuroendocrine immune systems are in balance, our white blood cells easily distinguish between food, self and non-self, but it is not easy. We are constantly being exposed to environmental toxins in a world that is out of balance. When our neuroendocrine immune systems are overwhelmed they can fall out of balance. The result is that many people are allergic to foods that others enjoy. Others have immune cells that are not able to distinguish properly between one's own healthy cells and pathogens. Their white blood cells mistakenly attack and destroy their own tissues as they lose their abilities to distinguish between self and non-self. For example, in type-1 diabetes, the pancreas is destroyed by the body's immune system. In atherosclerosis, macrophages enter blood vessels and attacks them, producing excess cholesterol. In cancer, mitochondria become disrupted and produce proinflammatory signals that lead to uncontrolled, malignant growth. Moreover, smoldering inflammation often occur in people who are asymptomatic [1].

Our neuroendocrine immune system must be able to distinguish between food, self and non-self [1]. When unbalanced, our network of immune responses can mistakenly identify our own tissues and cells as non-self. The neuroendocrine immune system produces biochemical signals that cause the body to attack parts of itself, producing smoldering inflammation that can lie undetected for years. Eventually, smoldering inflammation causes an autoimmune disease. There are many types of autoimmune diseases. The ones that cause the most morbidity and mortality are heart disease, cancer, diabetes, stroke and Alzheimer's disease. These are also diseases that are linked to obesity or metabolic syndrome. They are caused, in part, by our neuroendocrine immune systems being overwhelmed by chemical toxins that cause imbalance and smoldering inflammation. So, it is important to do research to try to do more research and learn more [1].

The US EPA monitors 186 air pollutants, most of which are organic [1, 20]. This includes six criteria pollutants: carbon monoxide, nitrogen dioxide, sulfur dioxide, particulate matter, hydrocarbons and photochemical oxidants [20]. They also monitor pollutants in environmental water and soil samples [21]. This includes pesticides [21]. The pollutants can cause many different diseases, including many types of cancer [1]. However, pesticides have saved many lives in the past. For example, DDT was used to suppress a typhus outbreak in Naples, Italy in 1944-1945. It also fought river blindness in Africa by killing the insect vector (black fly). Several other insecticides have helped to control malaria in Africa, the Middle East and Asia [1, 22]. DDT was one of the first insecticides to be widely produced. It is an excellent contact poison against moths, beetles, lice and a variety of other insects. However, it caused birds' egg shells to become very thin so they cracked easily. Many people feel the environmental movement started with the fight to ban DDT, since DDT and other dichlorodiphenylethanes can cause loss of weight, anemia, tremors, muscle weakness, and anxiety. The structure of DDT is shown in Figure 1. Another insecticide that was once very popular is heptachlor. It was sprayed on many farms throughout the Mississippi River delta to control boll weevils in the cotton crop, but has been banned for decades. It can cause many form of neurological toxicity, including headache, dizziness, loss of consciousness, and loss of short term memory [1, 23].

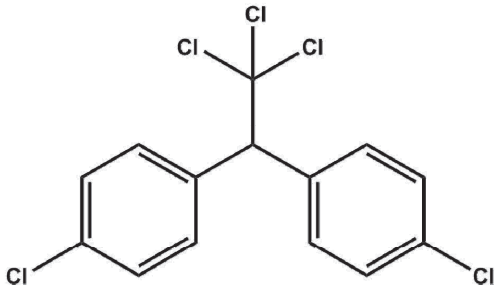

Figure 1. 2D structure of DDT, or 1,1'-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene).

Some insecticides, such as carbamates and organophosphorus esters, inhibit acetylcholinesterase [1]. It catalyzes the hydrolysis of acetylcholine, a neurotransmitter. Another class of insecticides, called pyrethroids, acts on voltage-sensitive Na^+ channels. They originally came from dried flowers of pyrethum, or chrysanthemum. Herbicides can kill weeds by inhibiting photosynthesis, respiration and nuclear division, as well as by inhibiting the synthesis of proteins, carotenoids and/or lipids [1]. Bipyridyl derivatives such as paraquat are especially toxic [24]. Paraquat has very high pulmonary toxicity due to lipids being peroxidized by superoxide radicals [24].

Detergents are another class of organic pollutants [1]. This includes the nonionic detergents called the nonylphenolpolyethoxylates, such as Triton X-100 (Figure 2). There are also anionic detergents, including linear alkyl sulfonates (LAS), such as sodium dodecylsulfonate (SDS), and linear alkyl benzene sulfonates (LABS). These are popular low cost detergents that are used in households and in many industrial detergent applications. The detergents that have a C_{12} (dodecyl) alkyl group are environmentally friendly, since they are biodegradable [1].

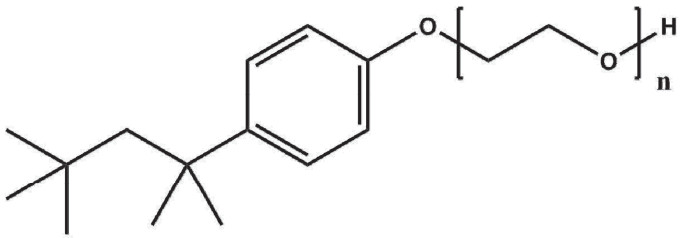

Figure 2. 2D structure of Triton X-100 nonionic detergent. It is a mixture of chemicals with different numbers of ethoxylate repeat units.

Another environmental toxin is bisphenol A (BPA) [1]. It is an endocrine disruptor. That is, it is an exogenous substance that acts like a hormone and disrupts the physiological function of endogenous hormones *in vitro* and in rodents *in vivo*. It is used to make polycarbonates, a type of plastic used to make baby bottles and other plastics [25]. It is also present in the liners of canned foods and is used to make epoxy resins for high performance coatings in the automotive and aerospace industries. When tested on rodents, high doses of BPA were found to have estrogenic properties and affect sexual development. However, humans are not exposed to such high levels. Still, even low doses caused enlarged prostates in the male offspring of pregnant mice. More importantly, human epidemiology studies have linked low doses of BPA to breast cancer and early onset of puberty. It may also be contributing to the world-wide epidemic of obesity [1]. For example, it was found that the higher the concentration of BPA in the urine of school-aged children, the higher the likelihood of obesity [26]. BPA may also help cause type-2 diabetes by accelerating the formation of a toxic amyloid protein [27]. In another study, it was shown that exposure to low doses of BPA (0.1 mg/kg/day) during critical early stages of perinatal development can cause endocrine and metabolic changes and adversely affected glucose homeostasis [28].

Most of the literature on BPA says that it comes from plastics [1]. It is used to make two main classes of polymers, or plastics: epoxies and polycarbonates. Almost all epoxies that are used as adhesives are quite mutagenic. So, when using glues or adhesives, wear gloves and wash your hands with soap and water afterwards. Also, epoxies based on BPA are also found in the liners of many canned foods and in some dental sealants. Polycarbonates are used to make strong, shatter-proof glasses. Like epoxies, they contain very small amounts of unreacted BPA. Most of it reacted to produce the plastics, but a few micrograms of unreacted BPA could leach out during normal wear and enter your body. However, many people argue that microgram amounts are too low to cause any damage. This has been argued back and forth for years. However, a recent study has uncovered a previously hidden connection [1]. Scientists used a new database, ChemProt, to evaluate not only new drugs, but also environmental chemicals based on their activity profiles against most known biological targets [1, 29]. They found an association between BPA and several types of cancer [1, 29]. This was based on literature data on the effects of microgram amounts of BPA.

While scientists and doctors were arguing, a completely new source of BPA has emerged – the thermal paper used to make most modern receipts [1]. It is mixed other chemicals, such as a fluoran leuco dye and

octadecylphosphonic acid, in special paper. When a light (laser) shines on parts of the paper, it turns it black, marking it with the numbers on the receipt [1]. Recent analysis found as much as 19 mg in a 12-inch long receipt [1, 30]. BPA was found in the urine of over 90% of the people tested in a recent study. The U.S. EPA has established the safe daily intake of BPA to be 0.05 mg/kg body weight per day, based on the assumption that the main source of exposure is through contaminated food [31]. So, a person weighing 80 kg, or 176 pounds, should not take in more than 4 mg of BPA daily. You don't have to handle very many receipts to reach that level. Even if you don't handle receipts, the people working at the cash register do – and they all handle your food and cash. Moreover, an infant can ingest even higher amounts by putting such a receipt in his or her mouth and sucking on it during their oral stage of development, when it may do much harm. Fortunately, you can wash BPA off with a little soap and water. BPA won't wash off with water alone – you need some soap to help dissolve and remove it. Soap and water will also wash off other pollutants, including phthalate esters.

BPA and nonylphenol are both endocrine disruptors that can increase the likelihood of developing metabolic syndrome [32]. They stimulate the accumulation of triacylglycerides in differentiated adipocytes that were derived from preadipocytes, in time- and concentration-dependent manners. They upregulated the expression of genes that code for proteins such as hormone-sensitive lipase, phospholipase A_2 and phospholipase C that are involved in developing metabolic syndrome. So, endocrine disruptors accelerate the differentiation of preadipocytes into mature adipocytes and the accumulation of lipids in the cell bodies [32].

Phthalate esters are also endocrine disruptors. They are esters of phthalic acid and are used as plasticizers [1, 33]. Phthalic acids are dibenzoic acids. The two –COOH groups can be *ortho*, *meta*, or *para*. The *ortho* isomer is most commonly used to make esters. When it reacts with 2-ethylhexyl alcohol, the diester di-2-ethylhexylphthalate (DEHP) is formed. They are added to plastics that would normally be hard and brittle, such as polyvinyl chloride (PVC) and the polyurethane in the dashboards of cars. Without an added plasticizer, dashboards would crack very fast. To protect them, phthalate esters are sprayed onto the plastic. This gives new cars a 'new car smell'. This is just one example. There are thousands more. Over 11 billion pounds of phthalate esters are produced each year. They are put into pharmaceutical pills and nutritional supplements, clothing, medical devices (IV tubing, gloves), body care products, baby shampoos and other products. Infants are exposed to higher concentrations because they put so many things in their mouths. Flooring that contains

PVC can be another large source of phthalate esters for infants. However, the main source of exposure to phthalate esters is the food and beverages that we eat and drink. Often, much of it is in the containers [1].

DEHP and other phthalate esters are used as plasticizers in plastic toys and other products that babies and infants put in their mouths [1]. DEHP is also known as dioctylphthalate. In rodents, DEHP is an endocrine disruptor. It lowers the production of testosterone in male fetuses. DEHP and other phthalate esters were found in human breast milk [33]. Even though phthalate esters were legal in children's toys in the USA until Congress passed a law in August, 2008, their use in children's toys in the European Union has been restricted for years [1]. As often occurs, the state of California was a leader in the USA on environmental issues. It passed its law before the Federal government passed the law prohibiting toys and child care products from having more than 0.1% DEHP, dibutyl phthalate or benzyl butyl phthalate, starting Jan 1, 2009. The Federal law also prohibits the presence of lead in children's toys and products [1].

BPA and EPA aren't the only environmental chemicals that are endocrine disruptors [1]. One of the most common classes of endocrine disruptors is the polybrominated diphenyl ethers, abbreviated as PDBE. They are used as flame retardants. Many of the common PDBEs were banned by the European Community in 2006. Several organochlorine pesticides, such as DDT and dieldrin are endocrine disruptors. Other common pollutants that are endocrine disruptors include polychlorinated biphenyls (PCBs), dioxins, polyaromatic hydrocarbons (PAHs), phenols and furans. Many of them bind to the aryl hydrocarbon receptor (AhR), which is part of a multimeric complex in the cytosol. The ligand-bound AhR dissociates and translocates into the cell nucleus, much like nuclear hormone receptors. There, it interacts with the aryl hydrocarbon receptor nuclear transporter (ARNT). This complex recruits other proteins to activate or repress transcription. Moreover, ligand-bound TCDD can induce functional regulatory immune cells (T_{reg} cells) that are important in autoimmunity. So, PCBs, dioxins and other environmental pollutants may help cause autoimmune diseases and cancer [1].

There are many other organic pollutants [1]. Some of the worst are not biodegradable. They are biorefractory (they resist decomposition by biological means) and are called persistent organic pollutants. That is, they are resistant to degradation by Bacteria or other organisms. Examples of biorefractory compounds include MTBE (methyl t-butyl ether), dioxins, PCBs, and other halogenated compounds such as perfluorooctane sulfonic acid, perfluorohexanoic acid and brominated compounds, such as tetrabromobisphenol A [1].

Environmental chemicals that are endocrine disruptors should not be confused with dietary estrogens, such as isoflavones [1]. These are chemicals that occur in soybeans. Isoflavones may prevent breast cancer and other types of cancer. Isoflavones and soy proteins may prevent heart disease and several forms of cancer. There has been some unjustified fear that isoflavones in soy milk might be bad for babies and infants. However, baby formulas made from soy have been used for centuries in vegetarian societies. Even in carnivorous societies, some women are not able to breast feed their babies, and their babies can be allergic to cow's milk. In such cases, soy based infant formula (SBIF) can be used and has been used to produce healthy children with no problem. In fact, an article in the peer-reviewed journal, *Nutrition*, concluded that, "there are no clinical concerns with respect to nutritional adequacy, sexual development, neurobehavioral development, immune development, or thyroid disease. SBIFs provide complete nutrition that adequately supports normal infant growth and development. FDA has accepted SBIFs as safe for use as the sole source of nutrition" [34].

There are also some metals that are environmental toxins [1]. The toxic metals on the EPA list include Sb, As, Be, Cd, Cr, Co, Pb, Mn, Hg, Ni, radionuclides and selenium compounds. There is more human exposure to Cd (cadmium) than what one might think. It is used as a stabilizer in polyvinylchloride, a ubiquitous plastic in many industrialized countries (especially the USA). Metal toxicity can be complicated by the fact that many metals persist in the body for a very long time (even a lifetime). Toxicity depends on the dose and the amount of time that the metal has been in the body. For example, the biological half-lives of lead and cadmium are 30 and 20 years, respectively. Cellular targets include enzymes and/or the membranes of cells and organelles. If a metal is part of a lipophilic compound (like tetraethyl lead), it will pass through the cell membrane and be slowly transformed to its inorganic salts. Some metals can interact with each other or with proteins, to increase their toxicity. Lead interferes with the calcium-dependent release of neurotransmitters. Metallothionin proteins form complexes with cadmium, zinc, copper and other metals. Lifestyle factors such as smoking and the consumption of excess alcohol can also affect metal toxicity. Some metals are carcinogens, including arsenic, beryllium, cadmium, hexavalent chromium and some nickel compounds. Beryllium can also cause a deadly lung disease [1].

Treatment of exposure to toxic metals can include chelation [1]. Chelating agents form complexes with metals. The first clinically useful chelating agent was BAL (2,3-mercaptopropanol), which was designed to detoxify arsenic, a possible chemical warfare agent. DMPS (2,3-

dimercapto-1-propanesulfonic acid) was developed [35] after that, and it has less toxicity. The calcium salt of EDTA can be used, but it is absorbed poorly when taken orally, so it must be injected parenterally. Penicillamine is a hydrolytic product of penicillin, and it has been used to remove copper lead, mercury and iron [1].

The toxicity of some metals is very dependent on the oxidation state and whether the metal is part of an inorganic or organometallic compound [1]. For example, chromium in the +3 oxidation state has very low toxicity, but hexavalent chromium (Cr^{6+} as part of H_2CrO_4, $Na_2Cr_2O_7$ or $K_2Cr_2O_7$) is highly toxic and is a known human carcinogen. Similarly, mercury can be very toxic (especially when breathing fumes of elemental mercury, or Hg^0) or almost harmless, when part of the mercury amalgam that is used in dental fillings. Methyl mercury is highly toxic [1].

Another example is arsenic, which can be part of an inorganic salt, or it can be part of an organometallic compound [1]. For example, roxarsone, or 4-hydroxy-3-nitrobenzene arsenic acid, is added to chicken feed to promote growth, kill parasites that cause diarrhea and improve the color of the meat [36]. Although roxarsone is less toxic than inorganic arsenic, it is converted to inorganic arsenic within the chicken and is excreted. Large doses (70 – 180 mg) of arsenic can cause acute illnesses and death. Symptoms include fever, anorexia, liver damage (hepatomegaly), melanosis and cardiac arrhythmia. Chronic exposure to lower doses can cause neurotoxicity, liver damage and peripheral vascular disease [1]. The US FDA analyzes chicken and many other foods for arsenic, mercury, other toxic metals, and many other toxic substances as part of the Total Diet Study [37].

Another persistent environmental pollutant and potent endocrine disruptor is tributyl tin (TBT) [38]. “It has been used as an anti-fouling agent in paints for marine shipping as fungicides on food crops, and as antifungal agents in wood treatments, industrial water systems, and textiles. Several organotin compounds are also used as heat stabilizers in the manufacture of polyolefin plastics, such as polyvinyl chloride (PVC). This brings them into closer contact with drinking water and food supplies. Tributyltin chloride and triphenyltin chloride are nanomolar agonist ligands for retinoid and peroxisome proliferator-activated receptors. These are nuclear receptors that play pivotal roles in lipid homeostasis and adipogenesis. There is an environmental obesogen hypothesis that predicts that inappropriate receptor activation by obesogens will lead to adipocyte differentiation and a predisposition to obesity especially in people who consume the typical high-calorie, high fat Western diet” [38].

Other organotin compounds are endocrine disruptors that can lead to obesity, so they are sometimes called obesogens [38]. They disrupt the normal developmental and homeostatic controls over adipogenesis and energy balance. Tributyltin chloride and triphenyltin chloride are nanomolar agonists for retinoid X receptors (RXRα, RXRβ, and RXRγ) and peroxisome proliferator-activated receptor γ. These are nuclear receptors that play key roles in lipid homeostasis and adipogenesis. So, there is an environmental obesogen hypothesis that proposes that inappropriate receptor activation by organotins will lead directly to adipocyte differentiation and a predisposition to obesity and/or will sensitize exposed individuals to obesity and related metabolic disorders if they consume the typical Western diet that is high in calories, as well as saturated and *trans* fats [38].

The adverse effects of poor nutrition and endocrine disruptors in the environment on obesity and human health begins during the perinatal period [39]. Obesity (more properly known as metabolic syndrome) is not caused by simply consuming more calories than you burn off by metabolism and physical activity. In contrast, it has been proposed that the recent large increase in obesity is also due to factors during the perinatal period. This includes problems with the nutrition of one's mother, her exposure to environmental obesogens and the synergistic interactions between these two factors. So, placental blood flow and nutrient transport to fetuses, as well as obesogenic components of the maternal and infant diets can influence one's body mass index (BMI) and susceptibility to diseases related to it. Also, developmental exposure to endocrine disrupting pollutants can disrupt healthy homeostatic control systems required to maintain a healthy BMI. These factors may help explain why 30% of the adults in the USA (and a high percentage in other countries) are obese and about 65% are overweight [39, 40]. More alarming is that the incidence of metabolic syndrome and diseases related to it (such as diabetes and other autoimmune diseases) are rising very fast in young children. So, the healthcare community is responding to this in an open collaboration to reduce this increase. One example of this is the creation of the International Society for Developmental Origins of Health and Disease. They are interested in the effects of maternal nutrition and exposure to obesogenic pollutants. However, the effects are not linear. That is, not only is a high birth weight a risk factor for obesity, but also is a low birth rate. In addition, even though leptin is an important hormone produced by adipocytes that increases satiety (the feeling of being full) and the utilization of energy in adults, it can also lead to obesity if a fetus experiences a surge in leptin when the mother over-consumes saturated

and *trans* fats. This is because a premature surge in leptin changes leptin programming and the way that the hypothalamus develops, which leads to obesity in adults. There is another example of nonlinearity – smoking tobacco. That is, smoking during pregnancy leads to low birth weight and slower intrauterine fetal growth retardation and complications in postnatal growth and development, including a strong tendency to gain weight later in life. In addition, obesogenic pollutants such as BPA can tend to decrease one's body weight as an adult, but tend to cause an increase in post-menopausal women who don't produce as much estrogen as they did previously. However, estrogen mimetics (like genistein) in soy can prevent obesity. Moreover, genistein and BPA have opposite effects on the methylation of nucleotides in genes. As a result, genistein tends to decrease one's tendency to become obese, while BPA increases it. In addition, genistein blocks the adverse effects of cadmium in some tissues [39].

Much of the data on the toxicities of environmental pollutants came from studies in which only one chemical at a time was tested, in accordance to reductionist thinking. However, we are exposed to many combinations of pollutants. So, it is important to note that some of them interact synergistically to cause toxicity [41]. This is especially important for products that contain a mixture of toxins. For example, antifouling biocides contain not only organic biocides, but also organometallic compounds and metals. In addition, sometimes pesticides and fungicides are administered together to protect crops from damage. Synergistic interactions between them cause higher toxicity than do the individual toxins [41]. In addition, the order in which one is exposed to toxins can be important [42]. That is, four toxins that bind to different targets exhibited very different toxicities, depending on the order to which they were administered. This is because each toxin is metabolized at a different rate, so they had different toxicodynamic recoveries. So, some of them had carryover toxicity when they were administered first. So, not only the dose, but also the timing makes the poison [42].

4.2 Epigenetic gene regulation: Linking early developmental environment to adult disease

Some environmental toxins can exert epigenetic effects that carry over to future generations [43]. That is, early environmental exposures on metastable epialleles and imprinted genes affect the fetal epigenome and subsequent susceptibility to diseases when they become adults. So, the field of environmental epigenomics involves studying the mechanisms by

which this occurs. The epigenome is especially susceptible to deregulation during gestation, neonatal development, puberty, and old age. However, it is most vulnerable to environmental insults during embryogenesis because the rate of DNA synthesis is high and the critical patterns of DNA methylation chromatin restructuring that are required for normal tissue development to occur in early development. In addition, stable epigenetic changes of certain genetic loci are potentially reversible in adulthood following exposure to histone deacetylase (HDAC) inhibitors, methionine, nutritional agents and environmental contaminants. So, one must consider not only the magnitude but also the timing of exposure. It's also important to note that some toxins, like diethylstilbestrol (DES) exert their toxic effects on not just the parent and child, but also grandchildren. On the other hand, the soy isoflavone genistein exerts beneficial epigenetic effects [43]. Similarly, Holocaust trauma has had significant effects on the children and grandchildren of the victims [44].

So, changes in one's epigenetics can play key roles in neurodevelopment and may increase the risk to psychiatric disorders later in life [45]. Adverse environmental influences in early life such as a lack of maternal care, exposure to alcohol and/or harmful narcotics as well as poor prenatal nutrition can alter one's epigenetic factors that can lead to neurodevelopmental disturbances that are related to psychiatric disorders [45].

4.3 Global climate change

Two of the most harmful environmental pollutants are carbon dioxide (CO_2) and methane (CH_4). They are greenhouse gases that warm up Gaia's atmosphere and subsequently the land, oceans and seas. As CO_2 dissolves in ocean water, pH drops, and it slows down calcification. This is causing serious damage to the world's coral reefs. Mountain glaciers near the equator supply fresh waters in Asia, Africa and South America. As they continue to lose ice through melting, there is less clean water in regions of the world that already have a serious lack it. In many frozen regions of our planet, melting tundra releases methane, or CH_4. Also, frozen methane deposits in the deep ocean are melting and releasing CH_4 into the atmosphere. We can be tempted to believe that global climate change is something that is happening to us because some unintelligent or greedy people caused it. This is based on reductionist thinking that was widespread before the advent of TQM. Instead of trying to fix the blame, we should focus on fixing the problem. If instead, we should change our outlook and realize that global climate change is something that is happening **for** us. We might be able to work together to solve or at least

minimize the problem. That is, global climate change is giving us the opportunity to actually do something about it. Even though TQM has made significant inroads in industry and healthcare, it has had less impact on the politics of global climate change, or global warming as most people in the USA call it. One might be tempted to focus on blaming people who refuse to acknowledge the fact that human activity is causing global climate change. However, that can be counterproductive. When people are made to feel evil and/or stupid, they often react stubbornly. Many people (especially those with military experience) will fight back when criticized. They may continue to do what others call offensive or even increase their behaviors that threaten all of humanity.

So, it's important to use the principles of TQM and systems thinking and remember that the people who are have the greatest potential to fix a problem are those who caused the problem. Moreover, it's important to remember the first rule of communication – know your audience. If you find yourself talking to someone who thinks that global climate change is a hoax, you might want to try a different approach. It might be better to emphasize how one can save money by turning down the thermostat during the winter and wear warm clothes instead. Similarly, money can be saved by turning up the thermostat in your home and work place during the summer and wear fewer clothes. Instead of telling someone that they should drive slower to save gasoline, it might be better to say that they can avoid serious traffic accidents and the injuries that occur. Similarly, spreading anger and hatred will not help. Moreover, happiness and positive thinking are healthy, but unhappiness and constant anger are unhealthy. So, it might be good to know that there was a very popular movie titled Armageddon, in which very masculine oil workers saved the world from being destroyed by an asteroid [46]. They didn't care why the asteroid was headed towards Earth. Instead of trying to blame God or some segment of society, they worked on solving the problem. They fixed the problem – not the blame. So, it would be great if we could get the fossil fuel industry to focus on fixing the problem of global climate change. Fortunately, many of them are. Large, very profitable companies that produce fossil fuels are working on alternative sources of energy. They might be much more concerned about staying profitable and adjusting to changes in the market than stopping global climate change, but they can still do some good. It's also important to realize that many people who call themselves conservatives oppose any limits to their excessive use of fossil fuels because they associate that with other changes in society that their political opponents favor. It can be very difficult to get a man to understand something if his job (or political career) depends on

him mis-understanding it. For example, the Republican chair of the US Senate Committee on the Environment and Public Works, James Inhofe, called global warming the "greatest hoax ever perpetrated on the American people". It can also be very difficult to get a man to understand or do something if he thinks it will cause him to go to Hell when he dies. So, we should focus on telling such men about how they can save money and forget about trying to address other important social agendas that they might think are evil. It might also be good to remember that there was a time when conservatives felt that it was important to respect and protect the environment. For example, there is a popular magazine called The Conservationist Magazine that many men read [47]. Many of them have modest incomes, live in relatively small houses and don't fly in airplanes. So, it can seem hypocritical to them when a rich politician from an allegedly liberal political party flies all over the world preaching about ending our addiction to fossil fuels while he or she lives in a large mansion that requires lots of fossil fuel to heat during the winter and cool during the summer.

However, when such politicians address friendly audiences who understand the terrible damage that global climate is causing and will continue to cause, they can provide important motivation. That could make up for the damage that is done when they fly around the country. Fortunately, there are also governors of states and mayors of cities in the USA who are working to pass local laws to help the environment. For example, Jerry Brown, the governor of California, has become an unofficial ambassador for the environment by working with the People's Republic of China to develop and implement clean, environmentally favorable forms of energy [48].

So, there are many things that we can do that will not only reduce greenhouse gas emissions, but also improve our health. One of the most important is to change the way we eat. We should stop eating meat and switch to a plant-based diet. We should also stop wasting food and putting it in landfills. For example, the expiration dates on many foods are usually set arbitrarily. Moreover, most grocery stores and supermarkets refuse to even try to sell food that has bruises or other discolorations. So, we should follow the excellent example of Denmark, where supermarkets sell expired and ugly fruits and vegetables at discounted prices. In addition, Brazilian scientists are constantly trying to find ways to reduce agricultural waste by finding new uses for the peels and seeds that were once just discarded. A recent book lists the many things that every individual can do to not just slow down global climate change, but even reverse it [49].

Unfortunately, throughout history people have complained that advances in technology would seriously damage the economy. People thought that cars would cause massive unemployment as the production of horse-drawn buggies and whips decreased. Even in the 21st century many people fear that replacing gas guzzling cars with hybrid or electric cars would damage the economy. Some men get very emotional and throw temper tantrums when they say that there is no such thing as global warming, or that humans are not to blame. It may seem very difficult to communicate with them and sell them on the importance of the environment or the fact that renewable energy is creating many jobs and is good for the economy. So, it is good to remember that there was also a time when the USA and the rest of the world was suffering from a major economic depression. During that time, Dale Carnegie wrote a book titled "How to Win Friends and Influence People" [50] that sold over a million copies and helped salespeople increase their productivity at a time when few people had any extra disposable income. Buying the book and following its advice was not a luxury – it was a necessity. One of the main themes of the book was that a salesperson (or anyone who wants to influence people) needs to focus on the needs of the client or customer. Instead of starting a conversation by telling the client (or politically conservative individual) what you want or think, start by asking him or her what they think, want or need. It's also essential to make the person you are trying to convince of something that they are important. Even if they might have terrible attitudes about Global Climate Change and the role of government in controlling it, they might live in small, modest housing, never fly anywhere and limit their use of fossil fuels simply to save money. So, at least in the USA, it's often better to talk about ways of saving money instead of ways of saving the planet. If we simply use systems thinking and recognize the common humanity in all of us, we can save lives and maybe even protect human civilization from destruction by Global Climate Change.

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APPENDIX

PRIMER ON THE NEUROENDOCRINE IMMUNE SYSTEM

A1 Introduction

As mentioned in Chapter 1 of volume 1, the nervous, endocrine and immune systems are no longer thought of as being separate from each other. They should be studied together using systems thinking and referred to as the neuroendocrine immune system. It should also include the microbiome. The many ways that they are linked were described. Still, many of the references that were cited to support this referred to the nervous, endocrine and immune systems as if they were separate. In addition, an understanding of basic terminology was assumed. So, the goal of this Appendix is to present some classical ideas and vocabulary that will help the non-experts in neurology, endocrinology and immunology understand the original references and the contents of this book better.

A2 The Nervous System

A2.1 Introduction

In the human nervous system, chemical and electrical signals are transmitted between neurons in complex networks and circuits in the brain [1]. This network and its circular organization are linked with other tissues and organs. They share several biochemical and signaling pathways. 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. So, the brain's connection with the endocrine system is part of classical endocrinology. The hypothalamus connects the nervous and endocrine systems through the pituitary gland, or hypophysis. 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 [1].

A2.2 Cells in the nervous system

The brain and spinal cord make up the central nervous system (CNS) [1]. It contains neurons and glial cells. The peripheral nervous system (PNS) contains all the sensory neurons. So, neurons are a principal component of the nervous system. They are electrically excitable cells that transmit and process information. Neurons are arranged in networks and circuits. They communicate with each other and with other types of cells through synapses. This is possible because neurons are polarized. There is a small electropotential with the inside of the cell membrane being slightly negative (about -70 mV) compared to the outside. When a neuron is stimulated by a neurotransmitter, the voltage difference changes and ions move across the cell membrane. There is an action potential, in which the inside becomes positive for a short time and then returns to a negative value, as ions move back across the membrane [1].

The sensory neurons in the PNS have one axon [1, 2]. The axons of sensory neurons are bunched into groups in the sensory or dorsal root ganglia. The dorsal root (or posterior root) is the afferent sensory root of a spinal nerve. Nerves are projections of neurons. Afferent nerves carry signals from sensory neurons to the CNS. Efferent nerves send signals from the CNS to the muscles and glands. Axons conduct signals that are generated by sensory receptors in the skin or other organs. The signals go through the dorsal root and into the spinal cord or cranial nerve nuclei. Some neurons have two axons, so they are called bipolar. Examples include sensory cells in the retina, olfactory mucosa and auditory nerves, as well as small nerve cells in the brain that are called granule cells. Other nerve cells are multi-polar. They have only one axon, but they have recurrent or collateral branches that feed back onto the same type of nerve cells [1, 2].

Neurons have microtubules that maintain their polarized shape [1, 2]. The microtubules contain a protein called tau that helps form their rope-like structure, which is needed for neurons to communicate with each other and to guide nutrients and chemical messengers down the axons. Neurons also have spherical synaptic vesicles in their axons. Their diameters range from 400 to 1200 Å, where 1 Å equals $1x10^{-10}$ m or 10 nm. They store neurotransmitters when a neuron is in the resting state. When activated, synaptic vesicles release neurotransmitters into the synaptic cleft (or gap) where they bind to their cognate receptors on the postsynaptic cell [1, 2].

Neurons also have several synapses with branched processes called dendrites that extend from the soma [1, 2]. Most dendrites have even smaller projections, called spines. Some neurons called principal, relay, or

projection neurons send out long axons that carry signals to other centers. In contrast, intrinsic interneurons only process signals within a center. The principal and intrinsic neurons, together with the incoming input fibers, make up a triad of neuronal elements, which is the functional unit of neural circuits [1, 2].

Neurons can be excited by electric current and the movement of ions across their cell membranes [1, 2]. The most active part of the brain, the cerebral cortex, has many types of neurons, each with excitable membranes. Each cortical neuron connects with 1000 to 10 000 other neurons. The brain is a self-communicating organ. It spends most of its resources on communication among its own neurons [1, 2].

Neurons and their cell bodies are a major component of the gray (or grey) matter in the brain [1-3]. Gray matter routes sensory and motor stimuli to interneurons of the CNS. It also performs mental calculations and stores memories. This is done in the cerebral cortex, which is a densely compacted area of neurons on top of our brain. This is also where we make our mental decisions and where most of the gray matter of the brain is located. There is also gray matter below the surface of the brain, in compact shapes called nuclei. Examples include the thalamus, striatum, putamen, globus pallidus and caudate nucleus. The gray matter also contains capillaries and glial cells, which can be either astroglia or oligodendrocytes. The average adult human brain contains about 10^{11} neurons and about 10^{15} synapses [1-3]. However, synapse is a general term for connections between not just neurons, but also between cells in the immune system [1, 4].

So, each neuron has a cell body and longer axons (up to 1 meter) that extend out from the cell body [1, 4]. These axons conduct electrical signals away from the soma and to axonal termini, called synaptic knobs. Most axons have many branches, allowing signals to be sent to many other neurons through their dendrites and spines. When axons are packaged in bundles, they form nerves. Dendrites are long, thin, and highly-branched. They receive signals from other neurons through synapses. So, neurons send signals through axons and receive them in dendrites. Signaling between neurons occurs by transmitting chemicals (neurotransmitters) from an axonal terminus to a dendrite. It goes across a synaptic gap, or cleft, of about 0.02 μm (20 nm) long [1, 4].

In humans and other vertebrates, the axons are coated with an electrical insulation, called the myelin sheath [1, 5]. This allows low currents to pass safely through axons at speeds up to 120 m/s. However, when the myelin sheath is damaged, it can lead to diseases such as multiple sclerosis, or MS. The myelin sheath is formed by glial cells, the most abundant cells in

the brain. They act like glue to hold neurons in their proper place and are [1, 5]. About 90% of the cells in the human brain are glial cells [1, 6]. Glial cells are classified as either microglia, astocytes, Schwann cells, or oligodendrocytes. Most of the glia and the neurons originate in the neuroectoderm of the developing embryo. However, microglia are part of the immune system and enter the brain through the circulatory system in early development [1, 6].

Microglia are specialized macrophages that are capable of phagocytosis, or the cellular process in which the cell membrane engulfs solid particles and forms an internal phagosome, or food vacuole [1]. Microglial cells can move around in the brain and multiply when the brain is injured. They can sense pathological changes and develop into macrophages and perform immunological functions. When present in healthy brain tissue, they are not macrophages. They help maintain the integrity of synapses. They sense defunct synapses and eliminate them. They also control the production of new synapses [1].

The myelinated axons make the white matter, which connects various parts of gray matter and carries nerve impulses [1]. Although it was once thought that white matter was merely passive infrastructure, we now know that it actively affects learning and can be involved in mental illnesses [1, 5]. In addition, white matter controls the signals that neurons share, and helps different brain regions work together. For example, astrocytes play active roles in brain function and information processing. Although astrocytes are not excitable by electric stimulus (as are neurons), they are excitable by Ca^{2+} [1, 5].

Astrocytes allow neurons to function by providing energy and neurotransmitters [1, 4]. They also provide physical barriers between synaptic connections and remove excess neurotransmitters from the extracellular space. They help form synapses and modulate synaptic function by communicating with neurons [1, 4]. Signals from astrocytes also control the waveform of the action potential and influence synaptic strength [1, 7]. Astrocytes also communicate with blood vessels, causing increased blood flow during when neuronal activity increases [1, 8]. They also help terminate neurotransmission and recycle glutamate (a neurotransmitter). In addition, astrocytes in the visual cortex can process information [1, 9]. Like neurons, astrocytes respond to visual stimuli with distinct special receptive fields and sharp tuning to visual stimuli. Astrocytes that are stimulated by light also affect blood flow in blood vessels. Astrocytes are the most abundant type of glial cell in the brain and they communicate with neurons through neurotransmitters [1, 9].

Moreover, astrocytes communicate with each other through waves of Ca^{2+} ions, and they can communicate with neurons [1, 4].

We are born with almost all the neurons that we need [1]. In the first 2.5 – 3 years of life, the human brain grows as glial cells grow and divide. There are about 10-100 billion neurons in the adult brain, although brain size in humans is not directly related to intelligence. This is best illustrated by a disease called hydrocephalus, or water on the brain. Back in the 1940s, when a baby was born with this condition, nothing could be done except keep the baby comfortable until it died. Now, physicians can drain the water and babies can live, grow and mature to lead ordinary lives. The disease occurs when the fluid-filled spaces in the middle of the brain expand due to increased pressure, pressing the brain against the skull [10]. Fortunately, this is no longer a death sentence, as many of these babies can grow up to lead happy, healthy and intelligent lives. One of them was a student at Sheffield University and had an IQ of 126, earned a first class honors degree in mathematics; and had virtually no brain. The neocortex of the student's brain was a layer about 1/25th of an inch (1 mm) thick, lining his skull. His brain weight weighed about 0.090 kg. That is only about 7% of normal brain weight. This student was one of many hydrocephalitic people with almost no brain. So, intelligence and brain size are not related. A ‘normal’ brain size is not necessary for intelligence [10].

Moreover, young children with autism tend to have larger brains that do children who are not autistic [1]. Moreover, social insects like honeybees have far fewer neurons than we do, but have complex social behaviors. Honeybees not only have a ‘dance language’, but they can also learn abstract concepts, such as ‘same’ and ‘different’. Also, some birds migrate tens of thousands of miles, performing navigational feats that only highly trained humans could do when aided by sophisticated technology. Even some ‘lowly’ insects perform inter-generational migration. So, animal behavior and their brains are not like machines, in which ‘bigger’ means ‘better’ and ‘smaller’ means ‘inferior’ [1].

A2.3 Regions of the brain

The normal human brain has many local regions, or centers, and many pathways that connect them [1, 11]. At each center, input fibers have synaptic connections on the cell body, or soma (also known as the perikaryon) of the neuron. At the top of our brain is the cerebral cortex, also known as the neocortex. It has a large neocortex and a smaller allocortex. There are also temporal, occipital, parietal and frontal lobes.

The cerebral cortex sorts through the vast amount of information that we receive from our internal and external environments. It keeps us on time and enables our language skills. It sorts out the information that we receive from our auditory, visual and olfactory senses. The neocortex looks gray and has six layers. It is comprised mostly of soma. The neurons send axons deeper into the brain. They are covered with a white myelin sheath, thus making the white matter of the brain [1, 11].

There are several important levels of organization in the brain [1, 2]. First there is the the synapse, followed by small clusters of synapses in microcircuits. They form dendritic subunits within dendritic trees of individual neurons. These tree-like structures are patterns of outgrowths of neuronal dendrites. The whole neuron with its several dendritic subunits makes up the second higher level of organization. Third, interactions between neurons form local circuits, which perform functions that are characteristic of different regions of the brain. The inter-regional pathways, columns, laminae and topographical maps are the fourth level of organization. They use several regions of the brain to mediate different types of behavior. However, all these levels of organization are inter-woven into a circular organization. That is, they are not organized in a hierarchy, with one on the top and others below them (as in a pyramid), but in an inter-dependent group in which all are important (as in a circle) [1, 2].

The cerebral cortex can be further divided into different parts: the premotor cortex, motor cortex, primary somatic sensory cortex, parietal lobe, visual cortex and temporal lobes [1, 2]. Moreover, the anatomy of the brain can be divided into at least four parts and three pairs of lobes that are separated by deep, gray grooves. The parts are the right hemisphere, left hemisphere, cerebellum and brain stem. There are three upper lobes: the frontal, parietal and occipital. Beneath them is the temporal lobe, followed by the pons, cerebellum and brain stem. The three functions of the frontal lobes are tension, tact and tenacity. Some children with hyperactivity attention deficit disorder (ADHD) may have less activity in the frontal lobes. This leads to problems with tact and proper control of their social behavior. They also tend to have trouble with tenacity, or the ability to stick with a task. The back edge of the frontal lobe has a cortex that controls movements in the opposite side of the body. That is, the left hemisphere controls movement on the right side and the right hemisphere controls movement on the left side of the body. The premotor cortex is often thought of as being part of the motor cortex, which is in the frontal lobe. The premotor cortex helps guide our movements and controls some of the muscles in the body. The motor cortex works with the premotor

cortex to plan and control body movements. The two hemispheres are connected by a broad band of nerve fibers in the corpus callosum [1, 2].

The parietal lobes are also known as the association cortex [1, 2]. They are behind the frontal lobes, and in front of the occipital lobes. They are connected to the temporal lobe, where they can check for previous memories. That helps keep us oriented with respect to our internal and external environments. The primary somatosensory cortex, or somatosensory system is in the parietal lobe. It reacts to different sensory stimuli, including pain, touch, temperature and propioception (body position). So, the parietal lobe integrates sensory information of different types, including visual, which comes from the visual cortex [1, 2].

The occipital lobe is the visual lobe of the brain [1, 2]. The visual cortex is connected to the retina of the eye, which is also considered to be part of the brain. The occipital lobe receives signals from our eyes and converts them into images in the brain. Then, at the bottom of the brain are the cerebellum, pons and the brain stem. The temporal lobes are parts of the cerebrum. They are involved in speech, memory and hearing. The cerebrum is also known as the telencephelon or forebrain. Telencephelon refers to the embryonic structure from which the cerebrum develops. The dorsal telencephelon develops into the cerebral cortex. The ventral telencephelon develops into the basal ganglia, also known as the basal nuclei. They are located at the base of the forebrain. Basal gangia are linked with the cerebral cortex, the thalamus and brainstem. Its primary function is to control activities of the motor and premotor cortex, so voluntary motions can be done smoothly. Several neurological disorders are associated with basal ganglia dysfunction. This includes addiction, Parkinson's disease, Tourette's Syndrome, and obsessive-compulsive disorder. The cerebrum is also divided into two nearly symmetric hemispheres, separated by the corpus callosum [1, 2].

Then there is the limbic system, which helps form the border between the cerebral cortex and subcortical layers [1, 2]. It is near the center of the brain and contains the hippocampus, hypothalamus, thalamus, olfactory bulb, fornix, amygdala, anterior thalamic nuclei and limbic nuclei. So, the limbic system supports many functions. This includes emotion, behavior, long-term memory, and smell (olfaction) [1, 2].

Another area of the brain is the substantia nigra, which is black and is in the midbrain [1]. Part of the substantia nigra is the *substantia nigra compacta*, which is responsible for producing dopamine in the brain. It plays a vital role in reward and addiction [1].

The cerebellum is between the brain stem and the rest of the body [1, 2]. It integrates sensory perception, coordination and muscle control. At

the top of the brain stem is the pons, which relays sensory information between the cerebellum and cerebrum [1, 2].

There are clusters of nerve cells called peripheral ganglia outside the CNS [1, 2]. They are distributed throughout the body. They are part of the PNS, which contains the somatic and autonomic nervous systems (ANS). The somatic nervous system contains the axons of spinal motorneurons, which directly innervate the skeletal muscles. There are no ganglia in the somatic nervous system, so no information is processed there. As a result, each impulse leaving the somatic nervous system reaches the neuromuscular junction unchanged [1, 2].

The ANS is composed of the sympathetic, parasympathetic and enteric nervous systems [1, 2]. They each have peripheral neurons. So, they can not only relay information, but also process and modify it. In the sympathetic and parasympathetic nervous systems, the peripheral neurons are clustered into ganglia. In the enteric nervous system, peripheral neurons are in sheets called plexi, found in the wall of the gut. In the sympathetic and parasympathetic nervous systems, motor neurons (also called motoneurons) in the spinal cord send pre-ganglionic axons to the autonomic gangion cells, which send their postganglionic axons out to the smooth muscles and glands. The ANS regulates the glandular secretion, cardiovascular function, intestinal motility and many other physiological properties [1, 2].

These maintenance activities are autonomous – they are mostly done without conscious control or sensation [1]. The ANS affects the heart rate, respiration, salivation, digestion, perspiration, diameter of the pupils, the discharge of urine and sexual arousal. Even though most of its actions are involuntary, some ANS functions (such as breathing) work in tandem with the conscious mind. The sympathetic and parasympathetic nervous systems in the ANS work together to maintain a type of balance. They have opposite effects on the body. The sympathetic system is used in actions requiring quick responses. The parasympathetic system is used in actions that do not require immediate reaction. The main actions of the parasympathetic nervous system are summarized by the phrase "rest and repose" or "rest and digest" (in contrast to the "fight-or-flight" of the sympathetic nervous system). The parasympathetic nervous system uses acetylcholine (ACh) as a neurotransmitter. ACh has two types of receptors, muscarinic and nicotinic. The sympathetic nervous system also uses acetylcholine. The first synapse in the sympathetic chain uses nicotinic acetylcholine receptors. The targeted synapse is mediated by adrenergic receptors, adrenaline and noradrenaline (also known as epinephrine and norepinephrine) [1].

A2.4 Neurotransmitters, neurohormones and neuromodulators

Messages are sent to and from neurons in the form of neurotransmitters, which act as primary messengers [1, 3]. There are about 50 different neurotransmitters. They are synthesized and released presynaptically. This is different from a neurohormone, which travels in the bloodstream and exerts its effects at a distinct site. There are also neuromodulators, which have no intrinsic activity, but instead simply modulate ongoing synaptic activity. However, these reductionist definitions have become less strict as neuroscientists have embraced systems thinking. Dopamine is a neurotransmitter in the striatum, but is a neurohormone when it is released from the hypothalamus and travels to the pituitary gland, where it inhibits the release of prolactin. Serotonin is a neurotransmitter in the raphe nuclei of the brain, but in the facial motor nucleus it acts as a neuromodulator and as a neurohormone. Most neuropeptides have many different activities in the brain and gut and are neuromodulators. So, neurologists now describe the activity of a neuroactive agent at a specified site rather than giving it a definition based on reductionist thinking [1, 3].

There are five classes of neurotransmitters: ACh, monoamines, amino acids, peptides and purines [1, 3]. ACh is a neurotransmitter in the PNS, CNS, autonomic ganglia and at neuormuscular junctions in skeletal muscle. Cholinergic neurons are depleted in Alzheimer's disease. Monoamine neurotransmitters include dopamine, norepinephrine, epinephrine, histamine, serotonin and melatonin. Norepinephrine and epinephrine are hormones as well as neurotransmitters. Norepinephrine and epinephrine are stress hormones that are crucial parts of the fight or flight response. Epinephrine (adrenaline) also binds to receptors in the liver, where it activates the phosphoinositide signaling system. It then triggers the hydrolysis of glycogen to form glucose, which is released into the blood. Histamine has many roles in the body, including acting as a neurotransmitter. Antihistamines (H1 histamine receptor antagonists) produce sleep. On the other hand, destruction of histamine releasing neurons, or inhibition of histamine synthesis inhibits sleep. Histamine is also important in the immune response and in allergies [1, 3].

The two most abundant neurotransmitters in the human brain are glutamate and γ-amino butyric acid (GABA) [1]. Glutamate excites neurons (so it is called excitatory), while GABA inhibits them. GABA is the major inhibitory neurotransmitter in the brain and glutamate is the major excitatory neurotransmitter. At the same time, glutamate is a metabolic precursor of GABA. Other excitatory amino acid neurotransmitters include aspartate, cysteine and homocysteine. They

depolarize neurons in the CNS. Other inhibitory amino acids include glycine, taurine, and β-alanine, which hyperpolarize neurons. Neurons that use glutamate and neurons that use GABA are located throughout the CNS and send projections to other neurons throughout the CNS [1].

Another extremely important neurotransmitter is ACh [1]. In the brain, ACh modulates the excitability of other neurons. The cell bodies of neurons that use ACh are located in the basal forebrain and send projections to the cerebral cortex. There are also neurons in the pontomesencephalic region that use ACh. They send projections to the thalamus, cerebellum, pons and medulla. There are also cell bodies of other cholinergic neurons in the spinal cord, autonomic preganglionic nuclei and parasympathetic nuclei. They send projections to smooth muscle, cardiac muscles, skeletal muscles, autonomic ganglia and glands [1].

Glycine is an important inhibitory neurotransmitter in the medulla, spinal cord, retina and brain stem [1]. A synthetic amino acid, *N*-methyl-D-aspartic acid (NMDA), is an agonist of glutamate. It binds to a subset of glutamate receptors, called the NMDA receptors. Glycine is a modulator of NMDA receptors. There is also an important derivative of aspartate, *N*-acetylaspartate, which is the second most abundant molecule in the brain (after water) [1]. *N*-acetylaspartate is a neuronal marker and is involved in maintaining the fluid balance in the brain. It is also a source of acetyl groups that are needed to make lipids in the myelin sheath, and it is a precursor for the synthesis of the important neurotransmitter, *N*-acetylaspartylglutamate, a dipeptide [1, 12]. When there is a loss of neurons in patients with chronic pain, the concentration of *N*-acetylaspartate decreases [1, 13]. *N*-acetylaspartate also helps produce energy in mitochondria. The concentration of *N*-acetylaspartate is also depleted in the brains of people with brain injury, stroke and Alzheimer's disease [1, 13].

There are also neuropeptides [1, 12]. Vasopressin and oxytocin have nine amino acids. They are synthesized in the neurohypophysis, also known as the posterior pituitary. They are secreted into the pituitary portal circulation where vasopressin can act synergistically with corticotropin-releasing hormone to trigger the release of ACTH (adrenocorticotropic hormone). Oxytocin is secreted during labor, delivery and when breastfeeding. Vasopressin (also known as the antidiuretic hormone) facilitates water reabsorption in the kidneys, while oxytocin stimulates epididymal and uterine muscle contraction [1, 12].

There are tachykinin neuropeptides that have ten to twelve amino acid residues [1, 12]. They rapidly induce contractions in the gut. There are

genes that code for larger precursor polypeptides called preprototachykinins. There are two mammalian tachykinin genes. Different tachykinins are formed by differential splicing of the mRNA of preprototachykinins. The least common mRNA codes only for substance P, while the other two forms encode both substance P and NKA (neurokinin A), also known as substance K. The second tachykinin gene, on human chromosome 12, encodes the neurokinin B (NKB) precursor. Substance P has 11 amino acid residues. It is in small neuronal systems in many parts of the CNS. It is a potent depolarizing agent in the spinal cord. Tachykinins also excite neurons, evoke behavioral responses, are potent vasodilatators and cause many smooth muscles to contract. Tachykinin neuropeptides are also involved in inflammation. So, tachykinin receptor antagonists are being developed to treat asthma and irritable bowel syndrome [1, 12].

There are also a vasoactive intestinal peptide (VIP) and VIP-related peptides [1, 12]. VIP has 29 amino acid residues. VIP-reactive neurons are quite abundant in the neocortex. In the PNS, VIP-active neurons innervate the gut, lungs and the thyroid gland. Two other members of this family are called PHI-27 and PHM-27, and have 27 amino acids. At their N-terminus, there is a histidine (H) residue and at the carboxy terminus there is either an isoleucine (I) or a methionine (M) in PHI and PHL, respectively. Both are active in the gut. Next, there is the pituitary adenylate cyclase-activating peptide (PACAP), which is in the pituitary gland. Two other members of this class are found in the hypothalamus. They stimulate appetite and help regulate blood pressure. They have been called either hypocretin-1 and -2 or orexin A and B [1, 12].

There are also pancreatic polypeptide-related peptides [1, 12]. The first one discovered was given the name PPY (a neuropeptide that contains tyrosine, or Y). It was also found in the brain and named neuropeptide Y, or NPY. In many places, including the ANS, NPY coexists with either epinephrine or norepinephrine. On the other hand, a shortened version of NPY binds to a different receptor (Y_2) and reduces the release of norepinephrine. The full NPY binds to the Y_1 receptor and increases the sensitivity of smooth muscles to norepinephrine and is one of the most potent vasoconstrictors known. During fasting, the concentration of NPY in the hypothalamus decreases [1, 12].

There are also opioid peptides [1, 12]. They are made in the pituitary gland and the hypothalamus during strenuous exercise, excitement and orgasm. They include dynorphins, endorphins and enkephalins. Endorphins have the same pharmacological profile of morphine. There are at least 12 of them. The major endorphin agonist is β-endorphin. In addition, enkephalins are pentapeptides that are involved in regulating pain

and nociception, or the activity produced in the peripheral and CNS by stimuli that can damage tissue [1, 12].

Somatostatin is another important neuropeptide [1, 12]. It inhibits the release of growth hormone. It is widely distributed in the gastrointestinal tract (GIT) and in the δ cells of the pancreas, where it can suppress the release of both glucagon and insulin. It is present in relatively high concentrations in the mediobasal hypothalamus, with smaller amounts in other regions of the brain, such as the dorsal root ganglia, amygdala, hippocampus and neocortex. Somatostatin hyperpolarizes neurons and can open M current channels, which muscarinic cholinergic receptors close so they can excite hippocampal and other neurons. As a result, somatostatin enhances responsiveness to acetyl choline [1, 12].

Cholecystokinin is peptide that is a hormone in the gut, but is a neuropeptide in the brain [1, 12]. Like VIP, NPY and GABA, it's in the thalamocortical and thalamostriatal connections. Like dopamine and neurotensin it's also in the substantia nigra and like substance P and serotonin (5-HT), it's also in medullary neurons. Cholecystokinin is an opioid antagonist. So, antagonists to one of the cholecystokinin receptors enhance the analgesic effects of opiates [1, 12].

Neurotensin has 13 amino acid residues [1, 12]. It's a potent analgesic that also induces hypothermia, accentuates barbituate and alcohol sleeping time and increases the release of prolactin and growth hormone. Neurotensin is located primarily in the anterior and basal hypothalamus, the nucleus accumbens and the midbrain dopamine neurons, as well as some of the neurons in the brain stem and spinal cord [1, 12].

Corticotropin-releasing factor, or CRF, is also found in the hypothalamus [1, 12]. It has 41 amino acids and it controls the secretion of corticotropin. It also increases the frequency of action potentials of neurons that fire in bursts, such as the hippocampal pyramidal cells. CRF can also induce seizure-like activity in the limbic system, activate spontaneous locomotion and cause an anxiogenic response [1, 12].

Another neuroactive polypeptide is called the extracellular leucine-rich repeat fibronectin containing 1 (Elfn1), because it has a repeating amino acid sequence that contains many leucine residues [1, 14]. It is selectively expressed by oriens–lacunosum moleculare (O-LM) interneurons and regulates the probability of presynaptic release. That way it can direct the formation of highly facilitating pyramidal-O-LM synapses. That is, even though synaptic transmission may be unidirectional, synaptic connections can also have bidirectional signaling. Pyramidal neurons in the hippocampus form distinct synapses onto two types of interneurons that have different functions. Excitatory synapses that connect with O-LM

interneurons are facilitating and have a low release probability, but synapses that connect with parvalbumin interneurons are depressing and have a increase the release of neurotransmitters [1, 14].

A2.5 Small molecule neuroactive NO and CO

There are two especially unusual small molecules that are neuroactive, nitric oxide (NO), and carbon monoxide (CO) [1, 12]. They are gases at room temperature and are biosynthesized only when needed. NO is synthesized from arginine in a reaction that is catalyzed by nitric oxide synthetase. All the other neurotransmitters are synthesized in the soma and are stored near the terminus of the axon in presynaptic vesicles. NO forms complexes with iron in the water-soluble form of guanylate cyclase and stimulates it, so it can catalyze the production of cGMP. Next, cGMP activates cGMP-dependent protein kinases, which catalyze the phosphorylation of other proteins. In cerebellar neurons, excitatory amino acids cause the release of NO, as does stimulation of noradrenergic peripheral neurons. NO is also plays a role in long term potentiation and long term depression. Long-term potentiation is a persistent increase in synaptic strength following high-frequency stimulation of a synapse. It is important in learning and memory. Long-term depression is a persistent decrease in synaptic activity following lengthy stimulation. Moreover, NO has many effects outside the CNS and PNS. It is the endothelium-derived relaxing factor. The endothelium is the inner lining of blood vessels. When stimulated by NO, the endothelium signals the surrounding smooth muscle to relax, causing vasodilation and increased blood flow. NO is also produced by macrophages and neutrophils so they can help kill Bacteria and other pathogens. However, NO can contribute to ischemic and reperfusion injury [1, 12].

CO is produced by the conversion of heme to biliverdin, which is catalyzed by heme oxygenase [1, 12]. Like NO, CO raises the concentration of cGMP. Heme oxygenase is found with guanyl cyclase and cytochrome P-450 reductase, which donates electrons for heme oxygenase [1, 12].

A3 The Endocrine System

The endocrine system consists of cells, glands and tissues that secrete hormones into the bloodstream to affect physiological and behavioral function and activities [1]. This includes metabolism, tissue function, sleep, mood, growth and development. Like the nervous system, the

endocrine system sends and receives information in the form of biochemical messages. However, its effects are generally slower to get started and last longer than those of the nervous system. A series of glands secrete hormones. When several of them signal each other in sequence, they can be called an axis, such as the hypothalamic-pituitary-adrenal axis [1].

One of the most interesting parts of the endocrine system is the hypothalamus. It is a small part of the brain, located below the thalamus and above the brainstem [1]. It links the nervous and endocrine systems to each other through the pituitary gland, or hypophysis. It helps control body temperature, hunger, parenting and attachment behavior, as well as thirst, fatigue, sleep, circadian rhythms and other activities of the ANS. When stimulated by high amplitude oscillations (delta waves) from the thalamus or cortex in the brain, it secretes hormones that stimulate or inhibit the release of pituitary hormones. It stimulates secretion of growth hormone-releasing hormone (GHRH) and prolactin, and inhibits the release of thyroid releasing hormone (TRH). Neurons in the paraventricular nucleus of the hypothalamus release corticotropin-releasing hormone (CRH) and other hormones into the hypophyseal portal system, where they diffuse to the anterior pituitary. The hypothalamus also secretes orexins (also called hypocretins), ghrelin, gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), somatostatin, and thyrotropin-releasing hormone (TRH), along with the non-peptide hormone, dopamine (which can also act as a neurotransmitter). At the same time, the hypothalamus responds to many different internal and external signals. It responds to daylight, circadian and seasonal rhythms, odors, gonadal steroids, corticosteroids, autonomic input, stress, invading organisms, neural input from the heart, stomach and reproductive tract and biochemicals in the blood. This includes peptide hormones, leptin, gherlin, insulin, pituitary hormones, cytokines, glucose and osmolarity [1].

Thee is also a hypothalamic-adenohypophyseal (anterior pituitary) axis [1]. Somatostatin is produced by the neuroendocrine cells of the periventricular nucleus. It inhibits the release of GH and TSH from the anterior pituitary. GnRH, GHRH, TRH and CRH stimulate the anterior pituitary to secrete follicle-stimulating hormone (FSH) and leutenizing hormone. Somatostatin inhibits the release of growth hormone (GH) and thyroid stimulating hormone (TSH). CRH stimulates the release of adrenocorticotropic hormone (ACTH). Dopamine neurons in the arcurate nucleus of the hypothalamus secrete dopamine, which inhibits the release of prolactin from the anterior pituitary [1].

Along the hypothalamic-neurohypophyseal (posterior pituitary) axis, oxytocin (OXT) induces uterine contractions and lactation [1]. Vasopressin increases the permeability of cells in the distal tubule and collecting tubule of the kidneys. This allows water to be reabsorbed and urine to be excreted. The extreme lateral part of the ventromedial nucleus helps to control food intake and satiety. When stimulated, appetite increases. Several of the hypothalamic nuclei are sexually dimorphic. They are different in males and females. There is even a sexually dimorphic nucleus in the preoptic area. Also, males and females respond differently to ovarian hormones due to estrogen-sensitive neurons that are sexually dimorphic. Male and female brains have different distributions of estrogen receptors, due to differences in neonatal exposure to gonadal steroids [1].

The pineal gland is also an important part of the endocrine system. It looks like a small (5-8 mm) pine cone, thus its name [1]. It is in the epithalamus, near the center of the brain, between the two hemispheres and tucked into a groove where the two thalamic bodies are connected. It has a lobular functional part made of cells called pinealocytes that are surrounded by connective tissue. The pineal gland receives sympathetic and parasympathetic innervations. Also, some nerve fibers penetrate into the pineal gland through the pineal stalk (central innervation), while neurons in the trigeminal ganglion innervate the gland with nerve fibers containing the neuropeptide pituitary adenylate cyclase-activating protein (PACAP). This neuropeptide stimulates adenylate cyclase, which catalyzes the biosynthesis of cAMP. It is not just a hormone but also a neurotransmitter and neuromodulator. Also, there are follicles that contain a gritty material that is made of calcium phosphate, calcium carbonate, magnesium phosphate and ammonium phosphate. Increased concentrations of calcium and phosphorus deposits in the pineal gland have been linked with aging [1].

Darkness causes the pineal gland to secrete *N*-acetyl-5-methoxytryptamine, which is better known as melatonin [1, 15]. Light inhibits the secretion of melatonin. The concentration of melatonin in the blood varies in a daily cycle or circadian rhythm. In infants, it becomes regular at about three months [1, 16]. About 90% of it is eliminated in one pass through the liver. Melatonin levels decrease during aging [1, 17]. Many of its physiological effects are caused by binding to its receptor, while others are caused by its wide spectrum antioxidant potential [1, 17]. Melatonin is part of the system that regulates the sleep-wake cycle. It causes drowsiness and lowers the body temperature [1, 18].

Then there is the pituitary gland, or hypophysis. It is a protrusion about the size of a pea (0.5 g) protruding off the bottom of the hypothalamus [1]. The anterior lobe secretes ACTH, growth hormone (GH), beta-endorphin, luteinizing hormone (LH), follicle stimulating hormone (FSH), melanocyte stimulating hormone (MSH), prolactin, and thyroid stimulating hormone (TSH). The posterior lobe stores vasopressin, and oxytocin (OXT) [1].

GH (also known as somatotropin) is an anabolic steroid, so it stimulates growth, cell reproduction and regeneration [1]. It is a stress hormone that stimulates cell division and multiplication of chondrocytes in the cartilage. GH also stimulates the biosynthesis of insulin-like growth factor 1 (IGF-1) in the liver, through the JAK-STAT pathway. IGF-1 subsequently stimulates growth in other tissues. More IGF-1 is produced in target tissues, so it is both an endocrine and autocrine hormone. It stimulates osteoblasts, which leads to bone growth. GH also elevates the concentrations of free fatty acids and glucose in the blood. It increases calcium retention, while strengthening and mineralizing bones. It increases muscle mass and promotes lipolysis, while increasing protein synthesis, reducing glucose uptake in the liver, and promoting gluconeogenesis. GH stimulates the growth of all organs except the brain. It also helps maintain the function of pancreatic islets and stimulates the immune system [1].

The glycoprotein hormone TSH stimulates the thyroid to produce thyroxine (T_4) which is coverted into triiodothyronine (T_3), which stimulates the metabolism of almost every tissue in the body [1]. It is secreted throughout life, but even more is secreted during periods of rapid growth and development. About 20% of the conversion of T_4 to T_3 occurs in the thyroid. The other 80% is converted in the liver and other organs. TSH has two subunits. The α subunit stimulates adenylate cyclase, which catalyzes the production of cAMP. The β (*beta*) subunit is unique and determines the specificity for the cognate receptor [1].

This leads to the butterfly-shaped thyroid gland, which is located on the anterior (front) of the neck, below the thyroid cartilage (‘Adam’s apple’) [1]. It weighs 2-3 grams in neonates and 18-60 g in adults. Thyroid hormones play essential roles in the developing fetal brain. The thyroid gland has two cone-shaped lobes or wings, right and left (*lobus dexter* and *sinister*) that are connected by an isthmus. It is covered by a thin, fibrous sheet called the *capsula glandulae thyroidea*. Two parathyroid glands lie between the two layers of this capsule and on the posterior side of the lobes. The thyroid gland helps control how fast a body uses energy and makes proteins as well as influencing the sensitivity of the body to other hormones. It does this by producing T_3 and T_4, which are made from

tyrosine and iodine (I_2), which comes from seafood and iodized table salt. Some of it is made from free tyrosine, while the rest is made by adding iodine to tyrosyl residues in thyroglobulin (T_g). The iodine is trapped inside T_g by H_2O_2 that is made in a reaction catalyzed by thyroid peroxidase. When stimulated by TSH from the anterior pituitary, follicular cells reabsorb T_g and hydrolyze the iodinated tyrosyl residues in lysosomes, making T_3 and T_4. Once secreted into the blood, they are partly bound to thyroxine-binding globulin, transthyretin and albumin. Only about 0.03% and 0.3% of T_4 and T_3, respectively, remain free [1].

Only free T_4 and T_3 can bind to one of three cognate cytosolic receptors, TR-α1 (in cardiac and skeletal muscles), TR-β1 (in the brain, liver and kidney) and TR- β2 (in the hypothalamus and pituitary glands) [1]. They cross the cell membrane easily and enter the cytoplasm, where they bind to receptors, causing them to migrate to the cell nucleus. The receptors bind to hormone responsive elements (HREs) in DNA either as monomers, homodimers or heterodimers. They regulate the transcription of responsive genes. In the absence of thyroid hormones, though, TRs form inhibitory complexes with corepressor proteins that bind to HREs and prevent transcription [1].

However, T_4 can also bind to a membrane-bound protein (integrin) that activates the ERK1/2 extracellular signal-regulated kinase [1]. This affects ion transport systems, such as the Na^+/H^+ exchanger. This leads to many effects, including cell proliferation. The integrins are concentrated on tumor cells and cells of the vasculature, stimulating angiogenesis and some types of cancer, including gliomas. Also, T_4 acts on the mitochondrial genome by importing nuclear TRs [1].

To keep the effects caused by T_4 from lasting too long, negative feedback is activated when the concentration of T_4 in the blood serum gets too high [1]. TSH production is suppressed by excess T_4 and by other factors, such as rising concentrations of glucocorticoids and sex hormones (estrogen and testosterone), and physiological conditions, such as cold (so thermogenesis can occur) [1].

The thyroid also produces the peptide hormone calcitonin [1]. It helps regulate Ca^{2+} concentrations in the blood serum. It protects against the loss of Ca^{2+} from the skeleton when Ca^{2+} is mobilized during pregnancy and lactation. It stimulates the movement of Ca^{2+} into bones (inhibits osteoclast activity), in opposition to the effects of parathyroid hormone (PTH). It also inhibits the absorption of Ca^{2+} in the intestines and inhibits reabsorption of Ca^{2+} by renal tubule cells, so that it can be excreted in the urine. One of its physiological effects is like that of PTH. They both inhibit phosphate reabsorption by kidney tubules. Calcitonin secretion is

stimulated by an increase in the concentration of Ca^{2+} in the blood serum and by the peptide hormones gastrin and pentagastrin. The calcitronin receptor is a G-protein coupled receptor (GPCR) and is found in the kidneys and parts of the brain. It is coupled to adenylate cyclase, so it triggers the biosynthesis of the second messenger cAMP. Calcitonin is rapidly absorbed and eliminated. It takes only one hour to reach its peak concentration in blood plasma. It is mostly metabolized in the liver by proteolysis. It can also be used as a diagnostic marker for medullary thyroid cancer if the concentration exceeeds 5 and 12 pg/mL in females and males, respectively; 40 pg/mL in children under 6 months and 15 pg/mL in children age 6 months to 3 years [1].

The alimentary system is also part of the endocrine system. The pancreas, stomach, duodenum, liver, and kidneys all secrete hormones [1]. The pancreas has endocrine and exocrine tissues. The exocrine cells secrete digestive enzymes which are delivered to the intestines. Clusters of endocrine cells (called islets of Langerhans) exist in the pancreas. The α-cells in the islets make glucagon; β-cells make insulin; δ-cells make somatostatin; and γ-cells make pancreatic polypeptide — all of which are delivered into the blood stream. The stomach secretes the hormones gastrin, ghrelin, neuropeptide Y, somatostatin, histamine and endothelin. Gastrin is also made in the duodenum and pancreas. It stimulates the production of gastric acid by parietal cells. It also stimulates the growth and maturation of parietal cells, causes cells to secrete pepsinogen (the zymogen for the digestive enzyme pepsin), increases antral muscle mobility and promotes contractions. It also induces pancreatic secretions and emptying of the gallbladder. Gastrin binds to the cholecystokinin B receptors, which stimulate the release of histamine and induce the insertion of K^+/H^+ ATPase pumps into the apical membrane of parietal cells (which in turn increases the release of acid into the stomach cavity) [1].

Ghrelin increases food intake and fat mass and activates the reward network of the brain in the mesolimbic system [1]. Neuropeptide Y (NPY), like ghrelin, stimulates the appetite. It's also a vasoconstrictor and it makes adipose cells grow in fatty tissue. It increases food intake and the storage of energy as fat (triacylglycerides). 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. It activates type II glucocorticoid receptors and

blocks 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. It should be noted that a diet high in fat and sugar stimulates the release of NPY, causing abdominal fat to increase [19]. Also, elevated concentrations of NPY have been associated with resilience against and recovery from posttraumatic stress disorder [1, 20].

The duodenum is the first part of the small intestines [1]. It is a hollow tube about 25-38 cm long that connects the stomach with the jejunum. Enzymes in it catalyze the digestion of food that has already been partly digested, thus preparing it for absorption in the small intestine. The duodenum also helps to regulate the rate in which the stomach empties itself when it's stimulated by the peptide hormones secretin and cholecystekinin. These hormones also cause the liver and gall bladder to relesae bile and the pancreas to release bicarbonate and the digestive enzymes trypsin, lipase and amylase into the duodenum [1].

The liver secretes insulin-like growth factor (IGF), which regulates cell growth and development, as well as producing insulin-like effects [1]. The liver also affects metabolism by secreting fibroblast growth factors (FGFs). FGF 21 is a hepatokine (cytokine from the liver) that acts as a global starvation signal to modulate fuel partitioning and metabolism as well as repress growth by acting on the nervous system [21]. It binds to its receptor in the brain, β-Klotho, and lowers insulin concentrations, inhibits growth, alters light-dark cycle activity, increases systemic corticosterone levels and inhibits female fertility [22]. It also suppresses the expression of the neuropeptide vasopressin in the suprachiasmatic nucleus (SCN) of the brain, which also expresses receptors for ghrelin and leptin. It also contributes to neuroendocrine control of female reproduction by acting on the SCN in the hypothalamus to suppress ovulation during starvation [22]. The liver also secretes angiotensin and its zymogen, angiotensinogen. They cause vasoconstriction and release aldosterone from the adrenal cortex. The liver also secretes thrombopoietin, which stimulates megakaryocytes to produce blood platelets. The liver also secretes hepcidin, which inhibits iron absorption in the intestines and iron release by macrophages.

The kidneys are also an important part of the endocrine system. They are shaped like kidney beans and are located at the rear of the abdominal cavity [1]. They filter blood and excrete wastes like urea and ammonium (NH_4^+) salts, while reabsorbing water, glucose and amino acids. They produce urine and help with homeostasis. They regulate pH, electrolytes and blood pressure. They secrete renin, erythropoietin, calcitrol and

thrombopoietin. Renin, also known as angiotensin, catalyzes the hydrolysis of the protein angiotensinogen to make angiotensin I. Renin is secreted by the afferent arterioles from specialized granular cells of the juxtaglomelular apparatus in response to a decrease in arterial blood pressure, a decrease in NaCl (table salt) in the ultrafiltrate of the urine and activity in the sympathetic nervous system. Renin circulates in the bloodstream. After angiotensin I is produced, it is further hydrolyzed in the lungs to make the vasoactive peptide angiotensin II. This reaction is catalyzed by angiotensin-converting enzyme (ACE). Angiotensin II constricts blood vessels and acts on smooth muscles to raise the resistance that is made by these arteries on the heart. The heart works harder, increasing the blood pressure. Angiotensin II also causes the adrenal glands to release aldosterone, which causes the epithelial tubes in the distal tubule and collecting ducts of the kidneys to increase reabsorption of Na^+. It exchanges Na^+ and K^+ and reabsorbs water, thus maintaining the electrochemical charge while increasing the blood volume and blood pressure. The renin-angiotensin system (RAS) also acts on the CNS to stimulate thirst, thus increasing water consumption. The RAS also reduces the loss of urine by secreting vasopressin from the posterior pituitary gland [1].

When the RAS system becomes over-activated it can lead to hypertension [1]. This can be controlled by either ACE inhibitors (such as ramipril and perindopril) or by angiotensin II receptor blockers (ARBs, such as losartan, irbesartan or candesartan). ACE inhibitors or ARBs are also part of the standard treatment after a heart attack. Also, pediatric kidney cancer can be caused by juxtaglomelular tumors (reninoma), Wilm's tumor and renal cell carcinoma, all of which produce renin [1].

Erythropoietin (EPO) is an essential glycoprotein hormone that controls the production of red blood cells, or erythrocytes [1]. It promotes the survival of progenitors and precursors of red blood cells by inhibiting their apoptosis. Also, EPO is important in the brain's response to neuronal injury and in the process of healing wounds. It also helps protect the brain under hypoxic conditions, such as stroke. Moreover, EPO may improve memory, due to its effects on the hippocampus, synaptic connectivity, neuronal plasticity, and memory-related neural networks. It binds to the EPO receptor on the surface of red cell progenitors. The EPO receptor is also found in bone marrow as well as the peripheral and central nervous systems. EPO is also called hematopoietin and hemopoietin. It is also produced in perisinusoidal cells in the liver, primarily in the fetal and perinatal period. When exogenous EPO is used as a performance-enhancing drug it is called an erythropoiesis-stimulating agent. It can be

distinguished from endogenous EPO because they undergo different post-translational modifications [1].

Calcitrol (also known as 1,25-dihydroxycholecalciferol or 1,25-dihydroxyvitamin D_3) increases the concentration of Ca^{2+} in the blood by increasing its absorption from the GIT [1]. It also stimulates the release of Ca^{2+} from osteoblasts in the bones, which subsequently causes them to release the secondary messenger protein RANKL, which activates osteoclasts. Calcitrol is sold under the names Rocaltrol, Calcijex, Decostriol and Vectical. It can be prescribed to treat hypocalcemia, hypoparathyroidism, osteomalacia, rickets, renal osteodystrophy, and osteoporosis. It can also be used for people who are undergoing dialysis, due to kidney disease. It can also prevent corticosteroid-induced osteoporosis. The main adverse side effect is hypercalcemia [1].

Many of the effects of calcitriol are triggered when it binds to its receptor, also called the vitamin D receptor or VDR [1]. When calcitrol is not bound to it, free VDR is in the cytoplasm. After binding calcitrol, it translocates to the cell nucleus, where it becomes a transcription factor, promoting the expression of a gene that codes for a protein that binds Ca^{2+}. As the concentration of the Ca^{2+} binding protein increases, cells can actively transport more Ca^{2+} from the intestine across the intestinal mucosa and into the blood. To maintain the electropotential across the cell membrane, anions (mostly HPO_4^{2-} and $H_2PO_4^-$) are also transported. As a result, calcitrol also stimulates the intestinal absorption of phosphate [1].

Thrombopoietin is another glycoprotein hormone [1]. It is also called megakaryocyte growth and development factor (MGDF). It is produced in the kidneys and liver and regulates the production of platelets by the bone marrow. It stimulates the production and differentiation of megakaryocytes, the cells in the bone marrow that fragment into blood platelets. Thrombopoietin binds to a receptor (CD110) on the surface of blood platelets, thus reducing the exposure of megakaryocytes to the hormone. As a result, the increase and decrease in concentrations of platelets regulate the concentrations of thrombopoietin. When the concentration of platelets drops too low, it causes more exposure of thrombopoietin to the undifferentiated bone marrow cells. This leads to differentiation into megakaryocyes and further maturation of these cells. On the contrary, high platelet concentrations reverse this process [1].

Next are the adrenal glands, which sit on top of the kidneys [1]. They both have an outer cortex and an inner medulla. They produce hormones in response to stress. They make corticosteroids (such as cortisol) and catecholamines (such as adrenaline and noradrenaline). They also produce androgens in their innermost cortical layer. The adrenal glands affect

kidney function by secreting aldosterone, which acts on the distal convoluted tubule and collecting duct of the kidneys. It causes reabsorption of Na^+ to increase, while excretion of both K^+ and H^+ increases. The outermost layer, the zona glomerulosa, is the main site for producing aldosterone, a mineralcorticoid. The reaction is catalyzed by the enzyme aldosterone synthase, also known as CYP11B2. Aldosterone is largely responsible for the long-term regulation of blood pressure. The zona fasciculata, located between the glomerulosa and reticularis, produces glucocorticoids, including 11-deoxycorticosterone, corticosterone and cortisol. The adrenal medulla is in the core of the adrenal gland, and is surrounded by the adrenal cortex. It secretes about 20% noradrenaline and 80% adrenaline. The chromaffin cells of the medulla are the human body's main source of the circulating catecholamines (adrenaline and noradrenaline). These water-soluble hormones initiate the fight or flight response. To carry out its part of this response, the adrenal medulla receives input from the sympathetic nervous system. Because it is innervated by preganglionic nerve fibers, the adrenal medulla is a specialized sympathetic ganglion. Unlike other sympathetic ganglia, however, the adrenal medulla lacks distinct synapses and releases its secretions directly into the blood. Cortisol also promotes adrenaline synthesis in the medulla. It promotes the upregulation of phenylethanolamine *N*-methyltransferase, thereby increasing the synthesis and secretion of adrenaline [1].

The reproductive system (including the testes, ovarian follicle and corpus luteum) are also in the endocrine system, as are the placenta and uterus when a woman is pregnant [1]. The testes secrete androgens (mostly testosterone), as well as estradiol and inhibin. They stimulate the development and maintenance of male characteristics by binding to androgen receptors. This includes the activity of the male sex organs and development of male secondary sex characteristics. Androgens are also the original anabolic steroids and the precursors of all estrogens. The primary and most well-known androgen is testosterone. Dihydrotestosterone (DHT) and androstenedione are not as well known, but are equally import in male development. DHT in the embryo triggers the differentiation of the penis, scrotum and prostate. Later in life, DHT contributes to male balding, prostate growth and sebaceous gland activity. Circulating androgens can influence human behavior because some neurons are sensitive to steroid hormones. Androgen levels have also been implicated in regulating human aggression and libido [1].

Estradiol (also known as E2 and 17β-estradiol) is a sex hormone that readily crosses the cell membrane and binds to the estrogen receptor (ER)

in the cytoplasm [1]. The estradiol-bound receptor then enters the cell nucleus where it acts as a transcription factor. In contrast with other estrogens, estradiol binds to both the ERα and ERβ receptors. Some medications, like tamoxifen, are selective estrogen receptor modulators (SERMs). Estradiol is present in males and females. In the healthy adult male, its concentration can be between 14 - 55 pg/mL. In women in the follicular phase (day 5), its concentration is between 19 - 140 pg/mL. In preovulatory women, it can be from 110 - 410 pg/mL. In the luteal phase, it is between 19 - 160 pg/mL. In post-menopausal women, it ranges from 0 to 35 pg/mL. That is, in the normal menstrual cycle, it is a growth hormone for tissues of the reproductive organs. It supports the lining of the vagina, the cervical glands, the endometrium, and the lining of the fallopian tubes. It enhances growth of the myometrium. Estradiol is also needed to maintain oocytes in the ovary. During the menstrual cycle, estradiol is produced by the growing follicle. This triggers the events in the hypothalamic-pituitary axis that leads to the surge in luteinizing hormone, which induces ovulation. In the luteal phase, estradiol works with progesterone to prepare the endometrium for implantation. During pregnancy, estradiol increases as the placenta produces it by positive feedback [1].

Estradiol also affects bones [1]. When it is absent or present at too low of a concentration in post-menopausal women, it can cause osteoporosis. It also plays an important role in women's mental health, with links between the hormone level, mood and well-being. Sudden drops or fluctuations in, or long periods of sustained low concentrations of estrogen may cause significant mood-lowering [1]. Clinical recovery from postpartum depression, perimenopause, and postmenopause can occur after estrogen concentrations are stabilized and/or restored [1, 23]. In men, estradiol prevents sperm cells from undergoing apoptosis [1]. Estradiol also acts as an antioxidant in the brain, so it is neuroprotective. Estradiol has been given with progestins as part of hormone replacement therapy in post-menopausal women. When an ethinyl group is added to estradiol it becomes ethinyl estradiol. It is a major component of contraceptive drugs and devices [1].

Inhibin and activin are two closely related protein complexes that have very different effects [1]. Inhibin inhibits the synthesis and secretion of follicle stimulating hormone (FSH), while activin activates it. In women, FSH stimulates the secretion of inhibin from the granulosa cells of the ovarian follicles, so inhibin inhibits this. There are two inhibins. Inhibin A reaches its peak concentration in the mid-luteal phase. Inhibin B reaches

its peak concentration in the early- to mid-follicular phase, and a reaches a second peak at the onset of ovulation [1].

The ovarian follicles and corpus luteum secrete progesterone, androstenedione, estrogens (mostly estradiol) and inhibin [1]. Progesterone is a steroid hormone that is involved in the menstrual cycle, pregnancy and embryogenesis. It is a member of the class of hormones called progestogens. Progesterone is the major naturally occurring progestogen. It inhibits the enzyme momoamine oxidase (MAO), which catalyzes the oxidative breakdown of the neurotransmitter serotonin. It also enhances the function of the serotonin receptor in the brain. Progesterone is also important in fetal development. It converts the endometrium to its secretory stage to prepare the uterus for implantation. At the same time, it affects the vaginal epithelium and cervical mucus, making it thick and impenetrable to sperm. If pregnancy does not occur, progesterone concentrations will decrease, leading to menstruation. During implantation and gestation, progesterone decreases the maternal immune network so that the pregnancy will be accepted. It also decreases the contractility of the smooth muscle in the uterus and inhibits lactation during pregnancy. The concentration of progesterone decreases after the baby is born, thus triggering the production of mother's milk [1].

Progesterone, pregnenolone and dehydroepiandrosterone are neurosteroids [1]. Progesterone can be biosynthesized in the CNS and is a precursor to another major neurosteroid, allopregnanolone. Neurosteroids assist in synaptic function, are neuroprotective, and affect myelination. They also improve memory and cognitive ability. In females, androstenedione is released into the blood to provide a substrate that produces estrogen in granulosa cells. Also, androstenedione can increase serum testosterone concentrations over an eight-hour period in men who take it as a single oral dose of 300 mg per day [24]. Because androstenedione is partly converted to estrogens, people taking this as a dietary supplement may have estrogenic side-effects [1]. Androstenedione is also a weak partial agonist of the androgen receptor. However, in the presence of full agonists like testosterone or dihydrotestosterone (DHT), it is antagonistic, due to its lower intrinsic activity. So, it is an antiandrogen [1].

The heart and skeletal muscles are also endocrine organs [1, 25-27]. The atrium of the heart produces atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) that are collectively called cardiac natriuretic peptides (cNPs) [1, 25]. BNP is expressed at relatively high concentrations in the heart. The cNPs 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 also decreasing salt concentration and thirst, thus leading to a reduction in extracellular fluid volume. ANF also inhibits the secretion of vasopressin from the pituitary gland, thus decreasing water reabsorption in the kidneys. ANF and BNP exert their effects mostly through a natriuretic peptide clearance receptor (NPR-A). It is a guanyl cyclase-coupled receptor. CNP signaling occurs mostly though an NPR-B receptor. When the peptide ligands bind to their receptors, cGMP is biosynthesized and some of it enters the circulation and affects several enzymes and ion channels. This can lead to vasorelaxing effects, inhibition of reabsorption of Na^+ in the kidneys, steroidogenesis in the adrenal glands and phototransduction. There is also an NPR-C receptor that helps to remove or clear peptides and other biopolymers [1, 25].

All three cNPs prevent cell growth and proliferation [1, 25]. BNP inhibits, while ANF antagonizes vascular smooth muscle cell growth and promotes endothelial cell function in atherosclerosis. It also inhibits the proliferation of visceral preadipocytes. The biosynthesis and secretion of BNP and ANF increase during hemodynamic overload, which occurs in chronic arterial hypertension and congestive heart failure. All three cNPs antagonize the renin–angiotensin–aldosterone system. They also induce the hydrolysis of lipids in fat cells and pre-adipocytes. The CNP peptide is expressed at about the same concentration at all times (is constitutive) and acts as a vasodilator on blood vessels while inhibiting the growth of vascular smooth muscle cells. The concentration of CNP increases in patients with congestive heart failure. The concentrations of cNPs in the blood increase during heart failure, myocardial infarction, hypertension, left ventricular hypertrophy and pulmonary hypertension [1, 25].

At the same time, skeletal muscles are part of the endocrine system [1, 25]. Cytokines that are released by skeletal muscles are called myokines. They are released when muscles contract. They subsequently affect other tissues. Moreover, exercise causes an increase in cytokine levels, which can help 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 immune system, but also help mediate changes in metabolism that occur during exercise [1, 25].

Skeletal muscles also produce other myokines that help regulate cell growth, regeneration, differentiation, transformation, and death [1, 27]. For example, myostatin inhibits the repair of damaged muscle cells. Myostatin can limit muscle mass and may become a therapeutic target. There are also activins and inhibins that help regulate the secretion of FSH

from the anterior pituitary. Activins and inhibins are important in reproduction. Their concentrations in the blood can change and serve as biomarkers for ectopic pregnancy. Activin is like myostatin, in that it inhibits muscle growth. So, activin receptors are potential therapeutic targets for neuromuscular disorders [1, 27].

Skeletal muscles also produce a glycoprotein called follistatin [1, 27]. It inhibits the biosynthesis 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 [1, 27].

Bone morphogenic protein (BMP) is another hormone that is produced by skeletal muscles [1, 27]. It is 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 [27].

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 endocrine system [1, 28-30]. Bones undergo continual autopoiesis. Osteoclasts remove bone cells, while osteoblasts re-make them [1, 30]. Also, osteocytes are the last step in the osteoblast lineage. They help regulate the processes of bone remodeling and mineralization [1, 30]. That is, the bone marrow produces hematopoietic stem cells 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) [30]. In addition, bones interact with adipose tissue and muscles to regulate bone density and body fat [28-30]. Bone marrow also produces adipocytes [1, 29].

It was once thought that obesity can protect against osteoporosis, but recent epidemiological and clinical studies have shown the opposite [1, 28]. Not only obesity, but also reduced muscle mass (which often occur simultaneously in the elderly) are risk factors for osteoporosis. 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 concentrations 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. Members of the epidermal growth factor family can inhibit both adipogenesis and osteogenesis [1, 28].

Adipose tissue contains not only white and brown adipocytes, but also cells called either beige fat or brite fat (from brown and white) [1, 28]. Beige fat cells emerge when cells in visceral white adipose tissue (VWAT) transdifferentiate. VWAT 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 crosstalk 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 indicates 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, while also affecting muscle mass and bone strength [1, 28]. MSCs can modify and influence almost all the cells of the innate and adaptive immune systems [1, 31]. 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 [1, 31].

So, it has been proposed that there is a bone-adipose axis [1, 29]. The number of mature osteoblasts and adipocytes in the bone marrow depends 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. That is, cytokines in the IL-6 family exert their effects through transmembrane receptor complexes. 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, including 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 concentration of IGF-1 decreases in the elderly, it can lead to bone loss and osteoporosis. Also, a loss of estrogen can increase the number 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 code for have different functions in these different cells [1, 29].

Bone is also an energy-regulating endocrine organ [1, 29] that also regulates mineral metabolism [30]. Osteoblasts secrete osteocalcin, which helps to regulate energy metabolism by regulating insulin secretion by pancreatic β-cells and testosterone secretion by testicular Leydig cells. Osteocalcin also regulates fatty acid metabolism in the liver, and the insulin sensitivity of muscle and fat [1, 30]. There is an osteocalcin-bone-pancreas-testis axis that helps to regulate energy metabolism and the production of sex hormones [1, 30, 32]. Osteocalcin helps to regulate male fertility [1, 32].

Both osteoblasts and osteocytes secrete FGF23 [1, 32]. It affects the kidneys, parathyroid gland, choroid plexus, and pituitary gland [1, 32]. FGF23 helps to regulate the concentration of phosphate (HPO_4^{2-} and $H_2PO_4^-$) and 1,25-dihydroxyvitamin D_2 in the blood [1, 33].

The bone marrow also produces early outgrowth cells (EOCs) that produce soluble hormones that have antifibrotic effects that can reduce progressive organ fibrosis in chronic kidney disease (CKD) and heart failure [1, 34]. That is, fibrotic injury occurs in CKD and it has deleterious effects on the heart. The EOCs secrete factors that inhibit the production of pro-inflammatory TGF-β and collagen [1, 34].

Finally, it has been shown that phytoestrogens, saponins and flavonoids can affect the osteogenic and adipogenic pathways of differentiation [1, 35]. The shift towards osteogenesis occurs by inhibiting

the transcription factors PPAR-γ and C/EBPα. So, phytoestrogens, saponins and flavonoids that inhibit them may help to prevent or minimize bone loss and other metabolic diseases [1, 35].

The major endocrine systems are the TRH-TSH-Y3/T4 system, the GnRH-LH/FSH-sex hormones system, the CRH-ACTH-cortisol system, the renin-angiotensin-aldosterone system and the leptin vs insulin system [1]. The TRH-TSH-Y3/T4 system is also known as the hypothalamic–pituitary–thyroid axis (HPT axis). It is responsible for regulating metabolism. The hypothalamus senses the concentrations of T_3 and T_4 and responds by releasing TRH, which stimulates the pituitary gland to produce TSH. Then, TSH stimulates the thyroid gland to produce thyroid hormone until its concentration in the blood returns to the healthy homeostatic range. The GnRH-LH/FSH-sex hormones system is also called the hypothalamic–pituitary–gonadal axis and HPG axis. It is a crucial part in the development and regulation of many of the body's systems, including the reproductive and immune systems. Fluctuations in the concentrations of these hormones cause changes in the amounts of hormones produced by each gland. These fluctuations have both widespread and local effects on the body, such as controling development, reproduction, and aging. The CRH-ACTH-cortisol system is also called the hypothalamic-pituitary-adrenal axis or HPA axis. It controls reactions to stress and regulates digestion, the immune network, mood, emotions and sexuality, along with energy storage and expenditure. It is the common mechanism for interactions among glands, hormones, and parts of the midbrain that mediate the general adaptation syndrome, or the body's short- and long-term reactions to stress. The renin-angiotensin-aldosterone system regulates blood pressure and fluid (water) balance. The leptin vs insulin system helps to control energy homeostasis [1].

A4 The Immune System

From the outside of our skin to the inside of our bodies, we have many ways to defend ourselves [1]. Our first line of defense is composed of physical and chemical barriers, such as antimicrobial peptides and enzymes (like lysozyme) that are secreted by saliva, tears, the respiratory tract and the skin (a physical barrier). In the gut, commensurate (or commensal) Bacteria help prevent infection by pathogenic Bacteria. The ecosystem that comprises the human organism co-evolved with commensurate Bacteria that have established a niche for themselves, in which they have a competitive advantage over other Bacteria. The highest density of commensurate Bacteria in the human body is found in the GIT.

There is a mucus tract that covers it and influences the function of antigen presenting cells (APCs) and epithelial cells so that our dendritic cells (DCs) can tolerate food and commensurate bacterial antigens. This mucus tract contains mucins that prevent inflammation by forming a non-attached outer mucus layer that is inhabited by Bacteria. Mucins also form an inner mucus layer that adheres to intestinal epithelial cells. It inhibits inflammatory responses to DCs by generating signals that make them tolerant to antigens. DCs are just beneath the epithelial layer of cells and present foreign antigens to other cells in the immune system [1].

When these first lines of defense fail to block pathogenic bacteria or other immunogens, the innate and adaptive immune systems need to be activated [1]. Both use white blood cells (leukocytes), which are a diverse group of cells that mediate the body's immune response. They circulate through the bloodstream and lymphatic system and are recruited to sites of tissue damage and infection [1].

Acute inflammation is made up of antibodies and humoral factors in the cell-free blood serum and other bodily fluids, formerly known as humors [1]. This includes the complement system, coagulation system, iron-binding proteins (lactoferrin and transferrin), interferons, lysozyme and IL-1. Antibodies are made in B cells, which are made in the bone marrow. The innate immune system recognizes pathogen-associated molecular patterns (PAMPs), such as double-stranded RNA, lipopolysachharides and other molecules that are common in viruses, bacteria and fungi, but not in healthy human cells. The innate immune system also senses the presence of microbial pathogens by detecting cytoplasmic DNA. However, erroneous detection of dinucleotides can help lead to some autoimmune diseases [1].

The innate immune system also recognizes damage- or danger-associated molecular patterns (DAMPs) such as uric acid crystals and ATP that are released from damaged cells [1]. The innate immune system protects us from infection by other organisms in a relatively non-specific way. It recruits immune cells to the site of infection, activates the complement cascade, identifies and removes foreign substances and activates the adaptive immune system through antigen presentation. That is, the innate immune system recognizes Bacteria, fungi and other organisms, and breaks them down. It also 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 has many parts [1].

Invading cells are killed by phagocytosis using a subgroup of leukocytes that circulate in the bloodstream as monocytes [1]. They are converted into macrophages, which subsequently enter tissues during inflammation. There is much heterogeneity in phenotype, homeostatic turnover and function in macrophages that are present in different tissues. DCs are a distinct lineage of mononuclear phagocytes. They specialize in presenting antigens to T cells while initiating and controlling immunity. DCs activate and condition virus-specific T cells. They bridge innate and adaptive immunity and have additional roles in shaping the immune response to pathogens, vaccines, and tumors [1].

There is also a cell-based immunity that does not include complement or B cells [1]. Instead, it uses macrophages, natural killer (NK) cells, cytotoxic T-lymphocytes (made in the thymus) and many different cytokines that are released in response to an antigen, Bacteria, fungi, protozoans and cancer cells. Both the innate and adaptive immune systems have cell-mediated and humoral components [1].

The adaptive or acquired immune system acts after it is stimulated by the innate system [1]. Mature dendritic cells in the innate immune system form immunological synapses with T cells that have the CD4 cell surface glycoprotein on them. So, they are called $CD4^+$ T-cells. There are two main types of T cells: $CD4^+$, which oversee the immune response and $CD8^+$ cells which do much of the actual killing. The adaptive immune response is initiated by specific interactions between antigen-loaded, mature dendritic cells and naïve $CD4^+$ T cells in the lymph nodes. Dendritic cells link the innate and adaptive immune systems. They engulf exogenous pathogens and toxins, chop them into pieces and present them to T-cells. To do this properly, they must be able to distinguish between self and non-self. So, while our brains help establish our personal identities to the outside world, the immune system establishes our internal identities [1].

As the name implies, the adaptive immune system adapts to toxic challenges from viruses, Bacteria, protozoa, and other organisms [1]. Adaptive immunity uses cytokine-producing helper T cells (T_H). Naïve T_H cells are stimulated by antigen presenting cells (APCs) that have cognate antigens on their surface. The naïve cells differentiate into two different types of T_H cells, T_H1 and T_H2. The T_H1 cells secrete interferon-γ and promote cellular immunity. The $CD4^+$ T_H2 cells produce the cytokines IL-4, IL-5, IL-10 and IL-13, while producing humoral immunity. A small percentage of the cells acquire and retain a memory of one or more

antigens from the invading viruses and micro-organisms. This enables us to mount rapid, effective responses to them after being exposed to them once before [1].

The adaptive immune system uses white blood cells called lymphocytes [1]. The adult human body has about 2 trillion lymphocytes, constituting 20-40% of all the white blood cells. The peripheral blood contains 20–50% of them as circulating lymphocytes. The rest of them move within the lymphatic system. Natural killer (NK) cells are large and granular. Smaller lymphocytes are T and B cells. There are three general classifications of $CD8^+$ cytotoxic T cells: naïve cells, central memory cells and effector memory cells. Naïve T-cells have not yet been exposed to their cognate antigen, but memory cells have. Memory T cells can recognize Bacteria, viruses and cancer cells. Central memory cells express the cytokine CCR7 and IL-2. Effector memory T cells (T_{EM}) express the effector cytokines IFNγ and IL-4. They can reside permanently, or with low turnover rates in peripheral tissues and migratory cells that can move from the periphery and into the blood. Moreover, T_{EM} cells rapidly recognize and kill infected target cells, thus containing nascent infections. Human T lymphocytes can be genetically engineered to express almost any gene, so they can have the desired specificity. They can be used in adoptive immunotherapy for cancer [1].

There is also a network of antibodies in the immune system [1]. They are connected to other antibodies and antigens. Antibodies are always being made, even in the absence of infection. They help define self. Every day, about 100 precursors of immune cells enter the thymus and undergo several cell divisions to make about $10^7 – 10^8$ T-cells [1].

T-cells make cell surface proteins called immunoglobulins, or Igs [1]. Igs have two heavy chains and two light chains that are joined by disulfide bonds. Each chain is made up of structural domains called Ig domains. These domains of 70-100 amino acid residues are classified as variable, joining or constant. They are made from three types of genes, which code for the constant, diversity and joining regions. That is, part of an Ig molecule has a structure that is very similar to other Igs in its class. This is called the constant region. There are five types of mammalian heavy chains: α, δ, ε, γ and μ. They make the IgA, IgD, IgE, IgG and IgM antibodies. The constant region is joined with the variable, or diversity region of the Ig, which is on the outer part of the Y-shaped arm. It has a unique structure that gives the antibody molecule its antigenic individuality and is coded for by diversity genes. These unique regions are called complementarity determining regions (CDRs). They define the idiotypes, or antigen binding specificity of the antibodies. More than a

million idiotypes are made. Those that bind to self antigens are marked for destruction. Those that bind foreign antigens survive and make memory B and T cells. Only about 5% of the T cells survive the selection process that eliminates the cells that recognize self-antigens and bind them tightly. There is a selection-driven tolerance (self-tolerance) that helps establish a type of balance or homeostasis. If the elimination process becomes too lenient, some of the T-cells that recognize self survive and kill some of the body's own cells, causing an autoimmune disease. If the elimination process becomes too severe, some of the T-cells that recognize invading pathogens would be killed and the body could succumb to disease. This homeostasis is controlled, in part, by regulatory T cells (T_{reg} cells), whose development and maturation is controlled by transforming growth factor-β (TGF-β). So, altered TGF-β signaling can predispose people to allergic responses to antigens that may be present in the environment and foods [1].

Throughout our lives, our immune systems go through different states of homeostasis [1]. When we are in our mother's womb, we have an underdeveloped immune system that mostly uses T_H2 cells instead of the T_H1 cells that we use as adults. We are born with a set of immune cells, which make many antibodies and antigens. After we are born and are exposed to different antigens from the environment, our adaptive (acquired) immune systems adapt by making different repertoires of antibodies. Chemical messengers communicate between cells and tissues, to help make necessary changes, in response to changes in our internal environments. So, there is a network in which antigens make antibodies, which make more antibodies. The adaptive immune system does this, but it takes some time. So, as mentioned earlier, the innate immune system responds faster and activates appropriate changes in the adaptive immune system. TLRs, NLR proteins and dendritic cells link the two together. So, the immune network consists of nodes (cells and tissues) connected by chemical messengers (chemokines, cytokines and receptors). The reductionist thinking of the 20th century identified the nodes and the chemical messengers. Systems thinking of the 21st century added an emphasis on the relationships between the components, how they communicate and how they are organized. A person's immune response must be kept in balance to maintain health. This is done through a network of co-signaling molecules. It is coordinated by a network of ligand–receptor interactions on the cell surface, with both co-stimulatory and co-inhibitory capacities. The direction and outcome of immune responses are decided by the interplay of these complicated and often counterbalancing network interactions [1].

The innate and acquired immune systems have opposite and complementary properties [1]. The innate system responds fast and causes inflammation, redness and swelling while it produces heat. It also helps activate the adaptive immune system, which responds slower and relieves inflammation. These actions are mediated by four classes of mediators and five families of cells [1].

The five families of immune cells are: phagocytes, granulocytes, NK cells, lymphocytic T-cells and lymphocytic B-cells [1]. Phagocytes, granulocytes and NK cells are in the innate immune system, while lymphocytic T- and B-cells are in the acquired immune system. Like erythrocytes and thrombocytes (platelets), white blood cells are made from precursors in the bone marrow. The $CD4^+$ T cells can be activated by a variety of pathogens. Phagocytes recognize, engulf and destroy viruses, microorganisms and many foreign organic compounds. Granulocytes include neutrophils, eosinophils, basophils and mast cells. They have secretory granules. They are also called polymorphonuclear leukocytes. NK cells interact with many different types of cells and influence their fate. They are highly selective. They kill infected cells and tumors, while sparing healthy cells. Lymphocytic T-cells bind antigens that are presented by MHCs that are expressed by diseased cells. They protect us from infection. Lymphocytic B-cells recognize antigens in their native form and make antibodies. Macrophages engulf and kill microorganisms. They also secrete factors that affect tissue repair and remodeling. Monocytes are recruited to inflamed tissues and become macrophages. DCs link the innate and adaptive immune systems. They ingest and break down the various constituents of the cell. Mast cells shape the inflammatory milieu and control the activation state of many cells that are crucial for adaptive immunity. Eosinophils kill parasites, help regulate other immune cells, help destroy tumor cells, and promote the repair of damaged tissue. Neutrophils kill invading microorganisms. Basophils contain the anticoagulant heparin, which prevents blood from clotting too rapidly. They also contain histamine, which acts as a vasodilator, promoting blood flow to tissues [1].

Phagocytes recognize, engulf and destroy viruses, microorganisms and foreign organic compounds [1]. They are classified as either professional or non-professional, based on their ability to perform phagocytosis. The professional phagocytes are neutrophils, mast cells, monocytes, macrophages, and DCs. An estimated 5% of the circulating white blood cells are monocytes. They live just a few days, after which they develop into macrophages, which are in body tissues. Monocytes selectively destroy cells whose outer membranes have been tagged with labels, such

as antibodies, complement fragments and C-reactive protein. Monocytes circulate in the bloodstream, bone marrow, and spleen and do not proliferate in a steady state. They have chemokine and adhesion receptors that mediate their migration from blood to tissues during infection. They produce inflammatory cytokines while removing cells and toxins. They can also differentiate into inflammatory DCs or macrophages during inflammation. Inflammation and pattern-recognition receptors that are associated with pathogens trigger monocyte migration to tissues and differentiation to inflammatory DCs and macrophages. Macrophages reside in lymphoid and nonlymphoid tissues where help maintain tissue homeostasis by removing cells that have undergone apoptosis, while also producing growth factors [1].

Macrophages are present at low concentrations in all resting tissues, but they increase in response to inflammatory signals [1]. In cardiovascular diseases, accumulated macrophages are quite harmful. Monocytes that circulate in the bloodstream are recruited to inflamed tissues and become macrophages. However, the accumulation of M2 macrophages (also known as alternatively activated macrophages) occurs not through changing their precursors that come from the bloodstream, but by local proliferation of macrophages. The liver has specialized macrophages, called Kupffer cells. About 80% of the fixed macrophages in the body are in the liver, as Kupffer cells. They ingest and degrade particulate matter and they can act as antigen-presenting cells. Kupffer cells are also a source of cytokines [1].

DCs are cells with many thin, long arms [1]. They are located throughout the body, but are particularly abundant in interfaces between the external and internal environments. This includes the skin and the lining of the nose, lungs and GIT, where they are ideally placed to encounter invading pathogens. DCs can migrate into the bloodstream and reach infected tissues. When they sense a tagged cell that is harmful, they are activated. They ingest and break down the various constituents of the cell. They contain proteasomes, which 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 an HLA, also called a major histocompatibility complex (MHC) protein. The tagged DCs then migrate to a lymph node where they encounter T-cells that have a matching receptor that binds the HLA-peptide complex. The DCs bind to $CD4^+$ T cells, and become APCs [1].

Classical DCs (cDCs) process antigens and present cells [1]. They have high phagocytic activity when they are immature cells and high cytokine-producing capacity when they mature [1]. They can move from

tissues to the T cell and B cell zones of lymphoid organs. cDCs regulate T cell responses both in the steady state and during infection. They are generally short-lived and are replaced by blood-borne precursors [1].

Plasmacytoid DCs (PDCs) differ from cDCs in that they live longer and some of them carry characteristic immunoglobulin rearrangements [1]. They are present in the bone marrow and all peripheral organs. PDCs respond to viral infection with a massive production of type I interferons (IFNs). However, they also can act as APCs to control T cell responses [1].

The second family of white blood cells is known as granulocytes or polymorphonuclear leukocytes [1]. The subclasses are neutrophils, eosinophils, basophils and mast cells. They have granules that contain enzymes that catalyze the hydrolysis of DNA, RNA, triacylglycerides, lipids, complex carbohydrates and proteins. The granules also have an enzyme called lactoferrin, which binds iron and keeps it away from Bacteria. Since iron is an essential nutrient, the Bacteria die. The granules also have enzymes that catalyze the breakdown of bacterial cell walls and the contents of the cells. They also produce ROS, such as superoxide (O_2^-) and hypochlorite (OCl^-) [1].

Mast cells are dispersed throughout most tissues but are crucially located at the host's interfaces with the environment, such as the skin and mucosa [1]. They help recognize pathogens and other signs of infection. They function not only as sentinels, but also as modulators of innate and adaptive immune responses, as they influence the outcomes of diseases. At the earliest stages of infection, mast cells are important for communicating the presence of a pathogen to many cell types located nearby in the site of infection and also further away in draining lymph nodes. To facilitate these interactions, mast cells are strategically located at the interfaces between the host and the environment. They are close to blood vessels and lymphatic vessels, as well as nerve fibers and tissue-resident immune cells, including DCs. Armed with granules that contain mediators, mast cells are often the first responders (within seconds to minutes) following recognition of an invading pathogen. Activation of mast cells by pathogens can lead to degranulation and de novo synthesis of cytokines. Pre-packaged, insoluble mediators are rapidly released into the surrounding tissue. This gives mast cell-derived products a temporal advantage over those produced by other immune surveillance cells. After being stimulated, mast cells shape the inflammatory network and control the activation state of many cells that are crucial for adaptive immunity [1].

Neutrophils are the most abundant phagocytes [1]. They circulate in the bloodstream and migrate to sites of infection when guided by cytokines, chemokines and other mediators. They arrive within an hour after infection and have half-lives of only 7-8 hours. They kill not only invading microorganisms, but also the body's own cells, so it is important that they don't live long. Eosinophils are only about 5% of the white bloods, but they are important because they have enzymes that catalyze the hydrolysis of proteins, DNA and RNA. Eosinophils can kill parasites because their granules contain the unique, toxic protein called cathepsin. Eosonophils are also professional APCs. They help regulate other immune cells, are involved in the destruction of tumor cells, and promote the repair of damaged tissues. The chemokine IL5 is produced by basophils. This enables them to interact with eosinophils, causing them to grow and differentiate. Eosinophils can regulate local immune and inflammatory responses. They accumulate in the blood and tissue of patients who have an inflammatory or infectious diseases [1].

Basophils and mast cells have similar properties [1]. They both have receptors for IgE and granules that contain histamine, leukotrienes B and C, prostaglandin D_2 (PGD_2) and various cytokines tht mediate inflammation. However, mast cells are found in most tissues and are more abundant than basophils. They are activated when their IgE-bound receptors are cross-linked by an antigen. The IgE is provided by a B-cell. Mast cells are important in asthma, rhinitis and several types of allergies [1].

The third family of white blood cells are the NK cells [1]. They are very cytotoxic, so they must be carefully regulated in a healthy immune system. They are crucial components of the innate immune system, but they also infuence adaptive immune responses. NK cells interact with many different types of cells to influence their fate. The outer portions of the cell membranes of the cells being targeted contain different types of receptors. Some activate and others deactivate NK cells. Cytokines such as TNF-α, viral double-stranded RNA and IFNs can activate them. The granules in NK cells release perforin and proteolytic enzymes, which form pores in the membranes of invading cells. This kills them by catalyzing the hydrolysis of their proteins. NK cells also remove the body's own cells that become malignant or infected with viruses, or any other cells that are tagged with IgG [1].

Lymphocytic T-cells comprise the fourth family of white blood cells [1]. They originate in the thymus and protect us from disease throughout our lives. Several hundred billion of them reside in lymphoid tissues and circulate in the bloodstream. T cell receptors (TCRs) bind antigens that are

presented by MHCs that are expressed by infected cells. About 100 million are made each day. Each population contains millions of different clones, with their own type of TCR. They recognize and bind to HLA-peptide complexes on APCs. T-cells that are bound to non-self HLA-peptide complexes mature and proliferate. When the TCR binds to an antigen, a cascade of molecular signaling occurs and the cytoskeleton is reorganized. The actin cytoskeleton organizes and maintains signaling complexes. There is an exponential multiplication of those highly competent T-cells and an elimination of incompetent T-cells. A negative selection occurs in the thymus for T-cells that bind strongly to HLA proteins that have a self peptide attached. Also, T-cells are distinguished by two different cell surface proteins, CD4 and CD8. The T-cells that have CD4 receptors bind class II HLAs and those having CD8 bind class I HLAs. The $CD8^+$ T-cells are cytotoxic and the $CD4^+$ cells are T_H cells. They either activate other immune cells or release cytokines. DCs bind to $CD4^+$ T-cells, activating them. Antigen-specific $CD4^+$ cells then interact with antigen-specific B cells, which proliferate and eventually differentiate into long-lived cells that secrete antibodies [1].

Lymphocytic B-cells make up the fifth family of white blood cells [1]. They originate in the bone marrow. They recognize foreign matter, microorganisms and viruses and produce antibodies that attach to foreign antigens. Then, they cooperate with other immune cells to eliminate the toxic threats. These immunoglobulins have two heavy chain proteins that are linked non-covalently to two light chain polypeptides. Adults have about 10^{12} B-cells and about 10^9 clones. Each clone produces its own antibody, which can be in the IgG, IgM, IgA, IgD or IgE class. The B-cell clones that produce Igs which recognize non-self antigens multiply. An important difference between B and T cells is that B cells recognize antigens in their native form, while T cells recognize antigens that have been processed into peptide fragments that are complexed with MHC on the surface of APCs. T cells recognize this complex by using their cognate TCRs. On the other hand, antigens activate B cell receptor signaling. This results in the internalization, processing, and presentation of antigen to T lymphocytes in the context of cell surface proteins of the MHC class II family. Recognition of such processed antigen by the T cell receptor induces the formation of a stable association, or synapse, between the two cell types, resulting in the transmission of signals required for regulating the B cell response [1, 36].

To make so many different types of immunoglobulins, B cells mix and match the variable, diversity and joining segments (V, D and J) that are coded for by different gene segments [1, 37]. The genes that code for the

heavy chain undergo V(D)J recombination. The genes coding for the light chain undergo VJ recombination. The V(D)J exon also codes for the antigenic determinant, or CDR. This is the part of the antibody that enables it to bind very specifically to an antigen. The recombination starts with a recombination activating gene, called RAG1/2. It codes for an endonuclease, which catalyzes the hydrolysis (or cleavage) of V, D and J segments. V(D)J recombination first occurs in progenitor B cells, called pro-B cells. They assemble the IgH (heavy chain) V, D and J exons and produce μ [1, 37]. Next, the IgL (light chain) V and J exons are assembled in precursor B cells, to produce immature B cells. They express μ and IgL chains as IgM immunoglobulins on the cell surface [1, 36]. These IgM^+ B cells migrate to peripheral lymphoid tissues, such as the spleen and lymph nodes. There they participate in antigen-dependent responses, including 36 class switch recombinations that change the function of the antibody and generate different classes of antibodies [1, 36].

When a B-cell encounters an APC, it ingests the antigen [1]. The antigen is displayed together with an HLA II protein which binds to T_H $CD4^+$ cells that secrete cytokines, which stimulate B-cell proliferation. Some B-cells have relatively large nuclei and make many copies of their antibodies. Other B-cells are long-lived memory cells. Another subtype of B-cells produces lower affinity IgM antibodies. They form pentamers and bind to the surfaces of Bacteria. This produces aggregates that are attacked and destroyed by macrophages. Most B-cells are distributed in tissues throughout the body, including the lymph nodes, as are T-cells. A smaller fraction of B-cells circulates through the blood vessels. Activated B-cells are cleared in the liver and spleen, so they only survive a few days. Naïve B-cells, which have not been exposed to antigens and memory B-cells are protected and live much longer [1].

When a person with a healthy immune system is infected by a virus, Bacteria or pathogenic organism, some of the B cells produces antibodies that may persist for months or years, depending on how long protection against the antigen-producing pathogen is required [1, 38]. Next, pathogen-specific B cells persist in a resting state. They circulate in the body and can be reactivated to produce more antibodies when a person is infected again. However, these two types of B cells produce different antibodies. The class or isotype usually changes. It is no longer the IgM class that was produced initially. The affinity of the antibody for the pathogenic antigen is much stronger than in the initial immune response. The immune memory includes not just the antibody that is made, but also involves the B and T cells that combine rapidly to make more antibodies.

This repeats and magnifies the previously successful immune responses [1, 38].

Naïve T and B cells that express IgM and IgD are activated by an antigen in the primary antibody response, either directly or after being processed by a dendritic cell [1, 38]. Depending on how they are primed, activated T cells become one of several types of T_H cells, such as T_H1, T_H2 or T_H17. They each produce a distinct repertoire of cytokines. T_H 1 cells secrete IFN-γ, T_H2 cells secrete IL-4, and T_H17 cells secrete TGF-β. In addition, activation of T cells leads to T memory (T_M) cells being produced. Signals from T cells induce B cell proliferation and class switching. Activated B and T_H cells can also establish the places where the affinity of the antibody for the antigen will increase. T_H cells within germinal centers are different than early subsets of T_H cells. They secrete IL-21 in addition to other cytokines. Long-lived plasma cells and memory B cells are produced in the germinal centers. They express isotypes of immunoglobulins that reflect the type of T_H that was used in the initial priming. Different classes of antibodies appear in the memory compartments. They are specialized in clearing specific types of pathogens. This is based on the initial interactions between dendritic and T cells [1, 38].

The four major classes of immune system mediators are chemotactic agents, cytokines, C-reactive protein and antibodies [1]. The group of chemotactic agents called chemokines attract immune cells to the site of infection or inflammation. Chemokines are relatively small proteins (molecular weights of 8000 – 10 000) that are secreted by several types of immune cells and attract other cells. They bind to receptors on target cells, which stimulates their movement towards areas of increasing chemokine concentration. They attract monocytes, macrophages and dendritic cells of the innate immune system and the T- and B-cells of the adaptive immune system. Chemokines also include products of the complement system, which are fragments from the degradation of microorganisms and viruses, and other small molecules [1].

There are about 50 chemokines and 20 receptors for them [1, 39]. Chemokines have selective chemoattractive properties. They coordinate the homeostatic circulation of leukocytes as well as their movement to sites of inflammation or injury. There are four families of cytokines. They are based on the pattern of their first two cysteine (CySH) residues. The first two CySHs are adjacent to each other in the CC family. Many of them have older names, such as MCP (monocyte chemotactic protein), MIP (macrophage inflammatory protein), and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted). They are all ligands

that bind to receptors, so the new names are CCL1 to CCL28. Some other chemokines have one amino acid between the CySHs (CXC) and some don't have two CySHs in their binding site (SC). All of them bind to GPCRs (G-protein coupled receptors) [1, 39].

Upon binding to a GPCR, two of the subunits, $G\alpha_1$ and $G\beta\gamma$, dissociate [1, 40]. This leads to an influx of Ca^{2+} and activation of the PI3K (phosphatidyl inositol 3-kinase) and small Rho GTPase signaling pathways. When chemokines are released by endothelial cells, they tend to remain concentrated and stay at the original site of infection. The concentrations of cytokines (such as IL-6) can vary by about 1000-fold during infection or trauma, from about 10^{-12} M (pM) to 10^{-9} M (nM). When chemokines bind to their cognate receptors, they induce signaling cascades that cause a rapid increase in integrin binding, which causes the leukocytes to adhere firmly to the endothelium. Once they are inside tissues, chemokines direct the migration of leukocytes. Neutrophils and monocytes are also attracted to sites of inflammation by CXC and CC chemokines. When chemokines and their GPCRs are not properly regulated, autoimmune diseases, chronic inflammation and associated diseases such as cancer can emerge. When properly controlled, chemokines can attract immune cells to lymphoid tissues and sites of infection and help activate neutrophils and monocytes. Adaptive immunity starts with the interaction between naïve antigen-specific T cells and dendritic cells that are loaded with cognate antigens. The interaction is due to two CC chemokines (CCL15 and CCL21) binding to their receptor (CCR7). This process is induced by pathogens [1, 40].

Most pathogenic organisms enter through the mucosal surfaces [1, 40]. Mucosal associated lymphoid tissues (MALTs) are the largest immune compartment in the body. They protect mucosal membranes from the adverse effects of infections. They must tolerate antigens derived from food and airborne matter and still be able to initiate immune responses against harmful antigens that might break through the mucosal lining. When a vaccine is administered to a mucosal site, it is taken up by M cells, dendritic cells or epithelial cells and it is passed on to APCs, which migrate to specialized tissues, such as Peyer's patches, lymph nodes and tonsils. They attract immune cells, which migrate to the site of the mucosal infection [1, 40].

Many chemotactic agents are the products of the complement system, which is the large family of proteins that is made in the liver and some immune cells [1]. They include proteases that catalyze the hydrolytic breakdown of foreign proteins. Chemotactic agents provide reactive fragments that can bind to foreign and abnormal cells, which tags them for

destruction. They produce small peptide fragments that attract immune cells to sites of infection or inflammation [1].

Fragments produced by the degradation of microbes, viruses, blood clots or fibers are also chemotactic agents [1, 41]. For example, Bacteria contain CpG dinucleotides that stimulate both the innate and adaptive (acquired) immune system. They promote immune responses that are assisted by T_H1 cells. Lipopolysaccharides (LPS) are produced by Bacteria. They bind to TLR4. LPS can cause sepsis and cholestasis. TLR4 also responds to LPS. Other fragments include lipoproteins and glycolipids, which are recognized by TLR2. Many Bacteria contain flagella, which can be broken into the protein called flagellin, which is recognized by TLR5 [1, 41].

Another class of type of chemotactic agents is made up of other small molecules such as leukotrienes B_4 and C_4 [1]. They are derivatives of arachidonic acid, and can cause inflammation [1]. RONS that are produced by reactions catalyzed by NADPH oxidase are also small molecule chemotactic agents that act on neutrophils [42].

The second class of immune system mediators is cytokines [1]. They are protein growth factors that are secreted by immune cells. They regulate and coordinate the immune response, while playing a role in cell proliferation and inflammation. Subtypes include interferons, monokines (made by monocytes), lymphokines (made by lymphocytes), chemokines and interleukins (made by leukocytes and affect other leukocytes). Examples include TNF-α, which activates macrophages and T-cells; IL-1, which activates T-cells; IL-2, which activates T, B and natural killer cells; TGF-β, which controls T_{reg} development and immune homeostasis; and interferons, which activate antiviral defenses [1].

The IL-1 family of cytokines has 11 members (IL-1F1 to IL-1F11) that are encoded by 11 distinct genes in humans and mice. IL-1–type cytokines are major mediators of innate immune reactions [1, 43]. They control proinflammatory reactions in response to tissue injury by PAMPs that are released from damaged cells (such as uric acid crystals or ATP). So, they are crucial mediators of innate immune reactions, and their actions are tightly controlled [1, 43].

Macrophages and monocytes are sentinel cells of the innate immune system [1, 43]. They produce IL-1α and IL-1β, like many other cell types, such as epithelial and endothelial cells, as well as fibroblasts. IL-1α is anchored to the cell membrane and signals through autocrine or juxtracrine mechanisms, while IL-1β is secreted by an unconventional protein secretion pathway. It can act in a paracrine manner or systemically. IL-1α and IL-1β rapidly induce the expression of hundreds of genes in many

different types of cells, including monocytes, macrophages, chondrocytes and fibroblasts, as well as epithelial and endothelial cells. At the same time, IL-1α and IL-1β induce expression of their own genes in a positive-feedback loop that amplifies the IL-1 response in an autocrine or paracrine manner. Transcription is stimulated within 30 min of exposure to IL-1α or IL-1β and can be sustained for many hours [1, 43].

Type-I IFNs stop many types of cancers, viral infections and autoimmune diseases [1]. IFNs are glycoproteins that are released in response to viruses, Bacteria, parasites and tumor cells. They activate NK cells and macrophages. They up-regulate antigen presentation to T-cells, thus increasing the ability of uninfected cells to resist viral infection. Also, some IFNs are used to treat cancer. They are given in combination with chemotherapy and radiation [1].

The third type of immune system mediator is the C-reactive protein (CRP) [1]. The concentration of CRP increases about 100-fold after an infection. It binds to the C3b protein fragment of complement, which stimulates monocytes and macrophages. Elevated CRP is also an important indicator of risk for a heart attack. The concentration of CRP can be kept low by the Mediterranean diet [1].

More recently, another class of immune network mediators was identified - neurotransmitters, such as L-DOPA, catecholamines and serotonin. For example, naïve T cells express a serotonin receptor, 5-HT_7 [1, 44]. Its expression is up-regulated along with 5-HT_{1B} and 5-HT_{2A} receptors when the T cells are activated. In addition, serotonin is transmitted between dendritic cells and T cells [1, 45]. Thus, serotonin may play an important role in activating the immune system [1, 45]. This should be remembered when giving or taking SSRIs (selective serotonin reuptake inhibitors) to treat depression, especially if allergies should develop [1].

Immune responses have also been classified as humoral or cell-based [1]. The humoral immune response uses B cells, while T cells are involved in cell-mediated immune responses. The humoral immune response is activated by external pathogens and is mediated by antibodies that are secreted by B-cells. Cell-mediated immunity does not involve antibodies but instead involves the activation of macrophages, NK cells, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Cellular immunity eliminates infected cells while making T_{H1} and T_{H2} cells. Both B cells and T cells carry receptors that recognize specific targets. T cells recognize targets that are non-self (pathogens) only after antigens (small fragments of a virus or pathogenic

organism) have been processed and presented together with a self receptor–MHC protein complex [1].

There are two major subtypes of T cells: the killer cells and the T_H cells [1]. Killer T cells only recognize antigens that are coupled to Class I MHC molecules, while T_H cells only recognize antigens coupled to Class II MHC molecules. In contrast, the antigen-specific receptor on a B recognizes whole pathogens without any requirement for antigen processing. Each lineage of B cell expresses a different antibody, so the complete set of B cell antigen receptors represents all the antibodies that the body can produce [1].

Killer T cells kill cells that are infected with viruses, Bacteria, fungi, helminths or are otherwise damaged or dysfunctional [1]. Like B cells, each type of T cell recognizes a different antigen. Killer T cells are activated when their TCR binds to their specific antigens in a complex with the MHC Class I receptor. Recognition of this MHC-antigen complex is assisted by a co-receptor on the T cell, CD8. The T cell subsequently travels throughout the body in search of cells where the MHC I receptors display the antigen. When an activated T cell contacts such cells, it releases cytotoxins like perforin, which forms pores in the target cell's plasma membrane, allowing ions, water and toxins to enter. When another toxin called granulysin (a protease) enters, it induces the target cell to undergo apoptosis. It’s especially important that T cells kill host cells that contain viruses, to keep them from replicating. T cell activation is tightly controlled and usually requires a very strong activation signal. T cells regulate both the innate and adaptive immune responses and help determine which types of immune responses the body will need to destroy a specific pathogen. T_H cells have no cytotoxic activity. They don’t kill infected cells or clear pathogens directly. Instead, they control the immune response by directing other cells to perform these tasks [1].

B cells identify pathogenic organisms when antibodies on their surfaces bind to specific foreign antigens [1]. The antigen-antibody complex is taken up by B cells and is processed by proteolysis to make smaller peptides. B cells display antigenic peptides on their surface, together with MHC class II proteins. This combination of MHC and antigen attracts a matching $CD4^+$ T_H cell, which releases lymphokines and activates the B cell. The activated B cell then divides. Its offspring secrete millions of copies of the antibody that recognizes this antigen. These antibodies circulate in the bloodstream and lymph nodes. They bind to pathogenic organisms that express the antigen and mark them for destruction. Antibodies can bind to bacterial toxins or interfere with the receptors that viruses and Bacteria need to bind to when they infect cells.

When B cells and T cells are activated and undergo replication, some of their offspring become long-lived memory cells. Throughout a person's lifetime, these memory cells can remember each specific pathogen that it encountered and will be able to mount a strong response if the pathogen is detected again. This process is called adaptive because it occurs throughout one's lifetime as an adaptation to infection and it prepares the immune system for future challenges. So, immunological memory can be either a passive short-term memory or an active long-term memory [1].

Passive immunity only lasts somewhere between a few days and several months [1]. However, newborn infants have little exposure to foreign microorganisms and are particularly vulnerable to infection. Fortunately, their mothers provide several layers of passive protection. During pregnancy, IgG is transported from mothers to their babies through the placenta, so they will have high levels of antibodies even at birth, with the same range of antigen specificities as their mother. Also, breast milk contains antibodies and healthy Bacteria that are transferred to the gut of the infant. These maternal antibodies and Bacteria protect babies from pathogenic bacterial infections until the they can synthesize their own antibodies [1].

On the other hand, long-term active memory is acquired when B and T cells are activated [1]. Active immunity can also be induced artificially, through vaccination. The principle behind vaccination is to introduce antigens from one or more pathogenic viruses or Bacteria that will stimulate the immune system. That way, the patient will develop a specific immunity without catching the disease that is associated with them. Infectious diseases are major causes of preventable death, so vaccination is extremely important for public health. Most viral vaccines use live attenuated viruses. On the other hand, many bacterial vaccines are based on pieces of the Bacteria, including harmless components. However, most bacterial vaccines have additional adjuvants that activate the APCs of the innate immune system and maximize immunity [1].

The immune system also identifies and eliminates many cancer cells [1]. That is, people often have a few cancer cells throughout much of their lives. While they remain healthy and free from malignant tumors, their immune systems eliminate the relatively few cancer cells that they encounter. They can do this because the transformed cancer cells express cell-surface antigens that are not present on normal cells. These antigens are foreign to the immune system, so their presence causes immune cells to attack the transformed cells. If the immune system is not working as well as it should, some of the tumor cells can evade the immune system and become malignant cells that have fewer MHC class I antigens on their

surface, thus avoiding detection by NK cells. Some tumor cells also release proteins such like the cytokine TGF-β. They suppress the activities of macrophages and lymphocytes. Also, the immune system can develop a tolerance for tumor antigens, so it can no longer attacks the tumor cells. Moreover, some macrophages can promote tumor growth by generating cytokines and growth factors that help tumors develop further. Also, a combination of hypoxia in the tumor and a cytokine produced by macrophages can induce tumor cells to decrease the production of proteins that block metastasis thus spreading the cancer cells. So, maintaining a healthy immune system is important for preventing cancer morbidity and mortality [1].

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Abbreviations and Acronyms Used

Note: Some abbreviations stand for very different things, so both definitions are given below

2D-PAGE: 2D Polyacrylamide gel electrophoresis
5-HT: 5-hydroxytryptamine
25D: 25-hydroxyvitamin D3
AA: Arachidonic acid
ACE: Angiotensin converting enzyme
ACEIs: ACE inhibitors
Ach: Acetyl choline
ACTH: Adrenocorticotropic hormone
AD: Alzheimer's disease
ADHD: Hyperactivity attention deficit disorder
ADMA: Asymmetric dimethylarginine
AGEs: Advanced glycation end products
AhR: Aryl hydrocarbon receptor
AKI: Acute kidney injury
ALA: Alpha linolenic acid
ALS: Amyotrophic lateral sclerosis
AMD: Age-related macular degeneration
AMDR: Acceptable Minimum Distribution Range
AMPK: AMP-activated protein kinase
ANF: Atrial natriuretic factor
ANG-1: Angiopoietin-1
ANS: Autonomic nervous system
AngII: Angiotensin II
APC: Antigen presenting cell
ARE: Antioxidant response element
ARNT: Aryl hydrocarbon receptor nuclear transporter
As: Arsenic
ASG: Acylated steryl glucosides
AT: Adipose tissue
B: Boron
BALF: Bronchoalveoloar lavage fluid
BAT: Brown adipose tissue
BCFA: Branched chain fatty acyls

BDNF: Brain-derived neurotrophic factor
Be: Beryllium
BEST: Biochemical Efficacy and Safety Trial
BF_3: Boron trifluoride
BMI: Body mass index
BMP: Bone morphogenic protein
BNP: Brain natriuretic peptide
Br: Bromine
BrSM: Bioregulatory systems medicine
βTrCP: β-transducin repeat containing protein
BSSL: Bile salt stimulating lipase
^{o}C: Degrees Centigrade
C1INH: C1 esterase inhibitor
CAMK: Ca^{2+}/calmodulin-dependent kinase
cAMP: cyclic AMP
CancerLinQ: Cancer Learning Intelligence Network for Quality
CAPE: Caffeic acid phenethyl ester
CBER: Center for Biologics Evaluation and Research (part of the FDA)
CCD: Charge coupled detector
CCK: Cholecystokinin
CCP: Critical control points
Cd: Cadmium
CD: Cluster of differentiation
cDC: classical Dendritic Cell
CDC: Centers for Disease Control, 15
$CDCl_3$: Deuterated chloroform
CDER: Center of Drug Evaluation and Research (part of the FDA)
CDR: Complementarity determining region
CDRH: Center for Devices and Radiological Health
CE: Capillary electrophoresis
CE-MS: Capillary electrophoresis coupled to mass spectrometry
CFR: Code of the Federal Register
CGAP: Cancer Genome Anatomy Project
CGCI: Cancer Genome Characterization Initiative
CGEMS: Cancer Genetic Markers of Susceptibility
CGIAR: Consulting Group on International Agricultural Research
cGMP: current Good Manufacturing Practices
CI: Chemical ionization
CKD: Chronic kidney disease
CMH: Ceramide monohexoside
CML: Chronic myelogenous leukemia

CML: *N*-(carboxymethyl)lysine
cNPs: Cardiac natriuretic peptides
CNP: C-type natriuretic peptide
CNS: Central nervous system
Co: Cobalt
CO: Carbon monoxide
CO_2: Carbon dioxide
COX: Cyclooxygenase
CoQ10: Coenzyme Q10
CPI: Critical Path Initiative
CRF: Corticotropin-releasing factor
CRH: Corticotropin-releasing hormone
Cr: Chromium
Cr^{6+}: Chromium in the +6 oxidation state, hexavalent chrome
CRF: Corticotropin-releasing factor
CRP: C-reactive protein
CSF: Cerebral spinal fluid
CTL: Cytotoxic T-lymphocyte
CTRPs: C1q/TNF-related proteins
Cul3: Cullin-3 based ligase
CVD: Cardiovascular disease
CYP: Cytochrome P450
DAMP: Danger-associated molecular patterns
DC: Dendritic cell
DC: Direct current
DDT: 1,1'-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene).
DEHP: di-2-ethylhexylphthalate
DES: Diethylstilbestrol
DGDG: Digalactosyldiacylglycerol
DHA: Docosahexaenoic acid
DJ-1: A protein deglycase
DMF: Digital microfluidics
DNA: Deoxyribonucelic acid
DRI: Dietary Reference Intakes
ECM: Extracellular matrix
EEG: Electroencephalography
EGCG: Epigallocatechin-3-gallate
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ELCD: Electrolytic conductivity detector
Elfn1: Extracellular leucine-rich repeat fibronectin containing 1

ENHANCE: Enduring Happiness and Continued Self-Enhancement
eNOS: endothelial Nitric Oxide Synthase
ENS: Enteric nervous system
EOCs: Early outgrowth cells
EPA: Environmental Protection Agency and eicosapentaenoic aid (an omega-3 fatty acid)
EPAACL European Partnership for Action Against Cancer
EPAC: Exchange protein activated by cAMP
EPO: Erythropoietin
EPR: Enhanced permeability and retention
EPS: Expressed prostate secretions
ER: Endoplasmic Reticulum
ERK: Extracellular signal-related kinase
ESPEN: European Society for Clinical Nutrition and Metabolism
ESRD: End-stage renal disease
ETC: Electron transport chain
ETH: Eidgenössiche Technische Hochschule
ETS: Erythroblast transformation-specific family of transcription factors
EU: European Union
F^-: Fluoride
FAB: Fast atom bombardment
FDA: Food and Drug Administration
FD&C Act: Food, Drug and Cosmetic Act
FEP: Free energy perturbation
FHS: Framingham Heart Study
FKBP: FK506-binding protein
fMRI: functional MRI
FPP: Farnesyl pyrophosphate
FSH: Follicle stimulating hormone
GABA: Gamma-amino butyric acid
γGCS: gamma-Glutamylcysteine Synthetase
GAL: Green Aspiration Level
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GalCers: Galactosylceramides
GBS: Group B *Streptococcus*
GelMA: Gelatin methacryloyl
GETS: Genomics Evaluations Team for Safety
GHRH: Growth hormone-releasing hormone
GHz: GigaHerz (million cycles per second)
GIT: Gastrointestinal tract
GLP: Good Laboratory Practices

GLP-1: Glucagon-like peptide-1
GMO: Genetically modified organism
GnRH: Gonadotropin-releasing hormone
GP: Glutathione peroxidase
GPC: Glycerophosphocholine
GPCR: G-protein-coupled receptor
GR: Glutathione reductase
Grx: A small redox enzyme that uses glutathione as a cofactor
GSK: GlaxoSmithKline
GSK3: Glycogen synthase kinase-3
GSSG: Glutathione disulfide
GST: Glutathione S-transferases
GTPase: Guanosine triphosphatases
H: Histidine
H_2: Hydrogen gas
H_2CrO_4: Chromic acid
HbA_{1c}: Glycated hemoglobin
HCS: High content screening
HCV: Hepatitis C virus
HDACs: Histone deacetylases
HDL: High-density lipoproteins
HER2: Human epidermal growth factor receptor 2
Hg: Mercury
HIF1α: Hypoxia-inducible factor 1-α
hiPSCs: Human induced pluripotent stem cells
HIV: Human immunodeficiency virus
HIVE: High performance integrated virtual environment
HLA: Human leukocyte antigen
HPA axis: Hypothalamic-pituitary-adrenal axis
HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A
HMO: Human milk oligosaccharides
HMO: Health Maintenance Organization
HMP: Human Microbiome Project
HO-1: Hemoxygenase 1
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
hsCRP: High-sensitivity C-reactive protein
HTS: High throughput screening

I: Isoleucine
I_2: Iodine
ICG: Initiative for Chemical Genetics
ICP: Inductively coupled plasma spectroscopy
IDH: Isocitrate dehydrogenase
IDP: Intrinsically disordered proteins
IF: Interferon
IFM: interfibrillar mitochondria
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
iNOS: inducible Nitric Oxide Synthase
INSERM: French National Institute of Health and Medical Research
IOM: Institute of Medicine
IP_3: Inositol triphosphate
IPA: International Pharmaceutical Abstracts
iPOP: Integrated Personal -omics profiling
iPSCs: induced Pluripotent Stem Cells
IR: Infrared
IRRI: International Rice Research Institute
IVT: *In vitro* transcribed
JAK-STAT: Janus kinase-signal transducer and activator of transcription
JNK: Jun N-terminal kinase
^{o}K: Degrees Kelvin
Keap1: Kelch-like enoyl-CoA hydratase-associated protein 1
KRAS: Kirsten rat sarcoma oncogene
LabPMM: Laboratory for Personalized Molecular Medicine
LABS: Linear alkyl benzene sulfonates
LAS: Linear alkyl sulfonates
LC-HRMS: Liquid chromatography coupled to high resolution mass spectrometry
LC-MS: Liquid chromatography coupled to mass spectrometry
LDL: Low density lipoprotein
LED: Light emitting diode light emitting diode
LH: Luteinizing hormone

LIE: Linear interaction energy method
LMW: Low molecular weight
LOX: Lipoxygenase
LPS: Lipopolysaccharide
LT: Leukotriene
LysoPCs: Lysophosphatidyl cholines
M: Methionine
M2 macrophages: alternatively activated Macrophages
mAb: monoclonal Antibody
MAG: Monoacylglycerides
MALDI-TOF/MS: Matrix assisted laser desorption and ionization with time of flight mass spectrometry
MALT: Maternal mucosa-associated lymphoid tissue
MALTs: Mucosal associated lymphoid tissues
MAO: Monoamine oxidase
MAPK: Mitogen-activated protein kinase
MCAT: Medical College Aptitude Test
MCL: Mast cell leukemia
MCP: Monocyte chemotactic protein
MCP-1: Monocyte chemoattractant protein-1
MCSF: Macrophage colony stimulating factor
MD: Molecular dynamics
MDSC: Myeloid-derived suppressor cells
MEG: Magneto-encephelography
MEMs: Microelectronic mechanical systems
Mg: Magnesium
MGC: Mammalian Gene Collection
MGDF: Megakaryocyte growth and development factor
MGDG: Monogalactosyldiacylglycerol
mGluR: metabotropic Glutamate Receptor
MHz: MegaHerz
MI: Myocardial infarction
MIE: Molecular initiating event
MIP: Macrophage inflammatory protein
MM/PBSA: Molecular mechanics/Poisson-Boltzmann surface area
MMPs: Matrix metalloproteinases
MMR: Measles, mumps, plus rubella vaccine
Mn: Manganese
MPTP: Mitochondrial permeability transport pore
MRI: Magnetic resonance imaging
MRM: Multiple reaction monitoring

mRNA messenger RNA
MRP3: Multidrug resistant protein-3
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
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)
Na^+: Sodium cation
NAA: *N*-acetyl aspartate
NAC: *N*-acetylcysteine
Nbs: Nanobodies
NCI: National Cancer Institute
NCTR: National Center for Toxicological Research
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS: National Health Service (British)
Ni: Nickel
NIH: National Institute of Health (USA)
NIEHS: National Institute of Environmental Health Sciences
NIMS: Network target-based identification of multicomponent synergy
NKA: Neurokinin A
NKB: Neurokinin B
NK cell: Natural killer cell
NLM: National Library of Medicine
NLR: NOD-like receptor
NLRP3: NOD-like receptor family pyrin domain containing 3
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 6.02×10^{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
NO: Nitric oxide
NOC: Number of clusters
NOD receptors: nucleotide-binding oligomerization domain receptors
NOE: Nuclear Overhauser Enhancement
NOESY: Nuclear Overhauser Enhancement Spectroscopy
NotchIC: Notch intracellular
NPR-A: Natriuretic peptide clearance receptor
NPY: Neuropeptide Y
NQO1: NADPH:quinone oxidoreductase
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
O_2: Oxygen gas
$O_2^{\cdot-}$: Superoxide anion
OCG: Office of Cancer Genomics
OCl^-: Hypochlorite
•OH: Hydroxyl radical
OIR: Office of In Vitro Diagnostics and Radiological Health
O-LM: Oriens–lacunosum moleculare interneurons
ONC: Office of the National Coordinator for Health Information Technology
$ONOO^-$: Peroxynitrite anion
ORACL: Optimal reporter cell lines for annotating compound libraries
ORF: Open reading frame
OS: Oscillatory disturbed shear
OTC: Over-the-counter
OVRR: Office of Vaccines Research and Review
OXT: Oxytocin
P4 medicine: Predictive, preventive, personalized and participatory medicine
PACAP: Pituitary adenylate cyclase-activating peptide
PAH: Polyaromatic hydrocarbon
PAMP: Pathogen-associated molecular patterns (bacterial)
Pb: Lead
PBT: Proton beam therapy
PC: Phosphatidylcholine

PCBs: Polychlorinated biphenyls
PCR: Polymerase chain reaction
PD: Parkinson's disease
PDE3B: Phosphodiesterase 3B
PDE-4 and PDE-5: Phosphodiesterase types 4- and 5
PERM: Proteasome, Endoplasmic Reticulum and Mitochondria
PET: Positron emission tomography
PGC-1alpha, PGC-1α, Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
PGD_2: Prostaglandin D_2
PFPD: Pulsed flame photometric detectors
PGE2: Prostaglandin E_2
pH: Negative log of H^+ concentration
PH: Plekstrin homology domain
PIC/S: Pharmaceutical Inspection Co-operation Scheme
PID: Photoionization detectors
PI3K: Phosphatidyl inositol 3-kinase
PKC: Protein kinase C
PNS: Peripheral nervous system
POP: Persistent organic pollutant
PPAR: Peroxisome proliferator-activated receptor
ppb: parts per billion
ppm: parts per million
PPY: A neuropeptide that contains tyrosine, or Y
PRR: Pattern recognition receptor
PSA: Prostate specific antigen
PTH: Parathyroid hormone
PTK: Protein tyrosine kinases
PTM: Post-translational modifications
PTSD: Posttraumatic stress disorder
PUFA: Polyunsaturated fatty acids
PURE: Prospective Urban Rural Epidemiology
PVC: Polyvinyl chloride
QC: Quality control
QM/MM: Quantum mechanics/molecular mechanics
QRM: Quality Risk Management
QSP: Quantitative systems pharmacology
Q-TOF: Quadrupole time of flight mass spectrometer
R: Gas constant
R: Relaxed state of hemoglobin
RA: Rheumatoid arthritis

RAC: Recombinant DNA Advisory Committee
RAGE: Receptor for AGE (advanced glycation end products)
RANKL: Receptor activator of NF-κB ligand
RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted
RAS: Renin-angiotensin system
RAS: An oncogene that codes for the oncoprotein ras
Rbx1: Ring box protein 1
RDA: Recommended Daily Allowance
rDNA: recombinant DNA
Redox: Reduction and oxidation
RefSeq: Reference sequences
RES: Reticuloendothelial system
rf: Radio frequency
ROC: Receiver operator characteristic curves
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
RPG: Relative Process Greenness
RXR: Retinoid X receptors
S1P: Sphingosine-1-phosphate receptor
SAR: Structure-activity relationship
saRNA: small-activating RNAs
Sb: Antimony
SBIF: Soy based infant formula
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
Si: Silicon
Siah1: Seven in absentia homologs
SIM: Single ion monitoring
SMA: Spinal muscular atrophy

Sn: Tin
SODA: Sequential operation droplet array
SOP: Standard Operating Procedure
SR: Scavenger receptor
SSM: subsarcolemmal mitochondria
SSRI: Selective serotonin reuptake inhibitor
STAT3: Signal transducer and activator of transcription activator 3
SU.VI.MAX: Supplémentation en Vitamines et Minéraux Antioxydants
SVD: Single value decomposition
T: Tense state of hemoglobin
T1D: Type-1 diabetes
T2D: Type-2 diabetes
T3DB: Toxin and Toxin-Target Database
T_3: Triiodothyronine, a thyroid hormone
T_4: Thyroxine, a thyroid hormone
TAT: Targeted alpha therapy
TBT: Tributyl tin
TCM: Traditional Chinese medicine
TCR: T-cell receptor
T_{EM}: Effector memory T cells
TF: Transcription factor
T_g: Thyroglobulin
TGF-β: Tumor growth factor-beta
TI: Thermodynamic integration
TIL: Tumor infiltrating lymphocytes
TJ: Tight junction
TLR: Toll-like receptor
T_M cells: T memory cells
TMA: Trimethylamine
TMAO: Trimethylamine-*N*-oxide
TMS: Tetramethylsilane
TNF-α: Tumor necrosis factor-α
TQL: Total quality leadership
TQM: Total quality management
TR: Thyroxine receptor
T_{reg}: Regulatory T-cells
TRNT: Targeted radionuclide therapy
Tr1: Type 1 regulatory T cell
T_{reg}: Regulatory T cells
TRH: Thyroid releasing hormone
TSP: 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt

UCP: Uncoupling protein
UPR: Unfolded protein response
USAID: US Agency for International Development
USDA: US Department of Agriculture
UV: Ultraviolet
UVA: Ultraviolet-A
UVB: Ultraviolet-B
UV-Vis: UV-visible
VAT: Visceral white adipose tissue
VCAM-1: Vascular cell adhesion molecule-1
VDR: Vitamin D receptor
VECs: Valvular endothelial cells
VEGF: Vascular endothelial growth factor
VEGF-A: Vascular endothelial growth factor-A
VIC: Valvular interstitial cell
VIP: Vasoactive intestinal peptide
VLDL: Very low density lipoprotein
V_{max}: Maximum velocity in an enzyme-catalyzed reaction
VO_2 max: Maximal oxygen consumption
VSMC: Vascular smooth muscle cell
VWAT: Visceral white adipose tissue
WAT: White adipose tissue
WES: Whole-exome sequencing
WHO: World Health Organization
XIC: Extracted ion chromatogram
XSD: Halogen specific detectors
XSIT: X-inactive specific transcript
Y: Tyrosine

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