Translational Toxicology and Therapeutics

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Windows of Developmental Susceptibility in Reproduction and Cancer

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Part One

Introduction: The Case for Concern about Mutation and Cancer Susceptibility during Critical Windows of Development and the Opportunity to Translate Toxicology into a Therapeutic Discipline

1

What Stressors Cause Cancer and When?

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1.1 Introduction

Translational biomedical research seeks to move laboratory findings based on models *(in silico*, *in vitro,* and *in vivo*) into human clinical trials to more expeditiously develop specific therapeutics, and then back again to the laboratory to inform future discovery [1]. From the background of developmental toxicology, it is well known that toxicant exposures may affect critical events in reproductive development, ranging from early primordial germ cell determination to gonadal differentiation, gametogenesis, external genitalia, or signaling events regulating sexual behavior. Translational genetic toxicology takes advantage of this developmental perspective to assess potential germ line mutagenesis or to study the potential for cancer in the fetus or offspring or the adult as the result of environmental exposures. Translational toxicology must strive to identify applicable therapeutics that can safely and effectively identify and help to mitigate potential harm from natural as well as anthropogenic environmental exposures.

Human exposures to chemicals, physical agents, and social factors are inevitable, thus the human fetus and the adult are subject to exposures and effects that can have lifelong consequences. Particularly, during dynamic developmental intervals described as "critical windows of susceptibility," exposures may have robust and durable effects that drive long-term health outcomes, including metabolism, functional status of organ systems, and cancer risks [2]. These same dynamic developmental intervals should be seen as "critical windows of responsivity" during which favorable/protective interventions should also be highly impactful offering potential durable reduction in

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risks of multiple adverse health outcomes, including cancers. To reduce the lifelong occurrence of preventable cancers, timely protective interventions during "critical windows" should include not only minimization of untoward voluntary exposures and substances of abuse but also active use of protective generally recognized as safe (GRAS) interventions/therapies, including nutritional, dietary supplementation, or well-established/repurposed and/or generally recognized as safe and effective (GRASE) pharmaceutical drugs.

This introductory chapter will promote the elucidation of cell stage, life stage, and lifestyle knowledge of specific cellular and molecular targets of known developmental toxicants, develop a systematic integrated approach to the identification of mutagenic and reproductive toxicants, and discuss sensitive, specific, and predictive animal models, to include minimally invasive surrogate markers, and/or *in vitro* tests to assess reproductive system function during embryonic, postnatal, and adult life. It will argue that integrated testing strategies will be required to account for the many mechanisms associated with development that occur *in vivo*. A key organizing principle used throughout this book is to consider how exposures that incur risk or other exposures/life events that may reduce risk during particular windows of susceptibility/developmental transitions, and thereby impact cancer occurrence.

In consideration of any cause–effect relationship, typically one thinks of the simple questions: Who, what, where, when, and how? Admittedly, "How?" questions are generally the most difficult because that understanding is a synthesis of potentially causal pathways. We aim to consider that the "Who?" and "When?" questions could be seen as people being exposed at different intervals across their respective life spans. Thus, in addition to information regarding what exposures occur that influence cancer occurrence, what is and is not known about exposures to those agents during life span intervals such as childhood, adolescence, across the broader life span, and/or late in life? Assessment of such timing of exposure with cancer outcomes seems to be a critical element if we aim to develop protective interventional strategies. In other words, whether we aim to reduce exposures or advocate protective lifestyle or therapeutic interventions, we must know when those interventions would most effectively impact later cancer outcomes.

Although there are differences between human development and that of laboratory animal models, developmental models have been extremely useful in assessing risks for key human reproductive and developmental processes. Some of these models will be discussed in Chapters 2 and 3. However, such systems have not been fully integrated with models to assess germ line mutagenesis or to study the potential for cancer in the fetus or offspring as the result of environmental exposures. Again, Chapters 2 and 3 will address current proposals for experimental animal test system integration.

To delve into the impact of exposures during "windows of susceptibility/responsivity," we must take into account the unique susceptibilities of the fetus.

Relatively, new information suggests that some widely held notions relevant to fetal exposures are incorrect [3]. Thus, we now know that amniotic fluid can be reabsorbed into the fetal circulation by fetal swallowing as well as via the fetal intramembranous pathway. The latter pathway is thought to be the most important mechanism for the resorption of toxicants, such as ethanol, into the fetal circulation [4]. Together with swallowing, this is a recycling system, through which toxic substances are excreted into the amniotic fluid and reabsorbed into the fetal circulation, thus extending the duration of each exposure [5,6]. This and other information relevant to fetal exposure *in utero* will be discussed in Chapter 8.

1.1.1 General Information about Cancer

Each year the American Cancer Society estimates the number of new cancer cases and deaths that will occur in the United States that year. In 2016, a total of 1,685,210 new cancer cases were expected to be diagnosed and about 595,690 cancer deaths were projected to occur in the United States [7]. Among children up to 14 years of age, an estimated 10,380 new cancer cases were expected to occur in 2016.

Population-based cancer registration began in the United States in 1975. Since then, childhood cancer incidence rates have increased by 0.6% per year. In 2016, 1250 cancer deaths were expected to occur among children. Cancer is the second leading cause of death in children ages 1–14 years, exceeded only by accidents. Childhood cancer death rates declined a total of 66% from 1969 (6.5 per 100,000) to 2012 (2.2 per 100,000). According to the American Society, this was largely due to improvements in treatment and high rates of participation in clinical trials. From 2003 to 2012, the rate of cancer-caused deaths in children declined by 1.3% per year.

Siegel *et al*. [8] reported that during the period 2006–2010, the then most recent 5 years for which there were data, the delay-adjusted cancer incidence rates declined by 0.6% per year in men and were stable in women. At the same time, cancer death rates decreased by 1.8% per year in men and by 1.4% per year in women. The rate of combined cancer deaths per 100,000 populations has declined continuously for two decades, from a peak of 215.1 in 1991 to 171.8 in 2010. The 20% decline during this time period equates to the avoidance of 1,340,400 cancer deaths (952,700 among men and 387,700 among women). Siegel *et al.* reported that the magnitude of the decline in cancer death rates varies substantially by age, race, and sex, with no decline among white women of 80 years of age and older to a 55% decline among black men 40–49 years of age. Remarkably, black men experienced the largest drop within every 10-year age group. The authors noted that progress could be accelerated by applying cancer control knowledge across all segments of the population [8].

While the severity of cancers is often measured in number of deaths, the number of years of life lost (YLL) may be a more appropriate indicator of impact

on society [9]. These authors calculated the YLL of adult cancers in Norway for 2012 and for the prior 15-year period. Their results showed that cancer deaths in Norway in 2012 represented 25.8% of all adult deaths (28.7% in men and 23.1% in women). Cancer deaths represented 35.2% of all YLL, with a 5.0% higher fraction in females than in males (32.8% in men and 37.8% in women) [9].

The etiology of cancer is generally thought to be the product of gene and environmental interactions. Environmental exposures are typically low and to mixtures of constituents that occur indoors and outdoors. Goodson *et al.* hypothesized that low-dose exposures to mixtures of chemicals in the environment may be combining to contribute to environmental carcinogenesis [10]. They reviewed 11 hallmark phenotypes of cancer, with multiple priority target sites for disruption in each area and prototypical chemical disruptors for all targets. Dose–response characterizations and evidence of low-dose effects and cross-hallmark effects for all targets and chemicals were considered. In total, 85 examples of chemicals were reviewed for their actions on key pathways and mechanisms related to carcinogenesis. Although 59% of the chemicals caused low-dose effects, only 15% (13/85) were found to show evidence of a dose–response threshold. No dose–response information was found for the remaining 26% (22/85). The authors speculated that the cumulative effects of individual noncarcinogenic chemicals acting on different pathways in related systems, organs, tissues, and cells could synergize to produce carcinogenic outcomes. They concluded that additional research on carcinogenesis focused on low-dose effects of chemical mixtures needs to be rigorously pursued before the merits of their hypothesis can be further tested [10].

In a published poster abstract, Parkin and Paul [11] estimated the percentage of cancer in the United Kingdom in 2010 resulting from exposure to 14 major life style, dietary, and environmental risk factors. Prevalence and relative risks of exposure to factors, including tobacco smoking, consumption of four different dietary components (fruit and vegetables, meat, fiber, salt) alcohol use, occupation, infections, radiation, hormone use, overweight, physical exercise, and reproductive factors were used to estimate the number of cancers occurring in 2010 attributable to suboptimal exposure levels in the past. These 14 exposures were responsible for 42% of cancer in the United Kingdom in 2010 (males 44%, females 40%). Tobacco smoking was the most important, accounting for about 60,000 new cancers (18.5% of all cancer; 22% in men, 15% in women), with less than 2% being the result of exposure to environmental tobacco smoke. The four dietary components account for 9.4% of cancer (10.7% in men, 7.1% in women). In men, alcohol use (5.1%) and occupational exposures (4.7%) are next in importance and in women, overweight and obesity are next (nearly 7% of cancers). The study is cited because estimates of this kind provide a quantitative assessment of the impact of various exposures. However, they are not synonymous with the fraction of cancers that might reasonably be prevented by modification of exposures. As discussed by the authors, “this requires scenario

modeling, with assumptions on a realistically achievable population distribution of risk factors, and the timescale of change." For example, although 50% of colorectal cancer can be attributed to lifestyle (diet, alcohol, inactivity, and overweight), only about 25% is preventable within a 20-year timescale [11].

Langley *et al.* [12] proposed a new research paradigm, adapted from twenty-first century toxicology that involves the following initiatives:

1) Develop a "big picture" of human disease that integrates extrinsic and intrinsic causes and links environmental sciences with medical research using systems biology.
2) Introduce a disease-centric adverse outcome pathway (AOP) concept, analogous to toxicity AOPs, with the intention of providing a unified framework for describing relevant pathophysiology pathways and networks across multiple biological levels.
3) Create a strong focus on advanced human-specific research (*in vitro*, *ex vivo*, *in vivo*, and *in silico*) in lieu of empirical, animal-based studies.

Langley *et al.* [12] have asserted that integrating data on extrinsic and intrinsic causes of disease using a systems biology (or systems toxicology) approach provides a more comprehensive understanding of human illnesses. Such an approach involves the perturbation of a biological system and the use of molecular expression data gathered through the use of omics technologies to understand the responses that occur at the systems level [13–15].

The AOP concept links exposure, involving chemical structures and molecular initiating events, via a sequence of key events, to an adverse outcome [16]. In a genomic sense, AOPs link external influences (*the exposome*), including drugs, chemicals in consumer products, food, or the environmental media, occupational exposures, infections, behavior, stress, smoking, ageing, nutrition, and radiation exposure to genetic effects (*the genome*), including susceptibility genes, up- and downregulation of genes, germ line and somatic mutations induced by drugs, chemicals and/or radiation, inherited single nucleotide polymorphisms, gene copy number changes, insertions, deletions, exome changes, and the accumulation of DNA damage, as well as epigenetic effects (*the epigenome*), including changes in the localized or global density of DNA methylation; posttranslational modifications of histones; changes in noncoding microRNAs; and changes in chromatin structure, which together alter the regulation of gene expression. Defects in the epigenome can cause disease and may be specific to tissue or cell types. Both genetic and epigenetic effects are then linked to adverse effects at cellular, organ, and individual levels.

According to Langley *et al.,* cellular/organ pathways may locate in immune function, apoptosis, calcium homeostasis, oxidative stress, growth factor signaling, nerve degeneration, and so on. Individual-level effects include embryonic development, disease, and death [12]. We certainly concur with this thinking and applaud the proposed new research paradigm, recognizing that

systems biology and systems toxicology must ultimately be understood at the network level as will be further discussed in Section 1.5.

1.1.2 Stressors and Adaptive Responses

A general reality in biology is that living systems are inevitably subject to external stressors, and a general observation is that these complex biological systems respond by adaptation – if those stresses do not exceed some definable threshold. Such adaptation includes subsequent strengthening of various endogenous responses as well as development of more diversified responses. A semantic point may be made that there is a gradation of meaning where stressors might be seen as positive stimuli on one end of the scale, but potentially harmful or lethal insults on the other end of the scale. Exposures in the early life impact cancer risk across the life span, with some increasing that risk but others reducing it.

1.2 What Stressors Cause Cancer and When?

We must view this question at the cell level, the life stage, and from a lifestyle perspective. We should also ask the question: Is there an adaptive response to modest stress? Regarding development of a cancer-specific translational toxicology therapeutic portfolio; we note that there are biological concepts regarding adaptive responses to modest stressors (adaptive stressors) in contrast to those stressors that exceed one or more bounds of tolerance within which an adaptive response might range.

Our goal is to provide a general overview on windows of susceptibility/responsivity, including maternal and fetal metabolic milieu, childhood cancers and therapies, and transitions into adulthood.

If there is a plausible public health basis to advocate for implementation of certain mitigative risk-reducing interventions, what are the essential ethical considerations to be made for protective "treatments" of the young for prevention of some remotely future disease (cancers) that the individual may or may not otherwise experience? In Chapter 19 we have delved into this and numerous other ethical issues facing the new field of translational toxicology.

In addressing chemical and metabolic exposures of concern, we agree that risks and benefits need to be considered. In this volume, we include natural and anthropogenic substances, both carcinogenic and anticarcinogenic, in the diet with commentary regarding both the good and the bad potential effects of natural chemicals and the evidence supporting each. We note, particularly, the childhood cancers and therapies for those cancers that need to be addressed. Regarding an important window of susceptibility or responsivity, the

peripubertal interval and the well-documented effect of early onset of menarche or breast cancer risk serve as good illustrations that will be discussed.

We note that any number of exposures could have an impact on the risk of cancer occurrence (as well as other diseases), and its indolent or aggressive behavior and progression over time. Chapters 5–8 are devoted to such exposures.

Environmental chemicals and drugs are a source of major concern in human exposure scenarios. We are exposed daily to low levels of literally thousands of industrial and household chemicals in our indoor and outdoor environments. For the most part, these represent involuntary exposures; however, we voluntarily expose ourselves to known human carcinogens in consuming alcoholic beverages and tobacco products. Not only do we expose ourselves, but also our children and even our grandchildren.

We briefly consider smoking relative to transgenerational cancer and other disorders. Thus, Dougan *et al.* [17] have studied grandmaternal smoking during pregnancy and its possible association with overweight status in adolescence. After adjusting for covariates, their findings suggest that the association between maternal smoking and offspring obesity may not persist beyond the first generation. However, grandpaternal smoking may affect the overweight status of the granddaughter, likely through the association between grandpaternal smoking and maternal smoking.

Pagani *et al.* [18] examined the reported behavioral habits of 2055 families by sifting through data from the Quebec Longitudinal Study of Child Development. The investigators looked particularly at levels of household tobacco smoke exposure when their child was between the ages of 1 and 7. They then attempted to ascertain any possible correlations between the level of smoking and measurements of the child's waist circumference and body mass index (BMI) at age 10. Higher amounts of both are known to predict a higher risk of gaining excess weight and developing metabolic disorders, such as diabetes, later on in adulthood.

"By the age of 10, those children who had been intermittently or continuously exposed to tobacco smoke were likely to have waists that were up to three-fifths of an inch wider than their peers. And their BMI scores were likely to be between 0.48 and 0.81 points higher," stated lead author Dr. Linda Pagani, of the University of Montreal, in a press release. "This prospective association is almost as large as the influence of smoking while pregnant. The researchers noted that only occasional smoking exposure was independently associated with excess weight, after controlling for factors like their parent's mental health or income, with a 43 percent greater chance of a child becoming obese or overweight in such a household [18]."

For certain other exposures, the case for transmission of cancer risk to future generations, via both genetic and epigenetic mechanisms, is much stronger. Thus, in a study by Peters *et al.* on parental exposure to solvents and subsequent

brain tumors in their children, parents of 306 cases and 950 controls completed detailed occupational histories. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for both maternal and paternal exposure to benzene, other aromatics, aliphatics, and chlorinated solvents in key time periods relative to the birth of their child. Adjustments were made for matching variables, including child's age, sex and state of residence, level of parental education, and occupational exposure to diesel exhaust. Their results demonstrated an increased risk of childhood brain tumors (CBT) with maternal occupational exposures to chlorinated solvents (OR = 8.59, 95% CI 0.94–78.9) any time before birth. Paternal exposure to solvents in the year before conception was also associated with an increased CBT risk mainly attributable to exposure to aromatic solvents: OR = 2.72 (95% CI 0.94–7.86) for benzene and OR = 1.76 (95% CI 1.10–2.82) for other aromatics [19].

The International Agency for Research on Cancer (IARC) has classified 118 agents as known human carcinogens (http://monographs.iarc.fr/ENG/Classification/). IARC considers an additional 75 agents as probable human carcinogens and another 288 agents as possible human carcinogens. Some of these are actually complex mixture of agents. Typically, in order to delineate the relative contribution of its chemical constituents, a mixture must be separated and chemically characterized. Two of the more pervasive complex mixtures of mutagens and carcinogens are combustion emissions and tobacco smoke (including direct, side stream, and environmental exposures).

Combustion emissions resulting from the burning of fossil fuels, in generating electricity, in heating our homes, or in powering our vehicles, represent a substantial contribution to the total human environmental exposure. These emissions include both particulates and products of incomplete combustion that represent the original starting materials (e.g., coal and crude oil). Their combustion yields carbon, sulfur, lead, mercury, and other elements. Fossil fuels can be refined to reduce unwanted constituents, and this has been important in the development of cleaner industries and engine technologies. Even so, oxidized sulfur and nitrogen, elemental products, and volatile organic carbon products (VOCs) are mutagenic, carcinogenic, and otherwise hazardous to human health.

Tobacco smoke (even tobacco vapor) and all tobacco products are human carcinogens. Volatile vapors, nonvolatile compounds, and fine particles are deposited directly into the airways and the pulmonary alveoli. The Food and Drug Administration (FDA) has listed 93 harmful and potentially harmful constituents (HPHCs) of tobacco products and tobacco smoke (Federal Register/Vol. 77, No. 64/Tuesday, April 3, 2012). These constituents account for much of the carcinogenicity and toxicity that is observed in smokers. Other risk factors associated with smoking include hypertension, stroke, atherosclerosis, and myocardial infarction. Smoking also affects reproductive health, causing delay in conception, low birth weight, and advanced menopause.

In addition to xenobiotic chemicals and drugs, human exposures also include both natural and synthetic substances as well as basic nutrition and supplements. For example, the introduction of industrial farming practices in the United States to meet consumer and processed food product requirements for low cost food has come about with significant problems of microbial contamination (from feces) and antibiotic resistance that have not been encountered previously on such a large scale. Thus, infectious exposures and food safety issues are important categories of concern for human exposure, particularly in children who can be frequent consumers, especially of fast foods containing highly processed meats.

Similarly, excessive exposures to the physical agents in the environment, including sunlight, noise pollution, nonionizing radiation, radon gas, and diagnostic medical radiation can be of concern with regard to cancer etiology. Social factors must also be addressed and have been examined more frequently with the evolution of new knowledge in the field of epigenetics, as will be discussed in Chapter 11.

We suggest as an organizing principle, taking a pan-life span view of cancer and to view what causes and prevents cancer in a cumulative incremental way (see Figure 1.1).

This diagram aims to illustrate some of the various factors beginning prior to conception and extending across the subsequent life span that may drive lifetime risk of cancer(s) upward or downward. Some might plausibly have more impact during key developmental windows while others may be

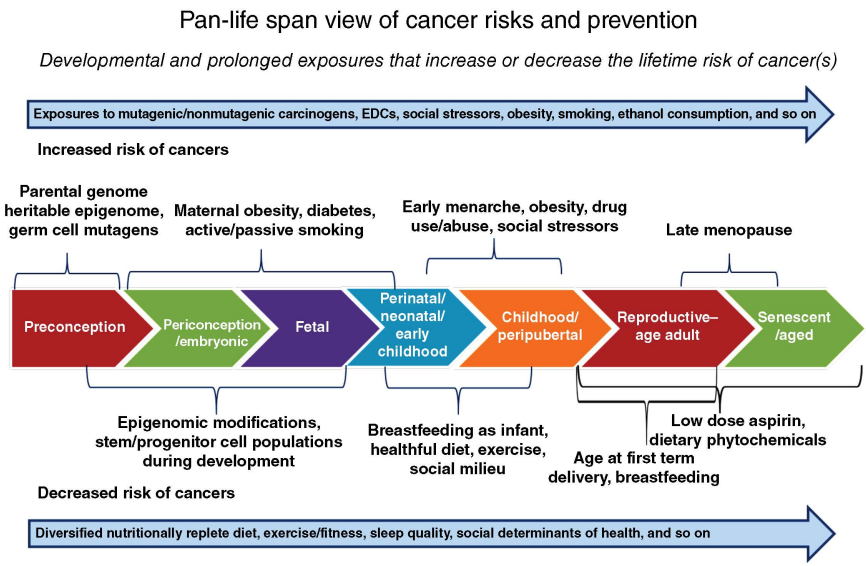

Figure 1.1 A pan-life span view of cancer risks and prevention.

cumulative and rather more subchronic or chronic in terms of either risk or protection. Any number of factors could be important such as biological sex, ethnicity, fitness as an adolescent or teen, assumption of tobacco smoking, discontinuation of tobacco smoking, age at first birth, age of puberty, other behaviors/lifestyle choices. For each cancer or group of cancers, there would be sets of risk factors and risk modifiers (mitigation). Some of these factors are discussed in greater detail in Chapters 9 and 10.

What are some of the considerations that relate lifestyle choices to cancer? A meta-analysis was undertaken by Garcia-Jimenez *et al.* to examine the association between diabetes, obesity, and cancer. Their results indicated that the interplay between hyperglycemia, increase in adipose mass, and inflammation that appears with obesity is critical in both diabetes and cancer, suggesting that obesity may link diabetes and cancer. Indeed, epidemiological evidence positively associates obesity with many site-specific cancers. The associations are strong for endometrial and kidney cancer but weaker for bladder, prostate, and stomach cancers. It may be important to note that highly prevalent lung cancers are inversely associated with obesity. According to Garcia-Jimenez *et al.*, type 2 diabetes (T2D) associates with most cancers that are linked to obesity. T2D represents >90% of diagnosed diabetes; studies that do not distinguish T1D from T2D follow a pattern similar to T2D. Significantly, most site-specific cancers that are positively associated with obesity show an even stronger association with T2D, suggesting that for those cancers T2D exhibits additional contributing factors [20].

What is the molecular basis of these kinds of associations? Genetically and biochemically there are many factors; however, one common denominator is Sirtuin 1 or SIRT1 (a member of the sirtuin family), which is a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase involved in removing acetyl groups from various proteins. SIRT1 performs a wide variety of additional functions in biological systems. Hubbard and Sinclair have reported that it deacetylates key histone residues involved in the regulation of transcription, including H3-K9, H4-K16, and H1-K26, as well as multiple nonhistone protein targets, including p53, forkhead box protein O1/3 (FOXO1/3), peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1a), and nuclear factor (NF)-kB. By targeting these proteins, SIRT1 is able to regulate numerous signaling pathways, including DNA repair and apoptosis, muscle and fat differentiation, neurogenesis, mitochondrial biogenesis, glucose and insulin homeostasis, hormone secretion, cell stress responses, and even circadian rhythm. The other sirtuins also play important roles in regulating mitochondrial reactions, glucose and insulin homeostasis, hepatic lipogenesis, DNA damage, telomere maintenance, inflammation, and the response to hypoxia [21].

Sun *et al.* have asserted that the dysregulation of SIRT1 can lead to ageing, diabetes, and cancer [22]. Using a ligand-based virtual screening of 1,444, 880 active compounds from Chinese herbs, they identified 12 compounds as

inhibitors of SIRT1. Three compounds had high affinity for SIRT1 as estimated by a molecular docking software program. Rahman and Islam have recently reviewed the biological functions of SIRT1 in obesity-associated metabolic diseases, adipose tissue, and cancer. In addition, they discuss the involvement of this enzyme in aging, cellular senescence, cardiac aging and stress, prion-mediated neurodegeneration, inflammatory signaling in response to environmental stress, development, and placental cell survival [23].

Another sirtuin is Sir2 or SIRT2, and its homologs are class III histone deacetylases. They are distinguished from class I and class II deacetylases by their requirement for beta-nicotinamide adenine dinucleotide (NAD+) as a cosubstrate [21]. In mammals, there are seven sirtuin homologs (SIRT1–7). SIRT1, SIRT6, and SIRT7 localize primarily to the nucleus; SIRT3, SIRT4, and SIRT5 localize to mitochondria; and SIRT2 localizes to the cytosol [24]. Although sirtuins were originally described as deacetylases, it is now evident that they have broader activity [24]. In addition to deacetylation, SIRT5 possesses desuccinylase and demalonylase activities [24], SIRT4 and SIRT6 are mono-ADP ribosyltransferases [6,24], and SIRT6 can deacylate long-chain fatty acids [25]. Indeed, it has been shown that the ability to catalyze long-chain deacylation is a general feature of mammalian sirtuins, and that in the case of SIRT6, long-chain fatty acids can enhance deacetylase activity [26].

1.2.1 Mutagenic MOAs

The term "mode of action" (MOA) encompasses a sequence of key events and processes beginning with the interaction of a chemical with a cell and proceeding through functional and structural changes that result in cancer. It is well established that mutations in somatic cells play a key early role in cancer initiation and may affect other stages of the carcinogenic process. All cancer cells acquire multiple mutations during carcinogenesis; therefore, mutation induction or acquisition can be key events at some stage in all cancers. Two important considerations in assessing evidence for a mutagenic MOA are (1) *when* the mutation occurs among the events that lead to cancer and (2) *whether* the action of the carcinogen as a mutagen is a key event in its carcinogenic process [27].

Mutagenicity of a chemical or its metabolite is an obligatory early event in a mutagenic MOA for cancer. This is in contrast with other MOAs wherein mutations are acquired subsequent to other key events (e.g., cytotoxicity with regenerative proliferation). With a mutagenic MOA for carcinogenesis, the chemical is expected to interact with DNA early in the process and produce changes in the DNA that are heritable. That a chemical carcinogen can induce mutation in one of a number of mutation assays is not sufficient to conclude that it causes specific tumors by a mutagenic MOA or that mutation is the *only* key event in the pathway to tumor induction. It should be pointed out

that the term "genotoxic" includes all effects on genetic information, whether or not the chemical interacts with the DNA. The term "mutagenic" implies interaction with DNA but not all carcinogenic chemicals that are capable of interacting with DNA will have a mutagenic MOA for cancer.

Yauk *et al.* reported the results of a 2013 International Working Group on Genotoxicity Testing [28]. This report will be discussed in detail in Chapter 2. The workshop key questions and outcomes were as follows: (1) Do genotoxicity and mutagenicity assays in somatic cells predict germ cell effects? Limited data suggest that somatic cell tests detect most germ cell mutagens, but there are strong concerns that dictate caution in drawing conclusions. (2) Should germ cell tests be done, and when? If there is evidence that a chemical or its metabolite(s) will not reach target germ cells or gonadal tissue, it is not necessary to conduct germ cell tests, notwithstanding somatic outcomes. However, it was recommended that negative somatic cell mutagens with clear evidence for gonadal exposure and evidence of toxicity in germ cells could be considered for germ cell mutagenicity testing. (3) What new assays should be implemented and how? There is an immediate need for research on the application of whole genome sequencing in heritable mutation analysis in humans and animals, and integration of germ cell assays with somatic cell genotoxicity tests. Focus should be on environmental exposures that can cause *de novo* mutations, particularly newly recognized types of genomic changes. Mutational events, which may occur by exposure of germ cells during embryonic development, should also be investigated. Finally, where there are indications of germ cell toxicity in repeat dose or reproductive toxicology tests, consideration should be given to leveraging those studies to inform of possible germ cell genotoxicity [28]. Additional information on mutagenic MOAs may be found in Chapter 2.

1.2.1.1 DNA Repair

DNA is subject to damage from environmental and dietary carcinogens, endogenous metabolites, certain anti-inflammatory drugs, and genotoxic chemo therapeutics. The prevention of mutations by DNA repair pathways led to an early appreciation of a role for repair in cancer avoidance. However, the broader role of the DNA damage response (DDR) emerged more slowly [29]. There are multiple DNA repair pathways, with subpathways providing lesion specificity. Nucleotide excision repair removes bulky DNA lesions; DNA nonhomologous end joining and homologous recombination repair DNA double-strand breaks; mismatch repair corrects mismatched base pairs; and base excision repair repairs damaged bases and links to single-strand break repair. Mutations in these pathways increase cancer susceptibility [29].

Cells respond to DNA damage by the activation of complex signaling networks that decide cell fate, promoting DNA repair and survival but also cell death. Whether it is to be cell survival or death depends on factors involved

in DNA damage recognition, and DNA repair and damage tolerance, as well as on factors involved in the activation of apoptosis, necrosis, autophagy, and senescence. The pathways that dictate the fate of the cell also have key roles in cancer initiation and progression. Furthermore, they determine the outcome of cancer chemotherapy with genotoxic drugs. Understanding the molecular basis of these pathways is important not only for gaining insight into carcinogenesis, but also in prescribing successful cancer therapy [30].

DNA damage triggers multiple cellular responses: It activates cell cycle checkpoints that provide time for the cell to repair the damage before it interferes with the replication machinery. Checkpoints prevent progression from G1 to S phase and from G2 to M phase, and an intra-S phase checkpoint regulates fork progression or origin firing. Many tumors have inactivated checkpoint responses. If repair fails or is saturated, the remaining DNA damage impedes replication and transcription, and the activated DDR signal cell death via downstream pathways. Therefore, the ability of a cell to survive DNA damage is proportional to the extent of damage, the repair capacity of the cell, the level of cell proliferation, the status of p53 and key DDR proteins including ataxia-telangiectasia mutated (ATM), ATR, and DNA-PK, the effectiveness of activating DNA repair genes (which is dependent on epigenetic silencing and cellular transcription factors), and the execution of downstream cell death pathways.

There are two DNA damage response signaling pathways: ATM-dependent signaling is activated by double strand breaks; and ataxia telangiectasia and RAD3-related (ATR)-dependent signaling is activated by single-stranded regions of DNA. DDR signaling can activate apoptosis and checkpoint arrest, and can influence DNA repair. Mutations in ATM signaling components confer cancer susceptibility. However, ATR-deficient mice show reduced capacity for tumor formation [29]. Multiple processes function to maintain the accuracy of replication and enhance recovery from replication fork stalling or collapse. Homologous recombination has a key role, and genes involved in this process are commonly mutated in cancers. Several mechanisms prevent DNA rereplication that can cause aneuploidy and subsequently genomic instability. Cancer cells need to maintain telomere length to survive since shortened telomeres lead to senescence. Activation of telomerase or an alternative pathway to maintain telomere length is common in cancers.

DNA repair capacity differs greatly among cell types, with human embryonic stem cells repairing most DNA lesions more effectively than differentiated cell types [31], whereas monocytes and muscle cells are defective in base excision repair [32,33] and some cancers show upregulation of repair, for example, metastatic melanoma [34], or highly variable MGMT repair activity such as in gliomas [35,36]. In simple terms, a low level of DNA damage activates DNA repair (with upregulation of repair genes XPF, XPG, DDB2, XPC, XRCC1, and others), whereas with high levels of DNA damage, repair is saturated, and

unrepaired DNA damage activates one of the death programmes, including apoptosis, regulated necrosis, and autophagy. Apoptosis represents a programmed cell death pathway that functions in some tissues during normal development but also prevents proliferation of damaged cells. Apoptosis can be p53 dependent or independent and p53 is commonly mutated in cancer [29]. It is not well understood how the cell switches between these pathways; however, it appears that the p53 phosphorylation status and antiapoptosis thresholds are key nodes in determining a cell's life or death following DNA damage. ATM and ATR seem to be the main decision makers, informing effectors such as p53 how to proceed. Increased drug resistance of tumors carrying mutations in ATM [37] illustrates the importance of ATM in initiating cell death pathways. Inactivation of p53 in cancer cells can lead to either drug sensitization or resistance, depending on the genotoxic agent employed.

Roos *et al.* [30] have suggested that targeting antiapoptosis proteins and pathways conceivably lowers the threshold for cell death for genotoxic and biological therapies. How specific DNA lesions activate and coordinate the complex interplay between survival and death is of fundamental importance for cancer therapy. The ultimate goal is to protect normal tissue during therapy with genotoxic anticancer drugs while sensitizing cancer cells to die. The protection of normal tissue has far-reaching implications for stem cells and for genome-compromised cells as the former have been shown to activate DNA damage-triggered apoptosis easily, and the elimination of the latter from the healthy cell population is a cancer prevention strategy.

1.2.2 Epigenetic MOAs

Epigenetics is the study of all mechanisms regulating gene transcription and genomic stability maintained throughout cell division, but not including the DNA sequence itself. Environmental epigenetics, also referred to as toxicoepigenetics, investigates the molecular biological processes that potentially link the environment to its impact on disease risk and outcome. This subject is discussed in detail in Chapter 13.

The epigenome modulates gene expression and cellular phenotype via chemical changes in DNA and chromatin that occur without modifying the DNA sequence. The epigenome is highly plastic and reacts to changing external conditions with modifications that can be inherited by daughter cells and across generations. Although this innate plasticity allows for adaptation to a changing environment, it also implies the potential of epigenetic derailment leading to so-called epimutations [38].

To date, DNA methylation is the best-studied epigenetic mechanism in which methyl groups are added to the cytosine base within cytosine–guanine dinucleotides (CpG sites). CpGs tend to be clustered in high-density CpG islands at the promoter of more than half of all genes. Unmethylated CpG

islands are found there in actively transcribed genes, whereas hypermethylation of the promoter results in gene repression. Over the last 5 years, our understanding is that methylation patterns across the gene (so-called intragenic or gene body methylation) may have a role in transcriptional regulation and efficiency. Genome-wide DNA methylation profiling studies support this concept, but whether DNA methylation patterns are a cause or consequence of other regulatory mechanisms is not yet clear. Shenker and Flanagan have examined the evidence for the function of intragenic methylation in gene transcription, its significance in carcinogenesis, and potential use in therapies targeted against DNA methylation [39].

DNA methylation changes have been associated with cancer, infertility, cardiovascular, respiratory, metabolic, immunologic, and neurodegenerative diseases. Experiments in rodents demonstrate that exposure to a variety of chemical stressors, occurring during prenatal or adult life, may induce DNA methylation changes in germ cells, which may be transmitted across generations with phenotypic consequences. A number of human biomonitoring studies show environmentally related DNA methylation changes mainly in blood leukocytes, but there are few studies on possible epigenetic changes induced in the germ line, even though sperm are readily accessible for analysis.

DNA methylation is a life-essential process as it modulates gene expression and drives cell differentiation in multicellular organisms. Synergistically with other epigenetic mechanisms, it allows cells and organisms to adapt to external changes, in a timely manner not matched by mutational mechanisms. Not surprisingly, DNA methylation is sensitive to external stimuli and, in contrast to mutations, is reversible. This duality presents a challenge in establishing possible links between environmental exposure and epigenetic changes that can have a long-lasting impact on cell function and ultimately health. Cancer is a good example of a disease associated with aberrant epigenetics, possibly triggered by environmental exposures.

Epigenetic marks are extensively altered in cancer but they may also change in normal tissues with age, which is the primary risk factor for most cancers. Xu and Taylor performed an epigenome-wide study to identify age-related methylation sites and examine their relationship to cancer and other underlying epigenetic marks. They analyzed DNA in 1006 blood samples from women aged 35–76 years from the Sister Study (http://www.niehs.nih.gov/research/atniehs/labs/epi/studies/sister/) and determined that 7694 (28%) of the 27,578 CpGs assayed were associated with age (false discovery rate, $q < 0.05$). Using independent data sets, they also confirmed 749 "high confidence" age-related CpG (arCpGs) sites in normal blood. Their findings suggest that as cells acquire methylation at age-related sites, they have a lower threshold for malignant transformation and this may explain in part the increase in cancer incidence with age [40].

Evidence exists that erroneous epigenetic marks play prominent roles in Alzheimer's disease, autoimmune diseases such as rheumatoid arthritis, and cardiovascular diseases, among others [38].

It should be noted that interindividual variation in methylation may also be a consequence of DNA sequence polymorphisms that result in methylation quantitative trait loci. Teh *et al.* [41] have investigated the genotypes and DNA methylomes of 237 neonates and found some 1500 punctuate regions of the methylome highly variable across individuals, termed variably methylated regions (VMRs), against a homogeneous background. Their explanation for 75% of VMRs was the interaction of genotype with different *in utero* environments, including maternal smoking, maternal depression, maternal BMI, infant birth weight, gestational age, and birth order. A prevalence of genetic over environmental determinants of interindividual variation of CpGs methylation has been recently reported in large Scottish and Australian cohorts. Finally, age is expected to be a major variable affecting the DNA methylation profiles in different tissues. In fact, recent studies aimed at exploring the importance of epigenetic changes to the ageing process and highlighting age-signatures of DNA methylation.

To fully understand the import of methylation signatures requires query of the human haploid DNA methylome containing approximately 30 million CpGs that exist in a methylated, hydroxymethylated, or unmethylated state. Notwithstanding this challenge, study of environmental epigenetics may be the best way to fully assess the impact of the exposome on human health. Indeed, the hypothesis of prenatal origin of adult-onset diseases is supported by the idea that mammalian tissue differentiation is mainly established during prenatal life, and that fundamental DNA methylation changes occur in the preimplantation embryo and during gonadal differentiation. Epidemiological mother–child cohort studies and maternal exposure assessment are needed to advance science in this area. Although the process of gametogenesis will only be completed after puberty, the bases of reproductive health are founded during prenatal life with primordial germ cell differentiation and gonad development. This requires that multiple exposure windows be considered to assess possible environmental effects on gamete genetic as well as epigenetic integrity [38].

The results of studies in rodents show that DNA methylation in germ cells can be altered by many different exposures during fetal as well as adult life. Limitations of these studies include the fact that more data are available on the male than on the female germ line, and only a few studies were at the whole genome scale, addressed the functional impact of epigenetic changes on gene expression and related cell pathways, and took into consideration dose–effect relationships. Even so, their results establish proof of principle that exogenous stressors may alter DNA methylation at developmentally important imprinted or metabolic genes [38].

Environmental exposure of the human germ line to mutagenic or epimutagenic agents may alter the reproductive capacity of the exposed individual and

may transmit damage to the following generation. Studies in rats and mice have shown that treatment induced not only DNA methylation changes in paternal sperm but also phenotype alterations in offspring. These observations suggest that DNA methylation profiles of gametes are not completely reset after fertilization but can be partly transmitted across generations. While studies have given conflicting results, several authors agree that direct transmission of methylation changes is not the only mechanism through which altered sperm methylation might affect the offspring phenotype and that sustained alterations of transcriptional regulatory networks early in development may likely result from a complex interplay between DNA methylation changes, chromatin modifications, and other epigenetic mechanisms. One implication of epigenetic inheritance systems is that they provide a potential mechanism by which parents could transfer information to their offspring about the environment they experienced [38,42].

From a clinical perspective, DNA methylation and other epigenetic changes in the sperm observed in subfertile patients are also important for reproductive environmental epigenetics because they seem to indicate a functional significance of DNA methylation changes in the male germ line. Thus, there is a need to conduct specific epigenetic analyses on the sperm of men exposed to reproductive toxicants, with the awareness that their PBLs might not be reliable surrogates for the relevant target cells [38].

On the basis of human somatic environmental epigenetics, rodent germ line epigenetic toxicological studies, and knowledge of the most environmentally relevant human reprotoxic agents, a priority list of environmental stressors for future human sperm epigenetic biomonitoring studies might be proposed: (1) dysmetabolism as a consequence of environmental and genetic factors, including their possible interactions, (2) endocrine disrupting compounds, and major lifestyle toxicants like tobacco smoke and alcohol with emphasis on prenatal exposure and mother child cohorts, and (3) prospective, long term, multigeneration follow-up surveys to take into account grandparental effects [38].

What are some of the other consequences of epigenetic inheritance? As already discussed, there is considerable controversy regarding epigenetic inheritance in mammalian gametes. Using *in vitro* fertilization to ensure inheritance exclusively via the gametes, Huypens *et al.* showed that a parental high-fat diet renders offspring more susceptible to developing obesity and diabetes in a sex- and parent of origin-specific mode. The "thrifty genotype" hypothesis postulated that metabolic thrift, the capacity to effectively acquire, store and use energy, is an ancient trait embedded in human genomes [43]. However, the prevalence rates for obesity and type 2 diabetes (T2D) have increased globally over recent decades at a pace that cannot be explained solely by genetic drift. Therefore, Huypens *et al.* experimentally tested whether epigenetic inheritance via gametes by itself could increase an offspring's susceptibility to develop obesity and T2D2. To this end, Huypens and colleagues fed isogenic

C57BL/6NTac mice a calorie-dense high-fat research diet (HFD), a control low-fat research diet, or normal standard chow for a period of 6 weeks. Parental (F0) HFD mice developed obesity, severe glucose intolerance, and fasting hyperinsulinemia. The authors concluded that the epigenetic inheritance of acquired metabolic disorders might contribute to the current obesity and diabetes pandemic [44].

1.2.3 Nongenotoxic Carcinogens, ROS, Obesity, Metabolic, Diet, Environment, Immune, Endocrine MOAs

Nongenotoxic carcinogens are chemicals that cause cancer without directly reacting with DNA. Despite their lack of mutagenicity, nongenotoxic carcinogens can influence the development and progression of cancer through a number of indirect mechanisms that (1) may increase cell proliferation and disrupt cell structures, (2) generate reactive oxygen species (ROS), (3) induce receptor-mediated signaling, (4) alter gene expression or epigenetic programming of cells, and (5) induce inflammation and modulation of the immune response. These diverse and complex secondary mechanisms by which nongenotoxic carcinogens induce neoplasia are often tissue and species specific. They rarely follow low-dose linearity, typically ascribed to genotoxic agents, and thereby they create difficulties for researchers and challenges in human health risk assessment for regulatory agencies. To illustrate the diversity and the complexity of evaluating nongenotoxic mechanisms of carcinogenesis, Chapter 12 examines an estrogenic toxicant and putative carcinogen used widely in a variety of consumer goods. The following introductory information is abstracted from Chapter 12.

A common mode of action for nongenotoxic carcinogens involves receptor-mediated effects. Steroids and xenoestrogens can cause cancer through hormone receptor-mediated interactions, including perturbed hormone balance, increased cell proliferation, and altered gene expression patterns. Estrogenic ligands, such as 17β-estradiol, bind estrogen receptors (ERs) and induce carcinogenicity by altering genomic and nongenomic regulation of transcription. More specifically, binding of estrogenic ligands to estrogen receptors α and β (ERα and ERβ), members of a nuclear receptor super-family, activates these complexes to bind estrogen responsive elements (ERE) in the promoter regions of target genes, thereby regulating their transcription [45]. Gene expression changes can also be induced independent of ERE elements through the interaction of ERα and ERβ with DNA-bound transcription factors [46,47]. Nongenomic signaling can also be induced by estrogenic ligand binding to membrane estrogen receptors or other estrogen binding proteins that induce kinase signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathway [46,47]. Collectively, these alterations induce changes in cell growth, differentiation, motility, and DNA damage response and repair that can

contribute to the development and progression of breast, ovarian, and endometrial cancers [46,48].

Laboratory studies in a number of model systems have confirmed the induction of ROS and DNA damage by oxidative DNA lesions such as 8-oxo-guanine [49–57]. In addition to generating ROS, bisphenol A (BPA) has also been shown to alter the antioxidant balance of cells depleting intracellular glutathione and altering the expression of catalase and superoxide dismutase [50,51,58–60]. Additionally, exposure of mice to BPA during pregnancy and continued exposure of the offspring during infancy has been shown to cause oxidative stress by decreasing antioxidant enzymes and increasing lipid peroxidation, leading to underdevelopment of the testis, brain, and kidneys of the offspring [50,52,61].

Two common ways nongenotoxic carcinogens induce oxidative stress are by generating ROS during their metabolism in the cell and/or by depletion of the antioxidant defense mechanisms in the cell that counterbalance both endogenous and exogenous ROS. BPA primarily induces ROS through the enzymatic (H_2O_2/peroxidase and NADPH/CYP450) and nonenzymatic (peroxynitrite/CO_2 and −OCl/HOCl) formation of BPA phenoxyl radicals [49]. These phenoxyl radicals can then be further converted by NADPH or intracellular glutathione to form superoxide, hydroxyl radicals, and H_2O_2 [49]. Generated ROS can then damage cellular macromolecules and induce DNA strand breaks, purine and pyrimidine lesions, and DNA proteins cross-links.

Recent work has demonstrated that the induction of oxidative stress by BPA induces a number of cellular changes that, when challenged by additional oxidative stress, induce an adaptive response, promoting cell survival [51]. This adaptive response was characterized by an initial compaction of cellular chromatin that prevents the excision of oxidatively induced DNA lesions followed by an up-regulation of DNA repair proteins that increases the repair of oxidatively induced DNA lesions [51]. These results demonstrate that induction of oxidative stress by BPA contributes significantly to its toxicity. These mechanisms need to be evaluated more thoroughly to understand the role they play in addition to the endocrine disrupting properties of BPA.

In addition to the induction of oxidative stress, inflammation and modulation of the immune response are also important mechanisms of action for some nongenotoxic carcinogens. The role that chemicals and chemical mixtures have on the cells of the human immune system is an emerging research area in environmental toxicology. Thompson *et al.* have reviewed the role that the innate immune cells and inflammatory responses play in tumorigenesis. Their focus is on the molecules and pathways that have been mechanistically linked with tumor-associated inflammation in the context of chemically induced disturbances in immune function as co-factors in carcinogenesis. Specifically, they consider the evidence linking environmental toxicant exposures with perturbation in the balance between pro- and anti-inflammatory responses.

Reported effects of bisphenol A, atrazine, phthalates, and other common toxicants on molecular and cellular targets involved in tumor-associated inflammation (e.g., cyclooxygenase/prostaglandin E2, nuclear factor kappa B, nitric oxide synthesis, cytokines, and chemokines) are presented as examples of chemically mediated target molecule perturbations relevant to cancer. Commentary is presented on areas of additional research required for development and integration of systems biology approaches to the study of environmental exposures and cancer causation [62].

The combination of inflammation and modulation of the immune response can lead to increases in the expression of growth factors and cytokines that ensure survival, while inducing inflammation and altering the immune response. Prenatal exposure of mice to BPA promoted the production of TH2 cytokines and was associated with a decrease in T regulatory CD4+ CD25+ cells [63]. Perinatal exposure to BPA also promoted the production of proinflammatory mediators through the dysregulation of mast cells [64]. Other links to mast cell degranulation, lymphocyte proliferation, and antibody response have also been reported [65,66]. Whether these inflammation and immune changes induced by BPA directly influence the progression and development of cancer has not been examined and the effects of these changes on allergic responses and asthma have not been conclusively verified [64,66].

Chronic inflammation is associated with an increased risk of cancer, and impairment of immune response, whether through immunosuppression or impaired surveillance, can contribute to tumor promotion [67,68]. Given the association of inflammation with cancer and the importance of immune surveillance in the removal of precancerous cells, more work is necessary to determine how BPA is influencing these responses. However, the robust responses of IL-6 and TNFα observed in a number of studies indicate that it may play an important role. Population studies support a role for BPA-induced inflammation, with an increase in C-reactive protein (CRP) levels observed in postmenopausal women [57], increase in IL-6 and CRP observed in premenopausal women with polycystic ovary syndrome [69], and increased levels of IL-6 and TNF-α observed in males [70].

Is BPA a transplacental carcinogen? Based on a recent study in mice it appears to be. Weinhouse *et al.* [71] explored the effects of exposure to BPA during gestation and lactation on adult incidence of hepatic tumors in mice. Isogenic mice were perinatally exposed to BPA through maternal diets containing one of four environmentally relevant doses (0, 50 ng, 50 μg, or 50 mg of BPA per kg diet) and approximately one male and one female per litter were followed until 10 months of age. Animals were tested for known risk factors for hepatocellular carcinoma, including bacterial and viral infections. Hepatic tumors were observed in exposed 10-month mice; 23% of offspring presented with hepatic tumors or preneoplastic lesions. A statistically significant dose–response relationship was observed, with an odds ratio for neoplastic and

preneoplastic lesions of 7.23 (95% CI: 3.23, 16.17) for mice exposed to 50 mg BPA per kg diet compared with unexposed controls. The authors concluded that early disease onset, the absence of bacterial or viral infection, and a lack of characteristic sexual dimorphism in tumor incidence support a nonclassical etiology. This is the first report of a statistically significant association between BPA exposure *in utero* and frank tumors in any organ. The results clearly link early life exposure to BPA with the development of hepatic tumors in rodents, with potential implications for human health and disease [71].

In a clinical investigation, Tarapore *et al.* examined the association between urinary BPA levels and prostate cancer and assessed the effects of BPA on induction of centrosome abnormalities as an underlying mechanism promoting prostate carcinogenesis. Their study, involving 60 urology patients, found higher levels of urinary BPA (creatinine-adjusted) in prostate cancer patients (5.74 mg/g [95% CI; 2.63, 12.51]) than in nonprostate cancer patients (1.43 mg/g [95% CI; 0.70, 2.88]) ($p = 0.012$). These findings suggest that urinary BPA level is an independent prognostic marker in prostate cancer and that BPA exposure may lower serum PSA levels in prostate cancer patients. Moreover, disruption of the centrosome duplication cycle by low-dose BPA may contribute to neoplastic cell transformation in the prostate [72].

Are there windows of susceptibility for epigenetic effects and are there opportunities for intervention? Day *et al.* showed that early puberty timing is associated with higher risks for type 2 diabetes and cardiovascular disease in women; it therefore represents a potential target for early preventive interventions. They characterized the range of diseases and other adverse health outcomes associated with early or late puberty timing in men and women in the very large UK Biobank study. Recalled puberty timing and past/current diseases were self-reported by questionnaire. Analyses were limited to individuals of White ethnicity (250,037 women; 197,714 men) and to disease outcomes with at least 500 cases (~0·2% prevalence) with careful correction for multiple testing (corrected threshold $P < 7.48 \times 10^{-5}$). In models adjusted for socioeconomic position and adiposity/body composition variables, both in women and men separately, earlier puberty timing was associated with higher risks for angina, hypertension and T2D. Futhermore, compared to the median/average group, earlier or later puberty timing in women or men was associated with higher risks for 48 adverse outcomes, across a range of cancers, cardiometabolic, gynaecological/obstetric, gastrointestinal, musculoskeletal, and neurocognitive categories. Notably, both early and late menarche was associated with higher risks for early natural menopause in women. In conclusion, puberty timing in both men and women appears to have a profound impact on later state of health [73].

Given that epigenetic transmission is across generations, what is the look-back period and what kinds of exposures must be considered? To begin to answer this question, Cohn *et al.* hypothesized that *in utero* exposure to DDT is associated with an increased risk of breast cancer. What is the extent of concern

for epigenetic effects from prior exposures? Many women were heavily exposed *in utero* during widespread DDT use in the 1960s. Cohn *et al.* designed a case-control study (involving $n = 118$ breast cancer cases, diagnosed by age 52 years and 354 controls matched on birth year) nested in a prospective 54-year follow-up of 9300 daughters in the Child Health and Development Studies pregnancy cohort. This study links measured DDT exposure *in utero* to risk of breast cancer. The primary participants were Kaiser Foundation Health Plan mothers who had received obstetric care in Alameda County, California, from 1959 to 1967; their adult daughters participated in the study. The daughters' breast cancer diagnosed by age 52 years (as of 2012) was the main outcome measured. The results showed that maternal *o,p*-DDT levels predicted daughters' breast cancer (odds ratio fourth quartile versus first = 3.7, 95% confidence interval 1.5–9.0). Lipids, weight, race, age, and breast cancer history did not explain the findings. Additional experimental studies are essential to confirm these results and discover causal mechanisms. The findings support classification of DDT as an endocrine disruptor, a predictor of breast cancer, and a marker of high risk for breast cancer [74].

Costello *et al.* studied the association between dietary patterns and risk of breast cancer in Spanish women, stratifying by menopausal status and tumor subtype, to compare the results with those of Alternate Healthy Index (AHEI) and Alternate Mediterranean Diet score (aMED). Costello *et al.* recruited 1017 incident breast cancer (BC) cases and 1017 matched healthy controls of similar age (±5 years) without a history of breast cancer. Adherence to the Western dietary pattern was related to higher risk of breast cancer (OR for the top versus the bottom quartile 1.46 (95% CI 1.06–2.01)), especially in premenopausal women (OR = 1.75; 95% CI 1.14–2.67). In contrast, the Mediterranean pattern was related to a lower risk (OR for the top quartile versus the bottom quartile 0.56 (95% CI 0.40–0.79)). While the deleterious effect of the Western diet was similarly observed in all tumor subtypes, the protective effect of the Mediterranean diet was stronger for triple-negative tumors (OR = 0.32; 95% CI 0.15–0.66 and P heterogeneity = 0.04). The results confirmed the harmful effect of a Western diet on breast cancer risk and provided evidence for the overall preventive benefits of a diet rich in fruits, vegetables, legumes, oily fish, and vegetable oils, particularly with triple-negative breast cancer [75].

Ferris *et al.* first used generalized equations to estimate a population average effect across all families ($n = 389$ cases, $n = 5643$ controls) followed by conditional logistic regression in order to examine within-family differences in a subset with at least two sisters discordant on ovarian cancer status ($n = 109$ cases, $n = 149$ unaffected sister controls). In the generalized estimation model, there was a reduced risk of ovarian cancer for ever use of oral contraceptives compared with never use (OR = 0.58, 95% CI: 0.37, 0.91), and in the conditional logistic model there was a similar inverse association, although it was not statistically significant (OR = 0.52, 95% CI: 0.23, 1.17). Ferris *et al.* examined this

association by BRCA1/2 status and observed a statistically significant reduced risk in gene noncarriers only. They observed a decreased risk of ovarian cancer with oral contraceptive use, supporting that this association observed in unrelated women extends to related women at higher risk [76].

1.2.4 Tumor Microenvironment MOAs

Potentially carcinogenic compounds may cause cancer through direct DNA damage or through multiple indirect cellular or physiological effects. As we have seen, the identification and investigation of these varied effects involves work in endocrinology, genetics, epigenetics, medicine, environmental health, toxicology, pharmacology, and oncology. Disruptive chemicals may contribute to multiple stages of tumor development via effects on the tumor microenvironment. The tumor microenvironment consists of complex interactions among blood vessels that feed the tumor, the extracellular matrix that provides structural and biochemical support, signaling molecules that send messages, and soluble factors, such as cytokines. It also consists of many types of host effector cells, including multipotent stromal cells/mesenchymal stem cells, fibroblasts, endothelial cell precursors, antigen presenting cells, lymphocytes, and innate immune cells.

Carcinogens can influence the tumor microenvironment through effects on epithelial cells, the most common origin of cancer, as well as on stromal cells, extracellular matrix components, and immune cells. Casey *et al.* have reviewed how environmental exposures can perturb the tumor microenvironment. They suggest a role for disrupting chemicals, such as nickel chloride, bisphenol A, butyltins, methylmercury, and paraquat, as well as more traditional carcinogens such as radiation, and pharmaceuticals, such as diabetes medications, in the disruption of the tumor microenvironment. Further studies interrogating the role of chemicals and their mixtures in dose-dependent effects on the tumor microenvironment could have important general mechanistic implications for the etiology and prevention of tumorigenesis [77].

Are certain cell types more sensitive to toxicants or more replaceable following damage than others? Recent work in the field of stem cell biology suggests that there is no single adult tissue stem cell hierarchy, and that self-renewal and repair requirements are unique to different tissues. Thus, stem cells may be uni- or multipotent and can exist in quiescent or actively dividing states. Activated "professional" stem cells may coexist with facultative stem cells, which are more specialized daughter cells that revert to a stem cell state under specific tissue damage conditions. Visvader and Clevers discuss stem cell strategies as observed in three solid mammalian tissues: the intestine, mammary gland, and skeletal muscle.

It is becoming increasingly clear that multiple stem cell types with different tissue renewal capacity can reside within the same tissue. Both transplantation

and lineage tracing assays have proved to be invaluable in dissecting stem cell compartments. The small intestine contains multipotent stem cells that are actively cycling to restore all cells within the crypt–villus unit. Adding an apparent layer of complexity, both uni- and bipotent epithelial stem cells appear to reside in the mammary epithelial tree. Functional diversity within a stem cell compartment is presumably established through the complex interplay between intrinsic and extrinsic signals [78]. Chapter 14 explores MOAs related to the tumor microenvironment and Chapter 15 explores MOAs related to dysregulated metabolism.

1.3 Relevance of Circulating Cancer Markers

Chapter 16 is devoted to the topic of this section. Developments in genomic techniques have provided substantial insight into the genetic complexity of malignant tumors. As will be discussed, there is increasing evidence that solid tumors encompass subpopulations of cells with distinct genomic alterations, that is, intratumor heterogeneity. Fisher *et al.* have asserted that intratumor heterogeneity is likely to have implications for cancer therapeutics and biomarker discovery, particularly in the era of targeted treatment. Evidence for a relationship between intratumoral heterogeneity and clinical outcome is emerging. The processes that exacerbate intratumoral heterogeneity, both iatrogenic and tumor specific, are likely to increase with the development and implementation of advanced sequencing technologies, and adaptation of clinical trial design to include comprehensive tissue collection protocols [79].

While there is accumulating evidence for substantial genetic diversity both within and between many common solid tumors, less is known about how such diversity is generated or its impact upon clinical outcomes such as response or resistance to anticancer therapies and the natural history of the disease. Tumor heterogeneity conceivably may impede the identification of predictive biomarkers, and the quest for personalized, or even curative treatment, and is an area of cancer research worthy of intensive and collaborative effort [79].

The identification of circulating tumor cells (CTC) in blood has emerged as one of the most intense areas of cancer research. CTC detection and enumeration can serve as a "liquid biopsy" and as an early marker of response to systemic therapy. The clinical relevance of CTCs as a prognostic factor is well established both in metastatic and early-stage breast cancer patients. The molecular characterization of CTC in breast cancer patients has a convincing potential to enable individualized targeted treatment and to spare these patients unnecessary and ineffective therapies. The elimination or decrease of CTCs following treatment is associated with improved clinical outcomes [80]. In addition, molecular characterization of single CTC holds considerable promise for predictive biomarker assessment enabling scientists to explore CTC heterogeneity. The application of reliable

single CTC isolation together with CTC molecular characterization using advanced next-generation sequencing technologies is opening new frontiers in the management of cancer patients [81].

Peeters *et al.* explored potential differences in the detection and prognostic significance of CTCs in metastatic breast cancer (MBC) based on immunohistochemical subtypes of breast cancer. They used the enumeration of CTCs with the EpCAM-based CellSearch system to determine the prognostic significance of these CTCs in patients with MBC. The EpCAM-based CTCs detected were not associated with any of the immunohistochemical subtypes of breast cancer in patients before first-line treatment; however, potentially clinically relevant differences were observed at very high CTC counts. Their results suggested a lower prognostic significance of CTC evaluation in HER2-positive patients with MBC [82].

Nygaard *et al.* examined the potential prognostic value of circulating cell-free DNA (cfDNA) in malignant disease. The level of cfDNA increases with malignancy but the biological mechanism is not fully understood. In a prospective biomarker trial in 53 patients with advanced non-small cell lung cancer (NSCLC), Nygaard *et al.* used positron emission tomography (PET) to examine the correlation between cfDNA and total tumor burden. There were no correlations between cfDNA and MTV ($r \leq 0.1$) or TLG ($r \leq 0.1$); however, cfDNA 475th percentile was correlated with shorter OS ($P \leq 0.02$) as confirmed by multivariate analysis. MTV 4the median was associated with a significantly shorter OS ($P \leq 0.02$). Nygaard *et al.* concluded that there was no significant difference in OS according to TLG ($P \leq 0.08$); thus, cell-free DNA may not be a simple measure of tumor burden, but seems to reflect more complex mechanisms of tumor biology, making it attractive as an independent prognostic marker [83].

Primarily, due to drug resistance, metastatic cancer patients face a prognosis largely affected by treatment failure. Circulating tumor-specific microRNAs (miRNAs) are promising biomarkers of tumor presence and recurrence, especially for diseases whose best chance of successful treatment requires early diagnosis and timely surgery of an already malignant but not yet invasive tumor such as colorectal cancer (CRC). Chemotherapy could miss CTCs, particularly a subpopulation of more aggressive stem-like CTCs characterized by multidrug resistance. Therefore, Gazzaniga *et al.* investigated the prognostic value of drug resistance and stemness markers in CTCs derived from 40 metastatic colorectal cancer patients that had been treated with oxaliplatin (L-OHP) and 5-fluoruracil (5-FU). Their results support the idea that isolating survivin and MRP5 CTCs may help in the selection of metastatic colorectal cancer patients resistant to standard 5-FU and L-OHP-based chemotherapy and for which alternative regimens may be appropriate [84].

MicroRNAs (miRNAs) are small (22 nt), tissue-based regulatory RNAs that are frequently dysregulated in cancer and have shown promise in cancer

classification and prognosis. Mitchell *et al.* [85] showed that such miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity. miRNAs originating from human prostate cancer xenografts enter the circulation, are readily measured in plasma, and can be used to easily distinguish xenografted mice from controls. This concept also applies to human cancer, where serum levels of miR-141 (a miRNA expressed in prostate cancer) can distinguish patients with prostate cancer from healthy controls.

Expression levels of miRNAs found to be differentially expressed in tumor versus normal colon tissues were investigated by Zanutto *et al.* [86]. They employed quantitative real-time PCR using plasma from CRC patients and from healthy donors and confirmed their results in independent case control series. Validated miRNAs were also measured in patients following surgery. Zanutto *et al.* identified four miRNAs differentially expressed between the compared groups. Two of these, miR-21 and miR-378, were validated and miR-378 expression was observed to decrease in nonrelapsed patients 4–6 months after surgery. Hemolysis did not influence the ability of miR-378 to discriminate CRC patients from healthy individuals. Zanutto *et al.* concluded that analysis of miRNA expression in plasma samples represents a useful noninvasive tool to assess the presence of CRC as well as tumor-free status at follow-up. They also concluded that plasma levels of miR-378 could be used to discriminate CRC patients from healthy individuals, irrespective of hemolysis [86].

Wang *et al.* [87] have reported that the circulating miRNAs, miR-17-5p, and miR-20a (miR-17-5p/20a) are elevated in the plasma of gastric cancer patients. However, the clinical significance of the circulating levels of these miRNAs, their prognostic predictive power, and their application in monitoring the effectiveness of chemotherapy remains unclear. To this end, Wang *et al.* measured plasma miR-17-5p/20a levels in unpaired preoperative ($n = 65$), postoperative ($n = 16$), and relapse ($n = 6$) groups of gastric cancer patients. Their results suggest that the levels of circulating miR-17-5p/20a may be a promising noninvasive molecular marker for pathological progression of gastric cancer, as well as prognosis and monitoring of results of chemotherapy.

Lu *et al.* [88] reported that nasopharyngeal carcinoma (NPC) has a distinctive geographic distribution and is characterized by its strong tendency of metastasis. They examined the microarray expression profiles of miRNAs in plasma samples of NPC patients to explore their clinical significance in disease development and progression. Their research identified 33 differentially expressed miRNAs between NPC patients and healthy volunteers and reported that plasma miR-9 may serve as a useful biomarker to predict NPC metastasis and to monitor tumor dynamics.

Shapira *et al.* [89] generated comprehensive miRNA profiles on presurgical plasma samples from 42 women with confirmed serous epithelial ovarian cancer, 36 women diagnosed with a benign neoplasm, and 23 comparable

age-matched women with no known pelvic mass. Twenty-two miRNAs were differentially expressed between healthy controls and the ovarian cancer group ($P < 0.05$) and six miRNA subset distinguished presurgical plasma from benign and ovarian cancer patients. Significant differences in miRNA profiles in presurgical plasma were observed in women diagnosed with ovarian cancer who had overall short survival when compared to women with overall long survival ($P < 0.05$). The preliminary data supported the utility of circulating plasma miRNAs to distinguish women with ovarian cancer from those with a benign mass and to distinguish women likely to benefit from currently available treatment for serous epithelial ovarian cancer from those who may not [89].

The science of using tumor molecular profiles to select clinical trial participants or to optimize therapy for individual patients is still in its infancy. However, the potential importance of methods that can integrate molecular, histopathological, and clinical information into a synergistic understanding of tumor progression cannot be overstated. While the possibilities are exciting, significant challenges remain before they can be effectively implemented with a strong evidence base and in a widely available and cost-effective manner [90].

1.4 Potential Cancer Translational Toxicology Therapies

Integrative medicine, as defined by Block *et al.* [91], is an approach to health and healing that "makes use of all appropriate therapeutic approaches, health care professionals, and disciplines to achieve optimal health and healing [91]." An integrative medicine intervention for cancer patients typically includes nutritional counseling, biobehavioral strategies, promotion of physical activity, and dietary supplements (including herbs, nutraceuticals, and phytochemicals). A comprehensive intervention of this type may contribute uniquely to improved cancer outcomes through its impact on a variety of relevant molecular targets, including effects on multiple cancer hallmarks [92,93]. Hallmarks that may be particularly affected include genetic instability, tumor-promoting inflammation, deregulated metabolism, and immune system evasion. Block *et al.* [91] characterize these hallmarks as metabolic since they are susceptible to manipulation by diet, exercise, and supplementation. Research on such comprehensive integrative approaches can contribute to the development of systems of multitargeted treatment regimens and help clarify the combined effect of these approaches on cancer outcomes [91].

Potential cancer translational toxicology therapies are discussed in Chapter 18. Examples of potential components of integrated therapies include incorporation of exercise into prenatal care that yields improvements in offspring metabolic disorders, cancer, and cardiovascular health and disease risk [94], and use of vitamin supplementation among middle-aged women, which is associated with substantial reduction in risk of all invasive cancers combined [95].

Maternal behaviors during pregnancy have been reported to impact offspring health in adulthood. Blaize *et al.* [94] explored the hypothesis that exercise during pregnancy can protect against chronic disease susceptibility in the offspring. To date, research has demonstrated that improvements in metabolic outcomes, cardiovascular risk, and cancer can occur in response to maternal exercise during pregnancy, including improvements in offspring metabolic disorders, cancer, and cardiovascular health and disease risk. Thus, overall, the current body of work supports the recommendations for exercise during pregnancy set by the ACOG and DHHS. Health care providers can use these data to educate pregnant mothers on the benefits of exercise during pregnancy for their offspring and encourage them to incorporate exercise into their prenatal care [94].

Regarding the use of vitamin supplementation among middle-aged women, higher serum 25-hydroxyvitamin D [25(OH)D] concentrations have been associated with a lower risk of multiple cancer types. McDonnell *et al.*[95] investigated whether the inverse association between 25(OH)D and cancer risk could be replicated, and if a 25(OH)D response region could be identified among women aged 55 years and older across a broad range of 25(OH)D concentrations. Age-adjusted cancer incidence was studied across a combined cohort ($N = 2304$) with 840 cases per 100,000 person-years (1020 per 100,000 person-years in the Lappe cohort and 722 per 100,000 person-years in the Grassroots Health cohort). Indeed, incidence was lower at higher concentrations of 25(OH)D. Women with 25(OH)D concentrations equal to or greater than 40 ng/ml had a 67% lower risk of cancer than women with concentrations <20 ng/ml (HR = 0.33, 95% CI = 0.12–0.90). In conclusion, 25(OH)D concentrations equal to or greater than 40 ng/ml were associated with substantial reduction in risk of all invasive cancers combined [95].

Gynecologic cancers constitute the fourth most common cancer type in women. Phytochemicals are a broad class of natural compounds derived from plants, a number of which exhibit useful bioactive effects toward these pathways. High-throughput screening methods, rational modification, and developments in regulatory policies will accelerate the development of novel therapeutics based on these compounds, which will likely improve overall survival and quality of life for patients [96].

Although growing evidence from trials and population-based studies has supported a protective role for dietary flavonoids in relation to risk of certain chronic diseases, the underlying mechanisms remain unclear. In particular, the impact of different dietary flavonoid subclasses on risk of epithelial ovarian cancer is also unclear with limited previous studies that have focused on only a few compounds. Some studies have focused on individual inflammatory biomarkers, but because of the limited specificity of any individual marker, an assessment of a combination of biomarkers may be more informative.

Cassidy *et al.* [97] prospectively examined associations between habitual flavonoid subclass intake and risk of ovarian cancer. They followed 171,940

Nurses' Health Study and Nurses' Health Study II participants to examine associations between intakes of total flavonoids and their subclasses (flavanones, flavonols, anthocyanins, flavan-3-ols, flavones, and polymeric flavonoids) and risk of ovarian cancer. Intake was calculated from validated food frequency questionnaires collected every 4 years. Participants in the highest quintiles of flavonol and flavanone intakes had modestly lower risk of ovarian cancer than that of participants in the lowest quintile, although the *P*-trend was not significant (HRs: 0.76 (95% CI: 0.59, 0.98; *P*-trend = 0.11) and 0.79 (95% CI: 0.63, 1.00; *P*-trend = 0.26), respectively). The authors concluded that higher intakes of flavonols, flavanones, and black tea (polyphenol) consumption may be associated with lower risk of ovarian cancer, but that additional prospective studies are required to confirm their findings [97].

1.4.1 Well-Established/Repurposed Pharmaceuticals

Drug repurposing (also known as repositioning) is the application of known drugs and compounds for new clinical indications. Colesevelam is an example of drug repurposing. Colesevelam was developed as an adjunct to diet and exercise to reduce elevated low-density lipoprotein cholesterol (LDL-C) in patients with primary hyperlipidemia. It has also gained approval to improve glycemic control in adults with type 2 diabetes mellitus. For other well-established drugs, such as aspirin, the number of indications has expanded based on continuing research, drug use, and experience.

Aspirin or acetylsalicylic acid (ASA) is a classic, nonsteroidal anti-inflammatory drug (NSAID) that is widely used to relieve minor aches and pains and to reduce fever. Aspirin is certainly one of the most established/repurposed pharmaceuticals and there is a significant body of epidemiological evidence demonstrating that regular aspirin use is associated with a decreased incidence of developing cancer. Langley *et al.* [98] have reported that aspirin has several mechanisms of action, independent of its inhibition of the enzyme cyclooxygenase (Cox) that may contribute to its anticancer effect. Thus, aspirin also influences cellular processes, such as apoptosis and angiogenesis that are crucial for the development and growth of malignancies. Evidence suggests that these effects can occur through Cox-independent pathways which places into question the rationale of focusing on Cox-2 inhibition alone as an anticancer strategy [98].

Randomized studies with aspirin primarily designed to prevent cardiovascular disease have demonstrated a reduction in cancer deaths with long-term follow-up. Concerns about toxicity, particularly haemorrhage, have limited the use of aspirin in cancer prevention. However, recent epidemiological evidence demonstrating that regular aspirin use after a diagnosis of cancer improves outcomes suggesting that it may have a role as an adjuvant, where the risk–benefit ratio will be different [98].

Regular aspirin use is associated with reduced risk of several malignancies. Epidemiologic studies analyzing aspirin, nonaspirin NSAIDs, and acetaminophen use and ovarian cancer risk have been inconclusive. Trabert *et al.* [99] reported analyses of pooled data from 12 population-based case–control studies of ovarian cancer, including 7776 case patients and 11843 control subjects accrued between 1992 and 2007. They found that aspirin use was associated with a reduced risk of ovarian cancer (OR = 0.91; 95% confidence interval (CI) = 0.84–0.99). Similar but not statistically significant results were obtained for nonaspirin NSAIDs, but there was no association with acetaminophen. In seven studies with frequency data, the reduced risk was strongest among daily aspirin users (OR = 0.80; 95% CI = 0.67–0.96). In three studies with dose information, the reduced risk was strongest among users of low dose (<100 mg) aspirin (OR = 0.66; 95% CI = 0.53–0.83), whereas for nonaspirin NSAIDs, the reduced risk was strongest for high dose (≥500 mg) usage (OR = 0.76; 95% CI = 0.64–0.91). In summary, aspirin use was associated with a reduced risk of ovarian cancer, especially among daily users of low-dose aspirin. These findings suggest that the 81 mg/day aspirin regimen proven to protect against cardiovascular events and several cancers could reduce the risk of ovarian cancer 20–34% depending on frequency and dose of use [99].

Epidemiological studies and other experimental studies suggest that ASA use reduces the risk of different cancers, including BC, and may be used as a chemopreventive agent against BC and other cancers. These studies have raised the tempting possibility that ASA could serve as a preventive medicine for BC. However, lack of in-depth knowledge of the mechanism of action of ASA reshapes the debate of risk and benefit of using ASA in prevention of BC. Studies by Maity *et al.* [100], using *in vitro* and *in vivo* tumor xenograft models, show a strong beneficial effect of ASA in the prevention of breast carcinogenesis. ASA not only prevents breast tumor cell growth *in vitro* and tumor growth in nude mice xenograft model through the induction of apoptosis, but also significantly reduces the self-renewal capacity and growth of breast tumor-initiating cells (BTICs)/breast cancer stem cells (BCSCs) and delays the formation of a palpable tumor. Moreover, ASA regulates other pathophysiological events in breast carcinogenesis, such as reprogramming the mesenchymal to epithelial transition (MET) and delaying *in vitro* migration in BC cells. The tumor growth inhibitory and reprogramming roles of ASA could be mediated through inhibition of TGF-β/SMAD4 signaling pathway that is associated with growth, motility, invasion, and metastasis in advanced BCs. Collectively, ASA has a therapeutic or preventive potential by attacking possible target, such as TGF-β, in breast carcinogenesis [100].

After skin cancer, prostate cancer is the most common cancer among men and it can often be treated successfully. More than 2 million men in the United States are prostate cancer survivors (American Cancer Society). The first description of the benefits of surgical castration in the treatment of prostate

cancer occurred 70 years ago. Despite advances in medical therapy (e.g., cabazitaxel, enzalutamide, abiraterone), androgen deprivation therapy (ADT) remains the cornerstone of treatment for advanced prostate cancer. The costs of ADT have risen dramatically, with uncertain survival benefits and substantial associated risks. At the same time, increasing numbers of men are undergoing prostate specific antigen (PSA) testing and prostate cancer is being diagnosed earlier [101].

Clinical studies of potent novel agents have shown survival benefits even in advanced disease, and with more aggressive treatment, men are remaining on ADT for much longer than might have been originally anticipated. The evidence is good that treatment of advanced prostate cancer by ADT results in improvements in symptoms in men with end-stage disease but, there is weak evidence for improvement in survival, except when ADT is combined with radical local treatment, particularly radiotherapy. As new agents enter clinical practice, a comprehensive research strategy is essential to optimize benefits while minimizing harm. According to Bourke *et al.* [101], it is only a matter of time before they will be considered in earlier disease, possibly as a new form of maximal androgen blockade.

Breast cancer is one of the most commonly diagnosed cancers in women. Fabian *et al.* [102] have reported that high intake ratios of the marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) relative to the omega-6 arachidonic acid reduced risk of breast cancer compared to those with low ratios in some but not all case–control and cohort studies. Plausible mechanisms include reduction in proinflammatory lipid derivatives, inhibition of nuclear factor-κB-induced cytokine production, and decreased growth factor receptor signaling as a result of alteration in membrane lipid rafts [102].

The research team at the Comparative Toxicogenomics Database (CTD) and a group of safety researchers at Pfizer developed a collaboration to text mine and manually review a collection of 88,629 articles relating over 1200 pharmaceutical drugs to their potential involvement in cardiovascular, neurological, renal, and hepatic toxicity. The CTD curates chemical–gene–disease interactions, and these detailed, contextualized, high-quality annotations curated from the past 70 years of scientific literature are now fully integrated with public CTD and phenotype interactions can be downloaded. Importantly, this curation can be leveraged for information about toxicity important to drug safety and enable researchers to develop testable hypotheses for drug–disease events. The availability of these curated chemical–gene–disease interactions will help facilitate development of new mechanistic screening assays for pharmaceutical compounds. This partnership demonstrates the value of public/private research data sharing and collaboration and the complementary needs of the pharmaceutical and environmental health science research communities (http://ctdbase.org/) [103].

With the recognition that histone deacetylases (HDACs) are a key component of the epigenetic machinery regulating gene expression, and behave as

oncogenes in several cancer types, there has been major interest in development of histone deacetylase inhibitors (HDACi) as anticancer drugs. A recent review by Ceccacci and Minucci [104] discusses results of the new research regarding the role of HDACs in cancer and the effect of HDACi on tumor cells, emphasizing haematological malignancies, particularly acute myeloid leukemia. HDACs can have opposite roles at various stages of tumor progression and in different subpopulations of cancer stem cells, which emphasizes the importance of investigating these attributes in conjunction with the clinical use of HDACi in cancer therapy.

Ceccacci and Minucci [104] reported that Pan-HDACi have given promising results in a small group of patients with selected haematological diseases, but their use individually has not been satisfactory. The difference in sensitivity to HDACi cannot be attributed to a single cause, making it difficult to envision a simple approach to patient stratification. This field deserves further study and remains a promising therapeutic avenue if selective HDACi prove to be more effective in the clinic with fewer side effects.

Studies in murine models of leukemia suggest that it is necessary to consider not only the differences among different classes of HDACs but also how the same molecules may act in "time" and "space." Ceccacci and Minucci have proposed a systematic study of the effects of HDACi and other epidrugs on the stem cell compartment versus the rest of tumor cells to devise treatment schemes to combine drugs targeting the different tumor cell subpopulations. Thus, the combination of epidrugs with DNA-damaging chemotherapeutic drugs, or proteasome inhibitors, has already shown promising results [104].

1.4.2 GRAS/GRASE, Diet, and Nutraceuticals

To reduce the occurrence of preventable cancers, we believe that timely protective interventions during "critical windows" should include not only minimization of hazardous voluntary exposures and substances of abuse but also the active use of protective GRAS interventions/therapies, including nutritional, dietary supplementation, including nutraceuticals, and/or well-established/repurposed pharmaceutical drugs. Repurposed pharmaceutical drugs must be safe and effective (GRASE) for the intended application. A drug is not considered a new drug by the USFDA *only* when it is generally recognized as safe and effective (GRASE). At a minimum, the general acceptance of a product as GRASE must be supported by the same quality and quantity of scientific and/or clinical data necessary to support the approval of a New Drug Application [105].

Diet clearly plays a major role in cancer risk. Lifestyle factors, including diet, have long been recognized as potentially important determinants of both susceptibility to and survival with many types of cancer. For details on diet factors in cancer risk and voluntary exposures – natural herbals, supplements,

and substances of abuse, refer to Chapters 5 and 6, respectively. Chapter 10 deals with dietary/supplemental interventions and personal dietary preferences for cancer reduction.

In this section we will address primarily the protective role of natural substances in the diet. For example, the association between coffee intake, tea intake, and cancer has been extensively studied, but associations are not established for many cancers. Certain other dietary agents, such as natural products, have been reported to show anticancer effects or potential beneficial effects for other diseases via a MOA that is plausibly relevant to reduction of cancer risk(s). However, the underlying mechanisms of these substances in human cancer often remain unclear. We have attempted to group studies according to putative mechanisms.

1.4.2.1 Suppression of Cell Proliferation and Induction of Cell Death

Epidemiological analysis has demonstrated a negative or inverse correlation between green tea consumption and the risk of non-Hodgkin lymphoma and prostate cancer. Recent studies show that epigallocatechin-3-gallate (EGCG), the major green tea polyphenol, suppresses the proliferation of cancer cells and induces cell death without adversely affecting normal cells. Several molecular mechanisms have been suggested to be responsible for this effect. Thus, the 67-kDa laminin receptor (67LR) was recently identified as the sensing molecule for EGCG [106]. This receptor overexpresses in cancer cells and plays a crucial role in the selective toxicity of EGCG.

Kumazoe *et al*. [106] focused on the molecular mechanism of EGCG and developing a novel strategy to amplify its effect. They identified 67LR as the sensing molecule of EGCG, and also revealed the downstream mechanism of EGCG-induced cell death and growth inhibition. Their studies demonstrated that 67LR acts as the cancer-overexpressed death receptor that induces the apoptotic signaling pathway. Their findings revealed that 67LR could be an attractive target for cancer chemotherapy and provide a rationale for the clinical value of EGCG as a 67LR-targeting drug. Based on the putative molecule mechanisms of EGCG-induced anticancer effect, PDE5, SET, and SphK1 act as resistant factors against EGCG, and also provided a novel strategy to amplify its anticancer effect [106].

Hashibe *et al.* [107] reported that previous investigations are not consistent on whether caffeine may be the source of possible associations between coffee and cancer risk. In the prostate, lung, colorectal, and ovarian cancer screening trial, of the 97,334 eligible individuals, 10,399 developed cancer. Cancers observed were 145 head and neck, 99 oesophageal, 136 stomach, 1137 lung, 1703 breast, 257 endometrial, 162 ovarian, 3037 prostate, 318 kidney, 398 bladder, 103 gliomas, and 106 thyroid. Hashibe *et al.* [107] reported that mean coffee intake was higher in lower education groups, among current smokers, among heavier and longer duration smokers, and among heavier alcohol drinkers. However, coffee intake

was not associated with the risk of all cancers combined (RR = 1.00, 95% confidence interval (CI) = 0.96–1.05). Tea drinking was associated with a slightly decreased risk of cancer overall (RR = 0.95, 95% CI = 0.94–0.96 for 1+ cups per day versus <1 cup per day). For endometrial cancer, a definite decreased risk was observed for coffee intake (RR = 0.69, 95% CI = 0.52–0.91 for greater than or equal to 2 cups per day). Caffeine intake from either substance was not associated with cancer risk in a dose–response manner. Hashibe *et al.* concluded that they observed a decreased risk of endometrial cancer for coffee intake, and a decreased risk of cancer overall with tea intake [107].

Plant food-derived polyphenolic compounds as a group are low in toxicity, and many of them have been proven to modulate key factors in cancer drug resistance, making them good candidates for reversing cancer resistance. Wang *et al.* [108] analyzed the combination effect of two chemopreventive polyphenols, curcumin (Cur) and EGCG, in reversing resistant breast cancer. Their results showed that EGCG significantly enhanced the growth inhibition and apoptosis in both doxorubicin (DOX)-sensitive and doxorubicin (DOX)-resistant MCF-7 cells induced by Cur. They believe that the mechanism may be related to the further activation of caspase-dependent apoptotic signaling pathways and the enhanced cellular incorporation of Cur by inhibiting the P-glycoprotein (P-gp) pump. They also suggested that Cur and EGCG in combination could enhance the toxicity of DOX, increasing the intracellular level of DOX in resistant MCF-7 cells. Their findings with the combination of Cur and EGCG encourage the treatment of human breast cancer resistance by combining two low-toxic chemotherapeutic agents from diet [108].

1.4.2.2 Anti-Inflammatory Effects: Insights from Various Diseases

The purpose of a study by McFarlin *et al.* [109] was to determine the effects of oral Cur supplementation (Longvida® 400 mg/days) on muscle and related activities, muscle soreness following exercise, creatine kinase, and inflammatory cytokines (TNF-α, IL-6, IL-8, IL-10) following eccentric-only dual-leg press exercise (EMID). Subjects ($N = 28$) were randomized to either Cur (400 mg/day) or placebo (rice flour), and were given supplements two days before to four days after EMID. The study demonstrated that Cur supplementation reduced biological inflammation but *not* quadriceps muscle soreness during recovery after EIMD. The authors suggested that observed improvements in biological inflammation may translate to faster recovery and greater functional capacity during subsequent exercise sessions, and that the next step would be to evaluate further the efficacy of an inflammatory clinical disease model [109].

In a cross-sectional analysis of 2375 Framingham Heart Study Offspring Cohort participants, Cassidy *et al.* [110] used an inflammation score (IS) to integrate 12 individual inflammatory biomarkers associated with intake of different flavonoid classes. Intakes of total flavonoids and their classes (anthocyanins, flavonols, flavanones, flavan-3-ols, polymers, and flavones) were calculated from validated

food frequency questionnaires. Individual inflammatory biomarkers were ranked, standardized, and summed to derive an overall IS (and subgroup scores of functionally related biomarkers). Their results remained significant after adjustment for physical activity, and vitamin C and fruit and vegetable intakes: Higher anthocyanin intake was inversely associated with all biomarker subgroups, whereas higher flavonol intake was associated with lower cytokine and oxidative stress biomarker concentrations. The authors concluded that these findings suggest that an anti-inflammatory effect may be a key component underlying the reduction in risk of certain chronic diseases associated with higher intakes of anthocyanins and flavonols [110].

During the past 15 years, there has been a substantial increase in interest in triterpenes (members of phytosterol family) due to their cholesterol lowering properties. Saleem [111] reported at least 25 clinical studies, 20 patents, and 10 major commercial triterpene-based products currently being sold worldwide. Lupeol, also known as Fagarsterol, is a triterpene found in vegetable oils, white cabbage, green pepper, strawberry, olive, mangoes, and grape. In the West, humans consume an average of 250 mg of triterpenes per day. The review by Saleem [111] provides a detailed account of preclinical studies conducted to determine the utility of Lupeol as a therapeutic and chemopreventive agent for the treatment of inflammation and cancer. These studies suggest that it is a multitarget agent with major anti-inflammatory potential involving key molecular pathways that include nuclear factor kappa B (NFκB), cFLIP, Fas, Kras, phosphatidylinositol-3-kinase (PI3K)/Akt, and Wnt/β-catenin in a variety of cells. Importantly, therapeutic effective doses of Lupenol exhibit no toxicity to normal cells and tissues [111].

Autism spectrum disorders (ASDs) have been associated with brain inflammation as indicated by microglia activation, as well as brain expression and increased plasma levels of interleukin-6 (IL-6) and tumor necrosis factor (TNF). Tsilioni *et al.* [112] reported that serum levels of IL-6 and TNF were elevated (61.95 ± 94.76 pg/ml and 313.8 ± 444.3 pg/ml, respectively) in the same cohort of patients with elevated serum levels of corticotropin-releasing hormone (CRH) and neurotensin (NT), while IL-9, IL-31, and IL-33 were not different from controls. The elevated CRH and NT levels did *not* change after treatment with a dietary formulation containing luteolin. The natural flavonoid luteolin has antioxidant, anti-inflammatory, mast cell blocking, and neuroprotective effects. In the study by Tsilioni *et al.* [112], the mean serum IL-6 and TNF levels decreased significantly ($P = 0.036$ and $P = 0.015$, respectively) at the end of the treatment period (26 weeks) as compared with levels at the beginning; these decreases were strongly associated with children whose behavior improved the most after luteolin formulation treatment. The results obtained indicate that there are distinct subgroups of children within the ASDs that may be identifiable through serum levels of IL-6 and TNF and that these cytokines may constitute distinct prognostic markers for the beneficial effect of the luteolin formulation [112].

The aim of a study by Dawson *et al.* [113] was to assess whether omega-3 polyunsaturated fatty acid supplementation alone or in combination with folic acid and B-group vitamins is effective in lowering homocysteine. Lowering homocysteine levels with folic acid and B-vitamins could interfere with cognitive decline and Alzheimer's. The Medline Ovid, Embase, and Cochrane databases were searched for randomized-controlled trial studies that intervened with omega-3 supplementation (with or without folic acid) and measured changes in homocysteine concentration. A total of 3267 participants completed 21 trials. Across all trials, omega-3 supplementation was effective in lowering homocysteine by an average of 1.18 μmol/l (95%CI: (−1.89, −0.48), $p = 0.001$). The average homocysteine lowering effect was greater when omega-3 supplementation was combined with folic acid and B-group vitamins (−1.37 μmol/l, 95%CI: (−2.38, −0.36), $p < 0.01$) compared to omega-3 supplementation alone (−1.09 μmol/l 95%CI: (−2.04, −0.13), $p = 0.03$). Omega-3 polyunsaturated fatty acid supplementation was associated with a modest reduction in homocysteine. The authors concluded that for the purpose of reducing homocysteine, a combination of omega-3s (0.2–6 g/day), folic acid (150–2500 μg/day), and vitamins B6 and B12 might be more effective than omega-3 supplementation alone [113].

1.4.2.3 Upregulation of Tumor Suppressor MicroRNAs

Hagiwara *et al.* [114] found that resveratrol exerts an anticancer effect by upregulating tumor suppressor microRNAs (miRNAs). In further study, they aimed to identify new dietary products that have the same ability to activate tumor suppressor miRNAs and therefore may serve as novel tools for the prevention and treatment of human cancers. They have described the generation and use of an original screening system based on a luciferase-based reporter vector for monitoring miR-200c tumor suppressor activity.

By screening a library containing 139 natural substances, three natural compounds – enoxolone, magnolol, and palmatine chloride – were identified as being capable of inducing miR-200c expression in breast cancer cells at 10 μM. Moreover, these molecules suppressed the invasiveness of breast cancer cells *in vitro*. Next, they identified a molecular pathway by which the increased expression of miR-200c induced by natural substances led to ZEB1 inhibition and E-cadherin induction. These results indicate that their method may be a valuable tool for identification of natural molecules that exhibit tumor suppressor activity in human cancer mediated through miRNA activation [114].

1.4.2.4 Regulation of Oxidative Stress

In their review paper, Gorrini *et al.* [115] discuss the controversial role of reactive oxygen species (ROS) in tumor development and in response to anticancer therapies, and the idea that targeting the antioxidant capacity of tumor cells can have a positive therapeutic impact [115]. As has been discussed

earlier, the regulation of oxidative stress is an important factor in both tumor development and response to anticancer therapies. Many signaling pathways that are linked to tumorigenesis can also regulate the metabolism of reactive oxygen species (ROS) through direct or indirect mechanisms. High ROS levels are generally detrimental to cells, and the redox status of cancer cells typically differs from that of normal cells. Because of metabolic and signaling aberrations, cancer cells exhibit elevated ROS levels. The observation that this is balanced by increased antioxidant capacity suggests that high ROS levels may constitute a barrier to tumorigenesis. However, ROS can also promote tumor formation by inducing DNA mutations and prooncogenic signaling pathways. These contradictory effects have important implications for potential anticancer strategies that aim to modulate levels of ROS [115].

Antioxidants are widely used to protect cells from damage induced by ROS. Sayin *et al.* [116] have asserted that the concept that antioxidants can help fight cancer is widely held in the general population and promoted by the food supplement industry, although clinical trials have reported inconsistent results. The authors show that supplementing the diets of mouse models of B-RAF- and K-RAS-induced lung cancer with the antioxidants N-acetylcysteine (NAC) and vitamin E markedly increases tumor progression and reduces survival. Furthermore, RNA sequencing revealed that NAC and vitamin E produce highly coordinated changes in tumor transcriptome profiles that are dominated by reduced expression of endogenous antioxidant genes. NAC and vitamin E also increase tumor cell proliferation by reducing ROS, DNA damage, and p53 expression in mouse and human lung tumor cells. The inactivation of p53 increases tumor growth to a similar degree as antioxidants and abolishes the antioxidant effect. Thus, antioxidants accelerate tumor growth by disrupting the ROS-p53 axis. Because somatic mutations in p53 occur late in tumor progression, antioxidants may accelerate the growth of early tumors or precancerous lesions in certain high-risk populations, such as smokers and patients with chronic obstructive pulmonary disease, who receive NAC to relieve mucus production [116].

Some trials show that antioxidants actually increase cancer risk. The study in mice by Sayin *et al*. reported that antioxidants accelerate the progression of primary lung tumors; however, little is known about the impact of antioxidant supplementation on the progression of other types of cancer, including malignant melanoma. Le Gal *et al.* [117] show that administration of NAC increases lymph node metastases in a mouse model of malignant melanoma but does not alter the number and size of the primary tumors. These results demonstrate that antioxidants and the glutathione system play a previously unrecognized role in the progression of malignant melanoma [117].

1.4.2.5 Activation of Signal Transduction Pathways

Lupeol is a pharmacologically active triterpenoid found in white cabbage, green pepper, strawberry, olive, mangoes, and grapes. Siveen *et al.* [118] evaluated the

effect of lupeol on the STAT3 signaling cascade and its regulated functional responses in HCC cells. The constitutive activation of STAT3, a signal transducer and activator of transcription signaling, has been linked with survival, proliferation, and angiogenesis in a wide variety of malignancies, including hepatocellular carcinoma (HCC). Lupeol effectively suppressed constitutive activation of STAT3 phosphorylation at the tyrosine 705 residue in a dose- and time-dependent manner. The phosphorylation of Janus-activated kinases (JAKs) 1 and 2 and the protooncogene tyrosine–protein kinase, Src, was also suppressed by lupeol. Thus, lupeol exhibited its potential anticancer effects in HCC through the downregulation of the STAT3-induced prosurvival signaling cascade [118].

Ascorbate, at millimolar concentrations, acts as a pro-oxidant, induces DNA damage and depleted cellular adenosine triphosphate (ATP), activates the ataxia-telangiectasia mutated (ATM)/adenosine monophosphate-activated protein kinase (AMPK) pathway, and results in mTOR (mammalian target of rapamycin) inhibition and death in ovarian cancer cells. The Akt/mammalian target of rapamycin (mTOR) signaling pathway serves as a critical regulator of cellular growth, proliferation, and survival. Akt aberrant activation has been implicated in carcinogenesis and anticancer therapy resistance. The combination of parenteral ascorbate with the chemotherapeutic agents carboplatin and paclitaxel synergistically inhibited ovarian cancer in mouse models and reduced chemotherapy-associated toxicity in patients with ovarian cancer. On the basis of its potential benefit and minimal toxicity, Ma *et al.* [119] recommended further study of intravenous ascorbate in combination with standard chemotherapy in larger clinical trials [119].

Piperlongumine (PL), a natural alkaloid present in the fruit of the Long pepper, is known to exhibit notable anticancer effects. Makhov *et al.* [120] investigated the impact of PL on Akt/mTOR signaling. Makhov *et al.* [120] examined Akt/mTOR signaling in cancer cells of various origins including prostate, kidney, and breast after PL treatment. They demonstrated for the first time that PL effectively inhibits phosphorylation of Akt target proteins in all tested cells. Makhov *et al.* then investigated the efficacy of *in vivo* treatment with PL and the autophagy inhibitor, chloroquine (CQ), using a mouse xenograft tumor model. The downregulation of Akt downstream signaling resulted in a decrease of mTORC1 activity and autophagy stimulation. Using the autophagy inhibitor, CQ, the level of PL-induced cellular death was significantly increased. Combination treatment with PL and CQ demonstrated a substantial antitumor effect in the xenograft mouse model. Their data suggest therapeutic opportunities to mediate cancer cellular death using PL, offering a new paradigm for both prevention and treatment of malignancy [120].

1.4.2.6 Mitigating Inherited Deleterious Mutations

Studies have shown that 3,3′-diindolylmethane (DIM) can upregulate BRCA1 expression in breast cancer cells. Haplo-insufficiency may

contribute to the development of breast cancer among women with a BRCA1 mutation. Thus, interventions that enhance BRCA1 expression may represent avenues for prevention. However, this has yet to be demonstrated *in vivo*. Kotsopoulos *et al.* [121] performed a study to evaluate the ability of orally administered DIM to upregulate BRCA1 mRNA expression in white blood cells. Eighteen women were enrolled in the study. Under the tested conditions, oral DIM was associated with an increase in BRCA1 mRNA expression in women having a BRCA1 mutation. Thus, the possibility exists of mitigating the effect of an inherited deleterious BRCA1 mutation by increasing the physiologic expression of the gene and normalizing protein levels. This approach represents a clinically important paradigm shift in the prevention strategies available to these high-risk women. Kotsopoulos *et al.* concluded that future studies with a larger sample size and higher doses of DIM are warranted [121].

Nucleostemin is a GTPase residing in the nucleolus that is considered to be an important cancer stem/progenitor cell marker protein due to its high expression levels in breast cancer stem cells and its role in tumor initiation of human mammary tumor cells. Tin *et al.* [122] proposed that nucleostemin might represent a promising therapeutic target for breast cancer. They used a new breast cancer cell line, 10AT-Her2, which is highly enriched in cells having a stem/progenitor cell-like character. 10AT-Her2 cells display a CD44+/CD24-/low phenotype with high levels of the cancer stem/progenitor cell marker protein nucleostemin, as well as active aldehyde dehydrogenase-1 (ALDH-1). 10AT-Her2 cells are highly sensitive to the antiproliferative apoptotic effects of indole-3-carbinol (I3C).

I3C is a natural anticancer indole carbinol found in cruciferous vegetables of the Brassica genus, such as broccoli and cabbage. I3C promotes the interaction of nucleostemin with MDM2 (murine double mutant 2), an inhibitor of the p53 tumor suppressor, and disrupts the MDM2 interaction with p53. I3C also induces nucleostemin to sequester MDM2 in the nucleolus compartment, thereby freeing p53 to mediate its apoptotic activity. Small interfering RNA knockdown studies of nucleostemin demonstrated functionally that nucleostemin is required for I3C to trigger its cellular antiproliferative responses, to inhibit tumorsphere formation, and to disrupt MDM2–p53 protein–protein interactions. In addition, expression of an I3C-resistant form of elastase, the only known target protein for I3C, prevented I3C antiproliferative responses in cells and in tumor xenografts *in vivo*, as well as disrupting the I3C-stimulated nucleostemin–MDM2 interactions. The results of Tin *et al.* [122] provide the first evidence that a natural anticancer compound mediates its cellular and *in vivo* tumor antiproliferative responses by selectively stimulating cellular interactions of the stem/progenitor cell marker nucleostemin with MDM2, freeing p53 to trigger its apoptotic response. Furthermore, their studies provide a new mechanistic template that can potentially be exploited for the

development of therapeutic strategies targeted at cancer stem/progenitor cells [122].

1.4.2.7 Mitigating Adverse Epigenetic States

While epigenetic drugs are being studied in cancer therapeutics, potential intentional use of epigenome modifying compounds to prevent cancers later in life raises scientific and ethical questions, some of which are now being openly considered in the neurosciences. Drugs that can reverse epigenetic states and alter behavior have been discussed from an ethical perspective by Szyf [123]. His thesis is that epigenetic drugs could be used not only in diseases such as dementia, Alzheimer disease, schizophrenia or major depression, for which ethical issues may be easier to address, but also within the spectrum of "normal" behaviors. In an experimental rodent model, an adult behavioral phenotype of anxiousness and hyperstress triggered by poor maternal care in early life was reversed with the histone deacetylase inhibitor trichostatin A7. Szyf [123] asks, "Could we justify using epigenetic drugs to alter phenotypes that are epigenetically controlled and might be socially disruptive but are not a disease *per se*? Would we use such approaches preventively to 'improve' behavioural phenotypes? Is it ethical to use epigenetic drugs to prevent 'criminality' in people or groups of people who display epigenetic marks of aggression or people who have already committed aggressive acts? What will be the regulatory limits for the use of epigenetic drugs for behavioural modifications of antisocial phenotypes? The possibility of epigenetic behavioural modifications raises the spectre of 'social engineering'. Who should have the authority to prescribe behaviour-modifying drugs for cases in which public security or goods are involved? What are the roles of parents and legal courts in such decisions when children are involved? What should the rules be for consenting adults? [123]."

It is widely accepted that performance-enhancing drugs are banned during athletic competitions. It is also clear that competitions that require cognitive skills affect every human and have a dramatically higher impact on human life course than entertainment sports. Szyf asks, "If epigenetic drugs could indeed enhance cognitive abilities in healthy people, should they be used? Would the use of 'epigenetic cognitive enhancement' drugs be considered unethical during exams and competition for jobs, grants and promotions? Do epigenetic cognitive enhancement drugs introduce an unfair element to the regular competition between humans for resources and rank?" Critically important is the possibility that a transient treatment with an epigenetic drug could result in long-lasting effects on cognitive skills. It would, in this case, be ineffective to police the use of drugs at the time of the competition. Furthermore, access to epigenetic cognitive enhancement drugs might enhance the gap that already exists between poor and rich communities within rich countries, as well as between rich and poor countries [123].

These and other ethical issues in translational toxicology are discussed in Chapter 19.

1.4.2.8 Paradigm for Study of Cancer Chemoprevention

Cancer chemoprevention involves the chronic administration of a synthetic, natural, or biological agent to reduce or delay the occurrence of malignancy. The potential value of this approach has been demonstrated with trials in breast, prostate, and colon cancer. The paradigm for developing new chemopreventive agents has changed markedly in the last decade and now involves extensive preclinical mechanistic evaluation of agents before clinical trials are instituted and a focus on defining biomarkers of activity that can be used as early predictors of efficacy. A mini-review by Steward and Brown [124] summarizes the current status of the field of chemoprevention and highlights new developments. Table 1.1 lists some potential mechanisms of chemoprevention. Some potential molecular targets for chemopreventive agents are shown in Table 1.2.

Progress in development of clinical chemopreventive agents has proceeded using a similar model to new drug development in cancer therapy, with sequential phase I, II, and III studies (see Figure 1.2).

Phase I studies have the primary aim of determining safety and pharmacokinetics such that a dose and regimen that is well tolerated by participants can

Table 1.1 Potential mechanisms of chemoprevention.

Mechanisms of tumor-blocking agents
Scavenging of free radicals
Antioxidant activity
Induction of phase II drug-metabolizing enzymes
Inhibition of phase I drug-metabolizing enzymes
Induction of DNA repair
Blockade of carcinogen uptake
Mechanisms of tumor-suppressing agents
Alteration in gene expression
Inhibition of cell proliferation, clonal expansion
Induction of terminal differentiation, senescence
Induction of apoptosis in preneoplastic lesions
Modulation of signal transduction

Source: Reproduced from Ref. [124] with permission of Nature Publishing Group.

Table 1.2 Selected molecular targets of potential chemopreventive agents (effects may be tissue and cell specific as well as dose dependent).

Gene expression	Transcription factors	Protein kinases	Enzymes	Others
Chemokines	NF-κB	IκBα		
Kinase	FTPase	ICAM-1		
Cyclin D1	AP-1	EGFR	Xanthine oxidase	VCAM-1
MMP9	Egr-1	HER2	Heme oxygenase	ELAM-1
COX2	STAT1	AKT	uPA	TF
5-LOX	STAT3	JAK2	GST	Bcl-2
iNOS	STAT5	TYK2	GSH-px	Bcl-χ
IL-12	PPAR-γ	JNK		P53
TNF	EpRE	PKC		
IL-6	CBP	Src		MDR
IL-8	β-catenin	PKA		Telomerase
				Cyclin D1

AP-1: activator protein 1; CBP: CREB-binding protein; COX2: cyclooxygenase 2; EGFR: epidermal growth factor receptor; Egr-1: early growth response protein 1; ELAM-1: endothelial-leukocyte adhesion molecule 1; EpRE: energy per resource element; GSH: glutathione; GST: glutathione-S-transferase; HER2: human epidermal growth factor receptor 2; ICAM-1: intercellular adhesion molecule 1; IL: interleukin; iNOS: inducible nitric oxide synthase; JAK2: janus kinase 2; JNK: c-Jun N-terminal kinases; MDR: multidrug resistance; MMP9: matrix metallopeptidase 9; NF-kB: nuclear factor-kB; PKA: protein kinase A; PKC: protein kinase C; PPARg: peroxisome proliferator-activated receptor-g; STAT: signal transducer and activator of transcription; TF tissue factor; TNF: tumour necrosis factor; uPA: urokinase-type plasminogen activator; VCAM-1: vascular cell adhesion molecule 1. *Source*: Reproduced from Ref. [124] with permission of Nature Publishing Group.

be defined. Some phase I studies may incorporate preliminary assessments of potential biomarkers of effect. Exposure is usually relatively short (up to 3 months). Choosing a starting dose and schedule is extremely difficult and may be guided by preclinical studies. Dose conversions can be used, which seek to achieve plasma concentrations in humans that should be safe and may approximate dose levels producing a biological effect in preclinical models. PK data from phase I studies provide the actual levels that are achieved in humans, and these can be refined in preclinical models that explore possible mechanisms of effect at clinically achievable concentrations. Studies may utilize existing drugs, such as aspirin, for which there is already extensive human data, and rapid progress to phase III trials can be contemplated in this situation.

Phase II trials typically follow, utilizing the optimal dose determined previously, with the aim of exploring in relatively few patients the impact of exposure on a selected biological endpoint. When potential biomarkers of effect are available, these can also be examined in the few patients. Phase II trials may

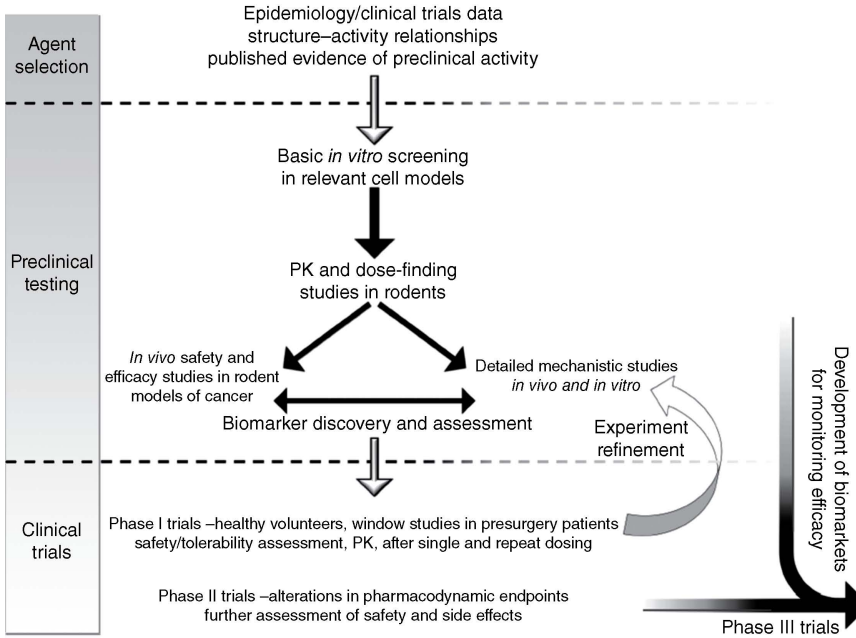

Figure 1.2 Stages in the preclinical and clinical development of potential chemoprevention agents. *Source:* Reproduced from Ref. [124] with permission of Nature Publishing Group.

incorporate a placebo (phase IIb) to better define subtle side effects and tolerability, and also to more accurately measure biological effects.

Phase III trials typically involve thousands of participants over a long period of time. Normally, there is randomization between the agent under investigation and a placebo. In chemoprevention trials, modification of a clinically relevant value, which is usually the incidence of malignancy, is the standard endpoint. Such trials may take many years and involve huge costs.

Given the time required for development of chemopreventive agents, recent interest has focused on phase 0 trials. These employ very low doses of the experimental agent and utilize new methodologies and technologies to study pharmacokinetics at a dose that minimize any risk of toxicity. It is anticipated that this approach will provide information to help determine a rational dosage regimen for future studies and will lead to early delineation of agents that have unfavorable bioavailability, metabolism, or distribution [124]. For detailed study on cancer prevention, please refer to Chapter 4.

Systems pharmacology is the name that is increasingly being used for the new systems-based approach that is being used to understand drug actions and for drug discovery. Systems pharmacology will take into account genomic variations

and molecular complexity in defining physiological and pathophysiological responses at the tissue, organ, and organism levels.

Systems-level analysis can be a powerful driver for understanding drug action. One can envisage three kinds of new knowledge coming from such analyses [125]:

- First is the identification of unanticipated adverse events that each drug might not produce on its own. Identification and prediction of such adverse effects could prove useful to guide physicians regarding which medicines can be coprescribed.
- The second kind of knowledge is the opposite of the first: identification of unanticipated beneficial effects by drug combinations, such as mitigation of side effects. This type of knowledge might lead to repurposing of approved drugs if their efficacy in suppressing adverse events could be established in rigorous clinical trials.
- The third kind of knowledge, which is the most forward-looking, is that network biology can be used for the discovery of new drugs. Network analysis can provide a rational basis for identifying targets, which, when modulated together by drug combinations, might be distinctively efficacious in treating complex diseases.

Combination therapy based on network biology could become efficacious for the treatment of progressive diseases, such as type 2 diabetes, kidney disease, congestive heart failure and, of course, many cancers. While the necessary knowledge is not yet available, the path forward can be readily seen. Large databases, such as FAERs, can provide empirical knowledge of good and bad outcomes associated with combination therapies in humans. As large amounts of genomic and molecular data are integrated with clinical data when electronic medical records become more widely used and molecular characterization of patients becomes more standardized, it will probably generate a wealth of systems-level information to analyze and generate hypotheses. These hypotheses might help with the design of studies to better understand the progression of diseases, and design new drugs or repurpose existing drugs that, in combination, are more effective for treating complex diseases.

For breast and ovarian cancer, steps have been taken to create a risk prediction model incorporating several of the known risk factors for computations. The Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA) is a risk prediction model that is used to compute probabilities of carrying mutations in the high-risk breast and ovarian cancer susceptibility genes BRCA1 and BRCA2, and to estimate the future risks of developing breast or ovarian cancer. Lee *et al.* [126] have described updates to the BOADICEA model that extend its capabilities, make it easier to use in a clinical setting, and yield more accurate predictions:

(1) updates to the statistical model to include cancer incidences from multiple populations; (2) updates to the distributions of tumor pathology characteristics using new data on BRCA1 and BRCA2 mutation carriers and women with breast cancer from the general population; (3) improvements to the computational efficiency of the algorithm so that risk calculations now run substantially faster; and (4) updates to the model's web interface to accommodate these new features and to make it easier to use in a clinical setting. Lee *et al.* [126] present results derived using the updated model, and demonstrate that the changes have a significant impact on risk predictions. All updates have been implemented in a new version of the BOADICEA web interface that is now available for general use: http://ccge.medschl.cam.ac.uk/boadicea/ [126].

1.5 Modeling and the Future

The integration of transcriptomic, metabolomic, epigenomic, and proteomic profiling technologies has helped to build the foundation of systems biology [13,14] and systems toxicology [15].

Systems biology has been used for several years across different scientific areas of biological research to uncover the complex interactions occurring in living organisms. Applications of systems concepts at the mammalian genome level are quite challenging, and new complimentary computational/experimental techniques are being introduced. Most recent work applying modern systems biology techniques has been conducted on bacteria, yeast, mouse, and human genomes. The systems biology view that complex networks underlie many diseases is being increasingly recognized and demonstrated for heart disease, kidney disease, diabetes, metabolic diseases, and cancers. To cast systems of interacting entities as networks is useful because it allows the use of graph theory, a branch of mathematics that analyses how complex systems are organized and how such organization enables system-level functions. Chapter 17 describes the omics technologies upon which translational toxicology modeling and the future of the field will depend.

Figure 1.3 illustrates the sequence of events between initial exposure to a toxicant and final disease outcome (left to right). Note that genetic susceptibility (red dot) influences every level of toxicological analysis. After exposure, the ADME (absorption, distribution, metabolism, and excretion) systems of the body control local concentrations of a chemical stressor in various body compartments. This is affected by genetics through the involvement of specific alleles encoding various transporters and xenobiotic-metabolizing enzymes among others.

Mathematical models, including exposure models, physiologically based pharmacokinetic (PBPK), and biologically based dose response (BBDR) models can be used to approximate the relevant processes. PBPK models are a set of differential equations structured to provide a time course of a chemical's

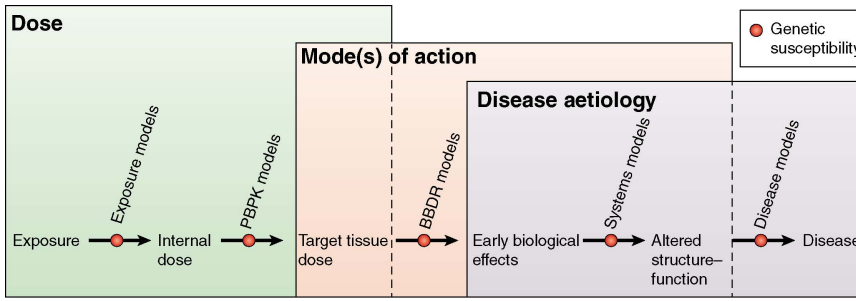

Figure 1.3 Computational models on the continuum between exposure and disease. *Source:* Reproduced from Ref. [15] with permission of Nature Publishing Group.

mass–balance disposition (wherein all inputs, outputs, and changes in total mass of the chemical are accounted for) in preselected anatomical compartments. BBDR models are dose–response models that are based on underlying biological processes. Once the target tissue is exposed to a local stressor, the cells respond and adapt, or undergo a toxic response; this process can be modeled with systems toxicology approaches. Finally, the disease outcome itself can be mimicked by genetic or chemically induced models of particular diseases. The colored boxes show the type of toxicologically relevant information that can be obtained from each set of models [15].

Figure 1.4 attempts to provide a framework for systems toxicology. This figure indicates the paths from the initial observation (rat in upper left) to an integrated toxicogenomics knowledgebase (blue cylinder), and so to systems toxicology (bottom right). The -omics data stream is shown by the clockwise path from rat to knowledgebase; and the "traditional" toxicology approach is shown in the anticlockwise path. The knowledgebase will integrate both data streams, along with literature-based knowledge; and by virtue of iterative modeling, will lead to a systems toxicology understanding. The framework involves "phenotypic anchoring" (to toxicological endpoints and study design information) and "sequence anchoring" (to genomes) of multidomain molecular expression datasets in the context of conventional indices of toxicology, and the iterative biological modeling of the resulting data [15].

Mathematical modeling in systems biology uses both bottom-up and top-down approaches to assemble information from all levels of biological pathways that coordinate physiological processes.

A top-down data driven approach integrates experimental data from various "omics" technologies. In a top-down approach, metabolic network reconstructions are performed using "omics" data (e.g., transcriptomics, proteomics) generated through DNA microarrays, RNA-Seq, or other modern high-throughput genomic techniques via appropriate statistical and bioinformatics methodologies. The top-down approach solves the problems through a large

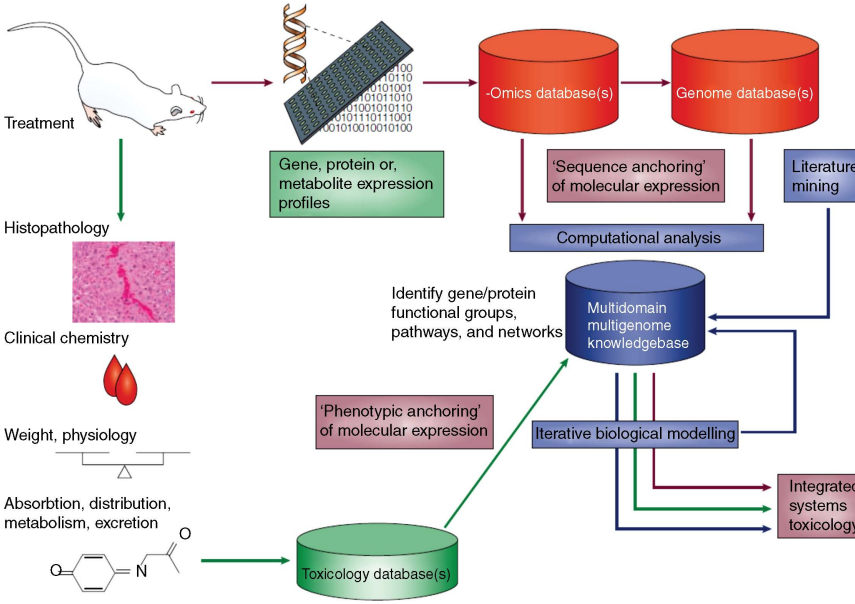

Figure 1.4 A framework for systems toxicology [15]. *Source:* Reproduced from Ref. [15] with permission of Nature Publishing Group.

number of entities. This approach does not emphasize the microscopic entities explicitly, but estimate their behavior at the macroscopic level, exemplified by ordinary differential equations (ODE) and partial differential equations (PDE). The ODE and PDE-based models are all population based, and the spatiality and topology that depend on individual interactions are, in general, ignored.

A model-based bottom-up approach depends upon a given model structure with kinetic parameters chosen such that an experimental observation can be reproduced quantitatively or qualitatively. A bottom-up approach typically encompasses draft reconstruction, manual curation, network reconstruction through mathematical methods, and validation of these models through literature analysis (i.e., bibliomics). The bottom-up approach is based on the synthesis of a complex from the activities on a lower system level; it emphasizes the microscopic level. This approach requires greater computational power in order to simulate a large number of significant entities in real world. From the model built by this approach, we can observe the interactions between entities specifically and study how they contribute to the emergence of global property. Cellular automata and (multi) agent-based methods are the most used bottom-up ones [127].

In order to ascertain the potential for exposures in early life to provide cancer-protective benefits across the life span, these two modeling approached must be unified in order to guide selection and assessment of plausible

protective interventions [125]. Mathematical models are frequently used to elucidate cellular design principles in order to understand complex biochemical networks preferably by using both approaches that can lead to a consistent description of cellular and molecular dynamics [128,129].

A third modeling theme in cancer that has its roots in developmental biology relates to stem cell division (proliferation) and whether cellular "bad luck" is primarily extrinsic or intrinsic in origin.

Recent research has highlighted a strong correlation between tissue-specific cancer risk and the lifetime number of tissue-specific stem cell divisions. Whether such correlation implies a high unavoidable intrinsic cancer risk has become a key public health debate with the dissemination of the "bad luck" hypothesis. Xu and Taylor [40] provide evidence that intrinsic risk factors contribute only modestly (less than ~10–30% of lifetime risk) to cancer development. First, they demonstrate that the correlation between stem cell division and cancer risk does not distinguish between the effects of intrinsic and extrinsic factors. They then show that intrinsic risk is better estimated by the lower bound risk controlling for total stem cell divisions. Finally, they show that the rates of endogenous mutation accumulation by intrinsic processes are not sufficient to account for the observed cancer risks. Collectively, they conclude that cancer risk is heavily influenced by extrinsic factors. These results are important for strategizing cancer prevention, research, and public health [40].

In contrast, Tomasetti and Vogelstein [130] point out that some tissue types give rise to human cancers millions of times more often than other tissue types. Although this has been recognized for more than a century, it has never been explained. They show that the lifetime risk of cancers of many different types is strongly correlated (0.81) with the total number of divisions of the normal self-renewing cells maintaining that tissue's homeostasis. These results suggest that only a third of the variation in cancer risk among tissues is attributable to environmental factors or inherited predispositions. The majority is due to "bad luck," that is, random mutations arising during DNA replication in normal, noncancerous stem cells. This is important not only for understanding the disease but also for designing strategies to limit the mortality it causes [130].

Our aim for the future is to promote the following objectives for future animal and human studies: (1) elucidate specific cellular and molecular targets of known toxicants; (2) design a systematic approach to the identification of mutagenic and developmental toxicants; (3) develop sensitive, specific, and predictive animal models, to include minimally invasive surrogate markers, and/or *in vitro* tests to assess function of cancer control systems during embryonic, postnatal, and adult life. While we will not be able to accomplish each of these objectives with our collective efforts on our previous book [131] or on this one, perhaps we can begin to lay the necessary foundations.

As for future protective developmental interventions, integrated testing strategies need to systematically account for the many mechanisms associated

with developmental events that occur *in vivo*. In order to apply the translational concept to mitigate environmentally induced toxicity, we are guided by the modest number of established and accepted therapeutics used primarily for fetal benefit and the limited number of dietary or supplemental interventions that have proven to be beneficial to or protective of the adult. These established or potential therapeutic interventions suggest that early steps in testing or implementing translational toxicology therapies during the *in utero* and early neonatal period will likely derive from GRAS options.

If we are to translate environmental health discoveries into safe and effective interventions, we must assert and characterize valid, applicable therapies, such as GRAS treatments, and eventually GRASE and other "ethical pharmaceuticals" for the protective care of highly vulnerable young patients. Since toxicology has repeatedly demonstrated that the fetus and child is more susceptible to adverse exposures than the adult, we believe we can create a safe and efficacious environmental health portfolio of interventional options to improve human health that include both reduction/avoidance of exposure and specific preventative/mitigative/restorative therapeutics.

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2

What Mutagenic Events Contribute to Human Cancer and Genetic Disease?

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2.1 Introduction

This chapter will lay the foundation for the reader's understanding of the translational toxicology of cancer and genetic disease resulting from mutations that can occur in somatic cells and in germ cells. As will be discussed later in the chapter, there are numerous data on mutation induction in somatic cells of rodents and humans and such mutations can lead to human cancer [1–5]; however, these data cannot readily be used to assess mutational risk of human germ cells because of the unique biological characteristics of human germ cells relative to those of other mammals or to somatic cells of either humans or other mammals [6–8].

In simplest terms, human genetic disease refers to disorders caused by mutations in one or more genes. Thousands of genetic disorders are known, and it is rare that any family is completely free of genetic disease, much less of cancer. In adults, 10% of hospital admissions are accounted for by genetic disease, and it is estimated that genetic defects are present in about 10% of all adults. Cancer is the most common genetic disease and it increases with age, driven by the accumulation of mutations over time.[1]

1 The online version of *Encyclopaedia Britannica* (https://www.britannica.com/) was an information resource for the topic of *human genetic disease* as discussed in Sections 2.1 and 2.2. The author of the 2015 *Encyclopaedia Britannica* section on "Human Genetic Disease" was Arthur Robinson. Irwin Fridovich was the author of a section on "Genetics of Cancer." The reader is referred to this resource for more comprehensive discussion of the topic.

Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer, First Edition. Edited by Michael D. Waters and Claude L. Hughes.

2.1.1 Childhood Cancer, Developmental Defects, and Adverse Reproductive Outcomes

It is good news that childhood cancers are relatively rare and many are curable; the risk of developing a cancer before age 20 is approximately 3 per 1000. The International Agency for Research on Cancer (IARC) has coordinated a worldwide study of the incidence of cancer in childhood. Contributors from over 50 countries have provided data on this topic in an IARC Technical Report [9]. A chapter in the report by Parkin [10] presents a summary of some of the major results. Childhood cancers are characterized according to 12 diagnostic groups, and incidence and frequency are defined primarily by tumor morphology. In this way it is possible to estimate risk of tumor types across different countries and ethnic groups, which can provide important information on the relative importance of genetic versus environmental factors in tumor etiology [11].

The bad news is that developmental defects and adverse human reproductive outcomes are not rare. About 3% of newborns in the United States have a clinically recognizable structural or genetic birth defect [12,13]. In developed countries worldwide, genetic defects are the major cause of failed pregnancies and nearly half of all miscarriages involve a fetus with an abnormal chromosomal complement. In tissues recovered from spontaneous abortions prior to the 13th week of gestation, there are chromosomal abnormalities in about half of abortions; this number declines to 5 per 1000 at term. During the first year of life, 20% of deaths are attributed to birth defects [14].

2.1.2 Newborn Screening for Genetic Disease

At birth, in most developed countries, newborns are screened for some of the more prevalent genetic diseases. Typically, a small blood sample is taken to test for metabolic disorders, the consequences of which can be prevented by appropriate interventions. However, even in the United States, the extent of mandated newborn screening varies from state to state with some states screening for as few as 3 conditions and others as many as 43. The federal Advisory Committee on Heritable Disorders and Genetic Diseases in Newborns and Children was established in 2003 with the goal of developing national policies and recommendations that should lead to more uniform and equitable newborn screening. In its report entitled "Newborn Screening: Toward a Uniform Screening Panel and System," the Health Resources and Services Administration (HRSA) identified 29 specific conditions that merit uniform and comprehensive screening [15]. Among the 29 conditions assigned to the core screening panel are three hemoglobinopathies associated with an Hb/S allele, six amino acidurias, five disorders of fatty oxidation, nine organic acidurias, and six unrelated conditions (congenital hypothyroidism, biotinidase deficiency, congenital adrenal hyperplasia, classical galactosemia, hearing loss, and cystic fibrosis).

2.1.3 Diagnosis of Genetic Disease

Diagnosis of genetic disease can be clinical, based on sets of symptoms that are well defined. Specific gene mutations can be diagnostic even if symptoms are not present. Frequently, multiple family members must be studied to complete a diagnosis and the findings may have relevance for the entire family. Treatment options frequently exist and some are as simple as removing exposure to constituents in the normal diet (e.g., phenylketonuria, PKU). At the other extreme, there may be no known treatment raising ethical issues surrounding termination of pregnancy and personal privacy. If such a situation is known to exist in a family and a couple wishes to have children, they may wish to participate in preconception counseling. Usually, a family history will be taken and used to construct a pedigree that can define whether and how a genetic disorder may be inherited; this will take place before a diagnosis is reached based on both the pedigree and the results of appropriate tests.

A medical geneticist can decide whether there is, in fact, a genetic component and calculate the risk of manifestation of the disease. The geneticist will determine whether inheritance (if any) is *single gene, chromosomal*, or *multifactorial*. If single-gene Mendelian inheritance is involved, the disease may be dominant, recessive, or sex linked. If the disease is autosomal and recessive, and a couple already has a child with the disease, they both are considered carriers and there is a risk that one-fourth of their future children will have the disease. If the disease is autosomal and dominant, and one parent is a carrier (whether symptomatic or not), there is a risk that half of their future children will inherit the mutation and may be affected. If neither parent carries an autosomal dominant mutation, but a child has been born with an autosomal dominant disease, the disease is presumed to have occurred via spontaneous mutation and the risk of future offspring of the noncarrier couple having the same disease is low. However, if a new mutation has occurred in the progenitor germ cells of one of the parents, an unknown proportion of parental sperm or eggs may carry the mutation. In this case, the sampling of blood is not likely to reveal what is referred to as *germ line mosaicism*. Risks of inheritance of X chromosome-linked diseases are more straightforward.

Genetic counseling for chromosomally inherited genetic disease typically involves a couple who has had a child with a chromosomal abnormality, either in *number* (e.g., monosomy, which is usually lethal, or trisomy) or in *structure* (e.g., deletions, duplications, translocations, or inversions) or a couple who has suffered multiple miscarriages. In either case, both parents are karyotyped and risk is assessed based on gain or loss of specific genetic material within both sets of chromosomes as well as other factors. If both parents display normal karyotypes, the risks of having additional children with a chromosomal disorder are typically low. However, it should be noted that karyotyping alone would not likely reveal gains or losses, unless they are extremely large. Chromosomal

microarray and or florescence *in situ* hybridization (FISH) would be needed to reveal most "submicroscopic" damage such as small deletions or duplications or copy number variants. Most inherited birth defects are not single gene or chromosomal but multifactorial. Based on population studies, if a couple already has one child with a multifactorial genetic disorder, the chance of having a second one with that disorder is about 3 in 100. The risk goes up to approximately 1 in 10 if they have had two affected children. Risks within specific families will vary from such population-based risk estimates. It should be noted that what has been described represents the simplest cases; the reality is that parental genotyping is not straightforward. Frequently, genotypes can only be approximated based on familial data. *Prior probability* based on Mendelian law (as described earlier) is combined with *conditional probability* based on family history and test results. With more complete data, the medical geneticist can provide the couple and the family with options based on available scientific evidence; that evidence usually comes from laboratory as well as from population studies in the literature.

We have previously mentioned the screening of newborns that can be mandated by law. The screening of the unborn fetus is quite another matter. There are societal fears that such information will be misused to intervene in the pregnancy; however, advanced knowledge of risks associated with certain genes may help physicians and parents understand a great deal about the health status of an unborn child. For this reason, relatively simple noninvasive prenatal tests, for example, involving ultrasound or maternal serum screening are usually offered to pregnant women. Maternal serum screening tests include, for example, the Triple Screen (performed ideally at 16–18 weeks) for levels of α-fetoprotein (AFP, produced by the fetus), β-human chorionic gonadotropin (hCG, produced within the placenta), and unconjugated estriol (uE3, produced by both the fetus and the placenta). High levels of AFP may indicate that the developing fetus has a neural tube defect (e.g., spina bifida or anencephaly). A more common reason for high levels of AFP is inaccurate dating of the pregnancy or a multiple pregnancy. Low levels of AFP and abnormal levels of hCG and uE3 may indicate that the developing fetus has Trisomy 21, Trisomy 18, or another chromosomal abnormality (see http://americanpregnancy.org/prenatal-testing/triple-screen-test/).

While the Triple Screen is not diagnostic and has a substantial false positive rate, it poses no risk to the mother or the fetus. Before invasive diagnostic tests are considered, a positive Triple Screen result should be followed by high definition ultrasound (particularly if a multiple pregnancy is suspected). Examples of invasive diagnostic tests include chorionic villus sampling (typically performed at ~10–11 weeks) or amniocentesis (typically performed at ~15–17 weeks). Invasive tests should be discussed thoroughly with all parties concerned as they entail definite risks and (as in the case of amniocentesis) may not yield results until 19 weeks or longer into the pregnancy. It should be noted that safer

diagnostic, but noninvasive, tests are under development, based, for example, on next-generation sequencing (NGS) of DNA and RNA from fetal cells isolated from the maternal circulation [16]. Next-generation sequencing is the latest major technological breakthrough in the continual search for more information about the human genome and DNA and RNA are rapidly becoming the prime targets of research for both mutation and gene expression analysis.

In the very near future, there will be a transition away from current methods because of major advantages in sample size requirements and availability of multiple sources of expendable tissues. As more genes are identified that pose a risk to human health, it will be possible to rapidly identify mutations that alter gene expression, which in turn can result in modification of proteins and metabolite profiles. Predictive genetic screening is rapidly becoming a reality and may be expected to rapidly change the practice of medicine, and especially that of medical genetics. Thus, even for large unphenotyped populations (e.g., in newborn screening), tests for metabolic disorders, now frequently based on measurements of protein or metabolites in blood samples, will be replaced by next-generation sequencing for mutations and gene expression, as well as by proteomics and metabolomics technologies.

The diversity of DNA sequence among individuals is thought to be the primary basis for differences in susceptibility to disease. Thus, the completion of the sequencing of the human genome and the subsequent resequencing of genomic segments among large numbers of individuals have led to rapid progress in understanding DNA sequence changes that underlie the rare, single-gene Mendelian disorders (http://www.ncbi.nlm.nih.gov/omim). However, common polygenic diseases have presented a greater challenge, and pathway approaches have been applied to study such diseases [8].

Genome-wide studies have provided valuable insights into the genetic basis of human disease, but they have explained relatively little of the heritability of most complex traits, and the variants identified through these studies have small effect sizes. This has led to the important and hotly debated issue of where the "missing heritability" of complex diseases might be found. In a "perspective" article, seven leading geneticists have discussed where this heritability may lie, what it could tell us about the underlying genetic architecture of common diseases, and how it could inform research strategies for uncovering genetic risk factors [17].

2.1.4 Familial and Sporadic Cancer

At the present time, unless there are symptoms or family history to suggest increased risk, adults are not tested for potential genetic disease. However, cancer is an exception and if close family members carry mutations, for example, associated with colorectal cancer or breast cancer, more frequent

surveillance via colonoscopies or mammograms, respectively, is recommended. Studies on specific cancer types have provided a great deal of our current understanding about predisposing mutations that confer susceptibility on individuals and subpopulations. While the popular belief is that "cancer runs in families," the fact is that most cancer cases are sporadic and not familial. In fact, 90% of cancers are sporadic and only about 10% are familial.

Some tissue types give rise to human cancers orders of magnitude more often than others. While this fact has been recognized for more than a century, it has not been explained. Tomasetti and Vogelstein [18] showed that the lifetime risk of cancers in various tissues is correlated with the total number of divisions of the normal stem cells maintaining that tissue's homeostasis. They suggested that only a third of the variation in cancer risk among tissues is attributable to environmental factors or inherited predispositions and that the other two thirds is simply due to "bad luck," that is, random mutations arising during DNA replication in normal, noncancerous stem cells. This assertion, important not only for understanding the disease but also for designing therapeutic strategies, has provoked a stream of scientific controversy. Recently, Wu *et al.* [19] provided evidence that intrinsic risk factors contribute only modestly to cancer development (less than ~10–30% of lifetime risk). They showed that the correlation between stem cell division and cancer risk does not distinguish between the effects of intrinsic and extrinsic factors and that intrinsic risk is better estimated by the lower bound risk controlling for total stem cell divisions. Finally, they demonstrated that the rates of endogenous mutation accumulation by intrinsic processes are not sufficient to account for the observed cancer risks and concluded that cancer risk is heavily influenced by extrinsic factors.

To gain an appreciation of sporadic versus familial influences on cancer, we will examine the etiology of three well-studied cancers: retinoblastoma, breast, and colon cancer. Retinoblastoma is a tumor of the eye that typically occurs in childhood. This very aggressive cancer clearly reflects both inherited germ line mutations and acquired somatic mutations. Current data suggest that 30–40% of all cases of retinoblastoma are inherited. The RB gene encodes a protein that normally functions as a tumor suppressor gene. If a child inherits one mutant copy of the RB gene, there is nearly a 100% chance of developing bilateral retinoblastoma because every retinal cell is subject to additional acquired mutations. This represents the "two-hit" hypothesis of retinoblastoma induction as well as the foundation of the two-hit theory on the genetic origins of familial cancer. Despite this argument, retinoblastoma is predominantly sporadic in nature as are our two additional examples, breast and colon cancer, although there is a clear familial component to each of these cancer types.

The onset of familial breast cancer is often before age 40, while sporadic breast cancer typically occurs later in life. In the case of familial breast cancer, inherited mutations in BRCA1 or BRCA2 are responsible for at least 50% of the cancers observed. In the general population, a woman has a 10% lifetime breast

cancer risk, but about half of women with mutations in BRCA1 or BRCA2 will develop breast cancer by age 50, and nearly 90% will develop breast cancer by age 80. BRCA1 mutations also confer a greater risk of developing ovarian tumors. Both men and women can transmit the BRCA1 or BRCA2 mutations to progeny, but females are much more likely to develop the disease.

Just as with breast cancer, familial colorectal cancer has been associated with mutations in genes that predispose to the disease. There are actually two forms of familial colorectal cancer: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch Syndrome). Both are autosomal dominant genetic disorders characterized by early onset. Individuals with FAP carry mutations in the adenomatous polyposis coli (APC) gene that codes for the APC tumor suppressor protein, while persons with Lynch Syndrome have mutations in DNA repair genes. Some individuals carry both types of mutations, and with the probability of acquiring additional somatic mutations, they have nearly a 100% chance of developing the disease over their lifetime.

New research indicates that there are at least three pathways involved with colorectal cancer initiation and development: chromosomal instability, microsatellite instability, and CpG methylator phenotype. Each pathway is discussed in detail in Ref. [20] as is new research indicating the importance of inflammation and microRNAs in colorectal carcinogenesis.

Perhaps more interestingly from the perspective of susceptibility, as illustrated in Table 2.1, multiple molecular markers have been identified that have implications for the progression of the disease in newly diagnosed patients [20].

In summary, inherited mutations and the accumulation over time of somatic mutations in relevant genes can act together to influence normal cell growth, initiate tumorigenesis, modify the tumor's blood supply, and facilitate metastasis (as illustrated in Table 2.1). These topics will be discussed later in Chapter 14.

2.2 Genetic Damage from Environmental Agents

In the modern world, we are constantly exposed to agents that may cause genetic damage. Ultraviolet and ionizing radiation, combustion emissions, industrial and household chemicals, drugs, and even food additives come to mind. In addition, there are viruses, fungi, bacteria, and plants that may cause genetic disease and/or promote cancer. Personal habits such as smoking or chewing tobacco, drinking alcoholic beverages, and the like also cause reproductive damage and cancer. Scientists and health practitioners tend to divide these agents into avoidable and unavoidable exposures, but they also recognize that continuously occurring endogenous DNA damage from the normal biological processes in healthy living cells is actually greater than exogenously

Table 2.1 Molecular markers and implications for disease behavior.

Gene	Effect on disease	References
CDK8 overexpression	Poor prognosis	[21]
K-ras cod. 12 mutation	Metastatic disease, poor prognosis, increased cancer-specific mortality	[22,23]
p-AMPK	Better survival among *p*-ERK positive	[24]
p53 expression	Better survival among nonobese	[25,26]
p21 loss	Better survival for patients >60 years	[27]
COX-2-positive tumors	Increased cancer-specific mortality	[26]
18q	Loss in non-MSI → decreased survival No loss → 5 year survival 96%	[28,29]
PI3KCA mutations	Increased survival among chronic aspirin users	[30]
Line-1 hypomethylation	Young age of onset and increased cancer and overall mortality	[31,32]
HIF1	High colorectal cancer-specific mortality	[33]
Cathepsin B expression	High colorectal cancer and overall mortality	[34]
MSI	Better prognosis and survival than CIN/MSS	[35–37]
Cyclin D1 overexpression	Low colon cancer and overall mortality	[38]
BRAF V600E	High cancer-specific mortality	[36]
CIMP-high	Low colon cancer-specific mortality	[36]
miR-203	Poor survival among Caucasians with stage IV and poor survival in blacks with stages I and II CRC	[39]
miR-21	Poor prognosis in patients with stage IV CRC	[39]
sTNFR-2 expression	Increased risk of CRC development, lower risk among those taking aspirin	[40]
Interleukin-6	Increased risk of CRC development, advanced CRC stage, and a worse prognosis	[41–43]
C-reactive protein	Association with increased risk of colorectal cancer, in particular in lean individuals	[44,45]

Source: Reproduced from Ref. [20] with permission of authors and publisher (http://creativecommons.org/licenses/by/4.0/).

induced environmental damage. Thus, most newly induced mutations arise as a result of endogenous rather than exogenous damage and "being alive is highly mutagenic."

Fortunately, living organisms have evolved defenses against many of the unavoidable exposures. Among these defenses is our ability to repair damaged DNA. To briefly pursue this example with our pervasive exposure to sunlight, UVB radiation penetrates and excites DNA in skin cells producing covalent bonds between adjacent pyrimidines resulting in the formation of pyrimidine dimers. Most of these dimers can be removed by nucleotide excision repair [46]. Those that escape this repair can cause mutations through errors in replication, or with sufficient damage they may induce programmed cell death (apoptosis).

Melanin from melanocytes is an additional bodily defense against sunlight, and the vehicle for tanning. Its photochemical properties dissipate the energy of the excited DNA. Melanocytes are found between the dermis and epidermis. When initiated, the uncontrolled growth of melanocytes can lead to melanoma, a deadly cancer that with adequate blood supply rapidly grows and spreads. Men are particularly susceptible to melanoma on their backs and women on their legs. Several predisposing genes have been found in families.

In some cases, the body's defenses are inadequate, especially if exposures are excessive. Natural toxins that are present in our food supply are a case in point. *Aspergillus flavus* is a fungus that produces aflatoxins, including aflatoxin B1, an extremely potent liver carcinogen. Levels of aflatoxin in the western diet are well controlled, but in Africa and parts of Asia this is not the case and liver cancer is prevalent.

Cancer-causing viruses are a particular scourge to human health. Common ones are hepatitis virus B and C, human papilloma virus, and herpes virus. Liver cancer is frequently associated with hepatitis B virus infection and exposure to aflatoxin. Viruses inject their genetic material into host cells and replicate themselves. If viral insertion occurs within the regulatory sequence or protein coding region of a gene that controls cell growth or division, or if the virus replicates and kills a sufficient number of cells, the infection can lead to cancer by enhancing replication and increasing the probability of mutation. Viruses also often carry oncogenes that do not require a host gene to be inactivated for cancer initiation (e.g., Rouss sarcoma in chickens).

For many of us, environmental chemicals and drugs are a source of major concern in human exposure scenarios. Literally thousands of industrial and household chemicals enter our indoor and outdoor environments, and we are exposed to low levels daily. These represent involuntary exposures; however, we voluntarily expose ourselves to known human carcinogens in consuming alcoholic beverages, smoking. and/or chewing tobacco – even smokeless tobacco. Some cultures in South Asia chew betel quid (areca nut wrapped in betel leaves) – a practice that induces oral and esophageal cancer among other maladies.

According to the International Agency for Research on Cancer (IARC), there are currently 118 known human carcinogens (http://monographs.iarc.fr/ENG/Classification/). In addition, there are 75 agents that are probable human carcinogens and 288 agents that are possible human carcinogens. Clearly, humans are typically exposed to a complex mixture of agents, and in order to delineate the relative contribution of the chemical components in a mixture, they must be separated and chemically characterized. Let us examine two of the more pervasive complex mixtures of mutagens and carcinogens, combustion emissions (from fossil fuels) and cigarette smoke (in all its forms), and ask the question whether these and/or other more specific agents pose a risk to human somatic cells or human germ cells.

Combustion emissions resulting from the burning of fossil fuels, in generating electricity, in heating our homes, or in powering our vehicles, represent a substantial contribution to human environmental exposure. These emissions include both particulates and products of incomplete combustion that represent the original starting materials. Coal and crude oil combustion yields carbon, sulfur, lead, mercury, and other elements. Depending on the origin of the oil or coal, different constituents in emissions may be elevated (e.g., vanadium in oil or sulfur in coal). Fossil fuels can be refined to reduce unwanted constituents, and this has been important in the development of cleaner industries and engine technologies. Even so, oxidized sulfur and nitrogen, elemental products, and volatile organic carbon products (VOCs) are mutagenic, carcinogenic, and otherwise hazardous to human health.

That urban/industrial air pollution contains a plethora of animal and human mutagens and carcinogens is well known. Thus, in 2002, Somers *et al.* [47] demonstrated that in laboratory mice exposed *in situ* to ambient air in a polluted industrial area near steel mills, heritable mutation frequency at tandem repeat DNA loci was elevated 1.5–2.0-fold compared with mice at a reference site 30 km away. This statistically significant elevation was primarily due to an increase in mutations inherited through the paternal germ line. In 2004, Somers *et al.* [48] showed that high-efficiency particulate air (HEPA) filtration of ambient air significantly reduced the heritable mutation rates at repetitive DNA loci in laboratory mice housed outdoors near a major highway and two integrated steel mills. These findings implicated exposure to airborne particulate matter as a principal factor contributing to the elevated mutation rates observed and added to the accumulating evidence that air pollution may pose genetic risks to humans and wildlife. Then, in 2008, Yauk *et al.* [49] showed that particulate air pollution in an urban/industrial setting caused a multiplicity of effects, including germ line mutations, DNA damage, and global DNA hypermethylation in mice exposed *in situ*.

Tobacco smoke (even tobacco vapor) and all tobacco products are human carcinogens. Volatile vapors, nonvolatile compounds, and fine particles are deposited directly into the airways and the pulmonary alveoli. The Food and

Drug Administration (FDA) has listed 93 harmful and potentially harmful constituents (HPHCs) of tobacco products and tobacco smoke (*Federal Register*, **77** (64), 2012). These constituents account for much of the carcinogenicity and toxicity that are observed in smokers. Other risk factors associated with smoking include hypertension, stroke, atherosclerosis, and myocardial infarction. Smoking also affects reproductive health, causing delay in conception, low birth weight, and advanced menopause [50]. Marchetti *et al.* have shown that side stream tobacco smoke induces mutations at an expanded simple repeat locus (Ms6-hm) in mouse sperm [51]. The relationship between noncoding tandem repeat instability and mutations in functional genes is unclear, but it is likely associated with male reproductive dysfunction. There are approximately 50 known rodent germ cell mutagens [52]; but, as yet, there is no definitive evidence of environmentally induced mutations in the human germ line. However, it is simply a matter of time as technology now exists to demonstrate such effects as will be discussed below.

2.3 Testing for Mutagenicity and Carcinogenicity

The field of science directly concerned with genetic disease and cancer is genetic toxicology. Mutations and genetic toxicity (genotoxicity to DNA) of all types, however caused, are within the purview of the field. Historically, the field of environmental mutagenesis (generally considered synonymous with genetic toxicology) was focused almost exclusively on induced mutations in the germ line (germ cells). Protagonists in the late 1960s and early 1970s spoke of an accumulating "mutagen burden" as a result of human exposure to environmental mutagens. While both somatic and germ cell mutations were studied, it was thought that mutations within the germ line were of far more concern [8,53]. When the premise that the "carcinogens are mutagens" was espoused by Bruce Ames and colleagues in 1973, and when somatic mutagens could readily be detected in short-term tests *in vitro*, practitioners of genetic toxicology altered their focus to develop predictive tests for carcinogenicity.

The fact that it is extremely expensive to test for carcinogens *in vivo*, requiring up to 2-year (lifetime) exposures in large numbers of experimental animals, led to a glut of newly developed short-term mutagenicity and genotoxicity tests in the 1970s and 1980s. The most prominent among these tests was developed by Ames *et al.* [54–56]. The Ames test is based on the use of mutant strains of the bacterium *Salmonella typhimurium*, with microsomes and cofactors added for metabolic activation, and can be performed in a matter of days.

Although the Ames test has been extremely successful, its utility has been largely limited to the detection of point mutations and multilocus deletions. Bacteria have circular chromosomes with a different organization than

eukaryotic chromosomes. Thus, although they have been used to study the formation of structural abnormalities, they cannot be used to identify complex forms of eukaryotic chromosomal damage, including gain or loss of whole chromosomes (aneuploidy), as will be discussed. For this reason, yeast, *Caenorhabditis elegans*, fruit flies, mammalian cells in culture, and laboratory animals have been used to screen for the broad range of genotoxicity, including chromosomal damage and aneuploidy. Short-term mammalian model systems can screen for genotoxicants, mutagens, and carcinogens within a matter of days to weeks. They are typically used in a battery of tests that include the Ames test. Because of the efficiency of these tests, the number of known mutagens now exceeds the number of known carcinogens by orders of magnitude.

An axiom of carcinogenesis is that both mutation and proliferation are required for cancer induction. *In vivo* testing for carcinogenicity is usually performed using both sexes of mice and rats (the US National Toxicology Program uses B6C3F1 mice and Fisher 344 rats). Following a 2-year exposure to a test agent, upward of 40 tissues per animal may be examined by trained veterinary pathologists for tumor induction. While originally considered a screening test, because of the costs and labor involved, only about 1500 chemicals have been studied in the rodent cancer bioassay [57]. As of 2007, the NCI/NTP Carcinogen Bioassay Program had tested 1547 chemicals, including about 560 tested by the National Toxicology Program [58,59]. In 2015, there were 582 NTP Technical Reports available on the bioassay of various agents [60], so the total of chemicals tested in the rodent cancer bioassay is still fewer than 1600.

It is well known that laboratory animal tests for carcinogenicity are not completely predictive of human carcinogenicity because animals and humans metabolize chemicals differently and have different repair capacities, and because chemicals are typically tested in experimental animals up to the maximum tolerated dose (MTD). At such high doses, cytotoxicity may kill cells directly, or may induce programmed cell death concomitant with enhanced regenerative proliferation, thereby increasing the opportunity for mutations in surviving cells. Fewer than 25% of known animal carcinogens are known human carcinogens even though most human carcinogens (with the exception of certain hormonal and immunosuppressive agents) have been demonstrated to be mutagenic in animal or human cells in culture [61,62].

EPA's Gene-Tox Program [63–67] defined the basic protocols and performance characteristics of the most useful short-term tests of the 1960s, 1970s, and 1980s, and began the process of gradually reducing the number of required screening tests to the handful still in use today. As discussed above, the Ames test was validated as a predictor of carcinogenicity [68,69]. However, it was soon learned that the test could not be used blindly, because the standard tester strains did not respond positively to carcinogenic inorganic metals or certain halogenated organics, and displayed poor specificity for nitrogen- and sulfur-

containing organics [70]. Furthermore, as carcinogenicity testing in animals proceeded, there was a gradual recognition of nongenotoxic (Ames negative) mechanisms of carcinogenesis [62]. This finding challenged the conventional interpretation of rodent carcinogenicity studies as well as the results of mutagenicity tests in terms of their role as predictors of human cancer [71].

Over the years, we have learned that of all compounds tested in rodents, about half are carcinogenic [72], and roughly half of these are putatively nongenotoxic [73]. Rat liver appears particularly sensitive to carcinogens that act via nongenotoxic mechanisms and has been studied extensively for this reason. It is clear, however, that both genotoxic and nongenotoxic chemicals induce cancer in a variety of target sites in rodents – the eight most frequent sites in both species being liver, lung, mammary gland, stomach, vascular system, kidney, hematopoietic system, and urinary bladder. There are species differences in carcinogenicity for certain chemicals in the liver, Zymbal's gland, and kidney [74]. However, there are no consistent differences in tissue distribution or pharmacokinetics between genotoxic and nongenotoxic agents, nor support for the idea that these two categories of agents induce tumors in different target organs [74].

2.4 Predictive Toxicogenomics for Carcinogenicity

Toxicogenomics (TGx) is a term for the combined technologies of transcriptomics, proteomics, metabolomics, and, more recently, epigenomics – tools used in the field of toxicology to study the expression of genes, proteins, metabolites, and epigenetic modifications, respectively. Gene expression profiling or transcriptomics measures the relative abundance of potentially thousands of RNA transcripts present in a sample. Typically performed using microarray technology (and, more recently, next-generation sequencing), each expression profile represents one tissue extract at one point in time for a single dose of a chemical. These expression profiles are interpreted collectively, with reference to tissue extracts from control animals, also studied as a function of dose and time, and compared with "training set" chemicals with similar modes of action.[2]

The toxicogenomics investigations *in vivo* carried out over a decade and reviewed in Waters *et al.* [61] have identified cancer-relevant gene signatures or biomarkers that discriminate among direct and indirect genotoxic carcinogens, nongenotoxic carcinogens, and noncarcinogens. The preponderance of

2 For an extensive discussion of the topic, please refer to *Toxicogenomics in Predictive Carcinogenicity*, Issues in Toxicology Series No. 28, ed. Michael D. Waters and Russell S. Thomas, Royal Society of Chemistry, Cambridge, UK, 2016, ISBN: 978-1-78262-162-1, EPUB eISBN: 978-1-78262-819-4, ISSN: 1757-7179, doi.org/10.1039/9781782624059, 503 pages.

accumulated evidence suggests that gene expression profiles reflect underlying modes or mechanisms of action and are therefore useful in predicting chemical carcinogenicity in rodents, especially in conjunction with conventional short-term tests for gene mutation and other forms of DNA damage [75].

Ellinger-Ziegelbauer *et al.*, in a series of studies [71,76,77], showed that a strong DNA damage response at the gene expression level suggests direct DNA modification, whereas increased expression of genes involved in cell cycle progression is more characteristic of indirect-acting agents.

Metabolism genes are prominently represented among gene expression signatures that discriminate various nongenotoxic modes of action: cytotoxicity and regenerative proliferation, xenobiotic receptor agonists, peroxisome-proliferator-activated receptors, or hormone-mediated processes [78,79]. Some modes of action of nongenotoxic carcinogenicity, such as the induction of oxidative stress, exhibit definitive signatures as early as 24 h following single dosing in animals [78–80]. But because there are multiple modes of action in the case of nongenotoxic carcinogens, noncarcinogens cannot be distinguished from them without time-consuming efforts to clarify what combinations of expression profiles relate to specific pathophysiological processes of carcinogenesis.

The majority of *in vivo* studies reviewed in Waters *et al.* [61] were in liver, with the notable exception of studies by Thomas *et al.* [57,81] in mouse lung. It is important to extend these investigations to other target organs and to identify within these organs the target cell populations from which tumors develop. For TGx studies to be broadly predictive, *in vivo* studies need to be performed simultaneously in several relevant metabolically active target organs. In such studies, it is important to distinguish between a tissue carcinogen and a tissue toxin since not all hepatotoxicants cause liver cancer.

Only five target tissues (liver, lung, mammary gland, kidney, and hematopoietic system) account for the positive responses of about 50% of the chemicals identified by the NTP as carcinogens [57]. Developing gene expression biomarkers for each of these tumor sites in mice and rats should provide an efficient means to prioritize chemicals for further testing. In the longer term, it may be useful to develop biomarkers for all 24 main cancer target tissues, as this may facilitate eventual replacement of the rodent cancer bioassay [57]. TGx methods may also be useful to better understand the mechanistic basis for species differences in target organs between rats and mice [74].

Applying the TGx approach in the preclinical phase of drug and chemical development could help discriminate compounds likely to be human carcinogens [57]. This could provide an assessment of product safety earlier in the development pipeline, leading to substantial monetary savings and reduced time to market. Indeed, the prevalence of potential nongenotoxic carcinogens in the drug development pipeline has been one of the primary drivers for the pharmaceutical industry to develop TGx approaches for predictive carcinogenicity. The work of Fielden *et al.* [78] and Nie *et al.* [79] suggest that

transcription profiling in appropriate target organs *in vivo* after short-term treatment (up to 14 days) has the potential to predict putative non-DNA-reactive mechanisms. Furthermore, it may be possible to use TGx methods to exclude DNA-reactive mechanisms for compounds for which positive results are observed only at high concentrations in *in vitro* gene mutation or chromosome damage assays. When such predictive approaches are combined with standardized test procedures in prospective interlaboratory validation studies, their accuracy and potential utility in carcinogenicity evaluation can be enhanced [82,83].

Commercial and industrial chemicals typically are not required to be tested for carcinogenicity unless evidence for adverse health effects is otherwise obtained. For those chemicals that do require further testing, TGx approaches would seem particularly valuable when used together with range-finding toxicity (14-day and 90-day) studies, as currently performed in conjunction with the rodent carcinogenicity bioassay.

As with conventional methodology, *in vitro* toxicogenomic approaches have major utility. A multilaboratory project coordinated by the Health and Environmental Sciences Institute (HESI) Committee on the Application of Genomics in Mechanism-Based Risk Assessment evaluated gene expression profiles of TK6 cells treated with model genotoxic agents using a targeted high-density RT-PCR approach [84]. The reproducibility of data across collaborating laboratories indicated that expression analysis of a relevant gene set is capable of distinguishing compounds that cause DNA adducts or double-strand breaks from those that interfere with mitotic spindle function or that cause chromosome damage as a consequence of cytotoxicity. This study therefore adds to the increasing body of evidence indicating that TGx analysis of cellular stress responses provides valuable insight into mechanisms of action of genotoxicants [71].

Relevance to human health is obviously the key issue for the future of predictive TGx studies. Chemicals that are both rodent and human carcinogens could be studied to identify biomarkers with more direct relevance to human health [57]. Compounds that do not produce positive test results in conventional genotoxicity assays and that do not exhibit biomarkers of genotoxicity in TGx methods are very unlikely to pose a genotoxic carcinogenic risk to humans. The same cannot be said for putative nongenotoxic carcinogens that are identified through the use of TGx methods. However, it should be possible in such cases to use TGx methods to characterize their likely modes of action by comparison with previously well-studied chemicals, as demonstrated by Fielden *et al.* [78,85] and Uehara *et al.* [80,86], and, with more experience, to predict relevance to humans.

The potential of omics technologies to explore transcriptional regulation (including epigenetics and microRNA) as well as downstream events (proteomics and metabolomics) in evaluating mechanisms of genotoxicity and carcinogenicity must also be investigated [87,88]. No single organization has the

resources to accomplish all of this independently. Therefore, collaborative efforts that include scientists from academia, industry, and regulatory agencies, such as the HESI Genomics Committee, the Critical Path Initiative in the United States, and the Innovative Medicines Initiative in Europe, are essential for developing standardized testing protocols and critically needed reference data [71]. If the TGx approach proves to be more broadly applicable through such efforts, it has the potential to become an efficient and economical alternative to the rodent cancer bioassay, potentially reducing the use of experimental animals while increasing the efficiency of predictive carcinogenicity testing.

2.5 Germ Line Mutagenicity and Screening Tests

As we have discussed earlier, Ames' studies indicating that "carcinogens are mutagens" [54] caused genetic toxicologists to turn from studies on germ cells to somatic mutation and cancer. As we shall see, even with the evolution of molecular genetics and next-generation sequencing, the field has been slow to return to its roots to apply these new tools to study germ line mutagenicity [8]. However, the field is clearly changing, and the critical need to refocus our testing efforts is immediately apparent. In this regard, the reader is referred to the International Workshops on Genotoxicity Testing (IWGT) Working Group report on "Approaches for Identifying Germ Cell Mutagens" [89] as an authoritative resource for further information on germ line mutagenicity and screening tests.

There are unique features that differentiate germ cells from diploid somatic cells: (i) Germ cells are haploid and meiosis only occurs in the germ line. (ii) They have a distinctive chromatin structure. (iii) Development and differentiation are prolonged. (iv) Eggs are arrested in meiosis prophase 1 from birth until puberty, and complete meiosis only after fertilization. (v) Major morphological changes occur in male germ cells, for example, as related to sperm motility. (vi) Sperm histones are replaced first by transition proteins and later by histones. (vii) Sperm are DNA repair deficient in the final haploid stages and egg DNA repair machinery takes over to repair damage sustained in the late-stage non-DNA repair proficient spermatids. (viii) There are sex-specific epigenetic features found only in the progenitor cells and early embryo. Therefore, there are agent- and sex-specific mechanistic effects related to embryogenesis and development in female versus male germ cells that do not occur in somatic cells *in vivo* or in cultured cells. Furthermore, evidence of chemically induced mutations in the germ line of rodents is not supported by human studies, raising question as to whether results of rodent studies can be extrapolated to humans. The fact is that conventional rodent germ line tests monitor a limited range of potential genetic damage, even in rodents. In addition, recent

investigations applying genomic technologies have shown critical genetic changes that cannot be observed by conventional methods [89].

While our knowledge is limited, the critical assumption has been made that testing for mutagenicity in somatic cells is adequate to protect the germinal tissues from similar exposures [90,91]. This assumption related to rodent model systems needs to be more rigorously tested than it has been in the past [92], especially since more recent test methods have demonstrated exceptions to the assumption [7,51], and many limitations are recognized in conventional rodent germ cell test methods. In some instances, human studies have led the way to the future of mutagenicity testing in germ cells as will be discussed.

The International Programme on Chemical Safety (IPCS) has developed a harmonized scheme for mutagenicity testing that states: "For substances that give positive results for mutagenic effects in somatic cells *in vivo*, their potential to affect germ cells should be considered. If there is toxicokinetic or toxicodynamic evidence that germ cells are actually exposed to the somatic mutagen or its bioactive metabolites, it is reasonable to assume that the substance may also pose a mutagenic hazard to germ cells and thus a risk to future generations" [75].

In order to address the assumption directly, it is critical that appropriate toxicokinetic or toxicodynamic data on chemical exposures as well as more appropriate germ cell tests be developed and applied. Some progress in test methods includes (i) transgenic rodent mutation assays (OECD guideline TG488) with recommendations for male germ cell mutation analysis); (ii) sperm and pedigree tandem repeat mutation analysis [93]; (iii) improved methods to quantify sperm DNA damage and chromatin effects [94]; and (iv) high-throughput screening (HTS) for aneuploidy in *C. elegans* eggs [95].

Human epidemiological studies and test results from modern assays provide support for the concern that rodent germ cell mutagens (e.g., paternal age, ionizing radiation, cigarette smoke, chemotherapeutic agents) are in fact human germ cell mutagens [96–102]. Human germ line mutagens are of great concern because of the fact that a single *de novo* mutation can potentially cause multiple disease phenotypes [103–107]. A given human genome contains around 100 loss-of-function variants, with as many as 20 of these resulting in complete loss of gene function [108].

Disease-associated *de novo* gene mutations occurring in the male germ line with increasing paternal age are considered equal in importance to the population burden of aneuploidy-related genetic disease associated with increasing maternal age [109]. Therefore, it is critical that tests be developed that are able to detect the full range of DNA and chromosomal events that may occur in germ cells, and potentially be transmitted to future generations. This includes premutational lesions transmitted by sperm to the ovum at fertilization that, if not repaired or misrepaired, can lead to *de novo* mutations. Endpoints of concern in this regard include gene mutations, chromosomal aberrations and aneuploidy, copy number variants, tandem repeat mutations, single-nucleotide

variants and deletions or insertions, and mutations in noncoding DNA. Mutations in noncoding DNA have been recognized recently for their importance to normal biological function [110,111].

Although male germ cells have been the focus of conventional assays, principally because of accessibility, female germ cell assays represent a major gap in testing capability. There are major differences between the sexes in competence within the various germ cell stages. This holds for both checkpoint control of the cell cycle and DNA repair, both related to the ability of environmental agents to induce heritable mutations. Male germ cells appear to have a more efficient meiotic checkpoint than female cells but male cells are repair deficient in postmeiotic stages when sperm chromatin condenses. Female germ cells, on the other hand, use stored mRNAs to retain the capacity for DNA repair until after fertilization [8].

The gold standard conventional germ cell assays, the heritable translocation test (HTT), and the specific locus test (SLT) are performed in the mouse. In the HTT, males are treated to induce chromosomal rearrangements (translocations) that cause sterility or semisterility in the F1 generation, thus demonstrating heritability (OECD Test Guideline 485) [112]. The SLT detects viable null mutations (at seven specific loci) ranging from base substitutions to large deletions [113,114]. Both tests measure genetic damage (of the types seen in human genetic disease) that is transmitted from treated parents through the germ line to the next generation. For practical reasons, including cost, extensive use of animals, and human labor, neither assay is performed any longer.

While it does not measure heritability, the dominant lethal test (DLT) is a routinely performed OECD guideline test [115,116] that measures genetic damage in germ cells sufficient to cause embryonic death. Following exposure of either rats or mice, usually males, a mating to virgin females is performed sequentially (usually every week) for a total of 10 weeks (rats) or 8 weeks (mice). An alternative protocol involves treating males throughout their spermatogenic cycle followed by mating at the end of exposure [117]. The contents of the uteri of pregnant females are examined after appropriate intervals to count live and dead embryos as well as total implants. The numbers of these events are compared (per female) between treated and control groups to determine the dominant lethal outcome. Chromosomal damage is thought to be the cause of preimplantation loss or embryonic death [118,119] but other causes (e.g., gene mutation, cytotoxicity, or teratogenicity) cannot be excluded. The test has not changed significantly since 1984 and it is still in use [120]. Chemicals that are positive in the DLT are also positive in the HTT that does measure a heritable effect [115,116]. Furthermore, the lowest effective doses that cause positive responses in the two tests are quite comparable; regression analysis for 15 mutagens tested in both DLT and HTT gave the following results: $r^2 = 0.92$; slope = 0.98; Y intercept = 0.05; $N = 15$ [92].

Another current test for chromosomal aberrations in male germ cells of mice and rats is the cytogenetic analysis of spermatogonial metaphases OECD TG 483 [121]. With this test, chromosomal effects can only be observed at the beginning of germ cell differentiation, so it is not known whether they are transmitted to mature gametes or offspring. It is possible to determine transmission by additional cytogenetic analysis of first cleavage zygote metaphases [122] and chromosome painting has greatly improved the analysis [123], such that stable balanced aberrations (e.g., reciprocal translocations) and unstable aberrations (e.g., acentric fragments, dicentric chromosomes) can be distinguished. Evidence has been gained to support assumptions about the fate of different types of chromosome aberrations by examining the zygote for chemicals tested in common between the DLT and HTT [119].

The transgenic mutation assay in rodents OECD TG 488 [124] is based on the detection of a mutation in a transgenic sequence that can be retrieved from any tissue (somatic cells in the standard assay) and subsequently expressed in bacteria [125,126]. When the assay is applied in the analysis of testicular cells and epididymal sperm, it provides a method to detect gene mutations in male germ cells. Importantly, the entire mutation spectrum (base substitutions, insertions/deletions, frameshifts) following chemical exposure can be monitored. If testicular tissues are examined as a part of the standard assay, protocol information on mutations in germ cells and somatic cells can be collected simultaneously reducing cost, animals, and time [127]. Recent studies have shown the utility of the transgenic mutation assay to assess the response of male germ cells to acute versus chronic exposures [128] and the sensitivities of the various stages of mouse spermatogenesis to mutagenicity [129].

Next-generation sequencing can readily be applied in conjunction with transgenic assays in rodents. In a very recent study, male gpt delta transgenic mice were treated with ENU in three dose groups (10, 30, and 85 mg/kg, i.p.), were mated with untreated females 10 weeks after the last treatment, and offspring were obtained [130]. The ENU-treated male mice showed dose-dependent increases in gpt mutant frequencies in their sperm, testis, and liver. Frequencies of inherited mutations increased with dosage more than 25-fold in the highest dose group. Genomic DNA of one family (parents and four offspring) from each dose group was used for whole exome sequencing, and unique *de novo* mutations in the offspring were detected. The mutation spectrum of the inherited mutations was characteristic of ENU-induced mutations (e.g., including A:T base substitutions) and no mutations were observed in the control group. The results, confirmed by Sanger sequencing, suggest that direct sequencing analysis may be a useful tool to investigate inherited germ line mutations, especially when applied in transgenic models.

Premutational and mutational changes detectable in sperm include DNA strand breaks and abasic sites in the comet assay [131], unscheduled DNA

synthesis (UDS) [132], chromatin packaging alterations in the Sperm Chromatin Structure Assay (SCSA) [133], and chromosomal effects using Fluorescent In Situ Hybridization (FISH) [134]. These tests can be applied in both laboratory rodents and humans, and can serve as prescreening tools for germ cell damage, although they do not assess heritable effects. The comet assay and the SCSA are already being applied in the clinical diagnosis of male infertility.

2.6 Reproductive Toxicology Assays in the Assessment of Heritable Effects

Conventional reproductive toxicology assays provide valuable information (often not considered) on reproductive effects, including the accessibility and toxicity of genotoxic chemicals and drugs to male and female germ cells.

2.6.1 Segmented Reproductive Toxicity Study Designs

There are two basic types of reproductive toxicity study designs, segmented studies and continuous studies. Whereas continuous cycle designs cover all stages from germ cell through fetal development and adulthood, segmented studies expose and evaluate limited aspects of development.

The OECD, FDA, and ICH have described protocols for reproductive and developmental studies according to the following nomenclature (http://www.toxikon.com/services/specialty-reproductive-toxicology.cfm):

Organization for Economic Cooperation and Development – OECD

414 – Prenatal developmental toxicity
415 – One generation reproduction toxicity
416 – Two generation reproduction toxicity
421 – Reproduction/developmental toxicity screening
422 – Combined repeat dose toxicity with repro screening test

Food and Drug Administration – FDA

Segment I – Reproduction toxicity
Segment II – Teratology in rats
Segment II – Teratology in rabbits
Segment III – Perinatal toxicity

International Conference on Harmonization – ICH

Stage A – Premating to conception
Stage B – Conception to implantation
Stage C – Implantation to closure of hard palate
Stage D – Closure of the hard palate to the end of pregnancy
Stage E – Postnatal developing to weaning
Stage F – Postweaning development of reproduction organs to puberty

For example, exposure of the fetus may be examined separately from postnatal stages and other critical developmental periods, as illustrated above, using different exposure and assessment windows. The International Conference on Harmonization (ICH) guideline [135] for reproductive toxicity Segment I studies begins exposure 4 weeks prior to mating in males, or 2 weeks in females, and continues from fertilization through to implantation. The OECD prenatal developmental toxicity study (OECD TG 414 [136) involves exposure from implantation through to parturition. Segment II involves exposure from implantation through fetal development and assesses both organogenesis and development [89]. In Segment III of the ICH pre- and postnatal developmental studies, exposure occurs from implantation and through lactation until weaning (Stages C to E). Segmented studies generally are not multigenerational studies. Various sampling times are used to assess developmental outcomes, for example, embryonic tissues can be sampled to assess skeletal damage, although this would be considered a teratogenic effect.

Segment III study designs are sometimes carried out to examine effects on the next generation (e.g., ICH pre/postnatal development studies). Exposed male and female pups are raised to maturity and mated to produce an F1 litter. Various outcomes are assessed in these unexposed F1 including survival, growth, general morphometric measures, and behavior. These F1 males and females are also mated to assess effects on fertility.

2.6.2 Continuous Cycle Designs

Most continuous cycle study designs evaluate multiple generations, and exposure continues across generations. The two main protocols are the National Toxicology Program's Reproductive Assessment by Continuous Breeding (RACB) [137] and the OECD multigeneration study, OECD TG 416 [138). Histopathology is evaluated in all parts of the reproductive and endocrine systems in the F0 that may be relevant to germ cell mutagenicity. At maturity, the F0 rodents are mated to produce an F1 generation that provides information on fertility and fecundity in the F0. Effects arising in the F1 generation, which is also exposed *in utero*, may be relevant to potential germ cell effects arising in the F0.

2.6.2.1 One-Generation Toxicity Study

A number of modifications to the multigenerational protocols discussed above have been made, including the one-generation reproduction toxicity study (OECD TG 415 [139) and the extended one-generation study design (enhanced pre- and postnatal studies) (OECD TG443 [140). Rodents are treated before mating through gestation in the modified one-generation study. However, the exposures are stopped at various intervals and the animals are either necropsied or mated to produce an F1 generation. The F1 are handled similarly as the F0, and mating is performed to produce F2 pups.

2.6.2.2 Repeat Dose Toxicity Studies

Repeat dose toxicity studies can easily be modified to assess potential germ cell effects. Acute and subacute (90-day studies) can be combined with reproduction/developmental toxicity screening tests such as OECD TG 408 and TG 422 [141,142]. Tissues examined in these studies include germ cells and sperm count, motility and morphology, and vaginal cytology can be used to indicate potential germ cell effects. Reduced sperm count and whole testis weight may reflect genotoxicity. Alterations in sperm morphology do not correlate with genetic toxicity nor do they impact male fertility[143,144].

The assays described above provide important data on toxicity to germ cells across developmental stages in males and females that are not sampled in conventional genetic toxicology studies. Furthermore, they can be invaluable in future genomic investigations on germ cell mutations and *de novo* mutations arising in offspring.

2.7 Assays in Need of Further Development or Validation

Some additional assays have been developed over the past decade or so and have been used to measure the effects of germ cell mutagens but are in need of further development and/or validation. The assays and their advantages and disadvantages are listed in Table 2.2 and described in the following sections.

2.7.1 Transgenic Rodent Gene Mutation Reporter Assay

The transgenic rodent assay (OECD TG 488) promises to be a very useful screening test for chemically induced male germ cell mutations [125]. Studies suggest a good correlation between mutagens detected with the TGR assay and the SLT [126]. Additionally, prototypical mutagens exhibit the expected dose–response in male germ cells for transgene mutations. Thus, it appears that the TGR loci respond appropriately and represent effects in occurring in other gene regions. However, the assay uses a reporter gene in a transgenic rodent and is limited to scoring mutations in a nontranscribed exogenous gene that is heavily methylated and there are some uncertainties that relate to the integration of somatic and germ cell testing with the TGR assay. Details regarding optimal sampling times for detection on mutations in sampling for germ cell versus somatic mutations are discussed in Refs [89,122].

While studies have indicated the need for care in extrapolating to other genomic regions [145], there is strong concordance between endogenous and reporter gene mutations [146]. The gene mutation assay may miss large deletions/insertions and rearrangements or CNVs. Since the assay is performed on sperm, potential inheritance is unclear. However, since it detects chemicals that are positive in the SLT [126], there is a high probability that heritable

Table 2.2 Summary of the advantages and disadvantages of existing assays in development or validation stages.

Endpoint transgenic rodent mutation
Advantages
Can be performed on most tissues enabling a comparison of somatic and germ cell sensitivity/specificity; neutral gene, scores gene mutation, OECD guideline, relatively simple (integrated into multiple test strategies)
Disadvantages
Need transgenic rodents, scores mutations in a nontranscribed exogenous gene, performed on germ cells not pedigrees, thus inheritance is unclear, may miss some types of mutations
Tandem repeat assays
Advantages
Endogenous loci, high spontaneous mutation rate, can be adapted to any species, some markers linked to diseases, sensitive at low doses, should be able to be integrated into other tests but validation has not been done
Disadvantages
Unclear indirect mechanism of mutation, noncoding markers, unclear relevance of tandem repeat mutation to gene mutations, small dynamic range, some technical challenges
Spermatid micronucleus
Advantages
Easily integrated into transgene mutation reporter assay and other toxicity tests, any species, can be directly compared with somatic MN to study germ cell specificity/sensitivity
Disadvantages
Currently laborious (but potential for flow cytometry), small database, not inherited
Sperm comet assays
Advantages
Can be performed in any species, relatively simple, can be compared with most somatic cell types, can detect a variety of DNA damage
Disadvantages
Difficult to integrate with other tests, high variability across laboratories and studies, biological relevance of endpoint unclear, technical issues, premutational damage only
Sperm chromatin structure
Advantages
Fast (flow cytometry approach), can be performed in any species, including humans, major validation exercises underway
Disadvantages
Germ cells only, premutagenic lesion (thus implications unclear), mechanisms causing changes in chromatin unclear, biological and technical variability results in differences across studies/laboratories

Source: Reproduced from Ref. [89] with permission of Elsevier.

mutations are detected and one study has demonstrated inheritance of *lacZ* gene mutations by offspring [147].

2.7.2 Expanded Simple Tandem Repeat Assay

ESTRs are a class of microsatellites that are long homogeneous arrays of relatively short repeats (4–9 bp); they spontaneously exhibit a very high replication-driven mutation rate involving length changes in germ line and somatic cells [148–150]. The very high spontaneous mutation rate makes the analysis of ESTR length change mutations an attractive approach for monitoring germ line mutation induction in mice. ESTR loci have been used repeatedly for analysis of germ line mutation induction in male mice exposed to ionizing radiation, chemical mutagens, and anticancer drugs [151–157], as well as environmental air pollutants [47–49,51,158]. ESTR mutations were originally detected in pedigrees by profiling DNA samples extracted from all parents and their offspring. Later, a more sensitive technique has been developed in which multiple samples, each containing approximately one ESTR molecule, are derived from diluted bulk sperm genomic DNA. Single-molecule PCR is then used to amplify the sample DNA allowing the detection of an indefinitely large number of *de novo* mutants in DNA sampled from sperm or other cell types [159]. This single-molecule sperm analysis technique dramatically reduces the numbers of mice required for the measurement of germ line mutation rates and avoids the wait for mating and birth. More importantly, this approach should be directly applicable in human studies [160–162].

In the offspring of mice exposed to X-rays of fission neutrons, the ESTR mutation rate in the germ line increases linearly with radiation dose [152–154]. An increase in ESTR mutation rate in mice is detectable at much lower doses than can be measured by standard genetic techniques and the dose–response is very close to that seen with conventional mutation assays including the SLT. Using ESTRs as the endpoint, statistically significant evidence for mutation induction is obtained by analyzing hundreds of mice; whereas other systems require thousands or greater numbers of mice. Offspring of male mice treated with either ethylnitrosourea (ENU) or isopropyl methane sulfonate (iPMS) displayed a significant increase in ESTR mutation rate [157], and increased sperm ESTR mutation frequencies were found following exposure of male mice to four commonly used anticancer drugs [156]. For the chemotherapeutic drugs, mutation induction was observed within a clinically relevant dose range; thus, the assay is sensitive and holds promise for assessing potential germ cell hazards in rodents and humans. An increasing number of repeat mutations are either associated with, or may cause, human genetic disorders [163].

As the observed increases in ESTR mutation rate in the germ line of exposed male mice are too high to be attributed to the total number of DNA damaged

sites within these loci, it has been suggested that ESTRs may reflect nontargeted events, where the initial mutagen-related DNA damage occurs elsewhere in the genome and indirectly increases the mutation rate at these loci [159]. As the mechanism of this nontargeted process remains unknown, ESTR loci can currently be regarded as a useful biomarker of exposure to mutagens. All mouse strains carry ESTR loci so that the assay can be integrated with conventional tests in mice. Dividing cells in the relevant phase of spermatogenesis must be sampled, which requires an additional set of mice. The ability to score ESTR mutations in testicular cells sampled during standard genetic toxicity testing has not yet been investigated but should be a subject of future research. It should be mentioned that microsatellite tandem repeats offer several advantages over ESTRs. An approach using microsatellites rather than ESTR is more likely to be used. See Ref. [164] for more details.

2.7.3 Spermatid Micronucleus (MN) Assay

MN are the product of chromosome damage and their analysis in somatic cells is the predominant *in vivo* assay used to confirm positive results *in vitro*. There are OECD guidelines for *in vitro* (TG 487) as well as *in vivo* (TG 474) somatic cell MN assays. The development of flow cytometry-based MN detection methods interrogating thousands of cells provide high sensitivity to detect small increases in MN *in vivo* [165] and *in vitro* [166].

An assay for detecting MN in spermatids of rats was developed by Tates [167]. A modified assay was used to detect MN in spermatids of mice originating during meiosis. About 25 chemicals were shown to induce significant increases in MN in exposed mice and, surprisingly, 4 chemicals (1,1,-dimethylhydrazine, diethylnitrosoamine, dimethylnitrosoamine, and beta-propiolactone) were positive in spermatids but negative in bone marrow [168]. An earlier IWGT workshop has addressed the utility of the MN spermatid assay and its possible integration with the analysis in erythrocytes [169,170]. Although very little work has been done, the MN spermatid assay could be integrated, for example, within the transgenic rodent assay in assessing *lacZ* mutations in sperm and/or seminiferous tubules.

Because of manual scoring, the spermatid MN assay is rarely used. However, a flow cytometry-based method is being developed in which spermatids are first isolated by flow sorting based on DNA content and then nuclear preparations are analyzed by flow cytometry to detect MN as described for the *in vitro* MN assay [89]. As with the somatic cell method, a flow cytometry approach would permit analysis of thousands of spermatids per sample providing needed sensitivity to detect small effects. In conclusion, the assay provides evidence of genotoxicity in germ cells even though the fate of a sperm cell carrying a MN is unclear, and it is unlikely that these would be inherited.

2.7.4 Sperm Comet Assay

The *in vivo* alkaline single-cell gel electrophoresis assay, also called the comet assay, measures DNA damage (strand breaks) in single cells [171] and is a sensitive assay for exposure to genotoxic agents both *in vivo* and *in vitro* [172,173]. Although it has been widely used with somatic cells, the assay has also been performed on mature sperm and on germ cells isolated from the seminiferous tubules [174]. The assay has been applied in multiple studies to demonstrate DNA damage in rodent sperm induced by exposure to genotoxic agents [175]. The assay is readily applicable to human sperm and, surprisingly, the experimental protocol for human sperm is in a more advanced stage of validation than in experimental animals. The OECD has developed Test No. 489 for the *In Vivo* Mammalian Alkaline Comet Assay. During the development of the OECD assay guideline, the inclusion of germ cells was discussed extensively [89,176]. It was decided, however, that the standard alkaline comet assay as described in the guideline is not appropriate for measuring DNA strand breaks in mature germ cells. Three factors were considered in taking this decision: (i) The proposed exposure regimen (three daily doses followed by sample collection 3–6 h later) does not work because it represents fully mature sperm with DNA highly compacted by protamines, which are extremely resistant to DNA damage [177,178]. (ii) The method for the analysis of germ cells collected from the seminiferous tubules is not fully validated and only a few studies have applied this approach [174]. A confounding issue is that cells collected from the seminiferous tubules contain two different germ cell populations (spermatocytes and elongating spermatids) in which DNA double-strand breaks are part of the normal process of development (meiotic recombination for the former, chromatin compaction in the latter) and variation in the proportion of cells that are analyzed between controls and exposed may produce a significant effect unrelated to exposure. (iii) After a prolonged exposure (i.e., 28 days), comet analysis in mature sperm could provide relevant information on whether a chemical induces DNA in germ cells. The comet assay in mature sperm is more complicated than in somatic cells because it requires an enzymatic digestion to relax the chromatin, and sperm are extremely rich in alkali labile sites, making it much more difficult to obtain reproducible results. To conclude, further development and validation is required before the comet assay can be routinely employed for regulatory purposes in the assessment of DNA damage in germ cells. And as mentioned earlier for other assays, the assessment of DNA damage via the comet assay in germ cells does not detect heritable effects.

2.7.5 Standardization of Sperm Chromatin Quality Assays

As with the comet assay, standardization and validation of assays for the quality of chromatin is more advanced in humans than in experimental animals.

Biomarkers of chromatin integrity in human sperm include chromatin template function, chromatin structure, structural damage (breaks and cross-links), and chromatin epigenome [94]. Three assays, the comet assay just discussed, the sperm chromatin structure assay (SCSA), and the terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL), are commonly used to assess sperm DNA integrity. The SCSA was developed 30 years ago [179] and is a flow cytometry-based assay that measures the sensitivity of sperm DNA to acid-induced denaturation. The extent of DNA denaturation is thought to be correlated with the presence of single-stranded DNA and is highly correlated with infertility [180]. The TUNEL assay measures DNA breaks *in situ*, assessed by the incorporation of dUTP at the break sites [181]. These assays measure different aspects of DNA integrity, and have different sensitivities, although they tend to correlate with each other. An international effort has begun to standardize the comet, SCSA/acridine orange and TUNEL assays [89]. When validated in humans, the assays can be applied to animal models to provide a rapid and sensitive approach to assess effects of environmental exposures on the integrity of sperm DNA.

Despite significant research on human sperm DNA integrity, our understanding of the mechanisms and consequences of sperm chromatin damage is limited. It is not clear what the implications of premutagenic lesions in chromatin are to offspring, although there are indications that sperm integrity contributes to a healthy pregnancy and the health of newborns [182–185]. There is no consensus on cutoff values for clinically abnormal parameters, and substantial biological and technical variability across species/studies/laboratories is observed.

2.8 New Technologies

Germ cell mutation research is experiencing a renaissance because of the availability of new genomics technologies and resultant information. These technologies bring excitement about what can now be done to answer questions from the past about germ cell risk for future generations [49,91,186]. Combined with new applications in the clinic genomic, next-generation sequencing approaches have demonstrated their power in identifying *de novo* mutations that cause severe human genetic disorders (106,187). Given that it is not currently possible to carry out a full cycle of gametogenesis *in vitro*, alternative models are being considered [89]. Below we also describe the potential utility of an HTS *C. elegans* model for egg aneuploidy that shows promise. A summary of the advantages and disadvantages of the new technologies is provided in Table 2.3.

2.8.1 Copy Number Variants and Human Genetic Disease

Stankiewicz and Lupski have asserted that approximately 12% of human genetic variation can be attributed to copy number variants (CNVs) [188]; others have

estimated up to 50 % [189]. CNVs represent structural variation that alters, and in many cases rearranges, the number of copies of specific segments of DNA. CNVs range in size from 50 bp to megabases [189,190] and account for a wide range of human genomic disorders [187,188,191,192]. High locus-specific mutation rates for genomics rearrangements are >1000-fold more frequent than point mutations [193]. Thus, a genome-wide analysis of CNVs (>100 kbp) in approximately 400 parent–offspring trios found a mutation rate of 1.2×10^{-2} CNVs per generation [194]. Not captured by existing test methods, *de novo* CNVs represent an important source of human genetic diversity.

In fact, the extent to which DNA structural variation, including duplication and deletion CNVs and copy number neutral inversions and translocations, contribute to human genome variation and disease has been appreciated only recently [195]. Because the complexity of structural variants was not envisioned, the frequency of complex genomic rearrangements, and how they come about, remained unknown. The concept that genomic diseases may be due to genomic rearrangements and not sequence-based changes, delineated a new category of conditions distinct from chromosomal syndromes and single-gene Mendelian diseases. Thus, mechanistic understanding of CNV/SV formation has provided new insights into the human genome and gene evolution and has increased our understanding of human biology and disease [195].

High-resolution array comparative genomic hybridization (or aCGH) and SNP (single-nucleotide polymorphism) microarray technologies [196,197] have been the technologies that have really made the detection and analysis of CNVs possible. These advanced technologies methods are now being used in the clinic to identify CNVs as sources of idiopathic diseases [187,198–202]. Surprisingly, little research has been performed, however, to explore the effect of mutagens on CNV formation. It has shown in human cells in culture that replication stress resulting from exposure to chemicals can lead to the formation of CNVs [203–205]. Exposure to hydroxyurea, aphidicolin, and low-doses of ionizing radiation results in the induction of CNVs via a replication-dependent mechanism, as opposed to replication-independent repair of double-strand breaks [205]. Increasing paternal age is also associated with increases in *de novo* CNVs in offspring through replication-based mechanisms [206]. Adewoye *et al.* [207] have demonstrated induction of germ line CNVs in offspring of mice exposed to radiation. Research to explore the effects of mutagens on germ cell CNVs is a critical avenue of research in view of their importance in human genetic disease. The technology is expensive and the analysis of parental genomes must be analyzed in addition to their offspring.

2.8.2 Next-Generation Whole Genome Sequencing

Next-generation sequencing is poised to revolutionize mutation analysis in all tissues. NGS technologies and associated bioinformatics tools have been

Table 2.3 Summary of the advantages and disadvantages of the newest technologies available to detect germ line mutations.

Endpoint copy number variant analysis using array CGH and SNP chips
Advantages
Major phenotypic effects, inherited mutation, relevant to human genetic disease
Disadvantages
New endpoint with no data in germ cell toxicology; currently expensive to measure; requires pedigrees; so far not suitable for measuring somatic mutation *in vivo*, so no direct comparisons can be made; needs extensive validation for work in toxicology
Whole genome sequencing
Advantages
Measures broad spectrum of mutations, inherited mutations, clear linkages to health can be made for certain mutations, any species including humans
Disadvantages
Expensive, currently requires pedigrees for interpretation (i.e., sperm analysis not ready yet), bioinformatics challenges, not applied in toxicology yet (no database), extensive validation still required
HTS for egg aneuploidy (*C. elegans*)
Advantages
Inexpensive, fast, established model organism in genetics, high degree of conservation in relevant pathways, detects effects in female germ cells
Disadvantages
Relationship to humans is unclear, limited to aneuploidy in eggs measured in embryos at this time, not validated

Source: Reproduced from Ref. [89] with permission of Elsevier.

developed to the point that they can now be applied to study the effects of mutagens on heritable germ cell mutations. As will be discussed in greater detail below, whole genome sequencing has demonstrated that increased transmission of *de novo* mutations to offspring is associated with increasing paternal age in humans [96]. Indeed, an increased prevalence of many diseases is observed in the offspring of older fathers [208]. These findings on human paternal age effects extend to microsatellite mutations [209] and CNV [206], although only to mitotic nonrecurrent CNVs.

Conover and Argueso [210] have pointed out that while gene CNVs are abundant in the human genome, and are often associated with disease consequences, the mutagenic pathways and environmental exposures that cause these large structural mutations are understudied relative to conventional nucleotide substitutions in DNA. The environmental mutagenesis research

community is seeking to remedy this deficiency, and there is a strong interest in the development of mutagenicity assays to identify and characterize compounds that may induce *de novo* CNVs in humans. Conover and Argueso [210] emphasized deep contrasts that exist between the proposed pathways that lead to nonrecurrent and recurrent CNVs: Nonrecurrent *de novo* CNVs originate primarily in mitotic cells through replication-dependent DNA repair pathways that involve microhomologies (<10 bp), and are detected at higher frequency in children of older fathers. In contrast, recurrent *de novo* CNVs are most often formed in meiotic cells through homologous recombination between nonallelic large low-copy repeats (>10,000 bp), without an associated paternal age effect. Given the biological differences between the two CNV classes, these authors believe that nonrecurrent and recurrent CN mutagens will probably differ substantially in their modes of action. Therefore, each CNV class may require their own uniquely designed assays to enable detection of the broadest possible spectrum of environmental CN mutagens.

Kong *et al.* [96] in a landmark proof-of-principle study used whole genome sequencing of 78 Icelandic trios (mother, father, child) to show that males pass on an average of two additional mutations to their offspring for each year of their reproductive life, suggesting that the father's age is a major factor in determining the number of *de novo* mutations in the child. More recent studies have investigated the maternal age effect on germ line *de novo* mutations [211] as well as the timing, rates, and spectra of human germ line mutation [212]. NGS is also being used much more routinely in the clinic. Rodent genome-wide mutation spectra and frequency should be compared with humans, and bioinformatics tools used to determine phenotypic consequences.

A strategy to develop appropriate sequencing methodologies for genetic toxicology applications is outlined in a manuscript from the ENvironmentally Induced Germline Mutation Analysis (ENIGMA) working group [91]. NGS germ cell studies currently require pedigrees for analysis, increasing the number of samples and time required. Once NGS technologies can accurately sequence a single-gamete genome, the situation will be improved. Further challenges involve storing the large amounts of data and applying the appropriate bioinformatics filters to remove sequencing artifacts without compromising sensitivity. Full genome sequencing has had limited application in toxicology; therefore, extensive validation and creation of a database will be required [91].

2.8.3 High-Throughput Analysis of Egg Aneuploidy in *C. elegans*, and Other Alternative Assay Systems

The Environmental Protection Agency has established the ToxCast Program to High Throughput Screening methods to identify chemicals that perturb molecular pathways relevant to human and environmental health [213,214]. The existing HTS assays assess the ability of a toxicant to initiate a DNA damage

response and are not effective in identifying tumorigens [215] and the detection of mutagens and aneugens is a major gap. Mutagenic effects on germ cells are not addressed.

A high-throughput assay has been developed in the roundworm *C. elegans* to measure chromosome segregation errors in eggs [95,216]. Roundworms have advantages including a large proportion of germ cells, a short generation time, and are suitable for culturing in 96-well plate format. *C. elegans* is an established model system in genetics and key meiotic pathways are conserved between *C. elegans* and humans. Aneuploidy is examined via X chromosome missegregation during meiosis and *C. elegans* embryos that inherit only one X chromosome that is distinguished by the expression of green fluorescent protein under the control of the X chromosome counting promoter xol-1. With robotic methods, the assay is completed in 4 days and hundreds of chemicals can be analyzed. This HTS assay is followed by other rapid methods such as DNA staining of the germ line and germ line apoptosis assay to ensure that aneuploidy originated from perturbation of germ line processes. Analysis of a selection of 50 chemicals from ToxCast phase 1 and known chemicals in the *C. elegans* assay revealed an accuracy of 69% in predicting the ability of chemicals to cause reproductive toxicity in rodents [95]. Importantly, the model can be expanded to apply whole genome sequencing or CNV analysis and it addresses a critical gap in examining the effects of mutagens on female germ cells. On the downside, the relationship of aneuploidy in *C. elegans* with the process in humans is unclear, as are pharmacokinetic and dynamic considerations, so the assay is limited to assessing aneuploidy in early embryos.

In a recent review, Ferreira and Allard have pointed out that alternative *in vitro* germ cell methods in model systems such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *C. elegans* have distinct advantages over traditional models. They discuss the benefits and limitations of each model, their application to germ cell toxicity studies, and the need for computational approaches to maximize their usefulness. Together, the inclusion of these alternative germ cell toxicity models, especially in large-scale, high-throughput applications, will be invaluable for the examination of germ cell toxicity in stages not easily accessible in mammals [217]

2.9 Endpoints Most Relevant to Human Genetic Risk

In reviewing the status of presently available tests and some in the pipeline, we have attempted to address the fundamental question posed in the title of this chapter. What mutagenic events contribute to human cancer and genetic disease? Research in the field has attempted to learn about the spectrum of mutational events that occur in humans, and that are relevant to human health. Modern genomic tools have presented an unprecedented opportunity to assess

genome-wide mutation rates across species [89]. Campbell and Eichler [189] have provided a comprehensive review of the mutational landscape of the human germ line. Full genome sequencing in human families has enabled direct measurement of rates of *de novo* mutations, showing that single-nucleotide variants (SNVs) range from $1\text{-}1.2 \times 10^{-8}$ SNVs per nucleotide per generation [96,218–220], and that 76% of SNVs originate in the paternal lineage. By contrast, for CNVs both the per locus mutation rate and the overall number of nucleotides affected per generation are considerably greater [189,193]. Thus, it has been estimated that one large *de novo* CNV (>100 kbp) occurs per 42 births in humans, compared to an average of 61 new SNVs per birth; however, the average number of base pairs affected by large CNVs is 8–25 kbp per gamete versus 30.5 bp per gamete for SNVs [189]. Furthermore, CNVs are sometimes caused by complex chromothripsis that involves multiple *de novo* rearrangements in a single event [221,222].

In addition to SNVs and CNVs, which may affect coding as well as noncoding DNA sequences, there are other types of relevant functional genomic changes that occur in the human genome, including small insertions and deletions, mobile element insertions, tandem repeat mutations, translocations, and aneuploidies [89]. Microsatellites exhibit proportionally higher *de novo* mutation rates than SNVs, providing an important source of genetic variation [209]. Campbell and Eichler [189] provide an insightful summary figure demonstrating the per generation rates of SNVs, indels, mobile element insertions (MEIs), large CNVs, and aneuploidies contrasted against the total number of base pairs affected per gamete (see Figure 2.1 [189). Note that an INDEL (INsertion/DELetion) is where a single base has been deleted, or inserted into one genome

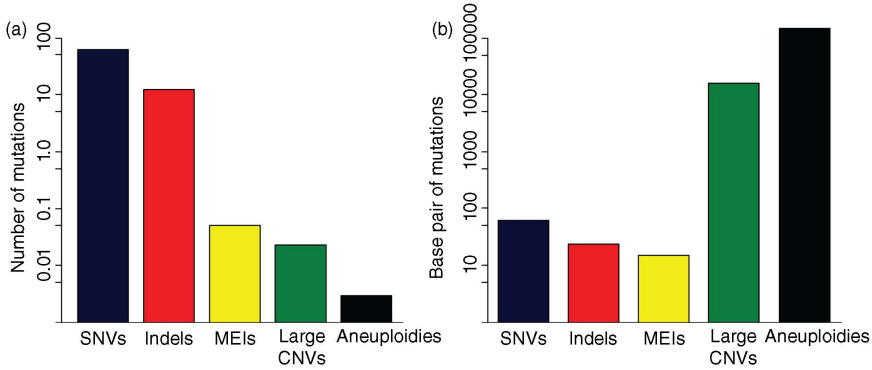

Figure 2.1 Comparison of the frequency and scale of different forms of genetic variation. *Source:* Reproduced from Ref. [189] with permission of Elsevier. (a) Average number of mutations of each type of variant per birth. (b) Average number of mutated bases contributed by each type of variant per birth. The *Y*-axis is log10 scaled in both panels.

relative to another. It is a symmetrical relationship, as a deletion in one corresponds to an insertion in another (thegenomefactory.blogspot.com/).

As shown in Figure 2.1, there is an inverse relationship between mutation size and frequency [189]. Numerically, there are more SNVs per genome than CNVs but the rate is much lower for SNVs and mutation affects only a single base pair. In contrast, large mutations such as CNVs or chromosomal aneuploidies are rare, and yet they affect thousands to millions of base pairs – affecting more base pairs per birth on average than SNVs.

Overall, the analysis of the rates and spectrum of human mutation reveals a diverse array of important genomic events that should be considered in genetic toxicology that are not currently captured in standard genetic toxicology batteries. Table 2.4 provides an overview of the endpoints that have been considered and indicates the assays that may be used to assess them [89].

Finally, it should be noted that human epidemiological studies have focused on phenotypic effects of induced dominant mutations occurring in the descendants of exposed parents [89]. Using genomics technologies, recent clinical research has shown that a large proportion of the mutations occurring in humans are recessive and not manifest phenotypically until several generations postorigination when mating occurs and a complementary mutation affects the

Table 2.4 Summary of the spectrum of *de novo* genomic changes occurring in humans and associated tests that can be used to measure them.

Endpoint	Relevant genetic toxicology test
Aneuploidy	Sperm and egg FISH, spermatocyte and oocyte cytogenetics, pedigree DNA microarray or deep sequencing, spermatid MN
Structural aberrations	Early embryo cytogenetics, sperm FISH, DLT, HTT, some can be identified by pedigree analysis using array CGH, spermatid MN, spermatocyte cytogenetics
Copy number variants	Pedigree array CGH (microarray) or deep sequencing
Small molecular rearrangements	Array CGH (as small as 500–5000 bp), pedigree deep sequencing
Small insertions/deletions	GPT delta transgene mutation (TGR assay), pedigree sequencing
Tandem repeat gains/losses	ESTR and microsatellite mutation analysis in sperm or pedigrees
Gene mutations	TGR (OECD TG 488), pedigree DNA deep sequencing
Noncoding mutations	Pedigree DNA deep sequencing, CNV analysis

Source: Reproduced from Ref. [89] with permission of Elsevier.

same locus [223]. This fact should be considered in clinical study designs so that such recessive mutations can be detected.

2.10 Worldwide Regulatory Requirements for Germ Cell Testing

Strategies and guidelines for regulatory toxicology testing, including requirements for germ cell mutation assays, were reviewed by Cimino in 2006 [224], and have not changed significantly. No jurisdiction requires germ cell testing in an initial test battery. Across regulatory agencies, genetic toxicology testing strategies can generally be separated into three tiers. Tier 1 contains required *in vitro* and somatic *in vivo* tests (as described earlier); tiers 2 and 3 contain germ cell tests (in the testes or spermatogonia) that can be requested for follow-up studies under certain conditions in many regulatory authorities, including the United States (EPA and (FDA), Canada (Health Canada), the United Kingdom (Committee on Mutagenicity: COM), Europe (Registration, Evaluation, Authorisation and Restriction of Chemicals: REACH), and Japan (Ministry of Health, Labour, and Welfare). India and Australia use only tier 1 assays and do not require any germ cell assays for regulatory purposes. Other countries generally follow strategies similar to the US EPA guidelines for industrial chemicals. For pharmaceuticals, the International Conference on Harmonization (ICH) Technical Requirements for Registration of Pharmaceuticals for Human Use does not require germ cell tests and assumes that *in vivo* somatic tests and carcinogenicity data will provide sufficient predictivity/protection for germ cell effects.

Eastmond *et al.* [75] have noted that the World Health Organization (WHO)/IPCS Harmonized Scheme says that if an agent is positive *in vivo* for somatic cell mutation, that agent can be selected for testing in germ cells; however, such testing is not required. In addition, WHO/IPCS identifies transgenic mouse models, the ESTR assay, the spermatogonial chromosome aberration assay, chromosome aberration analysis by FISH, the comet assay, and assays for DNA adducts as suitable assays in germ cells. In offspring, the WHO/IPCS tests include the ESTR assay, the DLT, the HTT, and the SLT.

The United Nations Global Harmonization Scheme (GHS) [225] identifies mutagens according to the categories noted in Table 2.5.

To date, 67 countries have implemented this IARC-like classification scheme (i.e., known, probable, or possible human carcinogens) and are in the process of integrating it into their relevant regulations. Within the European REACH strategy, an agent that is genotoxic in somatic cells based on the literature is evaluated to see if it is a potential germ cell mutagen based on bioavailability to the germ cells and appropriate *in vivo* data. If such an evaluation shows that the literature is insufficient to determine whether the agent is or is not a potential

Table 2.5 Categorization of mutagens by OECD/GHS/ECHA.

Category	Description
1A	Chemicals known to induce heritable mutations in germ cells of humans
1B	Chemicals that should be regarded as if they induce heritable mutations in germ cells of humans
2	Chemicals that cause concern for induction of heritable mutations in germ cells of humans

Source: Reproduced from Ref. [89] with permission of Elsevier.

germ cell mutagen, then that agent can be tested in a suitable germ cell genotoxicity assay. The number of chemicals for which testing in germ cell mutation assays was requested and/or evaluated under the Canadian Environmental Protection Act (CEPA) is similar to the number for which testing in rodent cancer assays was requested and/or evaluated for new substance assessments from 1994 to 2012 (G. Douglas, personal communication) [89].

In summary, germ cell mutation is a regulatory endpoint for many organizations and germ cell mutagens are classified in a manner similar to that of carcinogens by Health Canada, GHS, and the German regulatory agencies (MAK). Although germ cell mutation is an established regulatory endpoint, and more than 50 agents have been identified as germ cell mutagens in rodents, no agent has been regulated solely as a germ cell mutagen, or classified as a human germ cell mutagen. This situation is likely to change soon as accumulated data shows that cigarette smoke, air pollution, and ionizing radiation are likely human germ cell mutagens [52].

2.11 Conclusion

In this chapter, we have considered the mutagenic events that contribute to human cancer and genetic disease. We emphasized the importance of (i) protecting humans from heritable mutation hazards and risks through appropriate testing and (ii) determining the causes of *de novo* mutations in offspring. In addition, we highlighted the environmental exposures that are known to cause cancer and likely to cause genetic abnormalities and disease in humans. Our review of the advantages and disadvantages of the conventional assays for germ cell and heritable effects highlighted a number of gaps. We described several new assays that show great promise to help meet these needs. Recently recognized types of genomic changes, such as SNVs, MEIs, and, especially, CNVs, need to be explored to understand their relevance in germ cell genetic

toxicology. Such tests will require further development and validation, as well as research efforts to establish the best integrated testing strategies. The current database also needs to be improved and focused to identify the most effective approaches. This effort should include gleaning relevant data from conventional reproductive toxicology assays that historically have not been used for these purposes. Induced mutations that do not result in a known phenotype in the first generation must be studied for their disease-causing potential in future generations. In addition, intergenerational mutational events that result from exposure of germ cells during embryologic development and that can result in genetic disease should be investigated [226]. In conclusion, the application of new genomics technologies to evaluate animals, and particularly humans, exposed to mutagens via germ cells as well as somatic cells should be a priority.

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3

Developmental Origins of Cancer

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3.1 Introduction

An association between prenatal exposures and later risk of cancer has been recognized for many decades. In one of the earliest reports, the Oxford Survey of Childhood cancer in the late 1950s showed a relationship between exposure of pregnant mothers to diagnostic radiation and the development of leukemia and other cancers in the offspring [1,2]. Since that time, many studies have demonstrated increased risk of cancer later in life following *in utero* exposure to ionizing radiation [3–6] and various drugs and environmental chemicals, including the synthetic estrogen diethylstilbestrol (DES) [7,8], paternal smoking [9,10], dichlorodiphenyltrichloroethane (DDT) [11,12], and other pesticides [13–15]. This chapter explores biological features of the prenatal period that may increase or decrease sensitivity to carcinogens, including the ontogeny of xenobiotic metabolizing and DNA repair systems, reprogramming of the epigenome, and other developmental factors. Themes include issues of developmental plasticity in the embryo/fetus and how the developmental environment can have lifelong effects on health and disease, including risk of cancer, within the context of the developmental origins of health and disease (DOHaD) theory. The development of cancer after prenatal exposure to specific drugs and environmental agents will also be discussed, including a limited review of agents related to increased cancer risk following prenatal exposure in humans and laboratory animals. In addition, models that have been proposed for investigating the developmental origins of leukemia and breast cancer will be presented. Several governmental bodies including the United States Environmental Protection Agency [16,17], the

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California Environmental Protection Agency [18], and the Washington State Department of Ecology [19] have considered measures to account for the potential increased risk of cancer from developmental exposures, and their findings and approaches will be discussed briefly later in this chapter.

3.2 Current Trends in Childhood Cancer

Prenatal exposures can potentially manifest as increased risk of cancer at any age, but their relationship to childhood cancer has received more attention, in part due to the temporal proximity between exposure and outcome. Childhood cancers represent less than 1% of all cancers diagnosed each year, yet cancer is currently the second leading cause of death (next to accidents) in the United States for children age 5–14 years. According to estimates by the American Cancer Society, approximately 10,450 new cancer cases and 1350 cancer deaths in children (from birth to 14 years) and an additional 5330 new cases and 610 cancer deaths in adolescents (ages 15–19 years) are expected to occur annually in the United States [20]. Leukemia accounts for about a third of all cancers in children, most commonly acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML). Cancer of the central nervous system (CNS) accounts for approximately a quarter of childhood cancers, often affecting the cerebellum or brain stem. Other common forms of cancer found in children include neuroblastoma, lymphoma, and retinoblastoma. Among cancers diagnosed in children <15 years of age, about 6% comprise renal cancers. Among the renal cancers a majority (~95%) are embryonic in nature, belonging to the category of nephroblastoma (Wilms' tumor), which has the highest incidence during 0–5 years of age.

Both childhood and adult cancers may have a developmental origin. The prenatal period is a time of rapid cell division and growth, which may elevate the risk of DNA damage and misrepair. Xenobiotic metabolizing systems have varying developmental trajectories that affect the metabolism and distribution of chemicals in the embryo/fetus, potentially increasing or decreasing the carcinogenic potency of a chemical depending on the ontogeny of activating and deactivating metabolic enzymes. The prenatal period is also a time of dramatic reprogramming of the epigenome of the conceptus, a process that affects patterns of gene expression throughout life. Epigenomic and other effects of *in utero* exposures to environmental agents on the cellular and molecular biology of the conceptus may have latent consequences that alter the risk of cancer and other diseases later in life [21–23]. Because of the long latency period between prenatal exposures and the onset of adult cancer in humans, the contribution of *in utero* exposures to cancers in adulthood is an underexplored issue.

3.3 Potential Mechanisms of Prenatal Cancer Induction

Carcinogens may act in the embryo and fetus through mechanisms common to the induction of cancer in adults; additionally, the prenatal period may exhibit increased vulnerability to carcinogenesis for several reasons specific to the developing organism. Some chemicals act through genotoxic mechanisms, interacting with or altering DNA bases, while others act without direct interaction with DNA and are termed nongenotoxic carcinogens [24]. Genotoxic chemicals can be either direct acting or indirect acting, the latter requiring metabolic activation by drug metabolizing enzymes. Many, but not all, carcinogens cross the placenta [25], and direct-acting carcinogens appear to be more potent in cancer induction during early embryogenesis compared to indirect-acting chemicals [26].

Chemicals may act as initiators and promoters of cancer, or both. For example, some chemicals may form DNA adducts in fetal cells causing initiation and these cells remain dormant. At any subsequent life stage, exposure of these cells to a chemical promoter may cause cellular proliferation and fix a mutation [27]. Risk of cancer from prenatal exposure may be increased simply due to the longer period of time available for promotion after exposure to an initiator. *In utero* susceptibility to carcinogens is influenced by rapid cell proliferation. Occurrence of frequent and rapid cell divisions during prenatal development can result in enhanced fixation of mutations since there is little time available for repair of DNA lesions, while the clonal expansion of mutant cells gives a larger population of mutations. In addition, the immature status of the immune system in the growing fetus might compromise immune surveillance for cancer cells.

Some nongenotoxic carcinogens act through hormonal mechanisms and have been a focus of studies investigating developmental effects of endocrine disrupting chemicals [28]. There is increasing evidence that exposures to endocrine disrupting chemicals during development play an important role in hormone-sensitive cancers in women and cancer of the prostate gland in men [29]. For example, developmental exposure to endocrine disrupting chemicals, such as bisphenol A, has been linked to the development of mammary gland cancer in experimental animals [30], and prenatal exposure to DDT has recently been linked to increased risk of breast cancer in humans [11].

3.4 Ontogeny of Xenobiotic Metabolizing Enzymes and DNA Repair Systems

During the prenatal period, whether indirect carcinogens are metabolically activated or potential carcinogens are metabolically inactivated will depend in

part on the time of exposure and the ontogeny of drug metabolizing enzymes in the embryo and fetus. Enzymes involved in drug and chemical metabolism exhibit varied developmental trajectories in human tissues, with some metabolic capabilities emerging only after birth. Based on their developmental activities and expression patterns determined using specific probe substances and antibodies to the human enzymes, Hines [31] categorized the xenobiotic metabolizing enzymes as Classes 1, 2, and 3 enzymes (Table 3.1). Class 1 enzymes are expressed at high levels during the first trimester of pregnancy, but their expression is silenced or much reduced after birth; class 2 enzymes play an important role during fetal development and through adulthood, while class 3 enzymes, to which the majority of the enzymes belong, show negligible or very low levels during fetal stages, with a slight increase during second and/or third trimesters. Mature expression occurs from a few weeks after birth (e.g., CYP2D6) to 1 or 2 years of age (e.g., CYP1A2) or after reaching puberty (e.g., FMO3 and CYP2C9).

Expression of DNA repair enzymes during *in utero* development of the fetus also plays an important role impacting the immediate or later health outcomes such as cancer, malformations, or functional deficits [32]. Studies using rodent

Table 3.1 Classification of drug-metabolizing enzymes based on developmental trajectories.

Class 1	Class 2	Class 3	
ADH1A	CYP2C19	ADH1B	EPHX1
CYP3A7	CYP2B6	ADH1C	EPHX2
FMO1	CYP3A5	AOX1	GSTM1
GSTP	GSTA1	CES1	GSTZ1
SULT1E1	GSTA2	CES2	FMO3
SULT1A3	SULT1A1	CYP1A2	SULT2A1
		CYP2C9	UGT1A1
		CYP2D6	UGT1A6
		CYP2E1	UGT2B7
		CYP3A4	

Class 1 enzymes appear during the first trimester, remain active during gestation but have little to no postnatal expression; *Class 2* enzymes are expressed consistently during gestation through adulthood; *Class 3* enzymes are low or absent in the fetus and increase during 1–2 years postnatally, some not being maximal until puberty. ADH, alcohol dehydrogenase; CYP, cytochrome P450; FMO, flavin monooxygenase; GSTP, glutathione S-transferase pi; SULT, sulfotransferase; EPHX, epoxide hydrolase; GSTA, glutathione S-transferase alpha; AOX, alternative oxidase; GSTM, glutathione S-transferase Mu; GSTZ, glutathione S-transferase zeta; PON, paraoxonase; UGT, UDP glucuronosyl transferase; CES, carboxylesterase.
Source: Reproduced from Ref. [31] with permission of Elsevier.

models show that the expression of DNA repair genes varies depending on developmental stage, tissue type, and repair pathway. Although availability of such information in humans is limited, functional studies demonstrate ability of fetal tissues to repair DNA damage, suggesting the existence of at least some major DNA repair pathways during fetal development [32].

3.5 The Developmental Origins of Health and Disease (DOHaD) Theory

Studies during the late 1980s illuminated the potential effects of the early-life environment on lifelong health. Barker and colleagues [33] reported an inverse correlation between birth weight and the incidence of death from ischemic heart disease in men and women in the United Kingdom. Further studies confirmed these observations and extended them to show an association between lower birth weight and increased risk of the metabolic syndrome, including hypertension, stroke, and type 2 diabetes. These findings suggested that the *in utero* environment of the developing conceptus could have profound long-term implications for health and risk of disease. From these findings, a concept evolved called the DOHaD theory [34]. The DOHaD theory considers suboptimal developmental conditions to include over- or undernutrition, parental exposures to drugs or environmental pollutants, maternal stress, and maternal diabetes and obesity in the periconceptional or prenatal periods and to an as-yet undefined early postnatal period.

The implications of the DOHaD theory for toxicology have been considered, but research to date is limited [35–39]. Lifelong metabolic programming can occur through a number of mechanisms involving impacts on developmental growth trajectories, cell proliferation and differentiation, organ maturation, and paracrine and endocrine effects. Emerging science indicates that *in utero* exposures to a variety of drugs and environmental chemicals may lead to increased risk of adverse health effects, including cardiovascular disease, type 2 diabetes, obesity, stroke, renal disease, osteoporosis, Alzheimer's disease, and cancer in offspring over their life span. Evidence from experimental animals as well as epidemiological studies shows consistent support for the DOHaD theory [40]. Alterations to the epigenome are likely to play a central role in the developmental programming underlying the DOHaD theory, including the induction of cancer [22].

3.6 Epigenetic Regulation during Development

Superimposed upon the primary DNA sequence is a layer of "epigenetic" information that exerts control over the genome. Epigenetics has been defined

as "mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" [41]. Epigenetic mechanisms at the level of chromatin include chemical modifications falling into two main categories: (1) DNA methylation or hydroxymethylation [42,43] and (2) posttranslational modifications of the histone proteins that package the genome [44]. These chemical modifications influence transcription across developmental stages, tissue types, and disease states [45–47]. Noncoding RNAs are also considered to be epigenetic and can act during transcription, translation, or the posttranslational period [48].

Different subsets of genes exhibit different trajectories of expression across time, tissue type, and organs in the conceptus. Pluripotent cells of the cleavage stage embryo progressively differentiate along specific lineages to give rise to the tissues of the embryo and fetus. While regulation of differential gene expression by transcription factors is a key feature of development, it is now understood that gene expression patterns during development (as well as in the adult) can be defined by epigenetic modifications [49,50]. These epigenetic "marks" may be transient, such as histone modifications that, during cleavage, repress genes needed for later development, or long-lived, such as the DNA methylation and other chromatin modifications that result in X chromosome inactivation or the silencing of imprinted genes and transposons.

3.6.1 Critical Periods for Epigenetic Regulation

Epigenetic marks can be erased and reestablished at specific stages of the life cycle. There are two periods during which large-scale demethylations of genomic DNA are known to occur (Figure 3.1) [51]. One is during migration and proliferation of the primordial germ cells (PGCs); in the mouse embryo this takes place between gestation day 10.5 and 12.5. Imprinted genes are demethylated at this time [49,52], primarily at CpG islands in differentially methylated regions. Methylation is subsequently reestablished in a parent-of-origin manner during gametogenesis. Demethylation of DNA in the PGCs also serves to reactivate pluripotency-related genes needed in the early conceptus. It may be that a combination of passive and active demethylation processes is involved. Genomic demethylation is almost complete in PGCs, but some transposons remain highly methylated [53]. Tet demethylases are enzymes that can convert 5-methylcytosine to 5-hydroxymethylcytosine and other oxidation products that can result in demethylation by base excision repair.

The second period of widespread epigenetic reprogramming occurs shortly after fertilization. The sperm genome is highly methylated, yet after fertilization and removal of protamines (sperm proteins) from the paternal genome, many paternal alleles become demethylated. Demethylation of the paternal genome before the onset of DNA replication is followed by demethylation of both parental genomes by dilution once rapid DNA synthesis and cleavage begins.

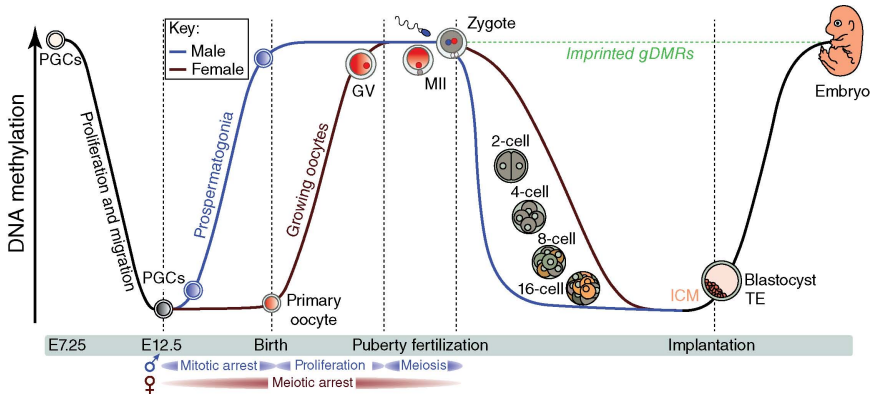

Figure 3.1 DNA methylation changes during developmental epigenetic reprogramming. There are two major periods of DNA demethylation, one during the proliferation and migration of primordial germ cells (PGCs) to the germinal ridge of the embryo, and another in the conceptus during early cleavage stages. Parentally imprinted genes escape this latter demethylation. E, embryo; gDMR, germ line differentially methylated regions; GV, germinal vesicles; MII, second meiotic division. *Source:* Reproduced from [51] with permission of Elsevier.

Despite maintenance of methylation in imprinted genes, total genomic methylation in the early embryo decreases, reaching a nadir at the blastocyst stage. General demethylation in the embryo at this stage may play a role in returning cells to pluripotency [54].

It is likely that the patterns and extent of epigenetic marks on the genome may be specified or altered, in part, by the prenatal environment. As these epigenetic marks can last a lifetime, it is plausible that epigenetic programming during development results in permanent changes in the physiology and response to later toxic exposures, and therefore, adult disease risk, including risk of cancer.

3.7 Mechanisms of Cancer in Offspring from Paternal Exposures

It is well known that genetic mutations can contribute to risk of cancer and that these mutations can be inherited from either parent or originate *de novo* in the conceptus. Damage to male gamete DNA resulting from radiation, chemical exposure, infection, or aging may be passed on to progeny if not repaired, and may increase cancer risk. More recently, it is becoming clear that epigenetic marks can also be inherited from either parent, and that histone modifications, DNA methylation, and noncoding RNAs in sperm play essential roles in successful fertilization and development of the conceptus [55,56]. Sperm

DNA is known to be sensitive to reactive oxygen species, and oxidative DNA damage can lead to mutations and epimutations [57]. Diverse chemicals and mixtures, including the fungicide vinclozolin, dioxin, some pesticides, and jet fuel, have been shown to induce heritable sperm epimutations in experimental animals [58]. As discussed further, cancer in offspring of smoking parents is related to paternal preconception smoking but less so to maternal smoking. In rodent models, paternal high-fat diet and overweight have been associated with increased incidence of breast cancer in female offspring [59,60]. The basis for this association is not understood, but an adverse effect on the sperm epigenome is plausible.

3.8 Parental Exposures Associated with Cancer in Offspring

Maternal and paternal exposures during the preconception or prenatal period have been associated with childhood or adult cancers in offspring. Selected case studies presented here are intended to highlight the diverse types of prenatal exposures that increase cancer risk later in life. For interested readers, there are additional parental exposures that have been associated with offspring cancer in humans that will not be discussed further here, including benzene (See [61,62] for meta-analyses of extant epidemiology studies), air toxics [63–65], and alcohol [66,67].

3.8.1 Radiation

The Oxford Survey of Childhood Cancers (OSCC) study was the earliest evidence of a statistical association between abdominal X-ray of pregnant women and childhood leukemia [1,2]. Further support comes from studies of Japanese atomic bomb survivors exposed *in utero* and in early childhood, who suffered an increased risk of diverse types of solid cancers in adulthood [68]. Risks appeared to be similar whether the exposures were *in utero* or during early childhood for most cancers, except Wilms' tumor diagnosed at 14 years of age. In several case–control studies, but not in cohort studies, a modest and consistent increase in cancer cases was reported with *in utero* exposure to diagnostic X-rays [69]. Several other studies have shown strong associations between maternal exposures to radiation and increases in solid tumors in offspring later in life. The International Agency for Research on Cancer (IARC) has concluded that "there is substantial evidence that suggests a causal association between exposure to diagnostic radiation *in utero* and childhood cancers" [69].

3.8.2 Diethylstilbestrol

Estrogen receptors are found on many cell types, and signaling through the estrogen receptor is involved in multiple developmental and physiological pathways. Estrogenic chemicals include naturally occurring steroids (e.g., 17 beta-estradiol), oral contraceptives such as ethinyl estradiol (EE), fungal products (e.g., zearalenone), environmental pollutants such as DDT, polychlorinated biphenyls (PCBs), bisphenol A (BPA), nonylphenol, kepone, plant products such as genistein (isoflavone), luteolin (flavone), resveratrol (stilbene) and coumestrol (coumarin), and the synthetic estrogen DES [70]. One of the earliest studies reported the use of DES as a drug of choice for preventing miscarriage and other pregnancy-related complications [71]. Often, miscarriage is preceded by a drop in estrogen levels in pregnant mothers, and supplementing these women with DES was thought to sustain pregnancy. It is estimated that from the 1940s until the mid-1970s, several million pregnant women were prescribed DES in the United States. In 1971, Herbst *et al.* [72] reported that the daughters of mothers who were treated with DES during pregnancy had higher risk of developing clear cell adenocarcinoma (CCA) of the cervix and vagina, a rare cancer in women. This seminal study revealing transplacental carcinogenesis by a widely prescribed drug has spawned decades of research on long-term effects in offspring following *in utero* exposure to chemical substances.

In addition to CCA of the cervix and vagina, several cohort studies conducted in the United States have shown increased risk of breast cancer in the daughters of DES-treated mothers compared to matched nonexposed control subjects [7,73–76]. In particular, these studies have shown that breast cancer incidence is twofold higher in daughters of DES mothers after 40 years of age. However, in a study involving DES daughters from The Netherlands, the risk of CCA of the vagina and cervix was significantly higher beyond 40 years of age, but no increase in breast cancer risk was observed [77]. See Section 3.9 for further discussion of prenatal DES exposure and breast cancer.

Studies using *in utero* exposure of experimental animals to DES at doses comparable to those received by humans showed similar tumor risk values between rodents and humans, supporting the utility of rodents as models for evaluating transplacental effects of DES (reviewed in Ref. [78]). This is in agreement with transgenerational transmission of risk for at least two generations, increase in risk at low DES doses, and altered risk of tumor initiation following *in utero* exposure to chemical carcinogens. It has been reported that prenatal exposure to DES in mice results in several genital tract alterations, including vaginal adenocarcinomas, adenosis, and uterine tumors [79,80].

Developmental exposure to DES has also been shown in mice to result in reduced Tet1 expression (Tet enzymes are involved with hydroxymethylation of cytosine bases) and reduced global 5-hydroxymethylcytosine levels in the adult uterus, and specific epigenetic modifications (DNA hypermethylation, DNA

hypomethylation, histone modifications) of genes related to developmental and cancer, including *Hoxa10, Ltf, Six1, c-fos, Nsbp1* and *Svs4* (reviewed in Ref. [81]).

3.8.3 Tobacco Smoke

Tobacco smoke is a human carcinogen that contains thousands of chemicals including >50 known individual carcinogenic chemicals, including aldehydes, hydrocarbons, aromatic amines, nitrosamines, and polycyclic aromatic hydrocarbons, most of which can cross the placenta [10]. Thus, it has been of great interest to determine whether maternal or paternal smoking or exposure to secondhand smoke is associated with increased cancer risk in offspring. There have been numerous epidemiological studies and meta-analyses conducted over the past two decades examining this question, and while the findings are not wholly consistent, the pattern that has emerged is one of a majority of studies finding a small or no significant increase in risk of cancer in offspring from maternal smoking during pregnancy, and a higher proportion of studies finding a positive association between paternal preconceptional smoking and offspring cancer. Interestingly, a few studies have reported a significant association of maternal exposure to secondhand smoke and increased offspring cancer incidence. The IARC has determined that paternal and maternal preconceptional tobacco smoke exposure and exposure during pregnancy are linked to increased risk of childhood cancer, based in part on studies showing a doubling of risk of hepatoblastomas in offspring when both parents smoke [82,83].

Leukemia is the most common cancer in children. Human and experimental animal studies indicate that childhood leukemia develops in a two-step process, including prenatal and postnatal events (see further). There are a number of epidemiological studies and meta-analyses in the literature, and they are in general agreement on linkages between parental smoking and childhood leukemias. As a whole, these studies elucidate significant relationships between paternal preconceptional smoking and childhood leukemia, while consistently showing weak or absent associations with maternal smoking.

Farioli et al. [9] found no significant association between paternal smoking in the periconceptional period or maternal smoking during pregnancy and the incidence of acute lymphoblastic leukemia in offspring. A study of parental smoking, CYP1A1 polymorphisms, and childhood leukemia found no significant association with smoking, and evidence for small differences between CYP1A1 genotypes [84]. Mattioli et al. [85] found no association of maternal smoking with childhood acute nonlymphocytic leukemia, but weak statistical evidence of an association with maternal secondhand smoke exposure and a significant association with paternal smoke exposure in the periconceptional period. In a study of maternal coffee and alcohol consumption during pregnancy and parental smoking, Menegaux *et al.* [86] reported no significant

association of ALL and parental smoking. Metayer *et al.* [87] examined the risk of childhood ALL by cytogenetic subtype, and found that paternal prenatal smoking combined with postnatal exposure to secondhand smoke was associated with a 1.5-fold increased risk of ALL. The effect was observed for B-cell precursor ALL with t(12;21) translocations, but not hyperdiploid B-cell ALL. Risk of ALL was increased with paternal periconceptional smoking in a meta-analysis by Milne *et al.* [88], and a dose–response was observed. Preconceptional paternal smoking but not maternal smoking was also associated with childhood ALL in a study by Orsi *et al.* [89].

Brain cancer is the second-most common cancer of childhood, and the leading cause of childhood cancer death. Studies of associations between parental smoking and childhood brain tumors (CBT) are largely negative. A large population-based case–control study found no effect of parental smoking on CBTs [90]. Norman *et al.* [91] reviewed case–control and cohort studies published during 1971–1995. The majority of the studies did not show increased risk of CBT with maternal smoking or secondhand smoke exposure. Likewise, no significant association between parental smoking and CBTs was found in the United States West Coast childhood brain tumor case–control study [92]. In the Surveillance of Environmental Aspects Related to Cancer in Humans (SEARCH) international (nine centers in seven countries) case–control study, odds ratios were calculated for all types of CBTs combined, four CBT histotypes in five age groups at each center [93]. There was no association between parental smoking prior to pregnancy, maternal smoking during pregnancy, or maternal secondhand smoke exposure and risk of CBT. Parental smoking before or during pregnancy showed no association with risk of CBT in a case–control study in Australia [94]. Interestingly, with small group sizes, odds ratios of CBTs in children under 24 months of age were 5.06 (95% CI 1.35–19.00) and 4.61 (95% CI 1.08–19.63). Further studies of risk of CBT by age of the child at diagnosis are needed to confirm this preliminary finding. Contrary to the fairly consistent negative findings of retrospective studies of parental smoking and CBT, a prospective study of 1.4 million Swedish births found that children of mothers who smoked during pregnancy had an increased incidence of brain tumors (hazard ratio 1.24; 95% CI 1.01–1.53), with the strongest relationship being observed in children 2–4 years of age at time of diagnosis [95].

A meta-analysis of studies on the association between exposure to maternal tobacco smoke during pregnancy and any childhood cancer [96] found a small increase in risk of all neoplasms (relative risk 1.04; 95% CI 1.03–1.19), but not of any individual tumor type, and no dose–response relationship was evident. The association of childhood cancers with paternal exposure was stronger, and significant for brain tumors (RR 1.22; CI 1.05–1.40) and lymphomas (RR 2.08; CI 1.08–3.98). In a study of 642 childhood cancer cases and matched controls, paternal preconception smoking was associated with a significant increase in

cancer risk, particularly for acute leukemia (two thirds ALL, one third acute myelocytic leukemia) and lymphoma. A dose response was evident, with paternal smoking of five pack-years prior to conception conveying odds ratios of 3.8 (CI 1.3–12.3) for ALL, 4.5 (CI 1.2–16.8) for lymphoma, and 1.7 (CI 1.2–2.5) for all cancers. In agreement with other studies, childhood brain cancer was not significantly associated with paternal preconception smoking [97]. Pang *et al.* [82] reported that the United Kingdom Childhood Cancer Study did not find an elevated risk of childhood cancer associated with paternal smoking. A meta-analysis of 18 published studies reporting both paternal smoking and childhood ALL risk found that paternal smoking was positively associated with childhood ALL for the preconception, pregnancy, and after birth periods, with a dose–response relationship evident for the preconception and after birth periods.

In a study that examined the relationship between parental smoking and cancer risk in adult offspring, Sandler *et al.* [98] included cancer cases between ages 15 and 59 at the time of diagnosis. All sites except basal cell carcinoma were included. Cancer risk in offspring of men who smoked was increased by 50%, while there was only a slight increase in risk of cancer in offspring of mothers who smoked. The relative risk for hematopoietic cancers was 1.7 when one parent smoked and 4.6 when both parents smoked. This study provides an interesting glimpse of the potential long latency between prenatal exposure and adult cancer.

3.8.4 Pesticides

Since the 1970s, pesticides have been implicated in the etiology of childhood leukemia, especially in rural areas where pesticides are used in agriculture. Parental occupational exposure to pesticides in agricultural operations or their use in the home has been associated with childhood leukemia, brain cancer, Wilms' tumor, Ewing's sarcoma, and testicular germ cell tumors [99,100]. In addition to these cancers, pesticide exposure has been associated with neuroblastoma, non-Hodgkin's lymphoma, soft tissue sarcomas, and cancers of the brain, colorectum, and testis [101]. Although many of the same kinds of cancers are detected in adults, the risks are greater in children. In children born to parents involved in farming, an association has been demonstrated with CNS tumors and leukemia [102]. Turner *et al.* [103] reported an association between residential exposures to pesticides, in particular insecticides, *in utero* or during childhood, and childhood leukemia. Other studies also reported positive associations of exposure to pesticides in households and childhood brain tumors [104] and lymphoma but not leukemia or solid tumors [105], astrocytoma and neuroblastoma [106], and childhood leukemia [107,108]. However, hematological malignancies have shown the strongest epidemiological association with pesticide exposure [109,110].

Several pesticides have been shown to induce epigenetic mechanisms in both *in vitro* and *in vivo* systems, as reviewed by Ref. [111]. These include endocrine disruptors (e.g., methoxychlor, vinclozolin), persistent organic pesticides (e.g., DDT, mirex), and metals (e.g., arsenic), which have been shown to alter DNA methylation, herbicides, such as paraquat and dieldrin, which are involved in histone modifications *in vitro*, and insecticides (e.g., dichlorvos) and some fungicides which are involved in altered microRNA expression *in vivo* [111].

3.8.5 Arsenic

Inorganic arsenic (iAs) is a known human carcinogen, exposure to which has been related to increased risk of skin, lung, bladder, and liver cancer [112], as well as other health effects. The World Health Organization (WHO) recommends that iAs in drinking water be at concentrations below 10 ppm [113], but this level is exceeded in many places worldwide, including parts of the United States. Inorganic arsenic crosses the placenta and has been shown to cause *in utero* growth retardation and neonatal mortality in laboratory animals [114]. In a systematic review and meta-analysis of the relationship between levels of iAs in groundwater and adverse pregnancy outcomes and infant mortality, groundwater iAs concentrations of >50 ppm were associated with increased risk of spontaneous abortion, stillbirth, and neonatal and infant mortality. There was also a significantly lower birth weight associated with iAs in drinking water [115]. Relationship of *in utero* iAs exposure to later development of cancer was not considered in this study.

Elevated exposure to iAs occurred in the northern Chilean city of Antofagasta when the water supply was changed to rivers that contained high concentrations of arsenic (up to 1000 μg/l). More than 250,000 people were exposed to high iAs concentrations in drinking water from 1959–1970 [116]. Studies involving evaluated iAs exposure of pregnant mothers or children during their early life found significant increases in lung, kidney, and bladder cancer as well as bronchiectasis and myocardial infarction [117,118] and fetal or infant mortality [119]. Exposure to moderate levels of iAs (<50 μg/L) was associated with lower birth weight in infants born between 1998 and 2000 [120]. Ongoing studies seek to elucidate the link between *in utero* or early-life exposure to iAs and later life risk of cancer.

Waalkes *et al.* [121] carried out prenatal exposures to arsenic in two strains of mice and examined the incidence and types of cancers in the offspring at adulthood. In one set, they treated pregnant C3H mice with up to 85 ppm sodium arsenite in drinking water from days 8 to 18 of gestation, and offspring were observed for up to 2 years. *In utero* exposure to arsenic produced a dose-dependent increase in liver carcinoma and adrenal cortical adenoma in male offspring and ovarian tumors and lung carcinomas and tumors together with preneoplastic hyperplasia of the uterus and oviduct in female offspring during adulthood. Further, prenatal arsenic exposure followed by postnatal exposure

to the tumor promoter, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in C3H mice induced excess lung tumors in both sexes and liver tumors in females. In a different experiment, male CD1 mice treated with iAs *in utero* developed tumors of the liver and adrenal glands and renal hyperplasia, while females developed tumors of the urogenital tract, ovary, uterus, and adrenal gland and hyperplasia of the oviduct. Prenatal exposure of CD1 mice to iAs followed by postnatal exposure to DES or tamoxifen induced carcinomas and papillomas of the urinary bladder and increased the liver tumor response in both sexes [121]. Thus, in both strains of mice, iAs has been shown to be a tumor initiator and complete carcinogen acting transplacentally.

While the mechanism underlying the induction of later life cancer by prenatal exposure to iAs remains to be elucidated, accruing evidence suggests a role for epigenetic developmental reprogramming. In a study of newborn cord blood samples, prenatal iAs exposure (drinking water levels) was compared with patterns of methylation of 424,935 CpG sites in 18,761 genes [122]. A total of 2919 genes exhibited iAs-related changes in CpG methylation. Gene expression was correlated with CpG methylation for a subset of the affected genes, and DNA methylation was associated with birth outcome metrics for seven genes. Laine and Fry [123] surveyed 12 studies of the relationship between prenatal iAs exposure and alterations to the fetal epigenome, transcriptome, and proteome. Across these human cohort studies, they identified a common set of affected genes, many of which are under putative regulation by tumor necrosis factor. There were 61 genes identified with differential CpG methylation by iAs, many known to play a role in cell cycle regulation or apoptosis.

3.9 Models for the Developmental Origins of Selected Cancers

Studies of the development of normal tissues and aberrant development leading to increased risk of cancer have led to the construction of conceptual models of the series of biological events and environmental exposures that can lead to carcinogenesis. Recent models published for breast cancer and leukemia demonstrate plausible pathways to cancer involving aberrant developmental processes and/or environmental insults. While there are certainly other plausible models for the developmental origins of cancer, these models are exemplary of the current thinking and research approaches surrounding the elucidation of developmental mechanisms of cancer.

3.9.1 Breast Cancer

The number of women diagnosed with breast cancer has been trending upward in the United States as well as Europe, and breast cancer is the leading cause of

cancer mortality in women worldwide [124–126]. The idea that breast cancer risk may be increased due to environmental exposures stems from the trend in rising incidence of the disease, the observation that the large majority of breast cancer patients have no family history of breast cancer, and the ubiquity of exposures to endocrine disrupting chemicals, including environmental estrogens. Furthermore, the increasing realization over the past several decades that the developmental environment can have profound influence on the risk of disease later in life has focused attention of the role of early-life exposures in the etiology of breast cancer. A congressional report entitled "Breast Cancer and the Environment: Prioritizing Prevention" (https://www.niehs.nih.gov/about/assets/docs/breast_cancer_and_the_environment_prioritizing_prevention_508.pdf) set as one of its two premises about breast cancer and the environment that "timing matters;" that is, there are periods of sensitivity to environmental exposures during which key events in normal breast development are occurring, and perturbation of these events may contribute to increased risk of cancer later in life.

Early-life exposures to DDT or DES have been linked to breast cancer in adult women, and serve as examples of early windows of sensitivity to chemicals that can perturb breast development and increase risk of breast cancer in adulthood. Conceptual models for the adverse outcome pathway between exposure to these and other endocrine disrupting chemicals and adult breast cancer have been proposed. DDT was a widely used pesticide during the 1960s in the United States and around the world, remains in use for malaria control in Africa and Asia, and is highly persistent in the environment. Most studies examining the relationship between DDT exposure and breast cancer have been negative. However, these studies for the most part did not consider life stage at the time of exposure to DDT. Cohn *et al.* [127], studying the Child Health and Development Studies (CHDS) pregnancy cohort, used age in 1945, when DDT was in wide use in the United States, as a proxy for earliest age at exposure to DDT. Exposure prior to 4 years of age was associated with the greatest risk of cancer diagnosed before age 50, and risk of breast cancer was only associated with DDT exposure prior to age 14. In a 54-year prospective follow-up of 9300 daughters in the CHDS cohort, maternal serum DDT concentrations 1–3 days after birth was predictive of breast cancer in daughters by 52 years of age (odds ratio 3.7, 95% CI 1.5–9.0 comparing 4th quartile versus 1st quartile maternal serum DDT) [11].

In vitro studies have demonstrated that nanomolar *o,p′*-DDT enhanced the HER2 tyrosine kinase activity in human MCF-7 breast cancer cells and led to increased MCF-7 proliferative foci; both effects were blocked by a HER2 antibody [128], suggesting that DDT carcinogenicity may require activation of HER2. Exposure of mice to the DDT metabolite, DDE, beginning at weaning, led to shortening of the latency to HER2-positive mammary tumors [129].

DES was prescribed to several million pregnant women worldwide to prevent miscarriage between 1947 and 1971 [130]. As already described, the critical discovery that led to DES being the seminal example of transplacental

carcinogenesis was the link to cervical/vaginal clear-cell adenocarcinoma in young women exposed to DES *in utero* (see Ref. [131] for review). A number of cohort studies have been conducted in the United States investigating the association of prenatal DES exposure and breast cancer, and these studies point to at least a doubling of breast cancer risk in DES daughters after the age of 40 [8,73]. A similar trend found in a recent European study [77] was not statistically significant, possibly because of the younger age of the European DES daughters in this study. After the discovery that DES was a transplacental carcinogen and a teratogen, animal models were developed that established causal links and expanded the range of health effects of DES. Estrogens are linked to increased breast cancer risk, leading to studies in animals of the effects of DES on mammary gland development and mammary gland tumorigenesis. Across a broad range of experimental designs (species, dosage, timing of dosage, route of administration, spontaneous versus carcinogen-induced tumors), animal studies showed that maternal exposure to DES at doses relevant to those taken by pregnant women increased the incidence of mammary tumors in offspring later in life [8]. Female offspring of pregnant rats dosed during the second and third week of gestation exhibited increased incidence of mammary tumors. In contrast, postnatal exposure prior to puberty reduced mammary tumorigenesis, similar to other estrogens.

Mammary gland bud development begins in humans at about 6 weeks of gestation, with budding and branching continuing to 20 weeks of gestation. Most mammary gland development is postnatal, with a rapid acceleration in female offspring at puberty, characterized by the development of terminal end buds into more differentiated structures, including terminal ductal lobular units and lobular alveoli. These various processes and transitions may represent sensitive targets for environmental insults. Rudel *et al.* [30] have presented a model of the pathogenesis of breast cancer following exposure to endocrine disrupting chemicals at different stages of the life cycle (Figure 3.2). The processes under way during succeeding life stages present different targets for disruption that in turn affect breast cancer risk late in life. In general, susceptibility to tumorigenesis is highest when exposure occurs while less differentiated terminal end buds are abundant, during adolescence [30].

Hilakivi-Clarke [8] proposed a model for the development of breast cancer following prenatal exposure to DES. This conceptual model posits that epigenetic changes underlie the increased risk of breast cancer in female offspring of mothers exposed during pregnancy, and also suggests that increased risk could be transmitted to future generations through epigenetic inheritance (Figure 3.3). *In utero* exposure to DES is known to alter the expression of DNA methyltransferases, promoter methylation, histone modifications, and microRNAs. Epigenetic changes have been associated with increased risk of breast cancer in women, including hypermethylation of tumor suppressor genes and changes in miRNA profiles in breast tumors compared to normal breast tissue.

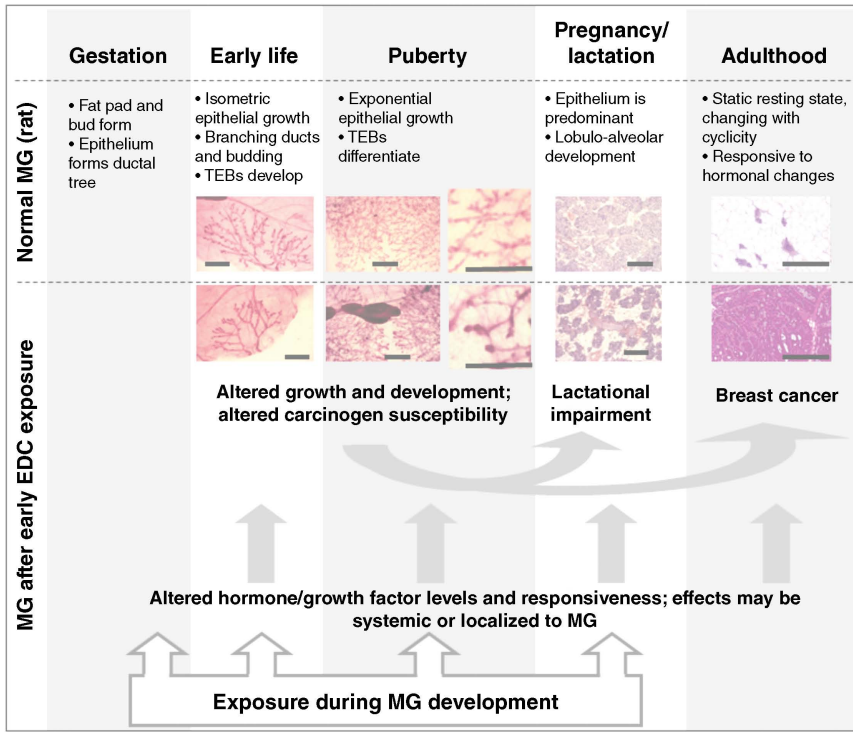

Figure 3.2 Stages of normal rat mammary gland (MG) development and effects of the environment on subsequent events. Early life EDC exposures can alter developmental programming at multiple stages of development and into adulthood, when effects on lactation or tumorigenesis occur. Effects on MG morphogenesis can be observed using MG whole-mount preparations. Mechanisms of adverse effects may act through altered gene imprinting or gene expression, disrupted endogenous MG signaling, or hormonal changes. Plausible (filled gray arrows) or more certain mechanisms (open white arrows) are indicated. (Photomicrographs for early life and puberty are from Refs [30] and [132], respectively. Photomicrographs of pregnancy/lactation and adulthood are courtesy of Dr. Suzanne Fenton.) Bars = 2 mm.

3.9.2 Leukemia

There has been a trend toward increasing incidence of childhood leukemia in recent decades, although few causes have been identified. As already discussed in this chapter, exposure to diagnostic X-irradiation *in utero* and parental smoking have been associated with increased risk of childhood leukemia, and substantial evidence for transplacental leukemogenesis also exists for *in utero* benzene and pesticide exposures. Because acute leukemias are the most common cancer of childhood, and the etiology is still poorly understood,

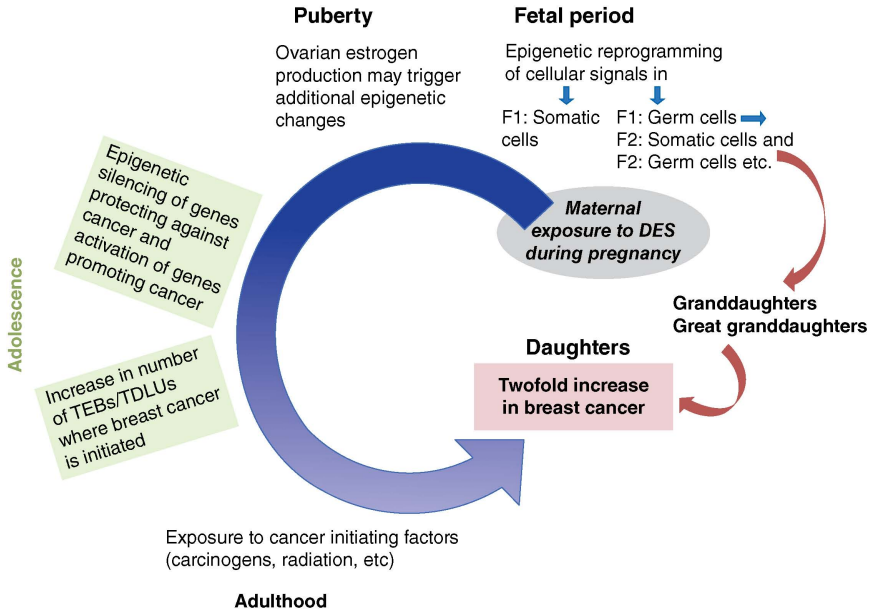

Figure 3.3 Schematic model showing prenatal exposure to diethylstilbestrol in pregnancy and cancer risk in daughters and granddaughters. *Source:* Hilakivi-Clarke, https://breast-cancer-research.biomedcentral.com/articles/10.1186/bcr3649. Licensed under CC BY 2.0.

efforts have been made to develop conceptual models of leukemogenesis, based on both clinical observations and animal studies.

Many childhood leukemias are thought to originate prenatally, with the first leukemogenic hit being genetic mutations or chromosomal abnormalities. Translocations (e.g., 11q23 translocations) and hyperdiploidy are observed in neonatal blood spots from children with acute lymphocytic leukemia [133]. Subsequent hits may be either prenatal or postnatal. One model of prenatal development of leukemia is seen in Down syndrome infants, who are at 10–20-fold increased risk of leukemia, with a 500-fold increased risk of megakaryocytic leukemia [134]. This model features two prenatal "hits" that give rise to a preleukemogenic state. The trisomy 21 condition that is the cause of Down syndrome is thought to be the first hit, present from conception, and the second hit is a GATA1 mutation in the fetal liver. A block in megakaryoblast differentiation and mutant cell clones can produce transient abnormal myelopoiesis, which may resolve, or with additional hits yet to be identified, progress to acute megakaryocytic leukemia (Figure 3.4). Another model, which has been proposed for non-Down syndrome childhood leukemia, includes an *in utero* first hit by exposure to an environmental agent, like benzene [135] or

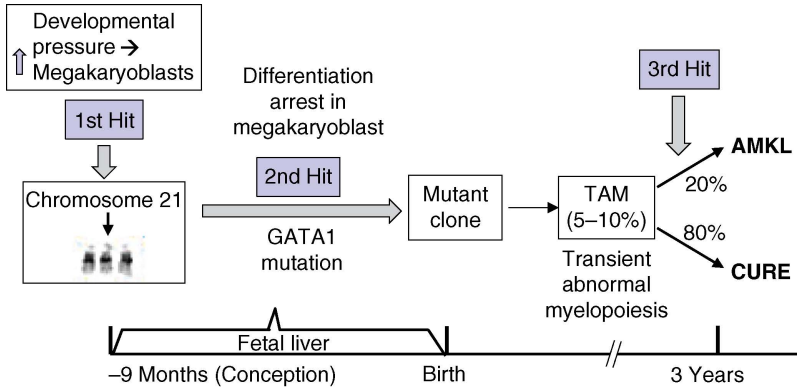

Figure 3.4 Stochastic model showing development of leukemia in Down syndrome (DS) patients. Megaloblasts from DS patients with constitutional trisomy with an acquired mutation in the transcription factor GATA1 with additional hits are likely to contribute to leukemogenesis. TAM, transient abnormal myelopoiesis; AMKL, acute megakaryocytic leukemia. *Source:* Reproduced from Ref. [134] with permission of Elsevier.

pesticides [136], causing oxidative stress, fragile site breakage or additional mutations, DNA double strand breaks and misrepair, and chromosomal translocations. Resulting chimeric proteins produce dysregulated gene expression and cell signaling. Secondary insults may be postnatal, including genetic or epigenetic alterations or aberrant immunomodulation. Enhanced proliferation and blocks to differentiation followed by clonal expansion then lead to overt leukemia (Figure 3.5).

3.10 Public Health Agencies' Views on Prenatal Exposures and Cancer Risk

3.10.1 The United States Environmental Protection Agency (US EPA)

In 1994, the National Research Council (NRC) recommended that "EPA should assess risks to infants and children whenever it appears that their risks might be greater than those of adults" [137]. In response to these NRC recommendations, the EPA developed the "Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens" [17]. The Supplementary Guidelines for risk assessment take into account the sensitivity of postnatal exposure to chemical carcinogens. They also examine the differential susceptibility for mutagenic carcinogens and recommend adjustments to the adult cancer slope factor when estimating cancer risk from early life exposure. In order to account for postnatal exposure to carcinogens during these vulnerable life stages,

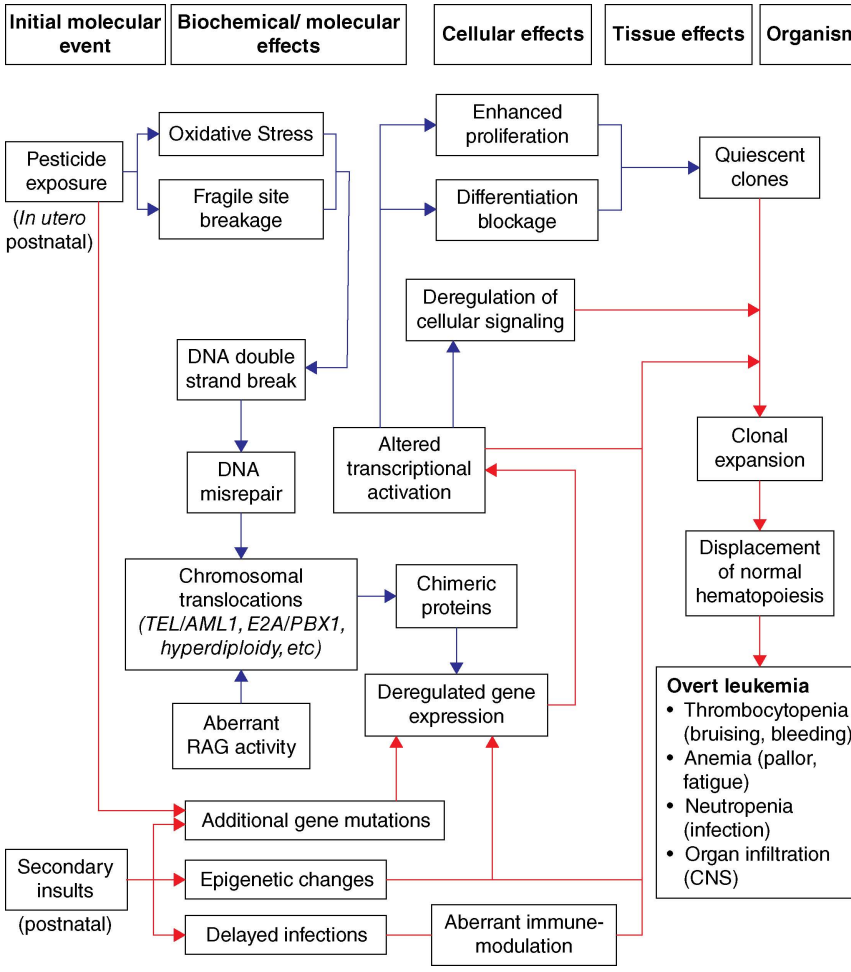

Figure 3.5 Chain of pathogenic events linking pesticide exposure to the development of childhood leukemia. Blue arrows indicate events related to the "first hit" and red arrows events relate to the "second hit." TEL, translocation-Ets-leukemia; AML, acute myeloid leukemia; PBX1, pre-B cell transcription factor 1; CNS, central nervous system; RAG, recombination-activating gene. *Source:* Hernandez, http://www.mdpi.com/1422-0067/17/4/461/htm. Used under license CC-By 4.0.

USEPA applies additional Age-Dependent Adjustment Factors (ADAFs) when calculating human cancer risk. These ADAFs, which are comparable to uncertainty factors, are applied to account for the particular vulnerabilities of the early postnatal period, childhood, and adolescence [17,138]. This EPA guidance does not explicitly address prenatal exposures, but allows for

additional consideration of life stage susceptibility as relevant scientific information on mechanisms of action during early life stages emerge: "Although the available studies (discussed previously) indicates that higher or lower cancer risks may result from early-life exposure, there is insufficient information or analyses currently available to determine a general adjustment at this time. As other modes of action become better understood, this information may include data on quantitative differences between children and adults" [17].

3.10.2 The California Environmental Protection Agency (CalEPA)

In 2009, the NRC released one of its reports entitled *Science and Decisions: Advancing Risk Assessment* (also called the Silver Book), wherein it was recommended that "EPA needs methods for explicitly considering in cancer risk assessment *in utero* exposure and chemicals that do not meet the threshold of evidence that EPA is considering for judging whether a chemical has a mutagenic mode of action" [139]. In 2009, the California Office of Environmental Health Hazard Assessment published a report entitled "*In Utero* and Early Life Susceptibility to Carcinogenesis: The Derivation of Age-at-Exposure Sensitivity Measures" [18]. This report considered and documented information on early-life susceptibility to carcinogens from human as well as experimental animal studies. In referring to early-life cancer susceptibility in humans, the CalEPA report documented studies with prenatal exposure to synthetic hormones such as diethylstilbestrol (DES) [72,140], X-irradiation [141], radioactive iodine [142], and immunosuppressive agents [143] (Table 3.2).

CalEPA [18] compiled early life exposure data for different chemicals initially from multilife stage exposure studies that had at least two groups of animals of which one group was exposed to chemicals during any of the different life stages, prenatal (conception to birth), postnatal (birth to weaning), juvenile (weaning to sexual maturity), while the second dose group is exposed preferably as adults (sexual maturity/breeding age). Experimental species included rats, mice, gerbils, and hamsters (Table 3.3). In studies where adult animals were not exposed, animals exposed as juveniles served as the referent group for comparison to the prenatal group. From the multilife stage exposure studies, 23 carcinogens were identified, of which 20 were genotoxic and the rest were nongenotoxic carcinogens. Of the 23 carcinogens, only 14 chemicals had prenatal exposure studies, which comprised 8 genotoxic and 6 nongenotoxic carcinogens. Cancer potency was estimated as an increase in risk with increasing cumulative dose. A life stage potency ratio (LP), which represents the inherent susceptibility of early life stages to carcinogen exposure, was calculated by dividing the early life stage exposure dose by that from an exposure conducted in adult animals. The results from these studies indicated that the prenatal, postnatal, or juvenile stage is sometimes, but not always, more sensitive to carcinogen exposure compared to the adults. This also suggests that

Table 3.2 Early life cancer susceptibility in humans.

Agent (reference)	Susceptible group	Case	References
Diethylstilbestrol (DES)	Fetus	*In utero* exposure arising from administration of DES during pregnancy resulted in an increased risk of cervical/vaginal adenocarcinoma in daughters, but not in mothers taking the drug	[72,140]
X-irradiation treatment for Hodgkin's lymphoma	Girls with developing breast tissue (10–16 years old)	10–16-year-old girls considerably more likely to develop breast cancer than those under age 10 similarly treated. Risk of cancer by age 40: 35%	[141]
Radioactive iodine fallout from the 1986 Chernobyl accident	Fetus/children	Increased risk of thyroid carcinoma was observed in children from Ukraine and Belarus exposed to radioactive iodine fallout. The greatest risk of thyroid carcinoma was observed in children aged five and under at the time of the accident.	[142]
Immunosuppressive drug treatment associated with organ allograft	Children ages 18 years or less	Children are more prone to develop posttransplant lymphomas and lymphoproliferative disorders than adults (53% versus 15%)	[143]

Source: Adapted from Ref. [18].

factors in addition to metabolic maturity may be contributing to prenatal susceptibility. For example, although safrole and benzidine require metabolic activation, they display greater susceptibility to *in utero* exposure.

CalEPA then conducted case studies with two genotoxic chemicals, diethylnitrosamine (DEN), which requires metabolic activation and ethylnitrosourea (ENU), which is a direct-acting carcinogen. The prenatal period was less sensitive to DEN exposure than the adult life stage, while ENU showed the opposite effect. This may be explained by the fact that DEN requires metabolic activation and cannot be metabolized by the embryo/fetus to the same extent as in adults.

Table 3.3 Multilife stage studies in experimental animals.

Chemical	Species	Exposure life stages				References
		Prenatal	Postnatal	Juvenile	Adult	
Genotoxic carcinogens						
Benzidine	Mouse	+[a]	+	+		[144]
Butylnitrosourea	Rat	+	+	+		[145]
Diethylnitrosamine (DEN)	Hamster	+[a]			+	[146][b]
		+[a]			+	[146]
Ethylnitrosourea (ENU)	Rat	+[a]	+	+		[147]
		+[a]			+	[148]
3-Methylcholanthrene (3-MC)	Mouse	+[a]			+	[149]
		+[a]			+	[150]
Safrole	Mouse	+[a]	+	+		[144]
		+[a]	+	+		[151]
Urethane	Rat	+[a]	+	+	+[b]	[152]
Vinyl chloride	Rat	+[a]	+		+	[153]
Nongenotoxic carcinogens						
Diethylstilbestrol (DES)	Mouse	+[c]			+	[154]
Dimethylnitrosamine (DMN)	Hamster	+			+	[155]
Di-*n*-propylnitrosamine (DPN)	Hamster	+[c]			+	[155]
		+[c]			+	[156]
1-Ethylnitrosobiuret	Rat	+[a]	+		+	[157]
2-Hydroxypropylnitrosamine	Hamster	+[c]			+	[156]
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Mouse	+[c]			+	[158]

a) Prenatal period more sensitive than adult in multilife stage studies.
b) Dosing initiated in later part of the juvenile period, from day 46 to 61.
c) Prenatal period less sensitive than adult in multilife stage studies.
Source: Adapted from Ref. [18].

3.10.3 Washington State Department of Ecology (WA DoE)

Consistent with the new scientific information and regulatory guidance provided by USEPA's *Guidelines for Carcinogenic Risk Assessment* [16] and *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure*

to Carcinogens [17] and CalEPA's methods and policies for making early-life stage adjustments to carcinogens with other modes of action [18], the State of Washington's Department of Ecology (WA DoE) has also evaluated the relevance of increased susceptibility of children from environmental exposures to carcinogens. The WA DoE explored sources of scientific uncertainty and variability that should be considered while updating and revising its Model Toxics Control Act (MTCA) cleanup regulation [19]. They determined that sufficient information exists based on physiological, behavioral, and anatomical differences between different life stages to indicate differences in exposure patterns and cancer potencies [19].

3.11 Conclusions

It is clear from the examples and discussions that exposures limited to the prenatal period are sufficient to induce cancer later in life in offspring in both humans and in animal studies. Human transplacental carcinogens identified by IARC include DES, ionizing radiation, and tobacco smoking. There are many more chemicals identified in laboratory animal studies. In an analysis of rodent studies on 15 chemical carcinogens, Hattis *et al.* [159] estimated a 5–60-fold increased carcinogenicity in the birth-to-weaning stage compared to adult exposure for mutagenic carcinogens, and an approximately fivefold increased risk from radiation exposure or direct-acting nitrosoureas during the fetal period, but not to mutagenic carcinogens requiring metabolic activation. Hattis *et al.* [160] conducted a quantitative likelihood analysis of life stage sensitivity of rodents to mutagenic carcinogens and estimated that for a "generic mutagenic carcinogen," mean lifetime exposure risk (including all life stages) was 2.8-fold higher (5–95% confidence interval 1.5–6-fold) than risk from adult-only exposure.

The prenatal period may be more, similarly, or less sensitive to the induction of cancer by chemical exposure than the adult, and the biological differences underlying life stage differences in susceptibility almost certainly include both pharmacokinetic and pharmacodynamic differences. The rapidly dividing cells of the embryo may be more sensitive to genotoxic carcinogens, and the presence of large numbers of stem cells and precursor cells at different stages of differentiation may also contribute to increased sensitivity [161]. Our growing understanding of the reprogramming of the epigenome during gametogenesis and early embryogenesis, and the elucidation of epigenetic changes occurring in carcinogenesis, has opened a fertile field for new understanding of the biological basis for sensitivity of the preconceptional and prenatal periods to carcinogen exposure. Such an understanding could also open the door for development of diagnostic/predictive biomarkers of later-life risk of cancer from prenatal exposures, allowing prospective identification of at-risk populations.

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Disclaimer: The views expressed in this chapter are those of the authors and do not necessarily reflect the views or policies of the United States Environmental Protection Agency.

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4

The Mechanistic Basis of Cancer Prevention

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4.1 Introduction

Cancer prevention is central to cancer control. Cancer control refers to all measures calculated to lessen the burden of malignant disease on the community by reducing the incidence of, or morbidity or mortality due to, cancer. All aspects of the clinical management of malignant disease, including improving the quality of life for those living with cancer and their carers, represent the immediate response to cancer diagnosis and a singular focus for community awareness. However, even as therapeutic intervention is revolutionized by the application of precision medicine [1], recognition of the likely burden of disease worldwide means, as summarized by the Director of International Agency for Research on Cancer (IARC), that we cannot treat our way out of the cancer problem [2].

4.2 A Mechanistic Approach

Prevention, in the present context, can be seen as encompassing all initiatives calculated to reduce cancer incidence. Traditionally, cancer prevention has been categorized as primary or secondary: the former referring to reduced exposure to carcinogens and the latter involving all other matters including, for example, the detection of premalignant disease through screening [3]. This approach is challenged by current insights regarding cancer etiology, specifically our recognition that only a subset of tumor types and perhaps an even smaller proportion of cancer cases are attributable to the impact of known carcinogens [4]. There are, however, cancer risk factors such as overweight/

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obesity that are considered to identify opportunities for cancer prevention. Accordingly, the opportunity is taken in this chapter to address cancer prevention with reference to current understanding of cancer etiology, including but by no means limited to the role of carcinogens.

4.2.1 Specifying Carcinogens

Accordingly, this discussion of cancer prevention is predicated, at least in the first instance, on etiology. Options to prevent cancer may be addressed on the basis of what is known about cancer causation, beginning with the conventional understanding of carcinogens. Carcinogens are particular chemicals or complex mixtures of chemicals, certain infectious organisms, and some types of electromagnetic radiation that increase the incidence of cancer in populations of humans and/or animals exposed to them [5]. Unless otherwise specified, the terms cancer, carcinogen, and carcinogenic as used in this chapter refer to humans.

Eliminating or reducing human exposure to particular carcinogens varies markedly in relation to whether exposure is involuntary and occurs in the absence of any direct decision to bring about such exposure [6]. Relevant circumstances include those at work, as a consequence of using a particular drug, being subject to pollution, and contact with carcinogenic contaminants in food or consumer products. Circumstances of exposure to carcinogens primarily as a consequence of individual decision-making are often identified as being determined by lifestyle. Exposure to carcinogens may occur as a consequence of active smoking, drinking alcohol, or deliberate sun exposure – relevant circumstances of exposure being largely, if not wholly, the responsibility of individuals.

Occasionally, separation of circumstances of exposure to carcinogens into those that are nominally unavoidable and those in which an individual acquiesces to a lesser or great extent can apparently lead to reliance upon semantics. Thus, active smoking is the prerogative of individuals, but exposure to secondhand smoke in many circumstances is unavoidable, and to that extent, not a matter of personal choice. Contact with hair dyes is an occupational exposure, and therefore unavoidable for hair dressers, but exposure to these products also involves consumer choice in relation to individuals who decide to dye their hair. A consequence of different circumstances of exposure to the same carcinogen is that preventive options in respect of a particular agent may likewise involve multiple specifications depending on the context.

4.2.2 Cancer Risk Factors Without Carcinogen Specification

Options for cancer prevention are not restricted to circumstances involving a specified carcinogen. Increased risk of cancer may be causally associated with

undertaking certain work in the context of which relevant carcinogens are implicated but not identified [7]. Increased risk of cancer consequent upon shift work or sedentary work does not implicate the role of an exogenous carcinogen (s). For the community at large, increased risk of cancer may be a consequence of overweight/obesity [8] or lack of physical exercise [9]. Despite prevention predicated on reduced exposure to a particular carcinogen(s), the scenarios mentioned in this paragraph prompt almost immediate recognition of preventive options not involving reduced carcinogen exposure.

There are multiple aspects of cancer etiology that are not immediately identified with the impact of exogenous agents. Hormonal and/or reproductive history mediate risk of certain cancers, primarily breast cancer [10]. Nonetheless, when populations are compared, variables such as age at menarche are shown to be influenced by nutritional status. Such considerations may implicate certain preventive options.

Heritable risk of cancer, including that mediated by highly penetrant single-gene defects such as mutation of BRCA1, implicates a particular approach to prevention – namely, the recognition of high risk individuals, and for those so identified, the provision of relevant services. Precision medicine, along with the prospect of an increasing proportion of the community being subject to genomic or comparable analysis, taken together with characterization of susceptibility by genome-wide association studies, offers new prospects for cancer prevention [11].

Finally, cancer may occur independent of any category of causative agent, with sex, age, race, and the like being the only evident risk factors. Sometimes identified as spontaneous disease, incidence rates for any tumor type being equated with the lowest recorded among diverse communities worldwide. Causation of such disease has been addressed as inherent to fundamental biological processes and the operation of chance [12]. By definition, primary prevention is excluded. To the extent that cancer prevention includes all measures calculated to reduce incidence and, particularly, mortality, cancer screening for either premalignancy or early-stage malignancy is the primary option for prevention of spontaneous disease.

4.3 Preventing Cancer Attributable to Known Carcinogens

4.3.1 Involuntary Exposure

4.3.1.1 Infectious Agents

The burden of cancer caused by infectious agents varies markedly between communities, accounting for a third of all cases in sub-Saharan Africa to about 3% in Australia and New Zealand [13]. Despite diversity, vaccination against HPV infection is as relevant to the prevention of cervical and related cancers in

the Australian community as it is to those in low- and middle-income countries where cervical cancer mortality is toward the top, if not the major cancer afflicting women. The efficacy of vaccination is successively being established, while new formulations are effective against an increasing broad spectrum of HPV subtypes [14].

The effectiveness of vaccination against hepatitis B virus is best illustrated in Taiwan and Singapore where in less than two decades hepatitis B antigen carriers dropped from 9.1 to 2.7% and the incidence of hepatocellular carcinoma dropped from 27 to 17% [15]. Introduction of HBV vaccination nationally in Gambia, following an IARC-supported intervention trial, has resulted in a fall in HBV infection from 15 to 20% when the project was initiated to less than 1% [16].

Infectious disease predisposing to cancer may be responsive to drug treatment. This year, several direct-acting antiviral agents to treat hepatitis C are anticipated to complete successful phase III trials and be commercially available [17]. Limited progress has been made in therapy for eradication of *Helicobacter pylori* infection because of resistant species. Data from randomized control trials suggest that searching for and eradicating *H. pylori* reduces the incidence of gastric cancer in healthy asymptomatic infected Asian individuals, but these data cannot necessarily be extrapolated to other populations [18].

4.3.1.2 Occupation

More single chemical substances have been recognized, and subsequently established as causes of cancer in humans, in an occupational context than in any other circumstance of exposure [5]. Thus, in respect of agents identified by IARC as carcinogenic to humans (group 1) until 2009, and therefore evaluated in the six *Monographs* volumes 100A–F, two volumes entitled "Chemical agents and related occupations" and "Arsenic, metals, fibres and dusts" concern occupational exposure, while, apart from chemicals, the volume addressing "Radiation" is largely concerned with occupational situations. Occupational carcinogens continue to be identified, particularly by singular circumstances of exposure giving rise to particular tumors as exemplified by 1,2-dichloropropane causing cholangiocarcinoma [19]. Most exposure to carcinogens in the workplace involves inhalational exposure, with dermal exposure also being relevant in some instances and ingestion and other routes of exposure being recognized in particular instances [20].

A range of measures are recognized as means to prevent occupational cancer [21], beginning with the abandonment of particular industrial processes or the replacement of carcinogenic chemicals with less hazardous substances. Failing that, closed systems may be adopted to prevent exposure, and this end may be achieved at least, in part, by the adoption of improved ventilation systems. Provision of personal protective equipment is considered the option of last resort. All such equipment must be properly maintained, specifically

including replacement of filters where appropriate. Exposure may also be reduced by the adoption of good hygiene practices, as facilitated by provision of clean work clothing and appropriate washing facilities.

Occupational cancer is wholly preventable by regulation that may be adopted in relation to any of the scenarios outlined above. In particular, statutory limitations on exposure, with reference, for example, to threshold limit values, provide for reduced exposure [22]. However, primary reliance on such regulatory determinations is not justified [23].

4.3.1.3 Drugs

While exposure to chemicals in the workplace is incidental, and in some cases, at least, involves minimal levels, drugs are, by definition, administered at levels known to have a physiological impact in some context. Safety of drugs, specifically with reference to avoiding of iatrogenic cancer, principally involves a focus on side effects and toxicity at the preclinical and phase 1 trial level of drug development, together with vigilance in relation to drugs in use [24]. Key considerations involve attention to functional groups as evidenced by molecular structural considerations and attention to appropriate testing for mutagenic activity arising in the course of metabolism [25].

Development of cancer consequent upon the use of particular drugs is rare, and discontinued use is the obvious preventive option, as has been the case with diethylstilbestrol and phenacetin. Pharmaceutical steroids as encountered in oral contraceptives and hormone replacement therapy have been variously demonstrated to increase and, in some instances, decrease risk of particular cancers [26,27]. The benefits accruing from such agents have warranted changes in formulation and constraints on usage as preventive measures. Likewise, with reference to the continued use of some hazardous pharmaceuticals, the largest category of cancer-causing drugs involve anticancer agents, ranging from alkylating agents to receptor-mediated agents, the carcinogenicity of which may primarily involve certain drug combinations. These agents identify the rare situation where monitoring of persons exposed in the context of pediatric cancer patients developing second malignancy is considered an acceptable option for cancer prevention [28].

Some natural products in traditional medicines, exemplified by plants containing aristolochic acids [29], are now recognized as carcinogenic. Otherwise, the scope of drugs for which some evidence of carcinogenicity exists is broad as exemplified by digoxin, hydrochlorothiazide, triamterene, primidone, and methylene blue [30]. No single formula may be endorsed as a preventive option. Rather, statutory authorities in all countries must strike a balance between particular risk data and the benefit accrued to relevant communities through using the drug concerned. In the present context, licensing and marketing of pharmaceuticals is properly recognized as a means through which cancer prevention can be achieved.

4.3.1.4 Pollution

The clearest burden of cancer attributable to pollution involves contamination of water by arsenic from natural and industrial sources in many countries, including Taiwan, Bangladesh, China, and some countries of Central and South America, with a level of concern being concentrations of greater than 10 μg/l [31,32]. Practicable means of reducing arsenic contamination of drinking water in southeast Asia center on well-switching, particularly to deep wells, rather than water treatment [33]. In Bangladesh where the problem is acute, both the means and the limitations on reducing arsenic levels are recognized [34]. Obviously, industrial pollution of water by arsenic is, at least in some instances, amenable to prevention by environmental protection legislation [35].

Although outdoor air pollution may be specified as causing lung cancer [36], prevention of this disease burden is most readily addressed with reference to specific pollutants. Diesel engine emissions are known to cause lung cancer, and are implicated as a cause of bladder cancer. Improved technology, characterized as low-emission, advanced-technology on-road heavy-duty diesel engines, is seen as contributing to reduced risk of cancer [37]. Technology is also recognized as fundamental to emission controls as addressing cancer in communities near polluting industry. Obviously, a range of respiratory diseases, and not cancer alone, are addressed by regulatory controls [38].

The need to reduce outdoor air pollution is preeminent in relation to pollutants apart from those specifically recognized as carcinogenic, and is achievable by statutory means to the betterment of health [39]. Reducing levels of industrial and vehicular air pollutants is a priority for countries with emerging economies [40]. Demonstrable improvement in prospects for industrial emissions can be achieved if appropriate control measures are adopted [41]. In 2012, the Chinese government launched a National Plan on Air Pollution Control in Key Regions setting out for the first time strict targets. To further improve air quality, the Chinese government adopted the first National Action Plan on Air Pollution Prevention and Control, which requires that, by 2017, specified particulate matter levels in cities above the prefecture level must be reduced by over 10% compared with 2012 levels [42].

4.3.1.5 Dietary Carcinogens

Carcinogen-contaminated food is exemplified by exposure to aflatoxins. Cancer causation by aflatoxins is related to the prevalence of hepatitis in the population exposed [43]. Food policy reforms in China resulted in a dramatic decrease in aflatoxin exposure, which, independent of hepatitis B virus vaccination, has reduced liver cancer risk [44]. Reduced exposure to aflatoxins in Africa is being achieved by improved grain storage and biocontrols [45].

Apart from aflatoxins, a range of fungal metabolites are implicated as possible causes of cancer and other toxic injury [46]. Fumonisins have been associated with liver and kidney cancer; ochratoxin A has been associated with kidney and

liver cancer, and sterigmatocystin is associated with liver and lung cancer. Preventive action in relation to such dietary contaminants primarily involves establishment of storage and other conditions that minimize growth of the respective fungi [47].

4.3.2 Tobacco Smoking

Tobacco smoke includes both a gaseous and particulate phase, and is a complex mixture of several thousand compounds that include, as the principal carcinogens, various polycyclic aromatic hydrocarbons and the nitroso derivatives of nicotine and nornicotine [48]. These chemicals are genotoxic carcinogens, and their impact on respiratory epithelium and other tissue is augmented, in respect of mediating malignant transformation, by substances causing inflammation and cellular proliferation following toxic injury. Tobacco smoke also contains nicotine that is addictive.

Tobacco smoking, specifically the smoking of cigarettes, is the major known cause of cancer. In 1981, Doll and Peto [49] ascribed approximately 30% of preventable cancer in the United States as caused by tobacco smoking; an estimate that has changed only marginally in the following 35 years [50]. Lung cancer has long been recognized in many developed countries as the major malignancy caused by smoking, but at least 14 other anatomical sites are involved including various organs of the aerodigestive and urinary tracts, together with one form of leukemia and also cervical cancer [51]. Tobacco smoking is established as a cause of lung and other cancer to the point of scientific certainty.

In virtually all communities worldwide, specifically among males, 75% or more lung cancer cases are attributable to tobacco smoking. This consideration, taken together with a 5 year survival rate for lung cancer of 15% or less, means that lung cancer mortality is a *de facto* indicator of tobacco usage occurring some 20–30 years before [52].

In many discussions of cancer prevention, tobacco smoking is the first and major topic considered as justified by both the burden of attributable malignancy and the scope of preventive initiatives [3]. Preventive measures evolved in relation to tobacco are now reckoned to encompass most, if not all, options that may contribute to changed behavior in relation to hazardous substances exposure, which is largely governed by personal choice. Reduced lung cancer rates have been attributed to tobacco control [53].

A further singular dimension to tobacco control involves the WHO Framework Convention on Tobacco Control – the first such international agreement adopted under the auspices of WHO [54]. The measures addressed in this international agreement have been enacted to varying degrees in different countries, each covering timeframes that also often vary between nations.

4.3.2.1 Measures to Limit Availability and Promotion

There is no general recognition of prohibition as a means to prevent cancer caused by smoking. Instead of banning tobacco products outright, in most jurisdictions their sale is restricted to adult customers; sale of the product to minors is illegal [55]. In many countries, tobacco products are often only available through a restricted category of outlets that may be subject to restricted opening times. The use of vending machines may be controlled.

Bans on the advertising of tobacco products, originally restricted to print and electronic media, and now involving electronic communications, are among the earliest measures recognized as contributing to tobacco control [56]. While promotion was initially recognized as involving sales promotion through advertising, the tobacco industry progressively resorted to other forms of promotion encompassing, for example, logo display in relation to sport, cultural, or other activity and promotion exemplified by the apparently incidental occurrence of smoking in the course of films and television programs [57]. Promotion overtly directed to youth has seen the marketing of flavored cigarettes [58]. Typically, litigation and specific legislation have seen these various initiatives at least subject to challenge, and prohibited in various jurisdictions.

The application of taxation policy as a means of raising the price of cigarettes is widely recognized as among the most effective tobacco control measures available [59].

4.3.2.2 Product Labeling, Health Warnings, and Usage Restrictions

Community awareness of the disease consequences of smoking has been achieved, in part, by warning labels mandated to be displayed on cigarette packs and the like [60]. The area of the pack and the graphic nature of such warnings have progressively increased over decades in most high-income countries. Most recently, in Australia, legislation to enforce plain packaging of cigarettes has been adopted [61] and other countries have indicated a commitment to this end [62]. The legislation has been challenged as an infringement of intellectual property rights and a restriction on international trade, thus far without effect.

Youth awareness of tobacco smoking as addictive and as causing respiratory disease, cardiovascular disease, and cancer is now a fundamental aspect of health education [63]. Community awareness of harm from tobacco smoking, including cancer causation, may be increased by media-based advertising supported by health authorities [64].

Inhalation of tobacco smoke by nonsmokers, sometimes specified by reference to environmental tobacco smoke or secondhand smoke, is recognized as causing a range of respiratory disease particularly in children [65]. A minor fraction of disease attributable to secondhand smoke is due to lung cancer [66]. Restrictions on tobacco smoking have progressively included, to a lesser or

greater extent in different countries, most workplaces [67], health care centers, public places, restaurants, bars, and other hospitality businesses [68] and some outdoor spaces, specifically where food is served [69].

4.3.2.3 Smoking Cessation

The tobacco control measures outlined thus far may be viewed as being effective by reducing the number of people who may otherwise have taken up smoking, though many of these measures may encourage present smokers to quit. The fact that tobacco smoking causes cancer critically includes reduced incidence of disease after smoking cessation. The benefit is age related, being most clear for people aged under 30, but still evident at any age that has proved amenable to investigation [70].

Since the matter was first subject to study, the limited success of individuals wishing to quit has provided clear evidence of the addictive impact of tobacco smoking. The situation has been markedly improved with the availability of nicotine replacement therapy [71]. Moreover, there are now a range of pharmaceutical products available to support smoking cessation [72]. Apart from smokers otherwise apparently in good health, the preventive benefit of smoking cessation specifically includes increased survival of persons diagnosed with almost all tumor types [73].

4.3.3 Alcohol Drinking

Approximately 3–5% of all cancer in high-income countries is attributable to the consumption of alcoholic beverages [6]. Drinking alcohol causes cancers of the oral cavity, pharynx, larynx, esophagus, liver, colorectum, and female breast. Cancer causation is mediated by ethanol, which, in addition to alcoholic beverages, is categorized as an IARC group 1 carcinogen [51].

In light of such causative information, it may be supposed that reduced consumption of alcohol is specifically identified as a central approach to cancer prevention. Quite obviously, this is not the case. The acute harm done by reckless and excessive consumption of alcohol is the focus of concern, beginning with the deaths and injury caused by drink driving and alcohol-fuelled violence [74]. A further immediate effect of alcohol consumption involves fetal injury, while chronic affects include addiction and cirrhosis. Initiatives to discourage high-level consumption of alcohol can be identified as contributing to cancer prevention, but are rarely framed in that context specifically.

In theory, nearly all of the measures developed in relation to tobacco control are applicable to the availability and use of alcohol. In practice, only a few matters have been actively pursued. Price, as subject to modification by taxation policy, influences alcohol availability [75]. Warning labels are an option, but unlike tobacco, where adopted, warning labels on alcohol typically do not refer to cancer causation as a reason for limiting intake [76].

4.3.4 Solar and Ultraviolet Radiation

Ultraviolet (UV) radiation causes all types of skin cancer, squamous cell carcinoma, basal cell carcinoma, and malignant melanoma. Risk is related to the circumstances of radiation, and varies in relation to a range of genetic factors [77]. Exposure to carcinogenic UV irradiation predominantly involves exposure to sunlight that may be incidental, specifically including occupational exposure, or deliberate. Other hazardous sources include UV-emitting tanning devices [78] and radiation emitted in the course of welding [79]. Skin cancer is subject to variation according to race, with Celtic heritage as indicated by fair skin, red hair, and blue eyes indicating marked susceptibility. Beyond that, risk of these cancers overwhelmingly involves white-skinned people [80].

Occupational exposure to sunlight is virtually unavoidable for a range of job descriptions that encompass, but obviously are not limited to, all categories of agricultural and forestry work; work maintaining road, electricity, and other services; many circumstances of construction and building work; on-foot supervision of pedestrian and vehicular traffic; and much employment centered on physical activity and recreation. Preventive measures may most immediately include employer provision of protective clothing (including eye protection) and sunscreen usage, all used in the context of a recognized hazard awareness and prevention program [81]. Beyond ensuring adoption by employees of "sun smart" practices, management initiatives may include adoption of work hours to exclude peak intensity exposure around midday.

Recreational exposure to sunlight is particularly challenging in communities in which a tan is recognized as a mark of good health. Behavioral change, predicated on the betterment of health-based education, may serve to underpin increased use of protective clothing and sunscreens [82]. Confirmation of the status of sunscreens as reducing risk of sunlight-induced cancer has been challenging in light of clear experimental evidence [83]. Simplistically based studies indicated that crudely assessed sunscreen use may serve as a *de facto* indicator of sun exposure and be positively associated with risk. In rigorously controlled circumstances, the protective effect is evident [84].

Means of sun avoidance are now established [85]. Prevention of unnecessary and harmful exposure of children to sunlight warrants particular mention as the one aspect of solar exposure [86] that may be viewed as appropriately addressed by regulation [87]. In Australia, for example, provision of shaded areas for child play is a statutory requirement, and there is similar enforcement of children's school clothing, specifically involving the wearing of hats [88].

The use of tanning devices is known to cause skin cancer independent of any requirement to extrapolate from findings involving solar radiation [77]. Prevention of cancer from tanning devices is in large part by statutory limits on the commercial provision of relevant services, at least to persons under 18, and in some jurisdictions involving an absolute prohibition [89,90].

4.4 Prevention Involving Complex Risk Factors

The term complex risk factors is used here to identify modifiable risk factors associated with cancer to the point of causation or to some lesser degree and that cannot be readily characterized as involving exposure to a known or immediately implicated carcinogen(s). Such circumstances of increased cancer risk are inherently challenging in relation to cancer prevention by comparison with cancer risks already discussed.

4.4.1 Workplace Exposures

Increased risk can sometimes be unequivocally identified with reference to particular job descriptions, most readily illustrated by reference to lung and bladder cancer being caused by work as a painter [91,92]. The relevant risk may be mediated by solvents, pigments, and/or some other components of paint or some exposure common to painters but not involving paint per se. Initiatives that may be rationalized in terms of some likely preventive effect are broad, extending from personal protective equipment through to monitoring the impact of altered technology and formulations [93]. Monitoring relevant workers in relation to indicators of exposure through to the early detection of disease may be considered [94].

4.4.2 Diet and Overweight/Obesity

As described more than 35 years ago in the assessment of attributable risk by Doll and Peto [49], the major role accorded to diet did not involve carcinogen-contaminated food but dietary composition, including, for example, fat intake. Estimates since that time have generally involved reducing the proportion of cancer cases attributable to diet and attributing cancer to overweight/obesity, particularly as this risk factor has become increasingly prevalent not only in high-income countries but also in other communities worldwide [6,8].

Independent of the impact of food contamination by dietary carcinogens such as aflatoxins, relatively few foods have been specifically identified as contributing to increased risk of cancer. Primary among these are red meat and processed meat [95]. For colorectal cancer, risk is incrementally related to intake. Sustained high intake of processed meat (over 100 g/day) and red meat (over 200 g/day) leads to the highest risk. By implication, reducing such intake would be preventive and dietary guidelines may be envisaged as contributing to this end. Immediate confirmation of a preventive intervention through randomized control trial is extremely challenging [96].

A critical factor in evaluating consumption of red and processed meat as presenting a carcinogenic risk to humans was that relevant epidemiological studies were designed to control for the impact of alcohol drinking, smoking,

and various parameters related to overweight obesity, sometimes addressed through BMI determinations or information regarding caloric intake. From a biological and epidemiological perspective, the requirement to distinguish between increased risk of cancer being directly associated with consumption of meat and any such risk being a consequence of overweight/obesity that occurs as a result of poor diet including high meat intake is imperative. Arguably, from a public health perspective, this distinction is a minor consideration, particularly if it can be established that avoidance of high meat intake results in decreased cancer risk [97].

The chronic disease is most markedly caused by overweight/obesity is type II diabetes [98]. By comparison, risk of certain cancers is almost an order of less magnitude. Behavioral change is fundamental to reducing the proportion of the population categorized as overweight/obese, and may be recognized as preventing cancer. In the United States, sugar-sweetened beverages, as a major dietary factor in the present context, have been subject to specific policies [99]. A recognized focus is the prevention of childhood obesity [100].

The extent to which dietary interventions may alter overweight/obesity in the first instance, and risk of malignant disease in the second instance, is being explored in a variety of clinical trials, many involving anticipated changes in the survival of cancer patients [101]. There is increasing and consistent evidence that adherence to cancer prevention guidelines is associated with the decreased risk of cancer [102].

4.5 Prevention Independent of Causative Agents or Risk Factors

4.5.1 Screening

Theoretically, population-based screening for premalignant or early-stage disease is a goal in respect of all common tumor types. In practice, the criterion of establishing reduced mortality through implementation of a screening program, together with the absence of harm consequent upon "false positive" notifications and overdiagnosis consequent upon detection of nonlethal disease, represents what may be insurmountable standards for most proposed screening protocols.

Screening for cervical cancer using the Papanicolaou smear is definitively established to reduce mortality [103]. The test detects premalignant cells rather than early-stage malignancy and therefore reduces incidence of cervical cancer. In high-income countries, the Pap test is rapidly being displaced by protocols based on detection of HPV [104]. Meanwhile, increasing evidence of the efficacy of vaccination against HPV infection offers the prospect of

primary prevention and decreased reliance on population-based screening for cervical cancer [105].

Setting aside any further consideration of cervical cancer, the two most widely adopted population-based screening procedures – mammography for breast cancer and fecal occult blood testing for bowel cancer – involve detection of early-stage malignancy. Upon their adoption, an increase in cancer incidence is anticipated preceding a decrease in mortality. The efficacy of mammography in reducing breast cancer mortality is related to the age range over which women are screened. In light of strong evidence of benefit from mammography from randomized control trial data, there is the immediate prospect of evaluations being based on current screening programs [106]. However, the prospect of overdiagnosis is increasingly recognized and may ultimately alter perspectives in relation to population-based programs [107].

Screening for colorectal cancer is evolving, a principal challenge being to engage a large proportion of the community identified as being at risk. Thus, in the United States, screening participation has been assessed as suboptimal, particularly among underserved populations such as the uninsured, recent immigrants, and racial/ethnic minority groups. Even so, a decrease of 30% in the US incidence of colorectal cancer between 2000 and 2010 among adults aged 50 and older has been attributed primarily to screening [108].

The prospect of population-based screening for prostate [109] and lung [110] cancers continues to be assessed. However, with the possible exception of the Pap smear, cancer screening tests continue to be mired [111].

4.5.2 Chemoprevention

Two decades ago, chemoprevention was seen as a dynamic and potentially central aspect of cancer prevention [112]. A primary focus involved the possibility that supplements containing β-carotene, α-tocopherol, selenium, and/or retinol may reduce incidence of many tumor types [113], an understanding largely predicated on case–control studies indicating the consumption of fresh fruit and vegetables markedly decreases risk of multiple cancer types [114]. This understanding has been set aside as results from prospective studies are accrued, while the negative results of supplement trials are exemplified by unequivocal evidence that β-carotene causes lung cancer [115].

The worth of cancer chemoprevention has been vigorously challenged [116]. Arguably, chemoprevention is most clearly demonstrable using pharmaceutical drugs such as tamoxifen and related agents to prevent second breast cancers. Thus, clinical trial results demonstrate anastrozole reduces breast cancer incidence by 53% in postmenopausal women [117]. Dauntingly, however, while tamoxifen may delay contralateral breast cancer, its use in this context does not reduce mortality [118].

The cancer chemopreventive role of aspirin has long been recognized [119]. Aspirin and other nonsteroidal anti-inflammatory agents prevent colon cancer specifically among those at familial risk [120]. Taking a broader perspective, the scope of the chemopreventive effects of aspirin is yet to be fully appreciated [121]. Moreover, new possibilities for chemoprevention in a range of contexts continue to arise and warrant investigation [122].

4.6 Conclusion

As noted earlier, this discussion of cancer prevention was developed on the basis of knowledge concerning cancer causation rather than, for example, current potential for most markedly reducing cancer incidence or a discussion predicated on particular methods of cancer prevention. No specific outcome was anticipated, but the completed review readily indicates an overall conclusion, namely, the more definitively an agent or risk factor is known to cause cancer, the easier is the task of envisaging preventive options. Conversely, the so-called spontaneous cancer presents the greatest challenge when preventive options are considered. Accordingly, the current state of cancer prevention encourages research directed toward the identification of circumstances upon which exposure to specified carcinogens accounts for the distribution of cancer in one or more communities. Such knowledge provides the best possible basis for the adoption of preventive measures.

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Part Two

Exposures that Could Alter the Risk of Cancer Occurrence, and Impact Its Indolent or Aggressive Behavior and Progression Over Time

5

Diet Factors in Cancer Risk

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5.1 Introduction

There is no doubt that diet plays a significant role in cancer risk. Lifestyle factors, including diet and exercise, have long been recognized as potentially important determinants of both susceptibility to, and survival with, many types of cancer. In addition to the significant role that diet plays in affecting adiposity, experimental and observational studies have indicated that diet may influence the cancer process in several different ways [1–4]. Most recommendations on cancer prevention and control that are endorsed by influential international groups such as the International Agency for Research on Cancer (IARC) focus on both diet and lifestyle factors, especially exercise [5]. Body fatness appears as a risk factor for several cancers [6]. While this has a genetic component [7], it is largely determined by poor diet and inadequate exercise. It is also affected by sex and geographic variables. Diets rich in high-calorie foods, such as fatty and sugary foods, may lead to increased calorie intake, thereby promoting obesity, while a high intake of sugary drinks has been specifically related to an increased risk of pancreatic cancer [8]. There is evidence that high intakes of fruit and vegetables may be especially effective in reducing the risk of cancers of the aerodigestive tract, and the evidence that dietary fiber (DF) protects against colorectal cancer (CRC) is convincing [9,10]. High intakes of red and processed meats have also been shown to associate with elevated risks of CRC.

Taking a range of evidence into account, the 4th edition of the European Code against Cancer recommends that, to reduce the risk of cancer, people's diets should be largely focused on whole grains, pulses, vegetables, and fruits, limiting high-calorie foods that contain high levels of sugar and/or fat, avoiding processed meat, and limiting red meat, foods high in salt, and alcohol consumption. This

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same diet pattern is also associated with improved overall survival after cancer diagnosis, especially for breast cancer (BC) and CRC patients [5].

5.2 Obesity

Excess adiposity or excess body fatness is generally considered to cover a spectrum of body mass indices (BMIs), whereby overweight is defined by BMI levels between 25 and 29 kg/m^2, while obesity is defined by BMI levels of 30 kg/m^2 and greater. There are differences in the associated risks depending upon both sex and hormonal status. Overweight or obese women after menopause have a higher risk of both breast cancer and endometrial cancer [11]. Excess body fatness itself induces a hyperestrogenic state, which is the likely cause of these elevated risks. Convincing associations have also been shown relating obesity to elevated risks of cancers of the ovary, endometrium, colon, oesophagus, gallbladder, pancreas, kidney, liver, and prostate [11].

In a study of 10,226 CRC cases and 10,286 controls of European ancestry, high BMI was associated with an increased colorectal cancer risk for women, but not for men [12]. However, the authors did not rule out whether abdominal obesity, rather than overall obesity, may be a more important risk factor for men than for women. For both sexes, high BMI, a year before diagnosis, was also associated with increased mortality for those individuals who had been diagnosed with invasive CRC [13]. This was true for two different molecular phenotypes of CRC.

Chronic local inflammation induced by acid reflux and gallstones is the likely cause of the increased risks of esophageal cancer and gallbladder postmenopausal cancer, while an increased risk of liver cancer appears to be associated with local inflammation induced by hepatic fatty infiltration [11]. Mechanistic hypotheses include elevated systemic or local tissue inflammation induced by adiposity, and effects of the associated elevated levels of leptin, insulin, and insulin-like growth factors, in association with depressed immune function, that are seen with excess body fatness.

Excess adiposity leads to increased circulating concentrations of a range of compounds that have been associated with increased cancer risk, including compounds affecting hormonal status, cellular proliferation, immune response, and inflammation [11,14]. This is especially important after menopause in women, where aromatase activity within adipose tissues has been shown to constitute the predominant source of endogenous estrogens. In such women, excess BMI increases the levels of circulating estrogens, while decreasing levels of sex hormone-binding globulin. These changes can be at least partly reversed by weight loss [11].

Murphy *et al.* reported differential relationships among men and women between specific adipose depots and obesity-associated cancer risk [15]. It is of

interest that the important mechanisms associated with obesity, and probably related to the consequent cancer risk, include effects on the gut microbiota and metabolic markers [16,17]. Fecal DNA was analyzed from a series of obese women, using quantitative metagenomic sequencing and analysis. The study also included a systematic search for bacterial genes associated with estimates of insulin resistance, inflammation, and lipid metabolism [16]. A number of bacterial species showed a relationship (either positive or negative), and some of these relationships were significantly modified by the intake of dietary carbohydrates and lipids.

5.3 Macronutrients

A preference for different foods and food groups has been associated with increased cancer risk. Lipids and carbohydrates come up strongly in many of these relationships. For example, in their studies on stomach cancer risk in a Northern American population [18], Hu *et al.* associated total macronutrient intake, and specifically intakes of total fat, saturated fat, and cholesterol with increased risk with the disease, while total DF showed an inverse association. The positive associations with intake of total fat and saturated fat appeared strongest in women, overweight or obese subjects, and ever smokers. In their studies associating macronutrient intake with the risk of urothelial cell carcinoma in the European Prospective Investigation into Cancer and Nutrition (EPIC) trial, an increase in energy intake from animal protein has been found to be associated with increased cancer risk, while an increase in energy from plant protein lowers the risk. For thyroid cancer, obesity and excess protein and carbohydrate consumption have been found to be key risk factors, especially in women [19]. In a Canadian study, high energy intake appeared to increase the CRC risk, while diets high in protein, DF, and carbohydrates more generally appeared to reduce the risk [9].

The 4-Corners Breast Cancer Study considered the influence of macronutrient composition on BC risk, in a comparison between Hispanic and non-Hispanic white women in parts of the United States [14]. For both of these groups, fat intake, especially saturated and monounsaturated fat, decreased risk, while total carbohydrate intake increased the risk. The Italian population studies by Franceschi *et al.* [20] also showed the risk of BC decreasing with increasing total fat intake but increasing with a higher intake of available carbohydrates. However, with these and many other studies, the nature of the carbohydrate source is critical. Such findings have been used to suggest a substantial risk, especially in southern European populations, of reliance on a diet largely based on starch. The data may also be complicated by body weight, since obesity has come across as a significant factor in cancer risk in a number of studies [6,19,21–25]. However, it is often difficult to pull apart the relative roles

of overnutrition in general, diet patterns associated with obesity, and/or physical inactivity [23,26,27].

5.3.1 Protein

Data relating protein consumption to cancer risk have shown mixed results. While a number of studies have related high protein intake to increased risk of cancers such as BC and CRC [19,28,29], others have suggested that higher protein intake may reduce the incidence of certain cancers, including hormone-related cancers of the breast and prostate, and also CRC [9,30]. Clearly, the results obtained depend upon the exact population studied, including their genotype and other elements of their normal diet. However, the nature of the protein also affects the results.

High intake of animal protein, especially protein from red meats, has been related to an increased risk of cancer. Indeed, the IARC has evaluated processed meats as human carcinogens, and red meats as probable carcinogens [31]. However, many of the reported effects may associate with cooking processes, rather than animal protein [29]. For example, in a New Zealand population, we showed evidence for a link between increased prostate cancer (PCa) risk and high temperature cooking, especially barbequing, likely to be associated with the formation of DNA-reactive carcinogens, including heterocyclic amines and polycyclic aromatic hydrocarbons [32]. Joshi *et al.* analyzed meat consumption, including portion size data and cooking methods for 3364 CRC cases, 1806 unaffected siblings, 136 unaffected spouses, and 1620 unaffected population-based controls, recruited into the CRC Family Registry [33]. They found an increased risk with cooking methods for certain cuts of meat, but not meat *per se*. This effect was strongest in those deficient in mismatch repair proficiency. Similar observations have also been made for BC and PCa [32]. Smoking of salmon was also shown to lead to the formation of various types of polycyclic aromatic hydrocarbons [34]. In a study from Taiyuan, China, DNA-reactive metabolites were also shown to be formed during the cooking of vegetables, wheat flour, and fruits [35].

Advanced glycation end products such as *N*(ϵ)-(carboxymethyl)lysine also occur in both cooked and uncooked foods, and have been associated with an increased risk of at least one type of cancer [36]. These are reactive metabolites, produced as a by-product of sugar metabolism [37]. Although they are present at low levels in unprocessed red meats, they increase significantly upon cooking, although this formation is reduced by marinating the meat [36,37].

The recent IARC evaluation concluded that the evidence for carcinogenesis by processed meat was significantly stronger than for unprocessed meats, and the former should be considered as human carcinogens [31,38]. Processed meats are those that have been modified by salting, curing, fermentation, or other processes to enhance flavor or preservation. *N*-Nitroso-compounds in

particular are DNA-reactive, and are often formed during processing of red meats [39,40].

Although high animal protein consumption does not appear beneficial to cancer prevention, a moderately increased intake of plant protein, especially from soy, has been suggested to be beneficial for PCa in some studies [41]. A community-based cohort in Japan asked whether soy or soy product intake was associated with all-cause mortality [42]. This value was found to be significantly higher in men with infrequent soy intake or with almost daily intake as compared with intake one to two times per week. Cancer mortality was higher among men who reported rarely eating soy. Gastric cancer risk was inversely associated with the high intake of soy foods in a Korean population, while BC risk was reduced by high intakes of total soy products, soybean curd, and soymilk [43]. Although many of the studies available provide only limited information on soy protein, because they consider soy products as a group, it is of interest that mechanistic studies support soy protein as having a plausible role in its own right. For example, Burris *et al.* showed convincing anti-inflammatory effects of a soy protein concentrate in apolipoprotein E-deficient mice [44].

The situation regarding soy is confused in relation to cancers that relate to hormones, especially PCa and BC, because it is not always clear whether soy protein or soy phytoestrogens are causing the effects seen (see Section 6.4). Lin *et al.* concluded that soy protein showed promise in reducing both PCa risk and progression [41]. However, a prospective randomized trial that studied men at high risk for PCa recurrence following radical prostatectomy randomized these to receive a soy protein supplement or placebo daily for up to 20 months. Although no decrease in the rate of recurrence was observed among men receiving soy protein, it has been commented that interpretation of trials such as this is difficult owing to a lack of dosing studies, and a wide variety of available soy products with differing composition and protein concentrations [45]. For BC, alternative protein sources suggested are from poultry or from legumes, with soy protein again appearing beneficial in some but not all studies [29,46].

5.3.2 Lipids

The available data on lipids appear mixed, partly because different studies have been inconsistent in the amount of information provided on the nature of the dietary fats consumed. A number of authors have reported that a high overall dietary fat consumption associates with higher risk of a number of cancers, and suggested that a reduction of overall fat intake could be beneficial [47,48]. For example, a recent meta-analysis of gastric cancer risk showed an overall increased risk associated with high total fat intake [49]. However, this study also provided evidence that specific subtypes of fats accounted for different effects. The increased risk was especially strong for high saturated fat intake, while inverse relationships were shown for certain types of polyunsaturated

fatty acids (PUFA) and also vegetable oils. High saturated fat intake has also been associated with an increased risk for breast cancer [47].

There is some evidence that elevated low-density lipoprotein (LDL), triglycerides (TG), and total cholesterol (TC) relate to high risk of CRC, although data have been inconsistent [50]. A meta-analysis of prospective cohort studies showed that BC risk among postmenopausal women, but not premenopausal women, was significantly reduced by elevated HDL cholesterol. The evidence suggested that serum levels of TG, but not TC and LDL-C, may be inversely associated with BC risk [51]. It is possible that genetic stratification might resolve apparent inconsistencies.

A key group of lipids associated with cancer risk are the omega-3 and omega-6 polyunsaturated fatty acids (*n*-3 and *n*-6 PUFA). The two long-chain *n*-3 PUFA, eicosapentanoic acid (EPA), and decosahexanoic acid (DHA) are at high levels in fish, especially oily fish. In their Japanese population that generally had a high dietary fish intake, Hidaka *et al.* found an inverse association of marine *n*-3 PUFA with pancreatic cancer risk [52]. However, while epidemiologic studies have shown that high *n*-3 PUFA intake is associated with reduced BC incidence among Asian populations, this relationship did not appear true for Western populations [53]. Inconsistencies also appear in different studies with different populations in relation to lung, CRC, and other cancers [52,54–57]. Part of the reason for these apparently conflicting data may be the ratio between *n*-3 and *n*-6 PUFA consumption. That is, there would seem to be benefits associated with not only increasing long-chain *n*-3 but also simultaneously decreasing *n*-6 PUFA, especially in Western civilizations. Such a rationale makes biological sense, given that *n*-3 PUFAs inhibit the inflammatory eicosanoids generated by *n*-6 PUFAs [58].

Since oily fish is the main dietary source of long-chain *n*-3 PUFA, their intake is usually measured in terms of fish and/or fish oil consumption. It is important to recognize that some of the apparent inconsistencies or negative effects may not necessarily relate to the fish or fish oil *per se*, but rather to other environmental factors such as pollution of the fishing area or oxidation of the isolated fish oils [59].

While apparently inconsistent data on associations between fat intake and cancer risk have been used as justifications for more and bigger studies, or lead to the conclusion that there is no association [57,60], another factor that might be justified in many studies is genetic stratification [61]. For example, an analysis of data from the CHARGE consortium showed that a variant in the fatty acid desaturase 1 (FADS1) gene modified the effects of dietary intake of long-chain *n*-3 PUFA on circulating fatty acid levels [61].

5.3.3 Carbohydrates

Carbohydrates comprise a large group of organic compounds occurring in foods and living tissues, containing hydrogen and oxygen in the same ratio as

water (2:1) [62]. These are generally divided into three main groups depending upon the complexity: sugars (mono- and disaccharides), oligosaccharides (short-chain carbohydrates), and polysaccharides. Since many of these can be broken down to release energy in the animal body, they are sometimes vilified in dietary recommendations to reduce the risk of chronic disease [63]. However, such recommendations do not recognize the complexity of the group and the range of different properties encompassed [62]. Spreadbury has suggested that dense acellular carbohydrates promote an inflammatory microbiota, and that these may be the primary dietary cause of leptin resistance and obesity [64]. He considered nutrition transition patterns and the health of those still eating diverse ancestral diets with abundant food to endorse the evidence suggesting that glycemic index or altered fat or carbohydrate intake does not appear to be the main cause of obesity. Instead, he proposed that refined flour, sugar, and certain types of fat are key attributors to obesity and other Western diseases. He extrapolated these data to suggest that whole foods containing carbohydrates primarily from root vegetables, leaves, and fruits will lead to more desirable endpoints.

The evidence for carbohydrate consumption in relation to cancer is variable, and it is recognized that there are some particular difficulties in available evidence for this group of compounds. As for many nutrients, there are difficulties in obtaining valid measures of long-term dietary intake. Particular problems relate to certain carbohydrates, especially sugars, in many manufactured foods, which can be hard to quantify in epidemiological studies [64]. Food composition tables do not generally distinguish the sugars occurring naturally in foods, such as fruits, from sugars added during manufacturing processes. Englyst *et al.* have suggested that some of the apparently contradictory data for carbohydrates in relation to the risk of diseases such as cancer should be addressed by grouping them into "available carbohydrates," which are digested and absorbed in the small intestine, and "resistant carbohydrates," which resist digestion in the small intestine and/or are poorly absorbed/metabolized [62]. They endorse the accumulating evidence suggesting that dietary restrictions on free or added sugars would be beneficial in protection against diseases such as cancer. However, they also highlight the evidence that resistant carbohydrates, especially the unrefined nonstarch polysaccharides (NSP) from plant cell walls, have a range of beneficial effects in protection against cancer [65].

We have highlighted the apparently contradictory evidence, suggesting that DF may either protect or enhance the risk of cancer development, especially CRC [66–68]. In its original definition, DF consisted only of plant cell walls, and these still comprise a major part of this group. However, they vary in their composition and properties. Some of the available evidence on DF and cancer is obscured by an ongoing lack of complete international agreement on the definition of DF [69]. The current definition lists three categories of carbohydrate polymers that are not hydrolyzed by endogenous enzymes in the small

intestine of humans. Although there has not been international consensus, the inclusion of carbohydrates with degrees of polymerization (DP) is in the range of 3 and 9. However, there appears to be a degree of agreement, suggesting that nondigestible carbohydrates with >DP3 should be considered as DF [69].

It is not only the chemical composition but also the processing of DF sources that affects their properties in relation to chronic disease [70–72]. Whole cereal grains contain three layers: the bran (outer layer), endosperm (middle layer), and germ (inner layer). The bran and germ contain concentrated amounts of DF, in association with various vitamins, minerals, and phytochemicals. During the refining process, the bran and germ are removed from the whole grain, leaving the endosperm that is primarily composed of starchy carbohydrates and low in nutrients. Although some nutrients, including certain B vitamins and iron, are added back to refined grains and flours during manufacturing, these represent only a fraction of what is initially removed from the grain. Refined grains are rapidly digested into simple sugars and absorbed into the bloodstream, whereas whole grains or less processed grains in the form of wheat bran have a range of properties that relate to cancer protection [72].

Available data suggest that DF containing suberin or lignin may be especially beneficial, although they are present in only small amounts in food plants [66]. DFs added to food include components of plant cell walls, such as pectins, resistant starches, and nondigestible oligosaccharides. Although these have been tested in animal carcinogenesis experiments, they do not always protect, and some may enhance carcinogenesis [65,66]. Few human intervention studies have been done on DF or sources of DF, with the exception of wheat bran, a good source of DF, which has been shown to protect in both animal models and human studies [72–74]. One of the ways in which this may be acting is by reducing the production and excretion of mutagens in stools [72]. Furthermore, phenolic components of intact wheat bran may have antioxidant and other beneficial effects [75–77].

The inclusion of oligosaccharides as DF is largely based on their physiological effects. At least in the United States, inulin, fructooligosaccharides, and other oligosaccharides are included as DF on food labels. This group includes several types of nutrient that may affect the composition of the colonic microbiota. It has been estimated that approximately 10^{14} bacteria of many different species colonize the human colon [17,78–80]. Bacteria have both positive and negative effects on carcinogenesis. They may enhance carcinogenesis by deconjugating and reducing bile acids, which are converted to active substances that promote cell proliferation and growth of adenomas [18,80,81]. Bile acids are associated with the digestion of fat, and the presence of bile acids in feces correlates with fat consumption [79]. Secondary bile acids (deoxycholic acid, lithocholic acid, 12-ketolithocholic acid), along with other fecal substances, are responsible for the initiation of colorectal carcinogenesis. Fecapentaenes are also highly potent mutagens and carcinogens originating from intestinal bacterial production [82].

Two geometric isomers, fecapentaene-12 and fecapentaene-14, are present in significant concentrations in the feces, with reported levels ranging from 5 μg to 6 mg/kg.

Although early studies implicated colonic bacteria such as *Helicobacter pylorum* as risk factors, especially for gastrointestinal cancers [83,84], it is increasingly clear that altering the composition or metabolic activity of the bowel microbiota through the use of DF might be important in reducing the prevalence of colorectal cancer [85]. Oligosaccharides are among the best characterized prebiotics, defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-bring and health" [77,86]. Inulin, oligofructose, lactulose, and resistant starch fulfill the definition. These properties are also shown by other carbohydrate-containing foods, including whole grain wheat and corn. While nondigestible oligosaccharides, such as oligofructose, have prebiotic effects, fermentable dietary fibers also have effects on the composition of the bowel microbiota [77,86].

5.4 Micronutrients

There are acknowledged difficulties in estimating dietary intakes of micronutrients in observational studies of cancer. Besides being part of the overall diet, they may be consumed as supplements, many of which have been found to show "U"- or "J"-shaped curves in which too much is as damaging to health as too little [41,76,87]. There are ethical dilemmas in simply allowing a population group to eat specific levels of a certain nutrient and waiting for cancer to develop. The best example of this may be the SELECT trial for cancer prevention, which considered a selenium (Se) supplement for cancer prevention, but found a result contrary to expectations [88], that is, the incidence of certain cancers was increased rather than showing the desired decrease. Such observations suggest there may be value in short-term biomarker studies, looking at the association between a specific dietary component and cancer risk, since these do not involve observation of diet and waiting for cancer to develop. Given the central importance of DNA, these may involve surrogate biomarkers such as DNA adducts or DNA damage [89,90] or changes in gene expression [91]. A range of various methods will be discussed in the following sections.

5.4.1 Vitamins

Vitamin A comprises a group of organic compounds that includes retinol, retinal, retinoic acid, several provitamin A carotenoids, and β-carotene. Results of a number of cross-sectional or case control studies originally led Peto

et al. [92] to suggest a cancer-preventive role for β-carotene. Most such studies showed a negative correlation between blood carotenoid levels and various biomarkers of DNA damage. However, some placebo-controlled carotenoid intervention trials using disease and mortality as outcomes have suggested a significant increase rather than decrease in mortality associated with vitamin A, β-carotene, or vitamin E supplements [93]. It is possible that this depends upon the concentration used in the supplement and also the population tested. Tissue culture studies have been used in order to shed light on the mechanisms, involving cotreatment with a DNA-damaging agent and various carotenoids. While the nonvitamin A carotenoids usually decreased the DNA damage, thereby promoting genomic stability that would lead to cancer protection, the provitamin A carotenoids had little or no effect at low concentrations, but increased DNA damage at higher concentrations [94].

B vitamins include niacin (vitamin B3), folate (vitamin B9), and vitamin B12. Choline is also included in this group, although it is not an essential nutrient unless the diet is also devoid of methionine and folate [95]. Folate is a key component of a number of root vegetables, including pulses such as red kidney beans, chickpeas, and lentils. It is essential in one-carbon metabolism, acting to supply the methyl units for DNA methylation [96]. There has been considerable variability in reports relating folate intake and the risk of various cancers including breast, prostate, bladder, lung, and CRC. For example, an elevated plasma folate, vitamin B12, and homocysteine have been associated with an increased risk of upper gastrointestinal (GI) cancers in a Chinese population. A dose–response meta-analysis of studies in BC used 14 prospective studies that reported data on 677,858 individuals [97]. Folate intake appeared to show little effect on the overall BC risk, but this analysis obscured the nonlinear relationship between folate intake and the risk of BC, whereby folate intake of 200–320 μg/day was associated with a lower BC risk, while the risk increased significantly with a daily folate intake of >400 μg [97]. The same relationship may not be true for all types of BC, since the European Prospective Investigation into Cancer and Nutrition (EPIC) study suggested that higher dietary folate intake was associated with a lower risk of sex hormone receptor-negative BC in premenopausal women [98].

In Chinese women, Kweon *et al.* found no statistically significant association of gastric cancer with dietary intake of folate, methionine, or B vitamins. However, among premenopausal women, the highest intake of folate was associated with increased gastric cancer risk, although there were no statistically significant associations observed among postmenopausal women [99]. Part of the variability seen across studies may be caused by different frequencies of certain genetic polymorphisms. For example, Jiang-Hua *et al.* found an association of methylenetetrahydrofolate reductase and methionine synthase polymorphisms with breast cancer risk, and this risk was modified according to the intakes of folate, vitamin B6, and vitamin B 12 [100]. Vitamins B also appear to

interact with alcohol consumption. Excess alcohol consumption has been associated with increased risks of several cancers, and this seems at least partly to act through its action as a methyl-group antagonist [101].

Vitamin C has been considered to be an antioxidant, which not only protects against the development of various cancers but may be considered in cancer treatment [102,103]. In human studies, the effects of vitamin C supplementation on various markers of genome stability depend on individual responses to vitamin C levels in the diet, and on concomitant exposure to oxidative stresses [104]. Vitamin C also protects against DNA damage, DNA strand breakage, and chromosomal aberrations [103,105].

The group of fat-soluble secosteroid hormones referred to as vitamin D are responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate, and zinc. In humans, the most important compounds in this group are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol) [106,107]. Very few foods contain vitamin D, and the synthesis of vitamin D (specifically cholecalciferol) in the skin is the major natural source of the vitamin. The dermal synthesis of vitamin D from cholesterol is dependent on sun exposure (specifically UVB radiation) [87,106].

The first study linking low vitamin D status to an increased risk of cancer was an ecological study linking colon cancer occurrence to annual mean daily solar radiation in the United States [108]. Ecological studies have provided important evidence for the role of vitamin D in cancer, as with many other diseases [87]. This type of study typically considers a large number of cases, and since people generally live in the same region for many years, UVB doses provide a reasonable proxy for vitamin D concentrations. There are over 15 types of cancer for which high UVB exposure and/or serum 25-hydroxyvitamin D [25(OH)D] concentrations have been found associated with reduced risk of cancer development. These are bladder cancer, BC, CRC, endometrial, oesophageal, gallbladder, gastric, lung, oral/pharyngeal, ovarian, pancreatic, prostate, rectal, renal, thyroid, and vulvar cancer, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and leukemia [109,110]. The evidence is stronger for more common cancers.

There is also increasing evidence that individuals with a higher circulating 25(OH)D concentration at the time of cancer diagnosis have better cancer-specific and overall survival rates, suggesting that individuals with cancer should raise their 25(OH)D concentrations [87,111]. Vitamin D is critical in the prevention of oxidative stress, chromosomal aberrations, and telomere shortening [112–116]. All these factors would be predicted to reduce genomic instability, tumor metastasis, and progression. Because 25(OH)D concentrations are higher in summer than in winter [117], differential survival between those diagnosed with cancer in summer compared with people diagnosed in winter would indicate a causal role for vitamin D in survival after cancer diagnosis. Evidence for this effect was initially reported for BC, CRC, PCa, and

Hodgkin's lymphoma in Norway [118,119]. Studies from the United Kingdom confirmed the data for BC while also adding lung cancer [120]. Similar results were reported for ovarian cancer in China and for brain tumors in Finland [121,122].

A number of studies on vitamin E have suggested that increasing vitamin E intake reduces the risk of pancreatic cancer, but this conclusion has not been supported by all the published studies. Peng *et al.* conducted a meta-analysis to assess the relationship between vitamin E intake and the risk of pancreatic cancer by combining the results from 10 observational studies (6 case-control studies and 4 cohort studies) [123]. They found a statistically significant inverse association between vitamin E intake and pancreatic cancer risk in both the case–control and cohort studies.

Other vitamins such as biotin (or vitamin H) and the vitamin-like coenzyme Q10 are also important in the maintenance of genomic stability and protection against cancer [124,125]. Vitamin K has been linked to prostate health in general, and showed a protective role with respect to advanced PCa in the Heidelberg cohort of the EPIC study [126]. It is important to recognize that there are considerable interindividual differences in the ability to absorb and metabolize all these vitamins [114,127]. Recognizing the optimal amount for an individual is of considerable importance.

5.4.2 Minerals

While a number of minerals are typically considered as toxicants, some of these are essential micronutrients, albeit usually with a narrow window of efficacy as compared with toxicity. These include iron [128], Se [19], and zinc [129]. Se provides a useful illustration of these complexities, since the population generally shows a "U"-shaped response curve, with both low and high selenium levels increasing genomic instability and cancer risk. The optimal form of Se, at the optimal level, may protect against DNA or chromosome breakage, chromosome gain or loss, damage to mitochondrial DNA, and detrimental effects on telomere length and function [130]. However, the optimal level of Se differs among individuals, and also with the form incorporated into the diet [19,131]. Various genetic polymorphisms may affect both the uptake and utilization of selenium among individuals [132]. The appropriate form of Se, at the appropriate concentration, has been shown to protect against PCa [89,132,133], gastric cancer [134], and CRC [135].

5.5 Phytochemicals

Phytochemicals, sometimes referred to as bioactives or non-nutrients, have been defined as "constituents in plant foods or dietary supplements, other than

those needed to meet human nutritional needs, which are responsible for changes in health status" [136]. A range of phytochemicals have been promoted as cancer preventive, including Korean red ginseng, curcumin from the Indian spice, turmeric, epigallocatechin gallate from green tea, genistein from tofu, diallylsulfide or *S*-allylcysteine from garlic, and capsaicin. This group includes various polyphenols, defined as having several hydroxyl groups on one or more aromatic rings, and divided into various groups according to chemical structure [137]. By far the best characterized of these are the flavonoids that are the dominant coloring pigment in plants, and itself divided into 13 different classes. Lignans are one of these classes, and are considered as phytoestrogens because of their estrogen-mimetic properties. Because they have distinctive properties, phytoestrogens will be considered separately from the other phytochemicals.

5.5.1 Phytoestrogens

Phytoestrogens are hormonally active compounds of which the main classes are lignans, isoflavones, and coumestans. Rye bread contains high amounts of DF and lignans, and other foods containing lignans in relatively high amounts are seeds (especially linseed), whole cereals, berries, tea, and some vegetables [138]. Only two lignans are considered to be of ultimate significance to human health: enterodiol and enterolactone. These biologically active lignans are formed in the human digestive tract through the interaction of intestinal microbes with dietary lignans [139]. Besides lignans, the other main classes of phytoestrogens are isoflavones and coumestans. Soy is an important dietary source in Asian countries, and soy products and legumes are the main source of isoflavonoid phytoestrogens, including genistein and daidzein. These are also metabolized by human intestinal microbes to the more potent estrogenic metabolite, equol, which has a greater affinity for estrogen receptors, antiandrogenic properties, and also antioxidant properties [140]. As previously identified, excess adiposity also affects hormonal status, and thus it is not surprising that the effects of phytoestogens depend in part upon BMI [11].

One well-studied phytoestrogen, genistein, shows both beneficial and adverse effects in various cancers, including breast, prostate, colon, liver, ovarian, bladder, gastric, brain cancers, neuroblastoma, and chronic lymphocytic leukemia [141]. It is apparent that while genistein may be beneficial in protecting against the development of a number of tumors, it can also favor cancer cell proliferation in a number of situations. By binding to estrogen receptors, genistein shows both weak estrogenic and weak antiestrogenic effects [142–144]. Genistein has also been shown to have antioxidant effects and may act in concert with other nutrients such as beta-carotene in beneficially affecting genomic stability [142]. Genistein also showed beneficial effects in combination with the DNA-damaging agent, bisphenol A [145]. However, in common with other estrogenic compounds

such as diethylstilbestrol, genistein has also been found to have adverse effects on cancer risk and survival after diagnosis [146].

Data on the effects of phytochemicals such as genistein on cancer risk in humans have been largely anecdotal for many years. However, in 2015, the EPIC study related prediagnostic polyphenol intake to BC survival in a European population. Specifically, this analysis considered flavonoids, lignans, phenolic acids, stilbenes, and other polyphenols in relation to all-cause and breast cancer-specific mortality. Among postmenopausal women, an intake of lignans in the highest versus lowest quartile was related to a 28% lower risk of dying from BC. However, the opposite trend was found in premenopausal women. There were no associations found for other polyphenol classes. Cotterchio *et al.* also related lignan intake to BC risk in a Canadian population, but emphasized that associations were strongly linked to BMI. In overweight, but not normal weight, premenopausal women, high lignan intake was associated with a lower BC risk [147].

5.5.2 Other Phytochemicals

Many polyphenols have been described as antinutrients because they may reduce the digestibility of proteins, not only through binding and precipitation but also through inhibition of digestive enzymes. Other polyphenols may form complexes with metal ions, reducing the intestinal absorption of minerals including iron and calcium. Thus, food processing measures are sometimes developed to reduce the polyphenol content of certain foods such as cereal grains [148]. However, except in extreme cases, there is reason to believe that undernutrition may actually lead to beneficial effects in terms of enhancing genomic stability and cancer prevention [137]. While some phytochemicals have been shown to have mutagenic effects, implying potential cancer risk, others have antioxidant and other potentially beneficial effects.

Curcumin is a polyphenol that is also the active ingredient in the spice, turmeric. In a rodent model of colorectal cancer, curcumin treatment led to downregulation of telomerase activity, cell cycle arrest, and induction of apoptosis [149]. Protection against DNA damage has also been shown by curcumin in combination with certain genotoxic agents. For example, in human hepatocyte LO2 cells, curcumin was able to protect against the adverse effects of Quinocetone (QCT), a compound that has been used as an antimicrobial feed additive in China. Pretreatment with curcumin significantly reduced the formation of reactive oxygen species, DNA fragmentation, and micronucleus formation [150]. However, in a different tissue culture model using Raji cells, curcumin increased reactive oxygen species (ROS) and cell cycle arrest, leading to structural chromosome abnormalities [151].

In vitro and *in vivo* studies coupled with clinical trials in recent years have supported the effects of curcumin in cancer prevention, as well as suggesting its

potential as an anticancer and anti-inflammatory agent [17]. Curcumin has been formulated into nanoparticles, liposomes, micelles, or phospholipid complexes in order to enhance its bioavailability and efficacy, but proof of its efficacy is currently weak.

Resveratrol (RSV) is another polyphenol that has been considered to be the beneficial component in red wine. High intakes of resveratrol have usually been considered beneficial to human health, including cancer-protective and anti-aging effects. For example, it is generally considered to be an antioxidant, and has shown a chemopreventive effect in different mouse cancer models [143,152,153]. In mammalian cells, RSV has effects on gene expression leading to the induction of telomere maintenance factors, without effects on cell proliferation [154]. However, in the HeLa colon cancer cell model, resveratrol has also induced DNA damage through prooxidant effects and DNA damage, leading to apoptosis [153].

Indole-3-carbinol and (−)-epigallocatechin-3-gallate (EGCG) from green tea are both examples of polyphenols that show strong evidence of protection against DNA damage through various epigenetic mechanisms [136,155]. Although EGCG has been shown to have anticancer effects, much of the data rely heavily on *in vitro* and animal studies [102]. However, EGCG is mostly metabolized if it is directly orally ingested. Thus, drug delivery systems have been developed in order to stabilize EGCG and enhance its anticancer effects. A 10-year prospective cohort study revealed that drinking ten 120 ml cups of green tea per day delayed cancer onset in humans by 7.3 years among females and by 3.2 years among males [5]. Subsequent studies by the same group showed that a similar regime, supplemented with tablets of green tea extract, significantly reduced the recurrence of colorectal adenomas in polypectomy patients [156]. ECGC has also been combined with the anticancer drug, paclitaxel in a coloaded liposome: a synergistic delivery that controls the invasiveness of MDA-MB-231 breast cancer cells, at least *in vitro* [157].

Some phytochemicals may have complementary activities in protection against DNA damage and carcinogenesis. For example, in broccoli, the isothiocyanate, sulforophane, and the polyphenol, quercetin, may complement one another in their epigenetic actions [158]. Daily administration of free sulforaphane showed beneficial effects in managing biochemical recurrences in prostate cancer after radical prostatectomy [159].

Duthie [160] suggested that the evidence for beneficial effects in cancer is particularly strong for berry phytochemicals, specifically anthocyanins, that modulate various biomarkers of DNA damage and carcinogenesis, in both *in vitro* and *in vivo* animal studies. However, again, evidence for cancer-preventive effects of these phytochemicals in human studies is currently weak.

5.6 Conclusions

There is no question that diet, especially dietary imbalance, is an important cause of cancer. While saturated fats and high caloric intake play important roles, other dietary components interact in various ways. Macronutrients often play an effect in the modulation of satiety, as well as other mechanisms that may promote or enhance carcinogenesis. Micronutrients often show a threshold intake level, and levels either below or above may be detrimental. Phytochemicals may also act as double-edged swords. A popular science journalist summed up dietary advice as "Eat food. Not too much. Mostly plants." [161]. In terms of cancer prevention, this may be a wise advice.

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6

Voluntary Exposures: Natural Herbals, Supplements, and Substances of Abuse – What Evidence Distinguishes Therapeutic from Adverse Responses?

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6.1 Introduction

Worldwide 50–80 million women [1] and 7.3 million American women alone are infertile [2,3]. Despite advances in assisted reproductive therapy (ART), pregnancy rates remain suboptimal [4,5]. Sadly, for almost half of all infertile women, the cause remains unknown. It has been suggested that development of the male reproductive tract, age of pubertal onset, semen quality, and fertility have all been adversely affected by exposure to environmental contaminants leading to the suggestion that human reproductive health is under siege [6–12]. While considerable attention has been focused on the impact of exposure to environmental contaminants, comparably less attention has been devoted to other potentially important chemical exposures. Specifically, voluntary exposure to chemicals such as alcohol and tobacco as well as the use of herbals and supplements promoted for their health benefits have, in general, received far less research attention. Moreover, potential beneficial effects of herbals and supplements are unclear and the clinical relevance of these exposures on human reproductive health is poorly defined.

Herbals and supplements are frequently sold over the counter without the requirement of a prescription. While the beneficial effects are widely promoted in the lay press, the impact of these compounds on health may be positive, negative, or neutral. Therefore, the focus of this chapter is the effects of exogenous chemicals on the reproductive system across the life span. In addition, where data permit, the relationship between exposure to these chemicals and adverse effects on the reproductive tract will also be explored.

Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer, First Edition. Edited by Michael D. Waters and Claude L. Hughes.

Experiments on animals and studies from tissue culture will be included where epidemiological studies provide evidence of a significant association to address issues of biological plausibility, dose–response, modes and mechanisms of action, and relevance to human health. We critically reviewed the epidemiological literature using PubMed and relevant search terms to evaluate the impact of natural chemicals in the diet, herbals, and supplements on human reproductive tract development and physiology over the life span and critical periods of development. We will focus on recent literature that has been published in the last 10 years since our previous authoritative review [13].

6.1.1 Alcohol

The adverse effects of maternal consumption of alcohol during pregnancy are well established. Since there has been an abundance of research reporting the assorted harms associated with alcohol use during pregnancy, linking maternal alcohol consumption with but not limited to spontaneous abortions, diseases, congenital anomalies, and infertility, this will not be described here. However, the pattern of alcohol consumption varies and includes moderate or occasional drinking of one to two drinks, whereas others engage in binge drinking. The consequences of different patterns of alcohol consumption are less clear and need to be considered separately as underling reasons and effects are likely to be different.

While previous research has acknowledged the relationship existing between maternal alcohol intake and various reproductive consequences, controversies still exist surrounding safe or moderate levels of alcohol intake during pregnancy. Of the various harmful interactions between alcohol and the reproductive system, spontaneous abortions have received the most research attention, with studies examining both moderate and excessive alcohol consumption during pregnancy. Results indicated that even moderate maternal alcohol intake during pregnancy was found to have increased risk of spontaneous abortions [14,15]. A dose-related increase in risk with adjusted hazard ratios demonstrating a large increase from 0.5 to 1.5 drinks/week (1.30, 95% confidence interval (CI) 1.02–1.65) to 2–3.5 drinks/week (1.55, 95% CI 1.09–2.22) was found during pregnancy, up to week 16, using Cox regression models [14]. There was no association found between moderate intake and spontaneous abortions beyond 16 weeks of pregnancy, which may reveal a less-sensitive time period of fetal development but necessitates further research to examine possible mechanisms. While no such dose–response relationship was observed by others, moderate maternal alcohol intake was associated with a two to three times higher adjusted risk of spontaneous abortions with 95% CIs [15]. In addition, paternal alcohol intake was associated with a two to five times increase in the adjusted risk of spontaneous abortions [15], contrary to historical research that showed no association between male alcohol intake

and spontaneous abortions. The reasons underlying these divergent findings are unclear.

As the number of maternal drinks consumed increased and examination of consumption on a per day basis, this pattern of increased risk continues with an increasing amount of alcohol. Not only was there a significant positive relationship between alcohol intake per day and spontaneous abortions [16] but also between increased binge drinking episodes during pregnancy and spontaneous abortions [17,18]. A significant positive relationship was found between spontaneous abortion and average absolute alcohol use per day across pregnancy (odds ratio (OR) 1.08; 95% CI 0.92–1.27) and was predicted by drinking frequency, for example, those mothers who drank 1 day per week were 2.59 times more likely to have a spontaneous abortion than those abstaining from alcohol [16]. In addition, they found that spontaneous abortions risk increased within this sample with increasing daily alcohol consumption, but this relationship may be due to the limiting sample of 302 high-risk, urban African American mothers [16].

The relationship concerning binge drinking proves to be slightly more complicated than regular consumption of alcohol concerning spontaneous abortions. The authors found that neither frequency nor timing of binge drinking to be associated with an increased risk of spontaneous abortions, but those women experiencing three or more binge drinking episodes during pregnancy showed an increased hazard ratio of 1.56 (95% CI 1.01–2.40) for stillbirth relative to their nonbinge drinking counterparts [17].

In summary, even occasional alcohol consumption before 16 weeks of pregnancy is associated with an increased risk of adverse outcome. Similarly, although the data are scant, and the relationship with binge drinking limited to adverse effects in those who binge drink more than three times, the data support the recommendation to avoid alcohol consumption when attempting to achieve pregnancy or when known to be pregnant.

6.1.2 Cigarette Smoking

Of the many environmental toxicants and lifestyle factors known to affect fertility and ovarian function, cigarette smoking is potentially the most clinically relevant and preventable toxic exposure in women [13,19], is a global health issue [20,21], and it targets for infertility prevention [22]. The health care costs in the United States associated with tobacco have been estimated to be approximately 193 billion dollars (*Wall Street Journal*). A well-documented health hazard, cigarette smoke also has serious consequences for reproductive health in women. The number of young women commencing smoking is increasing [23], suggesting that current smoking prevention strategies are ineffective in this population. Hence, we are faced with a growing population who are exposed to the single most preventable health risk in Canadians. The

increase in the number of young women who smoke coupled with the trend toward delayed childbearing [24] and the well-documented decrease in fertility with advancing age [25–27] highlight cigarette smoking as a serious public health issue. In the United States, the proportion of first births to women aged 30 years or greater has increased from 5% in 1975 to 24% in 2006 [22]. Hence, we predict that the young women who are smoking today will place even greater demands on fertility clinics in the future. This creates a major concern owing to the decreased number of ova retrieved in women who smoke [28] and the decreased success of ART in smokers [29,30]. The problem is compounded by the fact that most women are unaware of the risks to reproductive health attributable to cigarette smoking [31].

6.1.3 Herbals and Supplements

Several herbal supplements are recommended for the treatment of infertility and are available as over the counter medications. However, the literature supporting their use and their safety is surprisingly sparse. In the following sections, we review the available literature supporting the use of the most popular compounds and where available toxicity data are presented.

6.1.3.1 Melatonin

The effects of melatonin on infertility were described in 64 studies. After excluding review articles, foreign language articles, articles on nonmammalian species, and other irrelevant articles, seven remained for further investigation. Of these seven studies, results from human studies are provided in five, while the remaining studies were conducted using animal models.

Several recent clinical trials have shown that melatonin treatment of healthy infertile women increased fertilization and pregnancy rates [32–34]. One study has shown differences in serum concentrations of melatonin, malondialdehyde (MDA), and total antioxidant capacity (TAC) in fertile and infertile woman [35]. While serum melatonin levels were slightly higher in fertile women, the difference was insignificant ($p = 0.46$). However, there was a significant increase in MDA, a marker of oxidative stress, and a significant decrease in TAC in infertile women, indicating that oxidative stress may indeed play a role. Furthermore, they found a significant correlation between melatonin and TAC in fertile and infertile women and a significant but reverse correlation between melatonin and MDA in infertile and fertile women. While melatonin itself was not significantly different between fertile and infertile women, the correlation with markers of oxidative stress suggests a potential beneficial effect of melatonin treatment.

Five studies described the effects of melatonin in human models and its role in *in vitro* fertilization. Four of these studies looked at oral melatonin supplementation and its effects on oocyte quality and fertilization. In one study [36],

the beneficial effects of melatonin treatment on oxidative stress and oocyte quality were investigated. Follicular fluid of women undergoing IVF-ET was sampled, and the concentrations of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a measure of oxidative stress, was found to be significantly higher in women with high rates of degenerating oocytes. There was also a significant and negative correlation between intrafollicular 8-OHdG and melatonin. Therefore, 18 study participants were then given melatonin (3 mg/day), vitamin E (600 mg/day), or both melatonin and vitamin E, all of which significantly reduced 8-OHdG and hexanoyl-lysine concentrations. These authors subsequently recruited 115 patients with low fertilization rates (≤50%) in their previous IVF-ET cycle and divided them to received either melatonin (3 mg/day) or no treatment. While melatonin treatment improved fertilization rates compared with the previous IVF-ET (participants used as their own control), there was no significant difference between those treated with melatonin and controls. Tamura *et al.* further investigated the effects of melatonin treatment on mouse follicles. They incubated preovulatory follicles with hydrogen peroxide for 12 h to induce oxidative stress, a treatment that significantly reduced the percentage of mature oocytes that developed. The effects of hydrogen peroxide were significantly reduced by melatonin, showing that melatonin can reduce levels of oxidative stress that damage oocyte maturation.

Beneficial effects of melatonin were also reported in three additional studies [33,37,38]. The fertilization rates were compared between two cycles in the same patients, the first with no treatment and the second with treatment of melatonin (3 mg/day) for 2 weeks [33]. They found that the fertilization rate of ICSI was higher in the second cycle than that in the first cycle (69.3 versus 77.5%), and when limited to patients with fertilization rates below 60% it showed a dramatic increase (35.1 versus 68.2%). They further found that treatment with melatonin increased the rate of good quality embryos from 48 to 65.6%. In another study [37], administration of 4 mg/day myoinositol and 3 mg/day melatonin for 3 months prior to IVF in patients who had failed to conceive in previous IVF cycles due to poor embryo quality was explored. A significant increase in the number of mature oocytes, the fertilization rate, and the number of both total and top-quality embryos transferred was found. However, there was no increase in the total number of oocytes retrieved in this study. However, in another study [38], the effects of melatonin treatment on 60 women undergoing IVF, who were also suffering from sleep disturbances, were examined. They divided these women into treatment and control groups, and found that the use of melatonin (3 mg/day) starting on the 4th or 5th day of the menstrual cycle until the time of human chorionic gonadotrophin (hCG) injection significantly improved the mean number of the retrieved oocytes, the mean MII oocyte counts, and the G1 embryo ratio.

The use of melatonin supplementation of the *in vitro* culture medium in women receiving IVF with polycystic ovarian syndrome has also been

studied [39]. This study also showed promising results with implantation rates in the melatonin supplemented group being higher than those of the non-supplemented control group ($p < 0.05$). Taken together, these studies show promising results; however, melatonin has poor bioavailability, a short half-life, and is linked with central effects on sedation.

The effects of melatonin treatment on fertility have also been studied in controlled animal experiments. While several studies have looked into melatonin use in sheep, goats, and camels breeding out of season, we considered these studies irrelevant to a human health. The first relevant study looked at the effects of varying doses of melatonin (range 1 nM–2 mM) on folliculogenesis and oogenesis of *in vitro* cultured mouse ovarian follicles [40]. Secondary mouse follicles were cultured for 12 days, after which *in vitro* ovulation was induced by hCG/EGF. Oocytes were then collected, oocyte maturation was evaluated, and spindle and chromosomes were analyzed for normality. They determined that 2 mM melatonin is toxic, while 1 mM negatively influenced oocyte maturation capacity. They found that androstenedione and progesterone were increased in the presence of 100 μM of melatonin, while estradiol was unaffected. The highest dose that exerted no adverse effect on the measured parameters (10 μM) was recommended as a dose to be used in future studies to avoid negative outcomes. In a similar study [41], the effects of lower concentrations (0, 10, and 100 pM) of melatonin on *in vitro* cultured preantral mouse follicles. They found that after induced ovulation significant increases in follicle survival and diameter were seen in 10 pM melatonin group compared with controls, while the follicles in the 100 pM group were unaffected. Melatonin has also been shown to promote animal follicle development, oocyte maturation, and embryo development [41–43] *in vitro*.

In summary, several small clinical studies suggest a beneficial effect of melatonin on fertility in the absence of relevant adverse health effects. However, randomized control trials are needed and the optimal dose has yet to be defined.

6.1.3.2 Resveratrol

Resveratrol is a natural polyphenol and an antioxidant found in several foods such as grape skins, blueberry's, and red wine. It is hypothesized to increase fertility in women and as such has been included in some vitamin formulations for women attempting to conceive. We found 30 articles describing resveratrol and fertility, of which 3 were identified for further investigation. Of these, only one looked at humans and focused on resveratrol's effect on obesity-related infertility [44]. Obesity in women can lead to infertility due to oxidative stress resulting from increased levels of circulating oxidized low-density lipoprotein (oxLDL) and lipid peroxides. Resveratrol treatment was used to counteract the oxidizing effects of oxLDL's in a culture of granulosa cells taken from women undergoing IVF [44]. In this study, resveratrol treatment significantly reduced oxLDL-induced granulosa cell death from $62.6 \pm 22.7\%$ to $3.5 \pm 1.4\%$. However,

the number of study participants included in the analysis could not be ascertained.

The effects of resveratrol on fertility have also been investigated in animal models. The effect of resveratrol treatment on oocyte quality was examined in 16 obese versus16 wild-type mice [45]. Half the mice from each group were treated with 3.75 mg/kg resveratrol daily for 20 days while the mice were undergoing controlled ovarian hyperstimulation. Interestingly, they found a significantly higher number of oocytes were collected in wild-type mice, although the number was deceased in obese mice [19]. In another study [46], the effects of resveratrol on age-related infertility in mice were studied. Mice were fed 7.0 mg/kg/day resveratrol ($n = 25$ mice per group) for 6 or 12 months with age-matched controls and measured litter size, ovarian follicles, and oocyte quantity and quality. They repeated the experiment three times for consistency, and found that 12-month-old mice fed resveratrol retained the capacity to reproduce while age-matched controls could not. Furthermore, mice fed resveratrol for 12 months exhibited a larger follicle pool, and had an increased number and quality of oocytes. Resveratrol also affected embryo development *in vitro* in a dose-dependent manner. None of these studies investigated the toxicity of resveratrol. Resveratrol treatment also inhibited cigarette smoke condensate-induced adverse effects on follicle growth and steroidogenesis [47].

In summary, resveratrol is a naturally occurring polyphenol with antioxidant effects that has been shown to attenuate oxidative stress in cultures of granulosa cells; however, there is a paucity of literature to substantiate a beneficial effect on fertility. Similarly, the literature is relatively silent on the potential adverse effects associated with the use of resveratrol.

6.1.3.3 Dong Quai

Dong quai is involved in traditional Chinese medicine and is thought to potentially aid in fertility. Using search terms described above 40 studies were identified post 2006. Of these 40 studies only one study was related to fertility outcomes [48]. This was a case report in which dong quai was given to a woman with infertility related to the presence of an atypical polypoid adenomyoma. While dong quai was used to assist this woman in achieving pregnancy and she did conceive twice, she was unable to carry the child to term [48]. As this was a case report involving one woman only, it cannot be concluded that dong quai was responsible for conception. Hence, we conclude that there is no credible evidence in favor of the use of dong quai in fertility care.

In controlled animal studies of dong quai, the antioxidant, anti-inflammatory, and neuroprotective effects were explored. One study investigated the toxicity of dong quai in rats, finding no treatment-related toxicity at doses of 30, 100, and 300 mg/kg, p.o. once daily for 4 weeks [49]. The toxicity of Safrole-2′,3′-oxide, a metabolite of safrol that is found naturally in dong quai, includes cytotoxicity, DNA strand breaks, and micronuclei formation in both human cells *in vitro* and

in mice [50]. The absence of evidence of beneficial effects of dong quai and the lack of epidemiological studies designed to assess the potential adverse effects of this agent together with experiments studies with important toxic effects suggest caution around the use of this compound.

6.1.3.4 Eleutherococcus

Eleutherococcus is another herb known to be used as a treatment for infertility. Although we identified 32 articles published in the last decade, none discussed the plants' use in the treatment of infertility. One study looked into the toxicity of the plant in rats, finding that it has many potential benefits for the treatment of several diseases, such as hypertension and cancer [51]. In this study, there were also some upregulated endogenous metabolites and evidence of oxidative stress producing toxic effects [51]. Although this herb has been used by the infertile population, the lack of evidence to support a potential benefit and limited evidence of potential toxicity suggest that the use of this compound should be avoided.

6.1.3.5 Saw Palmetto

Twenty-one articles were identified, but again there were no studies investigating the plants' role in infertility. There were three studies looking into potential toxicity of the plant, two of which were randomized control trial on humans [52,53]. However, these trials were conducted exclusively on men as the primary function of the plant is in the treatment of prostatic hyperplasia. In the first 2008 study [52], 225 men were randomized to receive either saw palmetto berry extract (160 mg twice daily) or placebo over a 1-year period. No adverse effects or evidence of toxicity were observed; however, as the study used berry extract, it could not rule out potential toxicity of raw saw palmetto [52]. In a second randomized control trial, 369 participants were randomized to doses of 320, 640, and 960 mg/day of an ethanolic saw palmetto extract or placebo in an escalating manner at 6-month intervals for a total of 18 months of follow-up [53]. This study also showed no evidence of toxicity at doses up to three times the usual clinical dose during the 18-month period [53].

One animal study has also been conducted to investigate potential hepatotoxicity of saw palmetto [54]. In this study, 36 rats were divided into six treatment groups and were treated for 2 or 4 weeks with placebo or saw palmetto at doses of 9.14 or 22.86 mg/kg/body weight daily, a dose two and five times the maximum recommended human dosage. At 2 and 4 weeks the animals were killed and blood was collected to analyze hepatic enzymes. Results showed no significant difference in animal body weight, enzyme activity, or MDA formation at either time or dosage level. While there are no studies documenting a beneficial effect of saw palmetto use on any fertility outcome, the very limited data available fail to show evidence of an adverse effect.

Regardless, the current literature is inadequate to reach any conclusion on the safety of this compound.

6.1.3.6 Stinging Nettle

There were no published articles of stinging nettle relating to fertility among the 36 titles found in our search of the literature. Two studies have investigated the potential toxicity, though neither have done so in a human model. One study investigating extracts of stinging nettle associated with antidiabetic, anti-inflammatory, and antibacterial activity examined toxicity using artemia salina and Wistar rats [55]. They found that all extracts analyzed had an LC $50 > 1000\,\mu g/ml$ in artemia salina and that no mortality was observed during the 24-h period at the doses tested.

A second study aimed to determine the chemical composition of stinging nettle essential oil, and to evaluate its cytotoxic and genotoxic effects in human lymphocyte cultures *in vitro* [56]. A significant correlation was found between the concentration of 43 compounds in the essential oil and the following: chromosomal aberrations, micronuclei frequency, apoptotic cells, necrotic cells, and binucleated cells. While this could indicate potential toxicity, more case–control studies are needed.

6.2 Summary and Conclusions

While the literature relating to exposure to environmental contaminants and lifestyle factors such as alcohol and cigarette smoke is particularly robust, the literature describing either the beneficial or potential toxic effects of herbals and supplements for fertility is comparatively sparse. While the literature suggests a potential benefit of melatonin and resveratrol in ART, the literature describing potential adverse effects is weak. We also conclude that the literature supporting the use of herbals for reproductive health care needs cannot support this application. Moreover, the absence of a robust literature investigating potential adverse health effects of these compounds is troubling especially in view of the sensitivity of the conceptus to developmental exposure to exogenous chemicals.

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7

Voluntary Exposures: Pharmaceutical Chemicals in Prescription and Over-the-Counter Drugs – Passing the Testing Gauntlet

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7.1 Introduction

Pharmaceuticals have successfully treated a wide array of medical conditions from the common cold to cancer and everything in between. They take many chemical forms, including protein (biologics), nucleic acid, polymers, large macrocyclics, and small hydrocarbon molecules of 300 molecular weight or less; they can be naturally occurring compounds isolated from various organisms, or completely new synthetic entities. Recent advances in chemistry, cell biology, molecular genetics, and computational biology have facilitated the creation of new highly efficacious drugs at many cellular targets. But these technological advances have not been equally powered to understand and predict the complex mechanisms of cellular, organ, and organismal toxicity. Virtually all drugs exhibit a plethora of toxicological side effects reflecting the fact that, for all the good they might do, they are still mostly foreign and toxic to the body. One need only read the package insert of any marketed drug or over-the-counter drug product to see that each possesses a wide range of undesired side effects mostly of a "nuisance" nature but, in many cases (reviewed in Ref. [1]), serious enough to warrant a "black box warning" indicating very severe or even life-threatening effects. Repeated acute or long-term chronic exposures to these drugs must, therefore, be considered to have a clear risk component. Many more drugs drop out of the development process due to unexpected clinical organ toxicities than due to lack of efficacy [2,3]. Traditional approaches toward prediction of adverse drug effects have been only marginally successful but it appears as though new genomics and computational technologies have matured to the degree that they may confidently be applied to improving toxicity prediction both at the population and individual levels. This chapter attempts to provide a basic appreciation of the limitations of our current drug safety testing paradigm

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and the new technologies that might be brought to bear in their improvement. This chapter draws from and expands upon recent discussions of the future of toxicity prediction [4,5] as we go forward into the twenty-first century.

7.2 Testing of New Drug Entities for Genotoxicity

Drug-induced organ or system toxicities arise from multifactorial and usually poorly understood and unpredictable perturbations to normal cellular physiology, and such effects may not be observed until a drug is late in development or already marketed; at which point, depending on the indication and the relative risk/benefit analysis, further development may be halted or the drug pulled from the market. Determining if a drug is genotoxic, that is, capable of destabilizing the genome at the DNA sequence or chromosomal level, and therefore more likely to be carcinogenic, is more easily accomplished. Noting the positive relationship between the Ames test (see further) for mutagenicity and rodent bioassay results in a study of 300 chemicals, McCann *et al.* [6] proposed that a bacterial mutagenicity test be carried out on all new drugs and environmental compounds. With the realization that the Ames test, alone, was likely to "miss" clastogenic chemicals, it was proposed by the United States Food and Drug Administration [7,8] and the United States Environmental Protection Agency (EPA) [9] that a battery of genotoxicity tests, (see further) be run as a surrogate predictor of carcinogenicity.

The core test battery has changed somewhat over the years, and continues to do so, but most regulatory bodies have agreed to a harmonized approach of using a bacterial mutation assay (e.g., Ames test), an *in vitro* chromosome aberration assay in cultured mammalian cells and/or human lymphocytes, and at least one *in vivo* chromosome breakage test in rodents in most cases, for detecting chemicals with DNA damaging activity. For detailed descriptions of these assays specifically as they are used in the regulatory setting, see Ref. [10]. Briefly, the Ames bacterial reverse mutation assay measures the ability of a chemical to change the DNA sequence at specific genetic loci in strains of *Salmonella typhimurium* or *Escherichia coli* specially designed to detect chemical-induced base pair and frameshift mutation. Bacterial DNA, not encumbered with higher order protein structure as in eukaryotic chromatin, provides a sensitive method for the detection of drug/DNA interaction. The *in vitro* chromosome aberration assay conducted in cultured human lymphocytes or rodent cells, detects, in metaphase spreads, chemically induced simple and complex chromosome breakage (clastogenic) events as well as aneugenic events (addition or deletion of one or more whole chromosomes). This assay has the advantage of being able to visualize the actual chromosomal lesions, for example, simple breaks or complex rearrangements, allowing insight into the nature of the initial chemical insult. The mouse lymphoma assay (MLA) can

detect both mutation and, indirectly, chromosome breakage in cultured murine cells. Concern that this assay may generate misleading responses has led to a gradual movement away from its use, but it has been decidedly valuable in the safety evaluation of hundreds of drugs and other chemicals. The complexity of cultured mammalian cells relative to bacteria, and the multifactorial processes leading to chromosome damage in these cells can pose a challenge to understanding mechanisms and assessing risk associated with positive responses in these two assays. Finally, the *in vivo* rodent micronucleus assay measures the formation of DNA damage or chromosome loss in bone marrow cells of mice or rats exposed to test chemical orally or by intraperitoneal (IP) injection. Even though animals are dosed to near maximum tolerated doses (MTDs), test article exposures to target bone marrow cells are usually much lower than concentrations achieved in the *in vitro* assays and on this basis, the *in vivo* assay is considered less sensitive but most closely approximating human dosing.

A drug with genotoxicity revealed in the test battery is assumed to be a potential carcinogen while nongenotoxic drugs are considered unlikely to be carcinogens. Unfortunately, Nature is usually not that cooperative and the existence of a large compartment of apparent nongenotoxic carcinogens, as discussed in more detail further, has led to challenges to establish more rigorous empirical determinants.

Appendix A contains an updated and consolidated list of the genotoxicity and rodent carcinogenicity of 907 marketed drugs. Included are previously published data [11–14] and data from FDA package inserts on new drugs approved since the last review. This table is, to date, the most complete compendium of data relating to the genotoxicity and rodent carcinogenicity of marketed pharmaceuticals. Gaps in the data are found for (1) earlier drugs for which a complete genotoxicity analysis was not deemed necessary and was never done, (2) drugs with multiple positive genotoxicity findings that may have been conceded as probable carcinogens and were not tested in the bioassay, (3) drugs which did not undergo carcinogenicity studies because their therapeutic indication was for acute rather than long-term use, for example, analgesics, antibiotics, and (4) drugs developed for life-threatening indications (anticancer, certain antivirals) for which carcinogenicity was undesirable but not unacceptable in risk/benefit analyses. Several points, salient to the theme of this chapter are discussed.

Figure 7.1 indicates that the fraction of marketed drugs testing positive in the standard genotoxicity battery tests is 27 to nearly 40%. A dissection of the distribution of positive findings indicates that the greatest percentage of positives occurs in the chromosome aberration and MLA assays; positive Ames and *in vivo* micronucleus assays being consistently less frequent. The high overall percentage of marketed drugs with positive genotoxicity findings, to a large extent reflects, drug development prior to the advent of the formal FDA guidelines, which initiated the dialogue defining acceptability criteria for

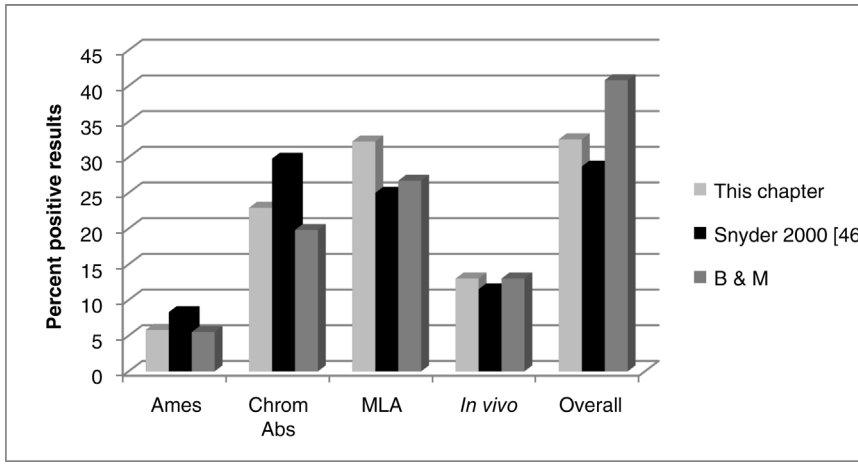

Figure 7.1 Distribution of positive genotoxicity assay findings.

genotoxic risk. But even today, a high percentage of drugs with positive genotoxicity findings continue to make it to the market (the last four years of new approvals 2012–2015, had about 25% drugs with at least one positive response, usually in a chromosome aberration assay; data not shown). Part of the reason for the continued approval of apparently genotoxic molecules is that many of these molecules are not recognized as carrying classical structural alerts (see further) and are "written off" as being false positive artifacts of no biological relevance. This may be wishful thinking, however, as many of these drugs may be noncovalent DNA binding agents (see further) not adequately considered in the learning sets of *in silico* programs for genotoxicity prediction. The interpretation of positive findings in *in vitro* chromosome aberration assays has been so problematic that the testing guidelines for new pharmaceutical entities have been amended to allow for the use of a second *in vivo* test to replace the *in vitro* chromosome aberration assays.

The relatively low percentage of Ames positives is due to the fact that a positive Ames test usually results in dropping that compound from further development [15]. The relatively low percentage of drugs testing positive in the *in vivo* assay reflects, pharmacodynamics and the usually much lower drug exposure to target organs than can be achieved in *in vitro* assays. A positive *in vivo* chromosome breakage finding is generally weighed more heavily than an *in vitro* chromosome finding. The *in vivo* assay has also been shown to have the highest predictive value for germ cell mutagenesis [16,17] important in reproductive and development toxicities.

The addition of new methods for detecting genotoxicity or increasing throughput of existing assays will not provide substantially greater confidence in understanding the nature of the DNA damaging activity of new drug

substances. In other words, is it due to direct action; is it secondary to physiological disturbance; is it due to perturbation of DNA metabolic processes? These are important distinctions since direct drug/DNA interaction, after correction for cellular DNA repair response, follows single hit kinetics, that is, damage is extrapolatable to zero dose implying no absolutely safe exposure is possible. Damage arising from metabolic perturbation is usually considered to be a thresholded phenomenon requiring a certain minimum cellular concentration of chemical, defined by the pathway or enzyme affected, to be achieved before damage is seen. While it is very difficult to make a meaningful calculation of just what the threshold concentration for any specific chemical in a given biological system might be, compelling assertion that one, in fact, does exist, is often important in regulatory decision-making.

Because the prediction of cancer risk associated with positive genotoxicity findings is so dependent on the etiology of the genotoxicity, it is imperative that we improve our ability to critically evaluate this.

7.3 Relationship between Genotoxicity Testing and Rodent Carcinogenicity

Table 7.1 is an evaluation of data from Appendix A showing calculations of the performance of genotoxicity testing in predicting rodent carcinogenesis. The data indicate that 43% (255/598) of all drugs with at least some genotoxicity data (positive or negative) are positive in at least one rodent carcinogenicity assay. This value is 39% (124/315) for the Brambilla and Martelli database [14] and 52% in an NCI/NTP study [18] in which compound selection was purposely biased toward carcinogens. These values seem high and raise the suspicion that much of the rodent carcinogenicity observed in the 2-year bioassay may be due to something other than direct genotoxicity; a likely alternative being mitogenesis induced by organ toxicity [19]. Consistent with this is the fact that 154 of 329 (47%) rodent carcinogens (50% in the Brambilla and Martelli database [14]) are nongenotoxic (Table 7.1). The sensitivity (percent true positives) of individual battery assays is similarly poor; the best response being the Ames assay at 77%, but still exhibiting a 47% false negative rate. Sensitivity is marginally improved for drugs with at least two positive genotoxicity results, especially if one is an Ames test (91% sensitivity). It has been previously concluded, and the present data confirm, that traditional genotoxicity testing is not highly predictive of rodent carcinogenicity and that this is due, to a great extent, to a large compartment of nongenotoxic carcinogens [20,21]. It is now evident that traditional genotoxicity testing has taken us about as far as we can go and that it is necessary to explore alternative molecular (genomic) approaches to carcinogenicity testing [18,22–24].

Table 7.1 Summary relationship between the genotoxicity of marketed drugs and rodent carcinogenicity.

Ames($N = 568$)	Positive carcinogenicity	Negative carcinogenicity
49 positives	38 (77% true positives)	11 (23% false positives)
519 negatives	243 (47% false negatives)	276 (53% true negatives)
Chrom Abs ($N = 447$)		
109 positives	71 (65% true positives)	38 (35% false positives)
338 negatives	151 (45% false negatives)	187 (55% true negatives)
MLA ($N = 189$)		
44 positives	28 (64% true positives)	16 (36% false positives)
145 negatives	69 (48% false negatives	76 (52% true negatives)
In Vivo ($N = 453$)		
53 positives	38 (72% true positives)	15 (28% false positives)
400 negatives	207 (52% false negatives)	193 (48% true negatives)
Ames/any other positive ($N = 32$)	29 (91% true positives)	3 (9% false positives)
Any two positives (but Ames neg) ($N = 32$)	23 (72% true positives)	9 (28% true negatives)
Overall ($N = 598$)		
255 positives	175 (69% true positives)	80 (31% false positives)
343 negatives	154 (45% false negatives)	189 (55% true negatives)
Overall Brambilla/Martelli ($N = 315$)		
124 positives	74 (60% true positives)	50 (40% false positives)
191 negatives	75 (63% false negatives)	116 (37% true negatives)

7.4 Can Drug-Induced Human Cancer Be Predicted?

DNA damaging anticancer and antiviral chemotherapeutic drugs can cause secondary tumors, most commonly hematological in nature, both by direct covalent DNA damage, such as caused by alkylating agents (e.g., bis chloroethyl nitrosourea; BCNU), DNA cross-linking agents (e.g., *cis*-Pt), and by noncovalent hydrogen bonding to DNA. Additionally, many non-DNA-targeted drugs act via perturbation of normal cell cycling processes causing DNA damage secondary to that perturbation. It is evident that certain drugs can cause mutation and chromosome breakage and that these specific drugs are likely to cause second site tumors. These drugs, partially listed in Table 7.2, are known to or expected to be rodent carcinogens.

Table 7.2 Drugs of concern for human cancer risk.

Drug–human cancer site	Genotoxicity	Rodent carcinogenicity[a)]	*In silico* genotoxicity
Pioglitazone-Bladder	Negative	Pos-Rat*14 fold	Negative
Acetaminophen-NHL	Positive	Pos-Rat	Negative
Statins-Breast	Negative	Pos-Rat, Mouse* 4-–11 fold	Negative
Omeprazole-??	Positive	Pos-Rat*10–30 fold	Positive
Gabapentin-??	Negative	Pos-Rat* 6–10 fold	Negative
Nortriptylin-Esophag/hepatic	Negative	?	Negative
Oxazepam-Lung	Positive	Pos-Rat,Mouse* 30 fold	Negative
Paroxetine/Fluoxetine-Testicular	Negative	Pos-Rat, Mouse* 20 fold	Positive
Hydrochlorothiazide-Renal	Positive	Negative	Negative
Nifedipine-Lip	Negative	Negative	Positive
Phenolphthalein-??	Positive	Pos-Rat, Mouse	Negative
Griseofulvin-Breast	Positive	Pos-Mouse	Positive
Metronidazole--Anal	Positive	Pos-Rat, Mouse	Positive
Phenobarbital–Sm Intestine	Positive	Pos-Mouse	Positive
Phenytoin–Esoph/liver/lung	Positive	Equiv-Mouse	Positive
Hyoscyamine-NHL	??	??	??
Sulindac-gall bladder	??	??	??
Cancer drugs, for example, cis-Pt	Positive	Pos-Rat, Mouse	Positive
Alkylating agents			
Mechlorethamine, Chlorambucil, Cyclophosphamide, Melphalan, Lomustine, Carmustine, Busulfan	Positive	Pos Rat, Mouse	Positive
Cross-linkers			
Cis-Pt, carboplatin	Positive	Pos Rat, Mouse	Positive
Topoisomerase Inhibitors			
Mitoxantrone, etoposide, doxorubicin, and related anthracyclines	Positive	Pos Rat, Mouse	Anthracyclines-Pos Topo binders-Neg

a) Approximate fold human exposure at which rodent cancer was observed.

Classical assessment of noncancer-targeted drugs for human carcinogenicity requires epidemiological studies that are very difficult to establish and perform as they are subject to confounding factors relating to normalization of specific drug exposures to various life styles, demographic, cultural, and numerous other factors. Table 7.2 lists drugs identified from the literature, as being, for whatever reason, possible human carcinogens [25–27]. Of the 17 noncancer targeted drugs evaluated epidemiologically, omeprazole, gabapentin, and phenolphthalein could not be confirmed in subsequent studies. The remaining 14 are best categorized as showing small nonstatistically significant elevations in human cancer frequency (generally less than twofold). As is often the case in human clinical trials and population-based follow-up trials, despite all efforts to sufficiently power each trial statistically, the number of patients exhibiting a particular toxicity may be insufficient to unequivocally assess a relationship to human cancer. Moreover, the long follow-up period required to show an effect of a new drug in elevating specific organ toxicities or cancer frequency, except for anticancer drugs in which second site tumors are often seen fairly quickly, precludes premarket prospective analysis.

But let us suppose that 5% of the population share a genomic signature which, for whatever reason (and it's not critical that this be understood mechanistically), sensitizes them to a specific drug. A putative drug-dependent doubling in absolute numbers of cancers from a background level of 2–4 per 1000 individuals would be a 0.2% increase, which would require a large N to be statistically detectable. If all the increase was attributable to the 5% polymorphism, this would be an actual 4% increase – much more easily detectable. So just knowing the population frequency of any gene or DNA sequence predisposing for specific drug-induced cancer not only facilitates epidemiological study interpretation but might also exclude the sensitive population from taking the drug in the first place. Thus, establishment of ways in which to identify genetic variation in the human population is critical in the marketing of safe drugs.

7.5 What Can Rodent Carcinogenicity Tell Us about Human Cancer Risk?

Table 7.2 indicates that of the 14 putative human carcinogens, 7 were genotoxic and 5 were nongenotoxic (two had no data); *in silico* genotoxicity prediction (see further) was consistent with genotoxicity for only 7 of 12 drugs. However, 9 of 11 (82% sensitivity) putative human carcinogens were rodent carcinogens (at least one species, one site). Although these numbers are very small, they suggest a correlation between rodent and human carcinogenicity. Certainly, the

fact that all anticancer drugs are also positive in the rodent carcinogenicity assay strengthens the confidence in this conclusion.

A nonepidemiological study looked at the predictivity of rodent carcinogenesis for human carcinogenicity, using drugs of "enhanced human cancer concern", as defined by wording in the "Warnings" or "Precautions" section of the package insert, as a surrogate for actual human carcinogenicity data [23]. Out of 44 drugs meeting the criteria for enhanced concern, 32 (72% sensitivity) were rodent carcinogens confirming the sensitivity mentioned in the epidemiology-based study. Inclusion of 243 drugs, not considered to be of enhanced concern, into the analysis resulted in an overall negative predictive value, that is, that fraction which was negative in both rodent assays and not being of enhanced concern, of 90%. However, the positive predictivity and hypothetical false positive rates were an unacceptable 20 and 80%, respectively. Nevertheless, these studies demonstrate the feasibility that rodent carcinogenicity studies may in some cases predict human carcinogenicity but the correlation is so weak as to not provide a high degree of confidence.

As already discussed, of the 663 drugs with rodent carcinogenicity data included in Appendix A, 341 (51.4%) were positive in at least one species at one site and 18.4% were positive in both rodent species. However, these values seem excessively high, as already mentioned there is no reason to doubt the data, although a very poor repeatability (57% of 121 replicates) of 2-year rodent bioassay results has been reported [28], so it is important to understand the underlying mechanism(s) of rodent cancer in the 2-year bioassay model. In actual practice, development of mechanistic data arguing against the conclusions of the bioassay is done on a case-by-case basis for regulatory submissions and, this often provides a compelling argument for regulatory approval.

The sum of the evidence suggests that many rodent cancers must be nongenotoxic in origin. This may or may not make the process irrelevant to humans depending on the mechanism. Drugs that may be actual genotoxic rodent carcinogens may also be irrelevant to humans if (1) the drug is being developed for acute rather than chronic use, (2) rodent and human physiology are not equivalent, (3) tumor types are not shared between species, and (4) employed doses are much greater in the rodent carcinogenicity study relative to projected clinical plasma levels. *Note*: Whenever possible, guidelines mandate dosing up to at least 25× the proposed human clinical exposure. On this point, 59 drugs that were carcinogens in both rats and mice were analyzed for fold projected drug plasma level as reported in the package insert. Twenty-seven (46%) exhibited tumor formation at plasma levels of 0.1–2-fold the projected clinical plasma level. Of the remainder, 26 (44%) were tumor bearing at 5–50-fold clinical plasma levels (Snyder, unpublished observations). This admittedly small sampling indicates that doses and

exposures employed in the 2-year bioassay may not always be in large excess to those used in patients.

7.6 Genotoxicity Prediction Using "Traditional" *In Silico* Approaches

The past decade has seen the rapid expansion of so called *in silico* models to predict both genotoxicity and carcinogenicity. These computational models fall into two categories: (1) SAR (structure–activity relationship) models based on known and chemically understood DNA reactive moieties, often referred to as "structural alerts" [29] numbering well over 100 and comprised of such chemical entities as alkylating agents, aromatic amines, fused planar tricyclics, and (2) QSAR, (quantitative SAR) which takes into account physicochemical characteristics that may affect DNA binding of the chemical. Detailed information about the formation of and inherent strengths and weaknesses of the major models (Leadscope, DEREK, MCase, etc.) can be found elsewhere [21,30–33]. Initial and, to some extent, prevailing enthusiasm for this technology was the hope that once the models were provided sufficient numbers of structures and accompanying data (genotoxicity, skin sensitization, carcinogenicity, etc.) to essentially saturate "chemical space" it might be possible to predict the activity of any novel compound without doing any actual testing, that is, based solely on chemical structure and physical properties. While strides have been made toward this goal, the apparent complexity of genotoxicities of even close structural analogs has reined in the optimism for a quick and easy fix. The sensitivity of DEREK and MCASE (MC4PC version) for predicting genotoxicity in the Ames, chromosome aberration, MLA, and *in vivo* micronucleus assays findings in a database of marketed drugs was 64, 36, 39, and 49% for DEREK and 45, 20, 30, and 19% for M4PC, respectively [12,34]. Use of both models together increased the sensitivities to 77, 42, 44, and 46%, respectively. But the increased number of false positive "calls" or "hits" also increased unacceptably. Only bacterial mutagenesis appeared to be modestly predictable consistent with the relative simplicity of that system. Similar limitations of these *in silico* systems have been previously discussed [4,35]. This balance of acceptable sensitivity at the expense of low specificity is a common feature of *in silico* systems and greatly reduces their utility *in silico* except in screening applications. The very low predictivities for drugs testing positive in chromosome aberration assays is interesting and, as discussed further, indicates apparently overlooked structural features for clastogenicity when the learning sets of these programs were created.

Evidence that *in silico* programs may have ability to distinguish between genotoxic and nongenotoxic rodent carcinogenicity, however, is shown in Figure 7.2.

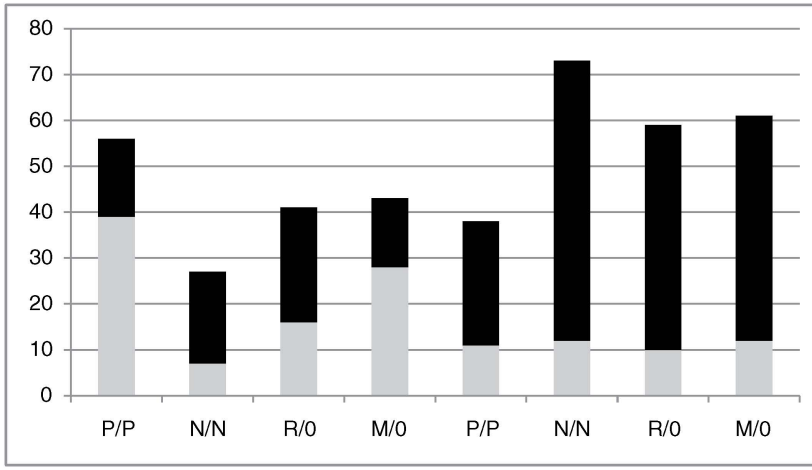

Figure 7.2 Structural alerts in genotoxic and nongenotoxic carcinogens and noncarcinogens. Column height is the number of drugs in each bin. The light part of each bar is the percentage of total drugs in each bar that carry structural alerts. The first four columns are genotoxic rodent carcinogens, the next four columns are nongenotoxic rodent carcinogens. P/P: rat and mouse carcinogens; N/N: rat and mouse noncarcinogens; R/0: rat carcinogen, mouse not known; M/0: mouse carcinogen, rat not known.

It is shown that noncarcinogenic drugs exhibited a low frequency (~10%) of structural alerts, as did all nongenotoxic carcinogens, whereas all groups of genotoxic carcinogens exhibited a higher frequency (15–40%) of structural alerts. Thus, an association occurs between *in silico* prediction and rodent genotoxic carcinogens but this relationship is, at present, too weak to be of significant value in drug discovery or development.

7.7 Covalent versus Noncovalent DNA Interaction

It is well appreciated that noncovalent (hydrogen bonding) interactions such as DNA intercalation and groove binding can be important sources of mutation (frameshift) and chromosomal breakage (reviewed in Ref. [36]) and, in fact, classical polycyclic planar intercalators, such as acridines and aminoanthracenes, are recognized as structural alerts in most models. Recent cellular studies, following up on earlier DNA studies [37] demonstrating that the DNA nicking activity of the groove binding drug, bleomycin, was dramatically enhanced in the presence of DNA intercalating agents, has confirmed the existence of a large number of functional intercalating agents not previously recognized as such due to the fact that they possess atypical nonfused ring structures and only partial planarity [38]. The likelihood that these molecules,

for example, antihistamines [39] and tamoxifen and analogs [40], may be legitimate intercalating agents, was strengthened by their computational docking behavior into DNA dinucleotide intercalation sites [41,42]. It was subsequently determined that many (65%) structurally nonalerting marketed drugs that tested positive in chromosome aberration assays were also possible intercalators as indicated by computational docking and/or a V79 cell-based bleomycin amplification assay [42,43]. For many of these, the presence of an N-dialkyl group enhanced genotoxicity presumably by increasing residence time of the intercalated drug on the DNA [44]. A more sophisticated docking study using both a charge-based and a structure-based docking program, 10 dinucleotide intercalation sites, and over 1350 marketed drugs [45] confirmed the early docking studies and further broadened the list of structures capable of noncovalent hydrogen bonding with DNA.

Atypical intercalators, like classical tricyclic intercalators, may elicit their genotoxic responses via inhibition of DNA topoisomerase II. This was suggested by cell-based studies in which it was demonstrated that the genotoxicity of known and suspected topoisomerase poisons, for example, bioflavonoids, was antagonized in the presence of catalytic topoisomerase inhibitors [46–49]. More recently, computational docking of over 1350 drugs into human DNA topoisomerase II ATP binding sites revealed several new classes of potential topoisomerase poisons, including steroids and vitamin D analogs [13,45]. Interestingly, the genotoxic but nonalerting benzimidazole proton pump inhibitors, for example, omeprazole and lansoprazole were shown to bind the ATP site strongly, consistent with their therapeutic mechanism, and providing a possible mechanism for their genotoxicity.

In silico models continue to enjoy some success in screening of drug candidates and assessment of impurities in drug product, but there would appear to be several hurdles that must be cleared in order to substantively increase their predictive performance. Among these would certainly be expansion of coverage of chemical space and inclusion of structural and functional features associated with noncovalent DNA binding.

7.8 Use of New Technologies to Predict Toxicity and Cancer Risk: High-Throughput Methods

Recognizing that traditional testing paradigms for genotoxicity and rodent carcinogenicity were not performing as well as they needed to and could not address the question of interindividual differences in human susceptibility to toxic stimuli, the European Union (EU), and US Federal agencies (EPA, NIH, NTP, and later FDA) independently initiated or accelerated programs to explore new approaches, including high-throughput analyses and/or genome-based platforms.

The US Federal collaborative toxicology research agreement was spawned in response to a National Academy of Sciences report calling for a shift of emphasis away from traditional testing algorithms to a more technologically driven and visionary approach. The primary focus of the resultant multiagency collaboration, known as "Tox21", is the collection, management, and ultimate analysis of vast amounts of data resulting from screening a library of more than 10,000 diverse compounds (including pesticides, pharmaceuticals, food additives, high production volume chemicals, fragrances, etc.) through a series of over 1000 high-throughput assays measuring anything from cytotoxicity in a wide variety of normal and transformed cell lines to radioligand binding, receptor binding, transporter assays, ion channel effects, and many more. These data are also stratified cheminformatically for SAR analysis. Most importantly, the data are all made available to the public to facilitate toxicity evaluation of new compounds. Recent reviews [50,51] provide excellent discussions of the specific goals and accomplishments of Tox21 to date. The applicability of Tox21 data to pharmaceutical discovery is obvious but, as discussed by Rovida *et al.* [51], it is too early to use this type of data in a regulatory setting.

Other, cell-based, high-throughput methods for screening for human susceptibility to chemicals are also being developed. The goal of these models is to identify specific genes associated with a toxic insult through systematic gene inhibition and observation of resultant cellular response. Knowing what genes are associated with a phenotype provides a starting point for gene selection in mRNA-based and pharmacogenomic studies. Among these models are the DT40 avian leucosis virus-transformed chicken B lymphocyte cell line in which one can produce stable gene-targeted reporter strains [52] deficient in, for instance, specific DNA repair genes or pathways, and a near-haploid human cell line KBM7 in which nearly 98% of expressed genes carry inactivating insertions [53,54].

7.9 Transcriptomics

The EU initiative, carcinoGENOMICS, in addition to advancing uses of genomic technologies, is aimed at addressing a mandate to use fewer animals in testing. Microarray- and PCR-based transcriptomics studies, proof of principles having been established from studies using databases such as the Iconix DrugMatrix [55], were carried out using a battery of liver-based *in vitro* cell systems. These studies demonstrated that one could distinguish based on mRNA patterns, cells treated with genotoxic-, nongenotoxic hepatocarcinogens, and noncarcinogens. The most reliable response was seen following treatment with genotoxic carcinogens [56–58].

Similar *in vitro* transcriptomics studies were simultaneously being conducted by labs in the United States and Europe. These interlaboratory studies

demonstrated distinctly different gene expression patterns in human TK6 and other cell lines treated with *cis*-Pt, etoposide, taxol, and NaCl; a direct DNA reactive agent, a topoisomerase inhibitor, an aneugen, and a cytotoxic agent, respectively [59,60]. Subsequent studies resulted in the identification of a 65-gene battery that could accurately classify toxicants as acting through genotoxic or nongenotoxic mechanisms [61,62]. This gene panel has been submitted to the FDA for consideration for use in regulatory applications and while it will most likely require refinement, provides a necessary first step in merging the science and regulatory aspects of twenty-first century drug discovery and development.

In vivo transcriptomics studies have also been conducted to assess early biomarkers of rodent cancer or specific organ toxicity (reviewed in Ref. [63]). Expression of a selected panel of genes in target organs of rats following 1–5 days of dosing with kidney toxicants [64] or genotoxic or nongenotoxic hepatic carcinogens [65–70] has been assessed. In both the kidney toxicology and liver cancer studies, there were clearly recognizable and reproducible differences between transcriptomics profiles obtained from untreated animals and those treated for as little as 1 day with toxicants or carcinogens. But while these mRNA profiles are intriguing, additional studies are required to identify and understand the mechanistic basis for those very early gene expression changes as they apply to the final endpoint seen after weeks or years of continuous insult in the traditional drug safety paradigm. Although time consuming and expensive, a validation of this approach would require collection of the standard panel of tissues, for potential mRNA analysis, from extra animals from 2-year bioassay studies, sampled after only a few days or weeks of drug treatment. Tissues showing abnormal toxicities or tumors at study termination might then be evaluated in the early sacrificed animals to establish any correlations between gene expression and the terminal endpoint. Because hyperproliferative response is a major source of nongenotoxic carcinogenesis, one obvious thing to look for might be early transcriptomic signs of proliferative responses, perhaps too subtle to observe histopathologically even with BrdU.

In a very recent study [71], it was demonstrated that both mRNA and microRNA expression patterns were distinguishable in primary mouse hepatocytes following short-term exposures to various genotoxic and nongenotoxic insults. MicroRNAs may provide still another means with which to establish the mechanistic consequences of xenobiotic-induced toxicities.

7.10 Single-Nucleotide Polymorphisms (SNPs)

The contribution of pharmaceuticals, even including anticancer and antiviral drugs, to the overall cancer load in humans is likely to be quite small [23] but we do not presently have the tools required to formally evaluate this.

Drug side effect profiles are often idiosyncratic; with nondose-dependent adverse effects arising in anywhere from 5% to 1:100,000 patients. Because of these low frequencies, serious adverse effects are often not recognized until after the marketing of a drug and quite a few otherwise highly efficacious drugs have been pulled from the market or have dropped out late in development due to these unexpected events [72]. The genetic basis of idiosyncratic toxicity should allow for the identification of patient populations that are expected to respond unfavorably and/or to obtain no therapeutic benefit from a given drug. By restricting use of drug in these people, the overall risk of the drug is substantially reduced and the drug may remain on the market for that patient subpopulation for which it is efficacious and safe. The possibility of prediction of adverse effects to a drug through individual genetic analysis (pharmacogenomics) has been made feasible through the sequencing of the human genome and the identification of a projected 10 million single-nucleotide polymorphisms (SNPs), sequence sites [73–78]. Greater than 100 specific associations of biomarkers with drug responses have already appeared in package inserts (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm) and several drugs are undergoing pharmacogenomic salvage operations after having run into toxicity or efficacy issues. These include abacavir (efficacy), carbamazepine (safety), bucindolol (efficacy), and lumericoxib (safety). While this approach has great promise, there are legal and ethical questions concerning the application of this so-called personalized medicine approach, which must be carefully resolved prior to a major roll-out of this technology.

7.11 Conclusions

The genotoxicity and carcinogenicity data on marketed drugs in Appendix A can be summarized by the following statements: (1) 30–40% of all drugs have at least one positive genetox finding; (2) approximately 40% of tested drugs are positive for carcinogenicity in at least one rodent species; (3) for all intents and purposes, genotoxicity is not strongly related to carcinogenicity; and (4) *in silico* models are not predictive of either genotoxicity or carcinogenicity. Much of this lack of correlation is not due to inherent failures in traditional testing paradigms but rather to inadequate means to distinguish nongenotoxic and genotoxic mechanisms for both DNA damage and carcinogenicity. Toward that end, much research has focused on developing gene signatures, specific patterns of cellular response to external stimuli, the presence of which in toxicology studies would be predictive of particular toxicities or cancer.

Progress has been multidirectional. Potential genes involved in response to xenobiotics have been identified in various novel systems such as the chicken lymphocyte DT40 and the near-haploid human cell systems in which selective

insertional mutagenesis is used to understand the role of specific genes in cellular response. In turn, the expression of these and other genes of interest in *in vitro* or *in vivo* test systems is evaluated by microarray-based transcriptomics. A set of genes reproducibly expressed in response to a specific type of stimulus, that is, a gene signature, could then be constructed. The goal would be the identification of gene signatures for a wide variety of toxicities that would serve as early predictors of organ toxicity and/or genotoxic and nongenotoxic carcinogenicity. The ultimate goal would be the demonstration that these signatures have human relevance such that human polymorphisms in one or more of the genes in any given signature might predict drug hypersensitivity.

Candidate gene and genome-wide association studies have already identified at least 30 genes or DNA sequences linked to various idiosyncratic toxicities associated with specific drug exposure [72]. The decision whether or not to use some drugs clinically, for example, abacavir and carbamazepine is already being made on an individual basis driven by the presence of specific HLA haplotypes associated with skin sensitization to these drugs. Non-HLA genes of apparent importance have been identified for statin myopathy, QT prolongation, and isoniazid-induced hepatotoxicity. The number of sequence- or gene-dependent adverse drug affects, including disposition to cancer, will increase dramatically as we continue to unravel the human genome. Pharmacogenomics studies, particularly those linking SNP sequences rather than genes, to adverse drug affects, will most appropriately be driven by the pharmaceutical industry. Some studies are already underway to try to salvage highly efficacious drugs with initially unrecognized and unacceptable toxicity profiles by applying genomics to identify and exclude from treatment that usually very small fraction of the population more likely to exhibit these adverse effects. This process is a long and costly one with much open debate over the practical and ethical pitfalls accompanying personal medicine. That notwithstanding, the human genome project has provided a means for a much deeper understanding of cell biology at all levels and the next decades should see tremendous advances in drug discovery, development, and safety.

Appendix A

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Abacavir	*[136470-78-5]*	N	P	P	P	P/P
Abiraterone	*[154229-18-2]*	N	N		N	0/0
Acamprosate	*[77337-76-9]*	N	N	N	N	N/N
Acarbose	*[56180-94-0]*	N	N	N	N	P/P
Acebutolol	*[37517-30-9]*	N	N			N/N

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Aceclofenac	*[89796-99-6]*	N	N		N	N/N
Acetaminophen	*[103-90-2]*	N	P	P	P	P/N
Acetazolomide	*[59-66-5]*	N	N comet		0/0	
Acitretin	*[55079-83-9]*	N			N	N/P
Aclidinium	*[320345-99-1]*	P		P	N	N/N
Acrivastine	*[87848-99-5]*	N	P	N	N	N/N
Actarit	*[18699-02-0]*	N				N/N
Acyclovir	*[59277-89-3]*		P		P	N/N
Adapalene	*[106685-40-9]*	N			N	P/0
Afatinib	*[439081-18-2]*	P	N		N	0/)
Albendazole	*[54965-21-8]*	N	N		N	N/N
Albuterol	*[18559-94-9]*	N	N		N	P/N
Alclofenac	*[22131-79-9]*	N			N	N/0
Alclometasone	*[66734-13-2]*					0/0
Alendronate	*[66376-36-1]*	N	E		N	P/P
Alfentanil	*[69049-06-5]*	N				0/0
Alfuzosin	*[81403-80-7]*	N	N	N	N	N/N
Aliskiren	*[173334-58-2]*	N	N		N	P/N
Allopurinol	*[315-30-0]*	N	E	N	N	N/N
Almotriptan	*[154323-57-6]*	N	P	N	N	N/N
Alogliptin	*[850649-61-5]*	N	N		N	P/N
Alosetron	*[122852-42-0]*	N	N	N	N	N/N
Alprazolam	*[28981-97-7]*	N	N		N	N/N
Alprenolol	*[13655-52-2]*	N				P/P
Alprostadil	*[745-65-3]*	N	N		N	0/0
Altretamine	*[645-05-6]*	P				0/0
Alvimopan	*[156053-89-3]*	N	N		N	N/P
Amantadine	*[768-94-5]*	N	N		N	0/0
Ambrisentan	*[177036-94-1]*	N	P		N	P/N
Amcinonide	*[51022-69-6]*					0/0
Amifostine	*[20537-88-6]*	P	N	P	N	0/0
Amiloride	*[2609-46-3]*	P				N/N
Aminolevulinic acid	*[106-60-5]*	P			N	0/0
Aminophylline	*[317-34-0]*		N			0/0
Aminosalicylic acid	*[65-49-6]*	N	E	N	P	N/N
Amiodarone	*[1951-25-3]*	N	N		N	P/N

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Amisulpride	*[21675-85-9]*	N	N		N	P/P
Amitriptyline	*[50-48-6]*	N	P		P-MN	0/0
Amlexanox	*[68302-57-8]*	N		N	N	N/N
Amlodipine	*[88150-42-9]*	N	N		N	N/N
Amobarbital	*[57-43-2]*					N/0
Amoxapine	*[14028-44-5]*					P/N
Ampicillin	*[69-53-4]*	N	N	N		N/N
Amprenavir	*[161814-49-9]*	N	N	N	N	P/P
Anagrelide	*[68475-42-3]*	N	N	N	N	P/0
Anastrozole	*[120511-73-1]*	N	N		N	P/P
Androstane	*[438-23-2]*	N				0/0
Androstanolone	*[521-18-6]*					0/0
Anecortave	*[7753-60-8]*			N		0/0
Atamestane	*[96301-34-7]*		N		N	0/0
Apixiban	*[503612-47-3]*	N	N		N	N/N
Apomorphine	*[58-00-4]*	P	P	P	N	N/N
Apraclonidine	*[73218-79-8]*	N	N	N	N	N/N
Apremilast	*[608141-41-9]*	N	N		N	N/N
Aprepitant	*[170729-80-3]*	N	N	N	N	P/P
Aprindine	*[37640-71-4]*	N				0/0
Aprotinin	*[9087-70-1]*	N				0/0
Aranidipine	*[86780-90-7]*	N				0/0
Argatroban	*[74863-84-6]*	N	N		N	0/0
Aripiprazole	*[129722-12-9]*	N	P	N	E	P/P
Armodafinil	*[112111-43-0]*	N	N			N/N
Artemether	*[71939-51-0]*	N	N		N	0/0
Asenapine	*[65576-45-6]*	N	N		N	N/P
Aspirin	*[50-78-2]*	N	E		E	N/N
Astemizole	*[68844-77-9]*	N	N		N	N/N
Atazanavir	*[198904-31-3]*	N	P		N	N/P
Atenolol	*[29122-68-7]*	N	E		N	P/N
Atomoxetine	*[83015-26-3]*	N	N	N	N	N/N
Atorvastatin	*[134523-00-5]*	N	N		N	P/P
Atovaquone	*[95233-18-4]*	N	N	N	E	N/P
Atropine	*[51-55-8]*	N				N/0
Auranofin	*[34031-32-8]*	N		P		P/N
Aurothioglucose	*[12192-57-3]*					0/P

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Axitinib	*[319460-85-0]*	N	N		P	0/0
Azatadine	*[3964-81-6]*					0/0
Azathioprine	*[446-86-6]*	P	P		P-MN	P/P
Azelaic acid	*[123-99-9]*	N	N		N	0/0
Azelastine	*[58581-89-8]*	N	N	N	N	N/N
Azilsartan	*[147403-03-0]*	N	P		N	N/N
Aztreonam	*[78110-38-0]*	N	N			P/0
Baclofen	*[1134-47-0]*					N/0
Balsalazide	*[80573-04-2]*	N	N	N	N	N/0
Barbital	*[57-44-3]*		E		P	P/0
Barnidipine	*[104757-53-1]*	N	N	N	N	N/N
Bazedoxifene	*[198481-32-2]*	N	N		N	0/0
Beclomethasone	*[4419-39-0]*	N	N		N	N/N
Bedaquiline	*[654653-81-3]*	N	N		N	N/0
Belinostat	*[866323-14-0]*	P	P		P	0/0
Benactyzine	*[302-40-9]*				P	0/0
Benazepril	*[86541-75-5]*	N		N	N	N/N
Bendamustine	*[3543-75-7]*	P	P		P	P/P
Bendroflumethiazide	*[73-48-3]*	N	E			N/N
Benorylate	*[5003-48-5]*	N				0/0
Benserazide	*[322-35-0]*	P				0/0
Benzoyl peroxide	*[94-36-0]*	N	N			0/P
Bepotastine	*[125602-71-3]*	N	N		N	N/N
Bepridil	*[64706-54-3]*	N	N		N	P/N
Betamethasone	*[378-44-9]*	N	P			0/0
Betaxolol	*[63659-18-7]*	N	N	N	N	N/N
Bexarotene	*[153559-49-0]*	N	N	N	N	0/0
Bicalutamide	*[90357-06-5]*	N	N		N	P/P
Bimatoprost	*[155206-00-1]*	N		N	N	N/N
Bisacodyl	*[603-50-9]*					0/0
Bisoprolol	*[66722-44-9]*	N	N		N	N/N
Bitolterol	*[30392-40-6]*	N		N		N/N
Bocepravir	*[394730-60-0]*	N	N		N	N/N
Bortezomib	*[179324-69-7]*	N	P		N	0/0
Bosentan	*[147536-97-8]*	N	N		N	P/P
Bosutinib	*[380843-75-4]*	N	N		N	N/N
Brimonidine	*[59803-98-4]*	N	N		N	N/N

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Brinzolamide	*[138890-62-7]*	N		P	N	P/P
Bromazepam	*[1812-30-2]*		P		N	N/0
Bromfenac	*[91714-93-1]*	N	N		N	N/N
Bromocriptine	*[25614-03-3]*	N	N		N	P/N
Brotizolam	*[57801-81-7]*	N		N	N	P/N
Budesonide	*[51333-22-3]*	N	N	N	N	P/N
Bumetanide	*[28395-03-1]*	N				E/0
Bupivicaine	*[18010-40-7]*					0/0
Buprenorphine	*[52485-79-7]*	N	N	N	N	P/N
Bupropion	*[34911-55-2]*	P			P-Abs	N/N
Buspirone	*[36505-84-7]*	N		N	N	N/N
Butaconazole	*[64872-76-0]*	N	N		N	0/0
Butenafine	*[101828-21-1]*	N	N		N	0/0
Butorphanol	*[42408-82-2]*	N				N/N
Cabazitaxel	*[183133-96-2]*	N	N		P	0/0
Cabergoline	*[81409-90-7]*	N	N		N	P/P
Cabozantinib	*[849217-68-1]*	N	N		N	0/0
Caffeine	*[58-08-2]*	N	P		E	N/N
Calcipotriene	*[112965-21-6]*	N	N	N	N	N/N
Calcitriol	*[32222-06-3]*	N			N	P/0
Camazepam	*[36104-80-0]*				N	0/0
Canagliflozin	*[842133-18-0]*	N		P	N	P/N
Candesartan	*[139481-59-7]*	N	P	N	N	N/N
Canrenone	*[976-71-6]*		P		P	0/0
Caprylidene	*[538-23-8]*	P				0/0
Capsaicin	*[404-86-4]*	N	N	P	N	0/P
Captopril	*[62571-86-2]*	N	N		N	N/N
Carazolol	*[57775-29-8]*	N			N	N/0
Carbamazepine	*[298-46-4]*	N	N		N	P/0
Carbidopa	*[28860-95-9]*	P		P	N	N/0
Carbutamide	*[339-43-5]*				N	0/0
Carfilzomib	*[868540-17-4]*	N	P		N	0/0
Carglumic acid	*[1188-38-1]*	N	N		N	0/0
Carisoprodol	*[78-44-4]*	N	P	P	N	0/0
Carteolol	*[51781-06-7]*	N			N	N/N
Carvedilol	*[72956-09-3]*	N	N		N	N/N
Casanthrol	*[8024-98-4]*	P	N		N	0/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Cefdinir	*[91832-40-5]*	N	N		N	0/0
Cefepime	*[88040-23-7]*	N	N		N	0/0
Cefixime	*[79350-37-1]*	N	N		N	0/0
Cefonicid	*[61270-58-4]*	N			N	0/0
Cefoperazone	*[62893-19-0]*	N	N		N	0/0
Cefotaxime	*[63527-52-6]*	N			N	0/0
Cefpodoxime	*[80210-62-4]*	N	N		N	0/0
Cefprozil	*[92665-29-7]*	N	N		N	0/0
Ceftaroline	*[400827-46-5]*	N	N		N	0/0
Ceftazidime	*[72558-82-8]*	N			N	0/0
Ceftibuten	*[97519-39-6]*	N	N		N	0/0
Ceftizoxime	*[68401-81-0]*	N			N	0/0
Ceftriaxone	*[73384-59-5]*	N	N		N	0/0
Cefuroxime	*[55268-75-2]*	N			N	0/0
Celecoxib	*[169590-42-5]*	N	N		N	N/N
Celiprolol	*[5698093-9]*	N	N		N	N/N
Ceritinib	*[1032900-25-6]*	N	P		N	0/0
Cerivastatin	*[145599-86-6]*	N	N		N	N/P
Cetirizine	*[83881-51-0]*	N	N	N	N	N/P
Cevimeline	*[107233-08-9]*	N	N	N	N	N/N
Chirocaine	*[27262-48-2]*	N	N	N	N	0/0
Chloral hydrate	*[302-17-0]*	P	P	P	P	N/P
Chloramphenicol	*[56-75-7]*	N		P		N/N
Chlordiazepoxide	*[58-25-3]*	E	N		P-MN	N/P
Chlormadinone	*[1961-77-9]*	N	P		P/Hep MN	N/N
Chlorophyllin	*[11006-34-1]*					0/0
Chloroquine	*[54-05-7]*	P	P		E-MN	0/0
Chlorothiazide	*[58-94-6]*	N	N			0/0
Chloroxine	*[773-76-2]*	N				0/0
Chlorpheniramine	*[132-22-9]*	N	P	N	N	N/N
Chlorpromazine	*[50-53-3]*	P	P			P/N
Chlorpropamide	*[94-20-2]*	N	E	N	P-MN	N/N
Chlorprothixene	*[113-59-7]*		N			0/0
Chlorthalidone	*[77-36-1]*	N				N/0
Ciclesonide	*[126544-47-6]*	N	N		P-MN	N/P
Ciclopirox	*[29342-05-0]*	N	P		N	0/P

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Cilastatin	*[82009-34-5]*	N			N	0/0
Cilazapril	*[92077-78-6]*	N	N			N/N
Cilostazol	*[73963-72-1]*	N	P	N	N	N/N
Cimetidine	*[51481-61-9]*	N	N			N/N
Ciprofibrate	*[52214-84-3]*	N	P			P/P
Cisapride	*[81098-60-4]*	N	N	N	N	N/N
Cisatracurium	*[96946-41-7]*	N	N	P	N	0/0
Citalopram	*[59729-33-8]*	P	P	N	N	P/N
Clarithromycin	*[81103-11-9]*	N	E	N	N	0/0
Clemastine	*[15686-51-8]*	N	N		N	N/N
Clenbuterol	*[37148-27-9]*	N	N	P	N	P/N
Clevidipine	*[166432-28-6]*	P	P	P		0/0
Clindamycin	*[18323-44-9]*	N			N	0/N
Clobazam	*[22316-47-8]*	N	N			P/0
Clobetasol	*[25122-46-7]*		N		N	0/0
Clodronate	*[22560-50-5]*	N				N/N
Clofazamine	*[2630-63-9]*	N			P	N/N
Clofibrate	*[637-07-0]*	N	P	N	N	P/N
Clomiphene	*[911-45-5]*	P	P		N	N/0
Clomipramine	*[303-49-1]*					N/N
Clonazepam	*[1622-61-3]*	N			N	N/0
Clonidine	*[4205-90-7]*	N			N	N/N
Clopidogrel	*[113665-84-2]*	N	N		N	N/N
Clotrimazole	*[23593-75-1]*				N	N/0
Clozapine	*[5786-21-0]*	N	N		N	N/N
Codeine	*[76-57-3]*	N	N	P		N/N
Colesevelem	*[182815-44-7]*	N	P		N	P/N
Colestipol	*[50925-79-6]*	N				0/0
Crizotinib	*[877399-52-5]*	N	P		P	0/0
Crofelemer	*[143465-45-6]*	N	N		N	0/0
Cromolyn	*[16110-51-3]*	N	N	N		N/N
Cyclizine	*[82-92-8]*	N				0/0
Cyclobenzaprine	*[303-53-7]*	N	N		N	N/N
Cyclodiol			P		P	0/0
Cyclotriol	*[135768-83-1]*		P		P	0/0
Cycloserine	*[68-41-7]*	N				0/0
Cyclosporine	*[79217-60-0]*	N	E		N	N/P

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Cyproheptadine	*[129-03-3]*	N	N			0/0
Cyproterone	*[2098-66-0]*	N	P		P-MN	P/P
Dabigatran	*[211914-51-1]*	N	N		N	N/N
Dabrafenib	*[1195765-45-7]*	N		N	N	0/0
Dalbavancin	*[171500-79-1]*	N-HPRT	N		N	0/0
Dalfampridine	*[504-24-5]*	N	N		N	P/N
Dalfopristin	*[112362-50-2]*	N	P		N	0/0
Dalteparin	*[9041-08-1]*	N	N	N	N	0/0
Dantrolene	*[7261-97-4]*	P				P/N
Dapagliflozin	*[461432-26-8]*	N	P		N	N/N
Dapsone	*[80-08-0]*	N	P	N	N	P/N
Darifenacin	*[133099-04-4]*	N	N		N	N/N
Deferasirox	*[201530-41-8]*	N	N		P	N/N
Deferipone	*[30652-11-0]*	P	P	P	P	0/0
Deferoxamine	*[70-51-9]*			P		0/0
Deflazacort	*[14484-47-0]*		P			0/0
Degarelix	*[214766-78-6]*	N	N		N	P/N
Delapril	*[83435-66-9]*	N				0/0
Delavirdine	*[136817-59-9]*	N	N		N	N/P
Depredone						0/0
Deserpidine	*[131-01-1]*	N				N/0
Desflurane	*[57041-67-5]*	N	N		N	0/0
Desipramine	*[50-47-5]*	N			P	0/0
Desloratadine	*[100643-71-8]*	N	N		N	P/P
Desogestrel	*[54024-22-5]*		N			0/0
Dexamethasone	*[50-02-2]*	N	P		P-MN	N/0
Dexmedetomidine	*[113775-47-6]*	N	P	N	P	0/0
Dexmethylphenidate	*[19262-68-1]*	N	N	N		0/P
Dexrazoxane	*[24584-09-6]*	N	P	P	P-MN	P/P
Dextromethorphan	*[125-71-3]*	N	N			0/0
Diazepam	*[439-14-5]*	N	P		P-MN	N/P
Diclofenac	*[15307-86-5]*	N	N	N	N	N/N
Dienogest	*[65928-58-7]*		N		N	0/0
Diflunisal	*[22494-42-4]*	N	N		E	N/N
Difluprednate	*[23674-86-4]*	N	N		N	0/0
Dihydralazine	*[484-23-1]*	P			P-MN	0/0

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Dihydroergotamin	*[511-12-6]*	N	P		N	0/0
Diltiazem	*[42399-41-7]*	N	N			P/N
Dimethisterone	*[79-64-1]*		N			0/0
Dimethyl fumarate	*[624-49-7]*	N	P		N	P/P
Diphenhydramine	*[58-73-1]*	N	P	N		N/N
Dipyridamole	*[58-32-2]*	N	N		N	N/N
Dipyrone	*[68-89-3]*	P	E			N/P
Disopyramide	*[3737-09-5]*	N				N/0
Disulfuram	*[97-77-8]*	N	P	P	N	N/N
Dofetilide	*[115256-11-6]*	N	N		N	N/N
Dolasetron	*[115956-12-2]*	N	N	N	N	N/P
Dolutegravir	*[1051375-16-6]*	N		N	N	N/N
Donepezil	*[120014-06-4]*	N	E		N	N/N
Doripenem	*[148016-81-3]*	N	N		N	0/0
Dorzolamide	*[120279-96-1]*	N	N		N	P/N
Doxacurium	*[133814-18-3]*	N	N	N	N	0/0
Doxazosin	*[74191-85-8]*	N	N		N	N/N
Doxefazepam	*[40762-15-0]*	N			N	P/0
Doxepin	*[1668-19-5]*	N	N		N	N/N
Doxercalciferol	*[54573-75-0]*	N	P	N	N	0/0
Doxycycline	*[564-25-0]*	N	P		N	0/0
Doxylamine	*[469-21-6]*	N	N		N	P/P
Dronabinol	*[1972-08-3]*	N	N		N	N/N
Dronedarone	*[141626-36-0]*	N	P		N	N/P
Droperidol	*[548-73-2]*		N			0/0
Drospirenone	*[67392-87-4]*	N	N		N	P/P
Droxidopa	*[23651-95-8]*	N	P		N	N/N
Duloxetine	*[116539-59-4]*	N	N		N	N/P
Dutasteride	*[164656-27-9]*		N		N	0/0
Dydrogesterone	*[152-62-5]*				E	0/0
Efavirenz	*[154598-52-4]*	N	N		N	N/P
Efinaconazole	*[64650-44-6]*	N	N		N	N/N
Eflornithine	*[67037-37-0]*	N	N		N	N/N
Eletriptan	*[143322-58-1]*	N	N		N	P/P
Elvitegravir	*[697761-98-1]*	N	E		N	N/N
Empagliflozin	*[864070-44-0]*	N	N	N	N	P/P
Enalapril	*[75847-73-3]*	N	N		N	N/N

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Enalaprilat	*[76420-72-9]*	N	N		N	0/0
Endralazine	*[39715-02-1]*	P			P-SCE	0/0
Enoxaparin	*[9005-49-6]*	N	N	N	N	P/0
Entacapone	*[130929-57-6]*	N	P	P	N	P/N
Entecavir	*[142217-69-4]*	N	P		N	P/P
Enzalutamide	*[915087-33-1]*	N	N		N	0/0
Epanova	*[0-3 fatty acids]*	N	N		N	0/0
Ephedrine	*[299-42-3]*	N	N			N/N
Epinastine	*[80012-43-7]*	N	P		N	N/N
Epinephrine	*[51-43-4]*	E	N			N/N
Eplerenone	*[107724-20-9]*	N	N	N	N	P/N
Epoprostenol	*[35121-78-9]*	N			N	0/0
Epremilast	*[608141-41-9]*	N	N		N	N/N
Eprosartan	*[133040-01-4]*	N	P	N	N	N/N
Eptifibatide	*[188627-80-7]*	N	N	N	N	0/0
Ergotamine	*[113-15-5]*	N		N		0/0
Eribulin	*[253128-41-5]*	N	N	P	P	0/0
Ertapenem	*[153832-46-3]*	N	N	N	N	0/0
Erythromycin	*[114-07-8]*	N	N	N		N/N
Escitalopram	*[128196-01-0]*	P	P	N		P/N
Eslicarbazepine	*[236395-14-5]*	N	P	P	N	0/P
Esmolol	*[81147-92-4]*	N	N			0/0
Esomeprazole	*[119141-88-7]*	N	P		N	P/N
Estazolam	*[29975-16-4]*	N			N	N/N
Estramustine	*[2998-57-4]*	N				0/0
Estradiol	*[50-28-2]*		P		P	0/0
Estradiol 2-OH	*[362-05-0]*		SHE-P			0/0
Estradiol 4OH	*[5976-51-4]*		SHE-P			0/0
Estriol	*[50-27-1]*		P			0/0
Estrone	*[53-16-7]*		P		P	0/0
Estrone, 16 alpha OH	*[566-76-7]*		SHE-N			0/0
Estrone, 2 methoxy	*[362-08-3]*		SHE-N			0/0
Eszopiclone	*[138729-47-2]*	N	P	N	N	P/P

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Ethacrynic acid	*[58-54-8]*	N				N/0
Ethambutol	*[74-55-5]*	N			P	0/0
Ethenzamide	*[938-73-8]*	N	P			P/0
Ethinylestradiol	*[57-63-6]*	N	P	P	E	P/P
Ethionamide	*[536-33-4]*	N	P	P	P-MN	N/N
Ethynodiol	*[1231-93-2]*				N	P/P
Etidronate	*[2809-21-4]*	N	N		N	N/N
Etodolac	*[41340-25-4]*	N	E	N	N	N/N
Etravirine	*[269055-15-4]*	N	N	N	N	N/P
Everolimus	*[159351-69-6]*	N	N		N	N/N
Exemestane	*[107868-30-4]*		P		N	0/0
Ezetimibe	*[163222-33-1]*	N	N		N	N/N
Ezogabine	*[150812-12-7]*	N	P		N	N/P
Famotidine	*[76824-35-6]*	N	N		N	N/N
Febuxostat	*[144060-53-7]*	N	P		N	P/P
Felbamate	*[25451-15-4]*	N	N		N	P/N
Felodipine	*[72509-76-3]*	N	N	N	N	P/N
Fenofibrate	*[49562-28-9]*	N	N	N	N	P/P
Fenoldopam	*[67227-56-9]*	N	P		N	N/N
Fentanyl	*[437-38-7]*	N	N	N	N	0/0
Ferumoxytol	*[722492-56-0]*	N	N		N	0/0
Fesoterodine	*[286930-03-8]*	N	N		N	N/N
Fexofenadine	*[83799-24-0]*	N	N		N	N/N
Fidaxomycin	*[873857-62-6]*	N	P		N	0/0
Finasteride	*[98319-26-7]*	N	P	N	N	P/P
Fingolimod	*[162359-56-0]*	N	N		N	N/P
Flavoxate	*[15301-69-6]*	N			N	0/0
Flecainide	*[54143-55-4]*	N		N	N	N/N
Fluconazole	*[86386-73-4]*	N	N	N	N	P/N
Flumazenil	*[78755-81-4]*	N	N		N	0/0
Flunarizine	*[52468-60-7]*	N	N		N	N/N
Flunisolide	*[3385 03 3]*		N			0/0
Flunitrazepam	*[1622-62-4]*	E			N	0/0
Fluocinonide	*[356-12-7]*		N		P	0/0
Fluopromazine	*[146-54-3]*	N	P		N	0/0
Fluoxetine	*[54910-89-3]*	N	N	N	N	N/N
Fluoxymesterone	*[76-43-7]*	N	P		P	0/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Fluphenazine	*[69-23-8]*	N			P-MN	N/0
Flurazepam	*[17617-23-1]*	N				N/N
Flurbiprofen	*[5104-49-4]*				N	N/N
Flutamide	*[13311-84-7]*	N				P/0
Flutazolam	*[27060-91-9]*				N	0/0
Fluticasone	*[90566-53-3]*	N	N		N	N/N
Fluvastatin	*[93957-54-1]*	N	N		N	P/P
Fluvoxamine	*[54739-18-3]*	N	N		N	N/0
Fondaparinux	*[104993-28-4]*	N	N	N	N	0/0
Formestane	*[566-48-3]*					0/0
Formoterol	*[73573-87-2]*	N	N		N	P/P
Fosfomycin	*[23155-02-4]*	N	N		N	0/0
Fosinopril	*[98048-97-6]*	N	P	N	N	N/N
Fosphenytoin	*[93390-81-9]*	N	P		N	P/P
Fospropofol	*[258516-87-9]*	N	N	P	N	0/0
Frovatriptan	*[158930-17-7]*	N	P	N	N	P/N
Fulvestrant	*[129453-61-8]*		N			0/0
Furazolidone	*[67-45-8]*	P	P		P	P/P
Furosemide	*[54-31-9]*	N	P	P	N	P/P
Gabapentin	*[60142-96-3]*	N	N		N	P/N
Galantamine	*[357-70-0]*	N	N	N	N	P/N
Gefitinib	*[184475-35-2]*	N	N	N	N	P/P
Gemfibrozil	*[25812-30-0]*	N				P/N
Gestodene	*[60282-87-3]*				P	0/0
Glatiramer Acetate	*[147245-92-9]*	N	P	N	N	N/N
Glibenclamide	*[10238-21-8]*	N	N		N	N/N
Glimepiride	*[93479-97-1]*	N	N		N	N/N
Glipizide	*[29094-61-9]*	N	N		N	N/N
Glyburide	*[10238-21-8]*	N			N	N/N
Glycopyrrolate	*[596-51-0]*	N	N		N	0/0
Granisetron	*[109889-09-0]*	N		N	N	P/0
Griseofulvin	*[126-07-8]*	N	P	P	P	0/P
Guanabenz	*[5051-62-7]*	P				N/N
Guanadrel	*[40580-59-4]*	N			N	P/N
Guanfacine	*[29110-47-2]*	N	N		N	N/N
Halobetasol	*[66852-54-8]*	N			P	0/0
Halometasone	*[50629-82-8]*					0/0

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Haloperidol	*[52-86-8]*	N	N		E	N/P
Halothane	*[151-67-7]*	N	N		P-MN	0/N
Heroin	*[561-27-3]*				P	0/0
Hexachlorophene	*[70-30-4]*	N	N		N	N/0
Hydralazine	*[86-54-4]*	P	P		E	P/P
Hydrochlorothiazide	*[58-93-5]*	N	N	P	N	N/N
Hydrocortisone	*[50-23-7]*	P			P	0/0
Hydroflumethiazide	*[135-09-1]*		N			0/0
Hydromorphone	*[466-99-9]*	N	N	P	N	0/0
Hydroquinone	*[123-31-9]*	P	P	P	P	P/P
Ibandronate	*[114084-78-5]*	N	N		N	N/P
Ibrutinib	*[936563-96-1]*	N	N		N	0/0
Ibuprofen	*[15687-27-1]*	N			P	N/0
Ibutilide	*[122647-31-8]*	N	N			0/0
Icatibant	*[130308-48-4]*	N	N		N	N/0
Icosapent	*[86227-47-6]*	N	P		N	N/N
Idebenone	*[58186-27-9]*	N	P	P	N	P/P
Idelalisib	*[870281-82-6]*	N	N		P	o/o
Ifosfamide	*[3778-73-2]*	P			P-MN	P/P
Iloperidone	*[133454-47-4]*	N	P		N	N/P
Iloprost	*[73873-87-7]*	N	N		N	N/N
Imatinib	*[152459-95-5]*	N	P	N	N	P/0
Imipenem	*[64221-86-9]*	N				0/0
Imipramine	*[50-49-7]*	N	P		P-MN	P/P
Imiquimod	*[99011-02-6]*	N	N	N	N	N/P
Indacaterol	*[312753-06-3]*	N	N		N	P/N
Indapamide	*[26807-65-8]*					N/N
Indinavir	*[150378-17-9]*	N	N		N	P/N
Indomethacin	*[53-86-1]*	N	N		E	N/N
Ingenol	*[30220-46-3]*	N	PSHE	N	N	0/0
Ipratropium	*[60205-81-4]*	N	N		N	N/N
Iproniazid	*[54-92-2]*	N			P	0/0
Irbesartan	*[138402-11-6]*	N	N		N	N/N
Isocarboxazid	*[59-63-2]*	N			P	0/0
Isoniazid	*[54-85-3]*	P	E		N	P/P
Isosorbide	*[652-67-5]*	N	N	N	N	N/N
Isotretinoin	*[4759-48-2]*	N	N		N	P/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Isradipine	*[75695-93-1]*	N	N		N	P/0
Itraconazole	*[84625-61-6]*	N	N	N	N	P/N
Ivabradine	*[155974-00-8]*	P	P		N	N/N
Ivacaftor	*[873054-44-5]*	N	N		N	N/N
Ivermectin	*[70288-86-7]*	N	N	N		N/0
Ixabepilone	*[219989-84-1]*	N	N		P-MN	0/0
Ketoconazole	*[65277-42-1]*	N			N	N/N
Ketoprofen	*[22071-15-4]*	N			E	N/N
Ketorolac	*[74103-06-3]*	N	P		N	N/N
Ketotifen	*[34580-13-7]*	N	N		N	N/N
Labetalol	*[36894-69-6]*	N				N/P
Lacidipine	*[103890-78-4]*	N	N		N	0/0
Lacosamide	*[175481-36-4]*	N	N	P	N	N/N
Lamivudine	*[134678-17-4]*	N	P	P	N	N/N
Lamotrigine	*[84057-84-1]*	N	N	N	N	N/N
Lansoprazole	*[103577-45-3]*	P	P		N	P/P
Lanthanum	*[7439-91-0]*	N	E		N	0/P
Lapatinib	*[388082-78-8]*	N	N	N	N	P/N
Lavaborole	*[174671-46-6]*	N	N		N	N/N
Leflunomide	*[75706-12-6]*	N			N	N/P
Lenalidomide	*[191732-72-6]*	N	N		N	0/0
Lercanidipine	*[132866-71-6]*	N	N		N	N/N
Letrozole	*[112809-51-5]*	N	P		N	P/P
Leuprolide	*[53714-56-0]*	N			N	P/N
Levalbuterol	*[34391-04-3]*	N	N			P/N
Levamisole	*[14769-73-4]*	N	N			N/N
Levetiracetam	*[102767-28-2]*	N	N		N	N/N
Levobetaxolol	*[93221-48-8]*	N	N	N		N/N
Levocarnitine	*[541-15-1]*	N				0/0
Levodopa	*[59-92-7]*	P		P	N	N/0
Levomethadyl	*[1477-40-3]*	N	N	N		N/N
Levomilnicipran	*[96847-55-1]*	N	N		N	N/N
Levonorgestrel	*[797-63-7]*	N				0/0
Lilopristone	*[97747-88-1]*	N				0/0
Lidocaine	*[137-58-6]*	N	N		N	N/0
Linaclotide	*[851199-59-2]*	N	N		N	N/N
LInagliptin	*[668220-12-0]*	N	N		N	N/N

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Lindane	*[58-89-9]*	N	E			N/P
Linezolid	*[165800-03-3]*	N	N		N	0/0
Liraglutide	*[204656-20-2]*	N	N		N	P/P
Lisinopril	*[76547-98-3]*	N	N		N	N/N
Lisuride	*[19875-60-6]*	P				N/N
Lomitapide	*[182431-12-5]*	N	N		N	N/P
Loperamide	*[53179-11-6]*	N				P/0
Loracarbef	*[76470-66-1]*	N	N		N	0/0
Loratadine	*[79794-75-5]*	N	N	P	N	N/P
Lorcaserin	*[616202-92-7]*	N	N		N	P/N
Losartan	*[114798-26-4]*	N	N		N	N/N
Lovastatin	*[75330-75-5]*	N	N		N	P/P
Lubiprostone	*[136790-76-6]*	N	N		N	P/N
Lucinactant	*[825600-90-6]*	N	N		N	0/0
Luliconazole	*[187164-19-8]*	N	N		N	0/0
Lumiracoxib	*[220991-20-8]*	N	P		N	N/N
Lurasidone	*[367514-88-3]*	N	N		N	P/P
Lynestrenol	*[52-76-6]*				P	P/P
Macitentan	*[441798-33-0]*	N	N	N	N	N/N
Mafenide	*[138-39-6]*			N		0/0
Manidipine	*[120092-68-4]*	N				N/N
Maraviroc	*[376348-65-1]*	N	N		N	N/N
Mebendazole	*[31431-39-7]*	P				N/N
Mecamylamine	*[60-40-2]*	N	N			0/0
Mechlorethamine	*[51-75-2]*	N	P			P/P
Meclizine	*[569-65-3]*					0/0
Meclofenamic acid	*[644-62-2]*	N	P		N	0/0
Medazepam	*[2898-12-6]*	N	P		N	0/0
Medroxalol	*[56290-94-9]*					N/P
Medroxyprogest	*[520-85-4]*		P		P	0/E
Mefloquine	*[53230-10-7]*	N			N	N/N
Megestrol	*[595-33-5]*		N		P	0/0
Meloxicam	*[71125-38-7]*	N	N		N	N/N
Melphalan	*[148-82-3]*	P	P		P-abs	P/P
Memantine	*[19982-08-2]*	N	N		N	N/N
Mepyramine	*[91-84-9]*	N	N	P	N	N/N
Mequitazine	*[29216-28-2]*	N			N	0/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Meropenem	*[96036-03-2]*	N	N		N	0/0
Mesalazine	*[89-57-6]*	N	N	N	N	N/N
Mesna	*[19767-45-4]*	N	N		N	0/0
Mestranol	*[72-33-3]*	N	P		P-Abs	P/P
Metaxalone	*[1665-48-1]*	N				N/N
Metformin	*[657-24-9]*	N	N	N	N	P/N
Methadone	*[76-99-3]*			P		N/N
Methapyrilene	*[91-80-5]*	N	P	E		P/0
Methimazole	*[60-56-0]*	N	E		E	P/E
Methoxsalen	*[298-81-7]*	P	P		P	P/N
Methyclothiazide	*[135-07-9]*	N	P			0/0
Methyldopa	*[555-30-6]*	N	N	P	N	N/N
Methylphenidate	*[113-45-1]*	N	P	N	N	N/P
Methylprednisolone	*[83-43-2]*					0/0
Methyltestosterone	*[58-18-4]*					0/0
Metoclopramide	*[364-62-5]*	N	N		N	P/0
Metolazone	*[17560-51-9]*	N				N/N
Metoprolol	*[51384-51-1]*	N	N		N	N/N
Metreleptin	*[186018-45-1]*	N	N		N	N/N
Metronidazole	*[443-48-1]*	P	P		P-MN	P/P
Mexiletine	*[31828-71-4]*	N				N/N
Mibefradil	*[116644-53-2*	N	N		N	P/N
Midazolam	*[59467-70-8]*	N	P		N	P/P
Midodrine	*[42794-76-3]*	N				N/N
Mifepristone	*[84371-65-3]*		N		N	Neg
Miglitol	*[72432-03-2]*	N	N		N	N/N
Miglustat	*[72599-27-0]*	N	N		N	P/P
Milnacipran	*[92623-85-3]*	N	N		N	P/N
Milrinone	*[78415-72-2]*	N	P	N	N	N/N
Miltefosine	*[58066-85-6]*	N	N		N	0/0
Minoxidil	*[38304-91-5]*	N	N		N	P/P
Mipomersin	*[629167-92-6]*	N	N		N	P/P
Mirabegron	*[223673-61-8]*	N	N		N	N/N
Mirtazapine	*[85650-52-8]*	N	N		N	P/P
Misoprostil	*[59122-46-2]*	N	N	N		N/N
Mivacurium	*[133814-19-4]*	N	N	N	N	0/0
Mizolastine	*[108612-45-9]*	N				0/0

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Moclobemide	*[71320-77-9]*	N			N	N/N
Modafinil	*[68693-11-8]*	N	N	N	N	N/N
Moexipril	*[103775-10-6]*	N	P		N	N/N
Mometasone	*[105102-22-5]*	N	P		N	N/N
Montelukast	*[158966-92-8]*	N	N		N	N/N
Moricizine	*[31883-05-3]*	N			N	P/P
Morphine	*[57-27-2]*	N	E		P-MN	0/N
Moxonidine	*[75438-57-2]*	N	N		N	N/N
Mupirocin	*[12650-69-0]*	N	N	N	N	0/0
Mycophenolate	*[128794-94-5]*	N	N	P	P	N/N
Nabumetone	*[42924-53-8]*	N	P		N	N/N
Nadolol	*[42200-33-9]*	N			N	N/N
Nafarelin	*[76932-56-4]*	N	N			P/P
Nalbuphine	*[20594-83-6]*	N	N	P	N	N/N
Nalidixic Acid	*[389-08-2]*	P	N	N		P/N
Naloxegol	*[854601-70-0]*	N	N		N	N/N
Naloxone	*[465-65-6]*	P	P	P	N	0/0
Naltrexone	*[16590-41-3]*	N	N	P	N	P/N
Nandrolone	*[434-22-0]*		P			0/0
Naphazoline	*[835-31-4]*					0/0
Naproxen	*[22204-53-1]*	N			P-MN	N/0
Naratriptan	*[121679-13-8]*	E	N	N	N	P/N
Nateglinide	*[105816-04-4]*	N	N	N	N	N/N
Nebivolol	*[152520-56-4]*	N	N		N	N/P
Nedocromil	*[69049-73-6]*	N		N	N	N/N
Nefazodone	*[83366-66-9]*	N			N	N/N
Nelfinavir	*[159989-64-7]*	N	N	N	N	P/0
Neomycin	*[1404-04-2]*	N	P			N/N
Nepafenac	*[78281-72-8]*	N	P	N	N	0/0
Nesterone	*[7759-35-5]*		N		N	0/0
Netupitant	*[298297-26-6]*	N	N		N	0/0
Nevirapine	*[129618-40-2]*	N	N		N	P/P
Niacin	*[59-67-6]*	N	N		N	0/N
Nicardipine	*[55985-32-5]*	N	N		N	P/N
Nicorandil	*[65141-46-0]*	N	N			N/N
Nicotine	*[54-11-5]*	N	P		P	N/N
Nifedipine	*[21829-25-4]*	N	N		N	N/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Nilotinib	*[641571-10-0]*	N	N	N	N	N/0
Nilutamide	*[63612-50-0]*	N	N		N	N/0
Nimesulide	*[51803-78-2]*				P	0/0
Nimodipine	*[66085-59-4]*	N			N	P/N
Nintedanib	*[656247-17-5]*	N	N		N	N/N
Nisoldipine	*[63675-72-9]*	N	N		N	N/N
Nitazoxanide	*[55981-09-4]*	P	N		N	0/0
Nitisinone	*[104206-65-7]*	N		P	P	0/0
Nitrazepam	*[146-22-5]*	N	N		N	0/0
Nitrendipine	*[39562-70-4]*					N/N
Nitrofurantoin	*[67-20-9]*	P	P	P		P/P
Nitrofurazone	*[59-87-0]*	P	P		P	P/P
Nitroglycerin	*[55-63-0]*	P			N	P/N
Nizatidine	*[76963-41-2]*	N	N	N	N	N/P
Nomegestrol	*[58691-88-6]*					0/0
Norelgestromin	*[53016-31-2]*		N			0/0
Norethisterone	*[68-22-4]*	N	P	P	E	P/P
Norethynodrel	*[68-23-5]*		P			0/0
Norgestimate	*[35189-28-7]*					0/0
Norgestrel	*[6533-00-2]*		P		N	0/0
Nortriptylline	*[72-69-5]*	N				0/0
Olanzapine	*[132539-06-1]*	N	N	N	N	P/P
Olmesartan	*[144689-63-4]*	N	P	P	N	N/N
Olodaterol	*[868049-49-4]*	N		N	P	P/P
Olopatadine	*[113806-05-6]*	N	N		N	N/N
Olsalazine	*[15722-48-2]*	N	N	N	N	N/N
Omacetaxine	*[26833-87-4]*	N	P		N	0/0
Omeprazole	*[73590-58-6]*	N	P	N	P	P/N
Onapristone	*[96346-61-1]*		N		N	0/0
Ondansetron	*[99614-02-5]*	N	N			N/N
Oritavancin	*[171099-57-3]*	N	N		N	0/0
Orlistat	*[96829-58-2]*	N	N		N	N/N
Oseltamivir	*[196618-13-0]*	N	N		N	N/N
Ospemifene	*[128657-22-7]*	N	N		N	P/P
Ouabain	*[630-60-4]*			P		0/0
Oxandrolone	*[53-39-4]*		P		N	0/0
Oxaprozin	*[21256-18-8]*	N	N		N	N/P

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Oxazepam	*[604-75-1]*	N	P	N	N	P/P
Oxcarbazepine	*[28721-07-5]*	P	P		N	P/P
Oxiconazole	*[64211-45-6]*	N	N		N	0/0
Oxprenolol	*[6452-71-7]*	N			E	N/N
Oxybutynin	*[5633-20-5]*	N				N/0
Oxycodone	*[76-42-6]*	N	P	P	N	0/0
Oxymetholone	*[434-07-1]*	N	N	N	N	P/0
Palonosetron	*[119904-90-4]*	N	P		N	P/N
Pamidronate	*[57248-88-1]*	N	N		N	P/N
Pantoprazole	*[102625-70-7]*	N	P	N	E-MN	P/P
Paricalcitol	*[131918-61-1]*	N	N	N	N	E/E
Paroxetine	*[61869-08-7]*	N	N	N	N	P/P
Pasireotide	*[396091-73-9]*	N	N		N	N/N
Pemetrexed	*[137281-23-3]*	N	N		P	N/N
Pemirolast	*[69372-19-6]*	N	N		N	N/0
Pemoline	*[2152-34-3]*					N/N
Penbutolol	*[38363-40-5]*	P			N	N/N
Penicillamine	*[52-67-5]*	P				N/P
Pentobarbital	*[76-74-4]*			P	P-MN	0/0
Pentosan	*[116001-96-8]*	N			N	N/N
Pentostatin	*[53910-25-1]*	P	N		P-MN	0/0
Pentoxifylline	*[6493-05-6]*	N	P		N	P/N
Perampanel	*[380917-97-5]*	N	N		N	N/N
Perazine	*[84-97-9]*		E			0/0
Perflexane	*[423-55-2]*	N	N	N	N	0/0
Pergolide	*[66104-22-1]*	N		P	N	P/P
Perhexiline	*[6621-47-2]*	N		P		N/0
Perindopril	*[82834-16-0]*	N	N	N	N	N/N
Permethrin	*[52645-53-1]*	N	E	N	N	N/P
Pethidine	*[57-42-1]*				P	0/0
Phenelzine	*[51-71-8]*	P			P-MN	N/P
Phenobarbital	*[50-06-6]*	P	P	P	N	N/P
Phenolphthalein	*[72-09-8]*	N	P		P	P/P
Phenoxybenzamin	*[59-96-1]*	P	P	P	E	P/P
Phentermine	*[1197-21-3]*	N				0/0
Phentolamine	*[50-60-2]*	N	N			P/P
Phenylephrine	*[59-42-7]*	N	N	P	N	N/N

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Phenylpropanol	*[14838-15-4]*	N		N		0/0
Phenytoin	*[57-41-0]*	N	E	P	E	N/E
Phytonadione	*[84-80-0]*	N				E/E
Pilocarpine	*[92-13-7]*	N	N		N	N/0
Pimecrolimus	*[137071-32-0]*	N	N	N	N	P/P
Pimozide	*[2062-78-4]*	N			N	N/P
Pindolol	*[13523-86-9]*	N			N	N/N
Pioglitazone	*[111025-46-8]*	N	N		N	P/N
Piperacillin	*[61477-96-1]*	N	N	P		0/0
Piracetam	*[7491-74-9]*	N	N			0/0
Pirbuterol	*[38677-81-5]*	N	N		N	N/N
Pirfenidone	*[53179-13-8]*	N	N		N	P/P
Piroxicam	*[36322-90-4]*	N			P-SCE	N/0
Pitavastatin	*[147511-69-1]*	N	P		N	P/N
Podofilox	*[518-28-5]*	N	P	N	P-MN	0/N
Polidocanol	*[9002-92-0]*	N		P	N	0/0
Polyestradiol	*[28014-46-2]*					0/0
Polythiazide	*[346-18-9]*	N				N/0
Pomalidomide	*[19171-19-8]*	N	N		N	0/0
Ponatinib	*[943319-70-8]*	N	N		N	0/0
Posaconazole	*[171228-49-2]*	N				N/N
Practolol	*[6673-35-4]*	N			N	P/P
Pralatrexate	*[146464-95-1]*	N	N		N	0/0
Pramipexole	*[104632-26-0]*	N	N		N	N/N
Prasugrel	*[150322-43-3]*	N	N		N	P/P
Pravastatin	*[81093-37-0]*	N	N	N	N	P/P
Prazepam	*[2955-38-6]*				N	N/N
Praziquantel	*[55268-74-1]*	N	E		E	N/0
Prazosin	*[19216-56-9]*	N	N		N	N/0
Prednicarbate	*[73771-04-7]*				N	0/0
Prednisolone	*[50-24-8]*	N		P		P/0
Prednisone	*[53-03-2]*	E	N	P	N	N/N
Pregabilin	*[148553-50-8]*	N	N		N	N/P
Primidone	*[125-33-7]*	P	N		N	N/P
Probenecid	*[57-66-9]*	N	N			N/P
Procainamide	*[51-06-9]*		N		N	0/0
Procarbazine	*[671-16-9]*	N		P		P/P

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Progesterone	*[57-83-0]*		N		P	0/0
Proguanil	*[500-92-5]*	N		N	N	0/0
Promazine	*[58-40-2]*	N	P			0/0
Promethazine	*[60-87-7]*	N	N			N/N
Propafenone	*[54063-53-5]*	N	N		N	N/N
Propantheline	*[298-50-0]*	N	N			0/0
Propofol	*[2078-54-8]*	N	N		N	0/0
Propranolol	*[525-66-6]*	E			E	N/N
Propylmesterolone	*[1424-00-6]*		N		N	0/0
Propylthiouracil	*[51-52-5]*	N	N			P/P
Pseudoephedrine	*[90-82-4]*	N	N			N/N
Pyrazinamide	*[98-96-4]*	N	P		P-MN	N/N
Pyridostigmine	*[155-97-5]*	N		E	N	0/0
Pyrilamine	*[91-84-9]*	N		P	P-MN	P/N
Pyrimethamine	*[58-14-0]*	N	P	P	P-Abs	N/N
Quazepam	*[36735-22-5]*	N		E	N	N/N
Quetiapine	*[111974-69-7]*	N	N		N	P/P
Quinapril	*[85441-61-8]*	N	N		N	P/N
Quinidine	*[56-54-2]*	N		N		0/0
Quinupristin	*[120138-50-3]*	N	N	N	N	0/0
Rabeprazole	*[117976-89-3]*	P	N	P	N	P/N
Raloxifene	*[84449-90-1]*	N	N	N	N	P/P
Raltegravir	*[871038-72-1*	N	N	N	N	N/N
Ramelteon	*[196597-26-9]*	N	P	N	N	P/P
Ramipril	*[87333-19-5]*	N			N	N/P
Ranitidine	*[66357-35-5]*	N	N	N	N	N/N
Ranolazine	*[95675-55-5]*	N	P		N	P/N
Rasagiline	*[136236-51-6]*	N	P	P	N	0/P
Reboxetine	*[98769-81-4]*	N	P		N	N/N
Regorafenib	*[755037-03-7]*	N	N		N	0/0
Remifentanil	*[132875-61-7]*	N	N	P	N	0/0
Repaglinide	*[135062-02-1]*	N	N		N	P/N
Reserpine	*[50-55-5]*	N	N	P	P	P/P
Retapamulin	*[224452-66-8]*	N	N	N	N	0/0
Retinoic acid	*[302-79-4]*	N	N		N	N/N
Rifabutin	*[72559-06-9]*	N	N		N	N/N
Rifamixin	*[80621-81-4]*	N	N		N	P/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Rifampin	*[13292-46-1]*	N	P		N	N/P
Rifapentine	*[61379-65-5]*	N	N		N	0/P
Riluzole	*[1744-22-5]*	N	E	N	N	N/N
Rimantadine	*[13392-28-4]*	N	N		N	N/N
Riociguat	*[625115-55-1]*	N	N		N	N/N
Ripazepam	*[26308-28-1]*					N/P
Risedronate	*[115436-72-1]*	N	P		N	N/N
Risperidone	*[106266-06-2]*	N	N	N	N	P/P
Ritonavir	*[155213-67-5]*	N	N	N	N	N/P
Rivaroxaban	*[366789-02-8]*	N	N		N	N/N
Rivastigmine	*[123441-03-2]*	N	P		N	N/N
Rizatriptan	*[144034-80-0]*	N	N		N	N/N
Rocuronium	*[143558-00-3]*	N	N		N	0/0
Rofecoxib	*[162011-90-7]*	N	N		N	N/N
Roflumilast	*[162401-32-3]*	N	N		PHamst	PH/N
Romidepsin	*[128517-07-7]*	N	N		N	0/0
Ropinirole	*[91374-21-9]*	N	N	N	N	P/P
Ropivacaine	*[84057-95-4]*	N	N	P	N	0/0
Rosiglitazone	*[122320-73-4]*	N	N	P	N	P/P
Rosuvastatin	*[287714-41-4]*	N	N	N	N	P/P
Rotigotine	*[125572-93-2]*	N	N	P	N	N/N
Rufinamide	*[106308-44-5]*	N	N		N	P/P
Ruxolitinib	*[941678-49-5]*	N	N		N	N/N
Salbutamol	*[18559-94-9]*	N	N		N	P/N
Salmeterol	*[89365-50-4]*	N	N	N	N	P/P
Sapropterin	*[62989-33-7]*	P	P		N	P/N
Saquinavir	*[127779-20-8]*	N	N		N	N/N
Saxagliptin	*[361442-04-8]*	N	N		N	N/N
Selegiline	*[14611-51-9]*	N	N	P	N	N/0
Sertindole	*[106516-24-9]*	N			N	0/0
Sertraline	*[79617-96-2]*	N	N	N	N	P/P
Sevelamer	*[52757-95-6]*	N	P		N	P/N
Sibutramine	*[106650-56-0]*	N	N		N	P/N
Sildenafil	*[139755-83-2]*	N	N		N	N/N
Simeprevir	*[923604-59-5]*	N		N	N	0/0
Simethicone	*[8050-81-5]*	N	N		N	N/N
Simvastatin	*[79902-63-9]*	N	N		N	P/P

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Sirolimus	*[53123-88-9]*	N	N	N	N	N/P
Sofosbuvir	*[1190307-88-0]*	N	N		N	N/N
Solifenacin	*[242478-37-1]*	N	N		N	N/N
Sotalol	*[3930-20-9]*	N			N	N/N
Spironolactone	*[52-01-7]*	N	N	N	N	P/0
Stanozolol	*[1048-03-8]*		P			0/0
Stavudine	*[3056-17-5]*	N	P		P-MN	P/P
Sucralfate	*[54182-58-0]*					N/N
Sulfamethoxazole	*[723-46-6]*	P	N			P/0
Sulfasalazine	*[599-79-1]*	N	N		P-MN	P/P
Sumatriptan	*[103628-46-2]*	N	N		N	N/N
Suvorexant	*[1030377-33-3]*	N	N		N	N/N
Tacrine	*[321-64-2]*	P	E		N	0/0
Tacrolimus	*[104987-11-3]*	N	N		N	N/N
Tadalafil	*[171596-29-5]*	N	N		N	N/N
Tafluprost	*[209860-87-7]*	N	N		N	N/N
Tamoxifen	*[10540-29-1]*	N	P		P	P/P
Tamsulosin	*[106133-20-4]*	N	N	N	N	P/P
Tapentadol	*[175591-09-0]*	N	E		N	N/P
Tasimelteon	*[609799-22-6]*	N	N		N	P/N
Tazarotene	*[118292-40-3]*	N	N	N	N	N/N
Tazobactam	*[89786-04-9]*	N	N	P	N	0/0
Tedizolid	*[856866-72-3]*	N	P	N	N	0/0
Tegaserod	*[189188-57-6]*	E	N		N	N/P
Telaprevir	*[402957-28-2]*	N	N		N	0/0
Telavancin	*[372151-71-8]*	N	N		N	0/0
Telithromycin	*[191114-48-4]*	N	N		N	0/0
Telmisartan	*[144701-48-4]*	N	N		N	N/N
Temazepam	*[846-50-4]*				N	0/N
Temozolomide	*[85622-93-1]*	P	P			P/0
Temsirolimus	*[162635-04-3]*	N	N		N	P/P
Tenofovir	*[147127-20-6]*	P	N	P	N	P/P
Tenoxicam	*[59804-37-4]*	N				N/N
Terazosin	*[63590-64-7]*	N	N		N	P/N
Terbinafine	*[91161-71-6]*	N	N		N	P/P
Terbutaline	*[23031-25-6]*					P/N
Terconazole	*[67915-31-5]*	N			N	0/0

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Terfenadine	*[50679-08-8]*	N	N		N	N/N
Teriflunomide	*[108605-62-5]*	N	P		N	N/N
Tesamorelin	*[218949-48-5]*	N	N		N	0/0
Testosterone	*[58-22-0]*		N		N	0/0
Tetrabenazine	*[58-46-8]*	N	P		P	0/0
Tetracycline	*[60-54-8]*	N	N	E	P	N/N
Thalidomide	*[50-35-1]*	N	N	N	N	N/N
Theophylline	*[58-55-9]*	E	E	E	E	N/N
Thiabendazole	*[148-79-8]*	P	P		N	P/N
Thioridazine	*[50-52-2]*	N			N	0/0
Thiotepa	*[52-24-4]*	P	P		P-MN	P/P
Tiagabine	*[115103-54-3]*	N	P	N	N	P/N
Tibolone	*[5630-53-5]*		N		N	0/0
Ticagrelor	*[274693-27-5]*	N	N		N	P/N
Ticlopidine	*[55142-85-3]*	N	N		N	N/N
Tigecycline	*[220620-09-7]*	N	N	N	N	0/0
Tilidine	*[20380-58-9]*					N/N
Tiludronate	*[89987-06-4]*	N	N		N	0/0
Timolol	*[26839-75-8]*	E	N		N	P/P
Tinidazole	*[19387-91-8]*	P	N	N	P-MN	P/P
Tiopronin	*[1953-02-2]*	N			N	0/0
Tipranavir	*[174484-41-4]*	N	N		N	P/P
Tirofiban	*[144494-65-5]*	N	N		N	0/0
Tizanidine	*[51322-75-9]*	N	N		N	N/N
Tobramycin	*[32986-56-4]*	N	N	N	N	0/0
Tocainide	*[41708-72-9]*	N	N	N	N	N/N
Tofacitinib	*[540737-29-9]*	N	P		N	N/N
Tolazamide	*[1156-19-0]*	E	N		N	N/N
Tolbutamide	*[64-77-7]*	N	N	P	E	N/N
Tolcapone	*[134308-13-7]*	N	N	P	N	P/N
Tolmetin	*[26171-23-3]*	N				N/N
Tolterodine	*[124937-51-5]*	N	N	N	N	N/N
Tolvaptan	*[150683-30-0]*	N	N		N	N/N
Topiramate	*[97240-79-4]*	N	N	N	N	N/P
Toremifene	*[89778-26-7]*	N	P		P-Hep Abs	N/P
Torsemide	*[56211-40-6]*	N	N		N	P/N

(*continued*)

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Tramadol	*[27203-92-5]*	N	N	P	P-MN	N/P
Trametinib	*[871700-17-3]*	N	N		N	0/0
Trandolapril	*[87679-37-6]*	N	N		N	N/N
Tranexamic acid	*[1197-18-8]*	N	N		N	P/P
Travoprost	*[157283-68-6]*	N	N	E	N	N/N
Trazodone	*[19794-93-5]*					N/0
Trenbolone	*[10161-73-8]*		P			0/0
Treprostinil	*[81846-19-7]*	N	N		N	0/0
Tretinoin	*[302-79-4]*	N	N	N	N	N/P
Triamcinolone	*[124-94-7]*	N	N			P/N
Triamterene	*[396-01-0]*	N	E		N	N/P
Triazolam	*[28911-01-5]*	N				0/N
Trichlormethiazide	*[133-67-5]*		P			0/0
Trientine	*[112-24-3]*	P	P		N	0/0
Trifluoperazine	*[117-89-5]*		N		P	0/0
Triflupromazine	*[146-54-3]*	N	N		N	0/0
Trilostane	*[13647-35-3]*					0/0
Trimegestone	*[74513-62-5]*		P			0/0
Trimethoprim	*[738-70-5]*	N	E			0/0
Trimetrexate	*[52128-35-5]*	N	P		N	0/0
Tripelennamine	*[91-81-6]*	N	E	N		N/0
Triprolidine	*[486-12-4]*	N				N/N
Triptorelin	*[57773-63-4]*	N	N		N	P/N
Troglitazone	*[97322-87-7]*	N	E	E	N	N/P
Trospium	*[10405-02-4]*	N	N	N	N	N/N
Ulipristal	*[159811-51-5]*	N	N		N	P/0
Umeclidinium	*[869113-09-7]*	N		N	N	N/N
Ursodiol	*[128-13-2]*	N	N			0/0
Valdecoxib	*[181695-72-7]*	N	N		N	N/N
Valproate	*[1069-66-5]*	N	N		N	P/P
Valsartan	*[137862-53-4]*	N	N		N	N/N
Vancomycin	*[123409-00-7]*			N	N	0/0
Vandetanib	*[443913-73-3]*	N	N		N	0/0
Vardenafil	*[224785-90-4]*	N	N		N	N/N
Vemurafinib	*[918504-65-1]*	N	N		N	0/0
Venlafaxine	*[93413-69-5]*	N	N		N	N/N
Verapamil	*[52-53-9]*	N	E		N	N/N

Name	CAS	Ames	Abs	MLA	*vivo*	Carc R/M
Vigabatrin	*[60643-86-9]*	N	N		N	N/N
Vilasidone	*[163521-12-8]*	N	P		N	N/P
Vinorelbine	*[71486-22-1]*	N	P	E	P-MN	0/0
Vismodegib	*[879085-55-9]*	N	N		N	0/0
Vorapaxar	*[618385-01-6]*	N	N		N	N/N
Voriconazole	*[137234-62-9]*	N	P		N	P/P
Vortioxetine	*[508233-74-7]*	N	N		N	N/N
Warfarin	*[81-81-2]*	N	E		N	0/0
Xylometazoline	*[526-76-3]*	N			N	0/0
Zafirlukast	*[107753-78-6]*	N	N	N	N	P/P
Zalcitabine	*[7481-89-2]*	N	P	N	P	N/P
Zaleplon	*[151319-34-5]*	N	E		N	N/P
Zanamivir	*[139110-80-8]*	N	N	N	N	P/N
Zidovudine	*[30516-87-1]*	P	P	P	P	P/P
Zileuton	*[111406-87-2]*	N	N	N	N	P/P
Ziprasidone	*[146939-27-7]*	P	P	E	N	N/P
Zofenopril	*[81872-10-8]*	N	N			N/N
Zoledronate	*[118072-93-8]*	N	E		N	N/N
Zolmitriptan	*[139264-17-8]*	P	P		N	P/N
Zolpidem	*[82626-48-0]*	N	N	N	N	P/N
Zonisamide	*[68291-97-4]*	N	N	N	N	N/N
Zopiclone	*[43200-80-2]*	N	E	P	N	P/P

N: Negative; P: Positive; E: Equivocal; Carc R/M: rat, mouse 2-year bioassay; 0 in carc column: not done; Abs: HPBL or hamster chromosome aberration assay; MLA: mouse lymphoma assay; vivo: *in vivo* rodent micronucleus assay.

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8

Children's and Adult Involuntary and Occupational Exposures and Cancer

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8.1 Introduction

The causal relationship between exposure and cancer was established for the first time at the end of the eighteenth century, when, in 1775, Sir Percival Pott published his observation about the link between the exposure to soot and cancer, later identified as squamous cell carcinoma, in chimney sweepers. This first step was at the same time a milestone in occupational toxicology, epidemiology, chemical carcinogenesis, and even in regulatory toxicology, since Pott's study led to the Chimney Sweepers Act in 1778. It was also the beginning of studies focusing on the exposure to carcinogens in the workplace, which, however, affected future research and the perspective of environmental exposures, considered as limited to carcinogens of industrial origin, present in the environment at lower concentrations.

While occupational medicine progressed throughout the eighteenth century, under the impetus of Bernardino Ramazzini, who is considered the father of modern occupational toxicology, it suffered a setback during the Industrial Revolution in nineteenth century and little was done until the beginning of the twentieth century.

Therefore, it took 150 years to initiate the first *in vivo* experiment to confirm Pott's observations. The experiment was performed in rabbits by Yamagiwa and Ichikawa in 1918 and confirmed in 1933. It came 20 years after the first report on chromosomal aberrations by Theodor Boveri, published in the same year when polycyclic aromatic hydrocarbons (PAHs) were identified, and was followed 20 years later by the discovery of DNA and the description of its structure.

Thus, the concept of genotoxic carcinogenesis was completely unwrapped in the first half of the twentieth century. It was developed, expanded, and

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reinforced in the following years, supported by progress in the knowledge of molecular genetics and in the development of molecular biology techniques.

Carcinogenesis was then described as a multistep process initiated by DNA damage, often a point mutation induced by reactive metabolites of exogenous chemicals (xenobiotics), sufficiently electrophilic to bind covalently to nucleophilic sites of DNA bases, forming adducts. The initiated cell could remain silent under the control of neighboring normal cells, or start proliferating under the effect of other chemicals, which may not react directly with DNA, but are able to affect one or more cell signals, inducing mitogenic effects. The tumor then progresses to malignancy, through further mutations affecting key genes involved in the control of homeostasis and other important biological traits.

To study this process and recognize putative carcinogens, several experimental assays were developed that were based on the initial concept of genotoxic carcinogenesis and on the need to highlight the risk from occupational exposures.

Therefore, while the *Salmonella* mutagenicity assay (better known as the Ames test, which was set up in the 1970s) is still recognized as one of the best reference assays for highlighting the mutagenic properties of chemicals and a model for future *in vitro* toxicology assays [1–3], the 2-year rodent carcinogenesis bioassay, whose current protocol was also developed in the same period, is under debate because of many limitations that would affect its applicability to meet the challenge of the new vision in cancer research in the twenty-first century [4].

The need for testing strategies to identify human environmental carcinogens became clear in the 1970s, when several pesticides were first recognized as carcinogens in rodents. Several governmental and regulatory agencies urged the need for reliable data to set policies for the prevention of risks for populations and for protection of human and environment health. The US National Toxicology Program was set up in response to this need, under the leadership of the US National Institute of Environmental Health Sciences. Testing guidelines have been developed since then by national and international agencies, with the aim to set standard rules to improve the repeatability of results and support the mutual acceptance of data.

On the basis of the results from experimental assays, thousands of chemicals have been studied and classified. Hundreds of chemicals have been banned, restricted, or never marketed, as they were found to be mutagenic and carcinogenic.

Nevertheless, several chemicals avoided the classification, since they gave negative results in standard tests. The list of unidentified chemicals include toxins for reproduction, such as thalidomide, chemicals inducing cancer in offspring as the consequence of prenatal, *in utero*, exposure, and chemicals

whose target organ or mechanism of action or toxicokinetics are not adequately represented in the animal bioassay, due to species peculiarities and interspecies differences.

The beginning of the twenty-first century was characterized by several milestones, which marked a turning point in the approach to environmental carcinogenesis.

The identification of cancer hallmarks added new insights in the comprehension of cancer onset and development [5,6]. The role of cancer hallmarks as the target of environmental agents was deeply exploited, providing a new picture of the complex interplay among molecular and biological endpoints, which need to be addressed to initiate and sustain the cancer process [7–17]. Accumulating evidence has shown the key role of inflammation and immune evasion to sustain the early steps of the cancer process deriving from environmental exposures, and the importance of genomic changes that do not affect directly the genetic code, thus suggesting a different nongenotoxic initiation of carcinogenesis [18,19].

The advent of the Genomic Era started the investigation of the role of genes and gene pathways in the onset and progression of disease and allowed the use of toxicogenomics to highlight the chain of events from exposure to effect as well as the gene–environment interactions.

New scientific discoveries and advanced technologies have opened the way for new approaches to hazard and risk assessment of chemicals, with the goal of better comprehension of the impact of involuntary exposures on human health.

As the awareness of the complexity of environment–health relationships has grown, it has also become clear that new approaches and integration of strategies would be needed to make the world safer and cleaner. Toxicology in the 21st Century (Tox21) and European Commission (EC) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation are both initiatives to fulfill this request.

Tox21 is a program supported by several governmental US agencies in order to develop better toxicity assessment of chemicals.

EC REACH Regulation is the European approach to fulfill the gaps in the knowledge of the toxicological profile of chemicals in the European market.

Since the beginning of the new century, research in the field of chemical carcinogenesis has accelerated. Better approaches to chemical testing and assessment, the introduction of omics technologies, and high-throughput screening (HTS) have allowed the identification of adverse effects at doses much lower than those usually used in the standard bioassay protocols, showing the importance to address the low-dose effects, that is, the biological changes that occur in the range of typical human exposures [20].

In the following paragraphs we will discuss the achievements and perspectives in a research and regulatory context of occupational and environmental exposures in the twenty-first century.

8.2 Occupational Exposures and Cancer

8.2.1 Occupational Cancer in the Twenty-First Century

Work-related cancer represents 32% of all work-related deaths, and it is the leading cause of death from injuries and illness at work [21].The association between occupational exposures and cancer has been estimated to be 3–6% of all cancers worldwide [22]. The World Health Organization (WHO) has estimated that the attributable fraction to work-related cancer averages 8.4% of all cancer deaths (13.8% male, 2.2% female) [21].

Since the first report on squamous cell carcinoma of the scrotum in chimney sweeps by Sir Percival Pott, several associations between occupational exposures and cancer have been described. The International Agency for Research on Cancer (IARC) has classified 118 agents, mixtures, and exposure situations as group 1 carcinogenic to humans (as of June 24, 2016), including all forms of asbestos, aluminum and coke production, iron and steel founding, the rubber manufacturing industry, as well as a number of agents found in the environment such as benzene, arsenic in water, cadmium, ethylene oxide, benzo[a]pyrene (BaP), silica, ionizing radiations, such as radon, and ultraviolet radiation including cosmetic tanning devices.

However, it is recognized that less than 2% of chemical or physical agents placed on the market in the past 30 years and commonly found in the environment have been adequately tested for carcinogenic properties. These chemicals include by-products that originate from manufacturing processes and to which workers are exposed.

An attempt to deal with the huge number of untested chemicals, chemical intermediates, and unknown by-products was made by the European Union with the EC Regulation 1907/2006 (REACH), a chemical legislation underpinned by the precautionary principle [23]. According to REACH, for each manufactured chemical, including existing chemicals, a complete registration dossier is required, reporting enough information to highlight toxicological and ecotoxicological properties, environmental impact and fate, and exposure. For occupational exposures, information about the chemical synthesis of the substance and its use as a chemical intermediate and/or in formulations, the industrial and professional uses are required to cover the full life cycle of the manufactured chemical [24].

While long-term industrialized economies have developed better management of occupational exposure and reduced the incidence of occupational diseases, emerging economies, newly industrialized countries, and less-developed countries are still facing significant occupational exposure to known carcinogens such as asbestos.

Asbestos represents a paradigmatic example for both occupational and environmental exposures. The exposure is strictly related to the incidence of

a rare tumor, the pleural mesothelioma. The association between exposure and disease is so strong that mesothelioma is considered a marker for asbestos exposure. Other rare tumors have been described in association with asbestos exposure. The large production and use of asbestos and asbestos-containing products have led to a large and diffuse contamination of the environment, to the point that asbestos still represents an environmental issue in countries where its production has ceased.

Thus, asbestos has been chosen as the prototype substance (a group of mineral fibers) to highlight the interaction between occupational and environmental exposure and to point out some questions concerning genotoxic and nongenotoxic carcinogenesis.

8.2.2 Past and Present Occupational Exposure to Asbestos

Asbestos has been classified as a human carcinogen by IARC in 1973. Since then, 55 countries have issued a ban on manufacturing and using all forms of asbestos. The association between the occupational exposure to asbestos and disease has been well established. Besides lung cancer and mesothelioma, prolonged exposures to asbestos may induce asbestosis and pulmonary efficiency decline. Fiber length, diameter, and biopersistence are critical factors influencing adverse outcomes. Despite the consistency of epidemiological data to demonstrate the strong cause–effect relationship with asbestos exposures, several countries are still producing, importing, exporting, and using asbestos. The worldwide production is estimated to be around 2.2 million metric tons per year. Five countries account for 99% of global production (Table 8.1) [25].

The rate of mesotheliomas in these countries is expected to increase in the next years, since the number of mesothelioma cases is proportional to the rate of asbestos production and consumption. For the same reason, the rate of mesotheliomas is expected to decrease in those countries where the production

Table 8.1 Production of asbestos in 2012.

Country	Annual production (metric tons)
Russia	1,000,000
China	400,000
Brazil	270,000
Kazakhstan	210,000
Canada	100,000

Source: Reproduced from Ref. [25] with permission from Elsevier.

of asbestos and asbestos-containing products has been banned. However, due to the long period of latency, ranging 10–50 years, with a median of 46 years, the incidence of mesothelioma has been constantly increasing during the last two decades and it is expected to peak in 2020–2025 [26].

The peak is related to past exposures, before the total ban. According to CAREX, the European CARcinogen EXposure database, 1.2 million workers were still exposed to asbestos in the period 1990–1993 (Table 8.2).

Data refer to the 15 countries that were part of the European Union at that time [27]. Moreover, several new EU members had continued producing asbestos until joining the European Union. Therefore, this is only a partial estimation of exposed workers still at risk to develop disease in the European Union.

Despite the total ban on asbestos, a consistent number of demolition and construction workers are still at risk of exposure [28]. This aspect should be taken into account for a more accurate risk/benefit evaluation of the need to replace asbestos-containing products, such as roofs or water pipes, which would imply a significant exposure for demolition workers.

Table 8.2 Exposed workers in the European Union in the period 1990–1993.

Industry/industrial process	Number of exposed workers
Construction	574,000
Personal and household services	99,000
Other mining	85,000
Agriculture	81,000
Wholesale and retail trade and restaurants and hotels	70,000
Food manufacturing	45,000
Land transport	39,000
Manufacture of industrial chemicals	33,000
Fishing	25,000
Electricity, gas, steam	23,000
Water transport	21,000
Manufacture of other chemical products	19,000
Manufacture of transport equipment	17,000
Sanitary and similar services	16,000
Manufacture of machinery, except electrical	12,000

Source: CAREX (CARcinogens EXposure) database. Reproduced from Ref. [27] with permission from *British Medical Journal*.

8.2.3 Toxicology of Fibers: What We Have Learned from the Asbestos Lesson

The identification of health effects related to particles, including fibers, requires supplementary information to highlight the toxicological profile. Length, aspect ratio, and surface area, as well as physicochemical properties, are all critical factors to predict the adverse effects. Particles toxicology, including fibers, was developed many years ago. However, the approach, which is still the basis of modern particles toxicology, has been developed in 1960–1970, by investigating the role of fiber type, length, and biopersistence in inducing mesothelioma following the experimental exposure to asbestos [29]. These early studies led to the fiber pathogenicity paradigm that focuses on the geometry of the fiber as the main characteristic to understand pathogenicity. Not all the fibers can induce adverse effects. Only fibers that are thin enough to deposit beyond ciliate cells, with a diameter equal to or less than 1 μm, and long enough to escape the macrophage-mediated phagocytosis, may induce pulmonary pathologies. However, according to the industrial hygiene definition, only fibers at least 5 μm or more in length, with a diameter equal to or less than 0.25 μm, and a length/width ratio 3:1 or 5:1, can be considered asbestos [30]. The fibers showing these characteristics cannot be engulfed by macrophages, generating impaired phagocytosis, referred as "frustrated phagocytosis," and affecting macrophage mobility. Asbestos fiber critical dimensions have been chosen on the basis of more than 30 years of experimental studies, epidemiological evaluation, as well as on the basis of a precautionary approach. The critical length was first set at 20 μm [31]. Then, it was demonstrated that fibrosis and carcinogenesis both were related to the exposure to fibers longer than 10 μm – the Stanton hypothesis [32], while asbestosis was independent of fibers length. Other studies demonstrated that carcinogenesis was related to fibers at least 8 μm in length [32]. Finally, it was reported that there was no "*convincing evidence*" that any adverse effect was related to fibers shorter than 5 μm, as previously reported by Davis *et al.* [31,33,34]. Some authors have pointed out that even shorter fibers may induce adverse effects, since they were found in tissues of exposed workers. However, the results from both experimental and human studies, which were performed to settle the so-called short fiber controversy, failed to demonstrate the toxicity of shorter fibers [31,35].

Biopersistence is another important factor, which may affect the clearance of fibers from the lung milieu. Long fibers that are not biopersistent can undergo breakage and degradation, which allows their removal through defense mechanisms. However, inflammation or cell proliferation can still originate, as the consequence of the release of toxic ions, during degradation. Biopersistent long fibers that reach pleura space may be retained at the pleura stomata space, then they accumulate and start an inflammation process [29]. Therefore,

biopersistence may actually play the key role in triggering adverse effects, making the difference in the exposure to different type of fibers [36].

The asbestos fibers more frequently encountered in the occupational exposures belong to two different groups of minerals: serpentine (chrysotile) and amphiboles (amosite, crocidolite). Chrysotile is less durable. It may split longitudinally, forming fibrils that are faster degraded in an acidic environment [29]. In fact, chrysotile is considered 6–60 times less potent than amphiboles [37]. Tumors would be the consequence of prolonged exposure to amphiboles or a combination of chrysotile and amphiboles. Several studies have tried to highlight the direct relationship between fiber type (i.e., chrysotile or amphiboles) and tumor type (i.e., lung cancer or mesothelioma). However, the adverse effect seems to depend rather on the rate of fiber translocation to the lung and from lung to pleura. The mechanism through which fibers are translocated from lung parenchyma to pleural space is not well understood. Interestingly, several studies have shown that both short and long fibers as well as particles are translocated into the pleural space [38]. While particles and short fibers may leave the pleura through stomata openings, long fibers are trapped in the pleura space, block the stomata, and start inflammation. Short fibers may be entrapped together with long fibers, which may prevent them from leaving the pleura through the stomata, concurring to increase tissue inflammation.

Is this process common to other occupational exposures?

The formation of black spots in the pleural space, as the consequence of the exposure to dust particles, has been revealed in miners as well as in urban area residents. Even if the accumulation of black spots is related to the extent of the exposure, in terms of dose and duration, most of the time black spots are not associated with any kind of pathology. The general population is exposed to respirable particles (environmental ultrafine particles) in ambient air. Occupational exposures to nanoparticles may occur as the consequence of industrial processes in the nanotechnology field.

In 2011, the EU Commission issued a tentative definition for nanomaterials, including either natural or manufactured nanoparticles "*in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm*" [39].

However, a difference exists between environmental and engineered nanoparticles. While environmental nanoparticles differ in their chemical composition, which is strictly related to the kinds of pollutants in the environment, engineered nanoparticles are purposely developed, their physicochemical properties are well known, and their toxicological and ecotoxicogical profiles can be identified.

This last point, however, is at the center of the scientific debate. The main focus of concern is related to the suitability of test systems to correctly classify

nanomaterials, in order to protect workers exposed to nanoparticles during industrial processes as well as to ensure the protection of both the environment and the consumers. Engineered nanomaterials may be generated from known substances that are made in the nanoscale to confer on them specific and desirable physicochemical properties (top-down approach). These kinds of nanomaterials have been suggested to retain the toxicological properties of the bulk substance, even if the nanosize confers the ability to reach biological compartments not attained by bulk compounds. New materials are generated through a bottom-up approach, by assembling single atoms and molecules into larger nanostructures.

Among bottom-up nanomaterials, carbon nanotubes represent one of the most important products in the nanotechnology industry. Carbon nanotubes can exist in the form of nanoparticles or, more often, as fibers. Because of the fibrous structure, carbon nanotubes have been predicted to induce the same effects as asbestos. Experimental studies have actually demonstrated that carbon nanotube long fibers can reach the peritoneal space [32,38] and exert length-dependent inflammogenicity when injected into the pleural space of mice [40], thus behaving like asbestos fibers. Do carbon nanotubes represent a risk for occupational and general population exposure? IARC has recently reviewed the possible cancer properties of carbon nanotubes and carbon nanofibers, reaching the conclusion that only for one product, the well-characterized Mitsui MWCNT-7, there is sufficient evidence in animal bioassays to classify it as "*possibly carcinogenic to humans*" [41].

Occupational exposures to carbon nanotubes and carbon nanofibers may happen throughout the product life cycle, from production to disposal. EU REACH Regulations pay great attention to the entire life cycle of substances, starting from manufacture, through formulation, end use, and degradation/transformation. It also takes into account those aspects that have played a key role in increasing the exposure to asbestos, such as the use at industrial site and the widespread use by professional workers [42].

It has long been debated whether nanoparticles are covered by EU REACH regulation and if it is appropriate and sufficient to protect human health and environment. REACH regulation actually sets out approaches that should be valid for all substances "*in whatever size, shape, or physical state*" and has improved the basic rules with recommendations specifically addressing the risk management of nanomaterials – in the Second Regulatory Review on Nanomaterials (2012) and in REACH review (2013). The EU Commission concluded that more specific requirements for nanomaterials have proven necessary to ensure that all information required to characterize the hazard and risk from the production and use of nanotechnology would be available. As a consequence, for many nanomaterials including carbon nanotubes and nanofibers, the available information still remains incomplete. Guseva Canu *et al.* recently reviewed the available information about occupational and environmental

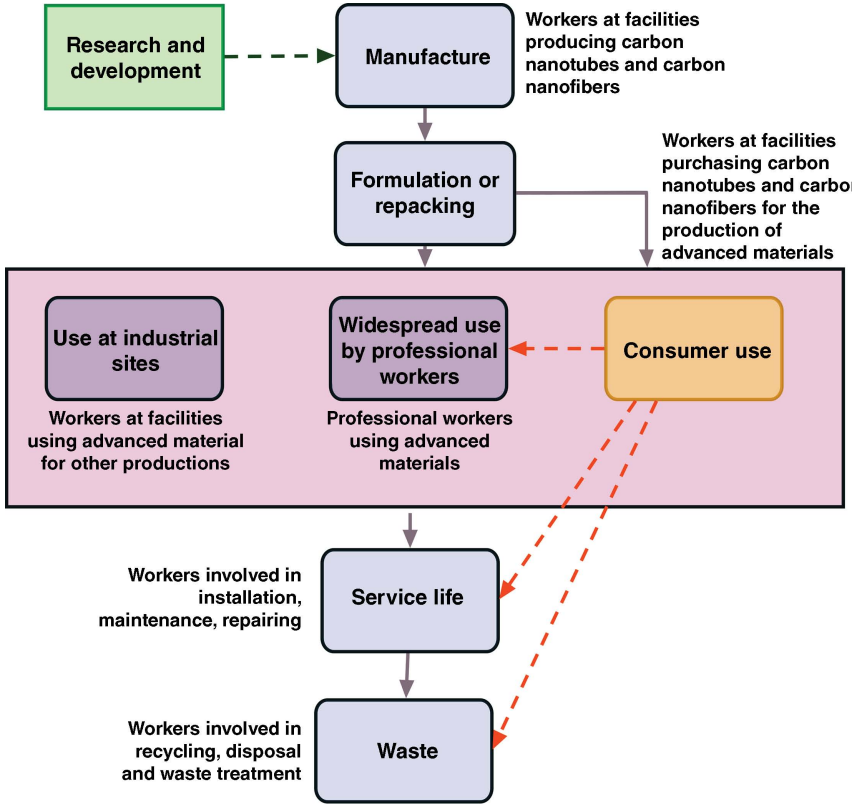

Figure 8.1 Possible workers exposures to carbon nanotubes and carbon nanofibers during the products life cycle.

exposures to carbon nanotubes and nanofibers, and concluded that "*release of CNT/CNF structures can occur during experimental processes or processes under development in research facilities, during incidental or accidental events, and during open production process*" [43]. Possible worker exposures to carbon nanotubes and nanofibers during the product life cycle are shown in Figure 8.1.

8.2.3.1 Mechanism and Mode of Action of Asbestos and Asbestos-Like Fibers in Carcinogenesis: The Role of Inflammation and Immune System to Sustain the Cancer Process

Asbestos is considered a complete carcinogen, inducing cancer through both genotoxic and nongenotoxic mechanisms. Asbestos has been reported to induce chromosomal aberrations, by interfering with the mitotic apparatus and generating micronuclei [44,45]. However, the damaged cells usually undergo blockage of the cell cycle and they rarely produce viable daughter

cells that are able to proliferate [45]. Thus, genotoxic events seem to occur on the pathway leading to an adverse outcome rather than starting the process.

The main initiating event may be related to the induction of oxidative stress, possibly through the production of reactive oxygen species (ROS), as the consequence of redox processes at the surface of the entrapped fibers. Even if oxidative stress is considered related to genotoxic carcinogenicity and ROS have been described as partly involved in the DNA damage from asbestos exposures [46], recent reports point out the key role of the oxidative stress in nongenotoxic carcinogenesis by triggering and sustaining tissue inflammation [18].

It is well recognized that inflammation plays a key role in the tumor process [17]. It has previously been reported that inflammation is the first adverse effect in lung carcinogenesis following exposure to environmental stressors [18]. This first event is immediately followed by the activation of the immune system, which, in turn, sustains the inflammation process in the lung tissue toward chronic inflammation. It has also been described that ROS may modulate the immune response as the first step followed by inflammation. This process is supported by the activation of one or more members of the Nox family that interplay with the p53 signaling pathway and the Wnt pathway [18,47,48]. The role of oxidative stress-dependent inflammation in asbestos-related cancer has been described before [8,17], suggesting that oxidative stress is the molecular initiating event in the adverse outcome pathway related to exposure to asbestos fibers.

Lung pathogenesis following the initiating event as the consequence of prolonged exposure to asbestos or asbestos-like fibers would not be different from the lung tumor process previously described [18]. Oxidative stress triggers acute inflammation in lung tissue, which evolves into chronic inflammation. Chronic inflammation may progress to noncancer diseases, such as interstitial fibrosis (asbestosis). Further genotoxic and epigenetic events may turn the status of chronic inflammation into lung cancer, through several morphological changes of the lung tissue, which include angiogenic squamous dysplasia and metaplasia. Lung cancer from asbestos exposures is strictly related to the dose of fibers.

Despite the increase in the incidence of mesothelioma, due to the high level of past exposure to asbestos in the workplace and the long latency period of this type of cancer, the key events in mesothelioma pathogenesis are still largely unknown. Available information from existing scientific literature has been organized according to the adverse outcome pathway (AOP) paradigm (Figure 8.2).

AOPs synthesize relevant information into sequential steps at different levels of biological organization, starting from the molecular level, with the identification of the molecular initiating event (MIE), to the identification of the response of the organism and the impact of the adverse outcome on the population. The AOP-based organization of toxicological information provides

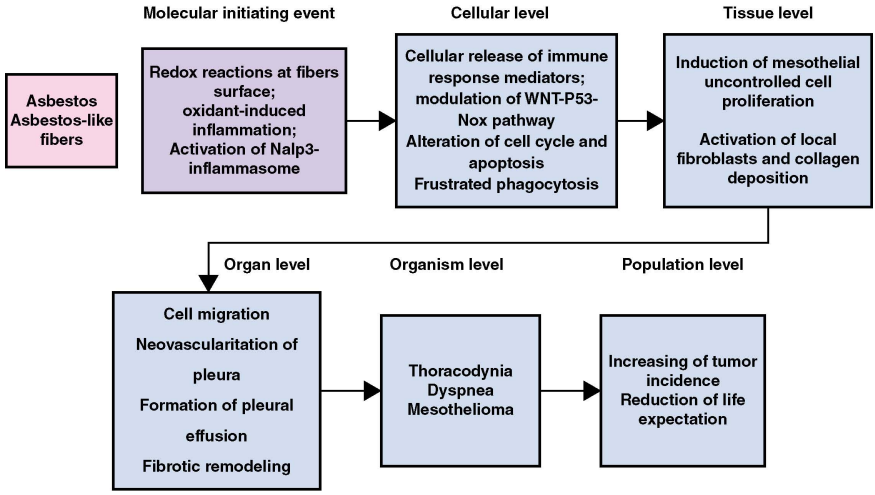

Figure 8.2 Chain of key events leading to pleural mesothelioma.

The existing knowledge about the key events leading to mesothelioma pathogenesis is organized into sequential steps at different level of biological organization, starting from the molecular level, with the identification of the molecular initiating event (MIE) through the identification of the response of the organism to the impact of the adverse outcome on population.

the biological basis for toxicity of chemicals and the effects related to chemical exposure [18,49,50].

8.2.4 Occupational Exposures and Rare Tumors

Rare cancers have been defined as those tumors that occur at less than 6 per 100,000 cases per year [51]. However, despite their low occurrence, it has been reported that more than 500,000 rare cancers are annually registered in Europe [52]. Several rare tumors are related to occupational exposures.

As we stated before, pleural mesothelioma is rarely diagnosed in the general population, while the association with asbestos exposure in the workplace is very strong. Rare cases of mesothelioma were diagnosed in nonworkers as a consequence of secondary exposures. Family members of asbestos workers developed mesothelioma after exposure to fibers brought home by workers on their clothes or hair. Asbestos, however, is related to other rare cancers including hypopharyngeal cancer, laryngeal cancer, squamous cell carcinoma of the esophagus, cancer of the small intestine, large cell lung carcinoma, and ovarian cancer. All these tumors are recognized by IARC as related to occupational exposure to asbestos as well as to other carcinogens such as iron and steel (pharynx), acid mists and rubber industry (larynx), carbon black,

sulfuric acid, PAHs (esophagus), organic solvents (small intestine and ovary), silica dust, diesel exhaust (ovary). The evidence for the association with the exposure to these chemicals is sometimes considered limited [52].

Other chemicals have recognized as more strictly related to specific rare tumors. Exposure to vinyl chloride is causally related to hepatic angiosarcoma. Benzene is associated with acute myeloid leukemia. Formaldehyde is related to chronic myeloid leukemia.

To date, no evidence has been reported for occupational exposures related to endocrine tumors, including carcinoma of the thyroid for which environmental exposures are thought to play the main role.

8.3 Environmental Exposures and Cancer

8.3.1 Environmental Exposures and Disease: Is This the Pandemic of the Twenty-First Century?

According to WHO definition, the environment is "*all the physical, chemical and biological factors external to a person, and all the related behaviors, but excluding those natural environments that cannot reasonably be modified*". WHO also calculated that 24% of global diseases and 23% of deaths are related to environmental factors. Most of these factors are environmental pollutants, agents that can affect both environment and human health.

Environmental pollution (and exposure) represents one the most complex topics in the field of risk assessment, as it is influenced by several factors that can contribute to the fate of pollutants in the environment. The study of occupational exposures in the workplace is facilitated by the accurate characterization of the agent, including the possibility to measure its typically higher concentration in a limited space and almost totally absence of other confounding exposures or at least the possibility to identify and consider them. Conversely, environmental pollution is the result of the spatial distribution and combination of chemicals in more than one environmental matrix, typically at low concentrations and the ability of chemicals to either accumulate or undergo degradation or chemical conversion to other constituents.

Both the rate of biological metabolism and the level of toxicity of metabolic products must be taken into account with occupational as well as environmental exposures. The chance that subjects in the general population may develop a disease causally related to a specific environmental exposure also depends on many factors and especially on the probability that the subject and the agent are spatially and temporally related.

The recognition of environmental pathologies, however, has increased during recent years concomitant with increasing industrial progress and the evolution of new technology. Some environmental pollutants are well known from a

toxicological point of view since they are products of anthropogenic activities, industrial products for which information from occupational exposures is available. These include asbestos or benzene, or products of combustion, such as PAHs and dioxins, which have long been studied. They may also include chemicals for which complete toxicological and ecotoxicological information is required by regulation before entering the market, such as pesticides due to the impact they could have on environmental and human health. However, for many chemicals, which were placed on the market years ago, very limited information is available. Some of them such as polychlorinated biphenyls (PCBs), whose production and use have been banned, persist in the environment and bioaccumulate in the food chain. This prevents the possibility of their complete removal from the environment. Some pollutants derive from natural sources, such as arsenic in well water, and contribute to the body burden. Environmental policies have reduced the concentration of several chemicals, considered as causing risk for human health and environment, by reducing industrial emissions and restricting their use. However, the concentration of some other agents, such as mycotoxins, has dramatically increased due to climate change. Emerging risks are related to those chemicals or agents for which new toxicological information has become available only after their widespread use, which allowed their increased presence in the environment. This is the case with many endocrine disruptors including phthalates and perfluorinated chemicals, largely present in waterways and other environmental media.

The evaluation of the risk from environmental exposures is complicated by the difficulty of monitoring all pollutants in all environmental media, by the scarce knowledge of the toxicological behavior of chemicals at low and very low doses and the lack of appropriate models to calculate the cumulative and aggregate risks related to complex mixtures and multiple exposures.

Several papers have been published recently addressing the complex matter of environmental exposures and related pathologies, trying to identify the gaps in our knowledge and the areas of potential intervention [53–55].

Some of the priorities, which have gained the highest attention during the last few years, are discussed in the following sections.

8.3.2 The Complexity of Environmental Exposures

Environmental pollution remains one of the major causes of health risk, especially in developing countries, where the environmental legislation is weaker or lacking. However, even in long-standing industrialized countries, where advanced technologies have reduced the emissions of hazardous pollutants and environmental policies set limits for each chemical of concern, it is difficult to completely manage the risk of environmental pollution.

Risk assessment practices have been developed with an aim to protect human health, and usually a conservative approach is used to protect the most sensitive

groups within a population, including children. For nongenotoxic carcinogens, it is generally assumed that a threshold dose exists, below which no adverse effect would be detected. The threshold dose is usually calculated from experimental studies, by identifying a "no observed adverse effect level" (NOAEL), extrapolating to humans and compensating for interindividual differences, by applying safety or uncertainty factors. Other approaches include the use of a benchmark dose, which is a dose corresponding to a specified change in the response. The lower bound on the benchmark dose or the NOAEL serves as the point of departure to calculate the "safe" dose for human exposure to the single chemical (e.g., Acceptable Daily Intake, Occupational Exposure Level, and Reference Dose) [56,57].

One of the major limitations of these approaches is represented by the extrapolation from dose–response curves, which are usually monotonic and assumed not to change shape at low doses. Several chemicals, which are usually present in the environment, show nonmonotonic dose–response (NMDR). NMDR is a biphasic dose–response defined as "*a dose response which changes direction from ascending to descending or vice versa and can occur at any part of the dose axis, not only at the low dose part*" [58]. However, NMDRs are often shown at low doses.

Moreover, synergistic effects within single chemicals copresent in a mixture at low dose may lead to adverse effects at concentrations close to the point of departure [59].

Therefore, the current approach to risk assessment may be inadequate to estimate the real risk from several environmental carcinogens copresent in a complex mixture at low doses.

Environmental pollutants of concern are usually ubiquitous and combined in complex mixtures whose toxicological behavior is difficult to predict on the basis of the results from hazard assessments on single chemicals.

The concentrations of single components vary according to the source. Mixtures contain chemicals known to be carcinogenic to humans as well as many noncarcinogenic chemicals that have been shown to exert effects at low doses, which are highly relevant to the process of carcinogenesis.

The current regulations worldwide establish the list of chemicals that should be identified and characterized in environmental samples and establish acceptable concentration levels for reference compounds, whose toxicological profiles have been evaluated in standard tests.

However, the complete chemical characterization of an environmental complex mixture is complicated by the limitation of chemical extraction procedures, the difficulty to analyze all the chemicals present as impurities or in trace amounts and the presence of unknown substances.

Therefore, the cancer risk assessment from environmental exposures should take into account the complexity of the global picture [59].

Cancer may arise from environmental exposures to chemical concentrations that are considered environmentally relevant, but not sufficient to affect

significant biological traits associated with carcinogenesis. However, single chemicals targeting specific cancer hallmarks may interplay in environmental complex mixtures. Thus, the adverse outcome may be induced at much lower doses than those at which the adverse effect has been observed in standard toxicological studies [20]. Moreover, considering the health risk posed by exposure to environmental mixtures, attention should be paid not only to the components of the mixtures but also to other environmental or individual risk factors that could affect the final outcome.

Is it possible to define a threshold for complex mixtures on the basis of the NOAEL or the benchmark dose calculated for single chemicals?

From a regulatory point of view, there is the need to identify a threshold for every kind of exposure.

An attempt to address the lack of appropriate toxicological information to define the potential risk of mixtures has been made by developing an approach based on the use of the threshold of toxicological concern (TTC) [60].

TTC is based on the distribution of several NOAELs from experimental studies, calculated for carcinogenic and noncarcinogenic endpoints. The lower fifth percentile of this distribution is used as the threshold of the mixture, assuming that unknown, undetectable substances would not have a NOAEL below this threshold [61].

The use of TTC in the regulatory context has been endorsed by several agencies, including the US Food and Drug administration for food packaging components, Joint FAO/WHO Expert Committee on Food Additives (JECFA) to evaluate the exposure to very low concentrations of substances in food, and the European Food Safety Authority (EFSA) for assessing food flavorings and for pesticide metabolites in groundwater [62]. EFSA also provided guidance for the use of TTC in the regulatory food safety context [63]. The European Medicine Agency (EMA) recommended the use of TTC for genotoxic impurities in pharmaceuticals and in herbal preparations.

TTC approaches have been applied to genotoxic/mutagenic toxicity [64] and used or proposed to overcome chemical mixture assessment challenges, including unknown constituents and synergistic effects, and to set exposure levels for pesticides, industrial chemicals, pharmaceuticals, aerosols, and personal care products with respect to a variety of toxic endpoints, including carcinogenesis and reproductive toxicity [62].

8.3.3 Environmental Impact on Early Stages of Life: Are Our Children at Risk?

Concern has been raised about exposure of children who can be affected at doses much lower than those considered safe on the basis of standard risk assessment approaches. Children are more sensitive due to the different ability to metabolize xenobiotics, thus affecting the rate of detoxification and

excretion. Newborns are even at higher risk since their metabolic pathways are still immature and remain immature for the first months of life [65]. Moreover, children's exposures to environmental pollutants (and other toxins) start much before their birth.

It is well known that certain exposures during the gestational period can determine defects at birth. Thalidomide probably represents the best known example. Children of mothers who had been prescribed thalidomide to alleviate nausea and other morning symptoms during their pregnancy were born without limbs. Ten years later, diethylstilbestrol (DES), another medication prescribed to pregnant women to prevent the risk of pregnancy complications, provided the first example of a chemical that did not induce defects at birth but later in the offspring [66].

Intrauterine exposure occurs through the placenta that, contrary to common belief, cannot exclude the transit of many toxins from mother to fetus. The placenta is responsible for important functions during the fetal development, such as the control of metabolism and fetal nutrition, gas and metabolite exchange, and endocrine control. However, the placenta itself can be affected by pharmaceuticals and other chemical stress factors of either maternal or environmental origin. Persistent chemicals can rapidly cross the placental barrier and reach the fetus. Fetal exposure has been confirmed by several studies reporting the presence of hundreds of toxins in the cord blood. These included neuroimmune and endocrine toxic chemical components that may influence critical steps of hormonal, neurological, and immunological development. Substances absorbed through the placenta are excreted into amniotic fluid and meconium, where several toxins have been found that have not been detected in cord blood, including pharmaceuticals, illegal drugs, heavy metals, and pesticides [65,67]. Accumulating evidence supports the concept that amniotic fluid is not "*just fetal urine anymore*", as pointed out by Underwood *et al.* [68], while it plays a role, and sometimes the main role, in sustaining the fetal exposure to toxic substances. This has been demonstrated in both experimental and human studies [69,70]. Amniotic fluid can be reabsorbed into the fetal circulation by fetal swallowing and via the fetal intramembranous pathway. The latter is thought to be the most important mechanism to reabsorb toxic substances such as ethanol [71]. Both mechanisms contribute to create a recycling system through which toxic substances are excreted into the amniotic fluid and reabsorbed into the fetal circulation, thus extending the duration of each exposure [71].

Besides transplacental exposure *in utero*, exchange of toxic substances between mother and child can continue through breastfeeding, thus allowing an extended period of child exposure from the embryonic stage to weaning.

Embryonic, fetal, and early postnatal life stages represent "windows of vulnerability," the period of higher susceptibility when exposures to toxins can cause permanent impairment of physiological functions and alterations in

organ architecture. These dysfunctions may affect the pregnancy outcome or be the cause of acute or chronic diseases, which became manifest at any point during the life span of offspring. Spontaneous abortion, intrauterine growth retardation, prematurity, and low birth weight are all adverse pregnancy outcomes, which are thought to be related to environmental pollution. Acute diseases are typical of early life and are usually represented by pneumonia afflictions and diarrheal disease. Chronic diseases include disorders of neurobehavioral development, adult and pediatric asthma, hypertension, obesity, diabetes, cardiovascular disease, and cancer.

This "fetal origin of disease" hypothesis suggests that environmental factors during programmed development affect genetic expression profiles in such a manner that they influence susceptibility to chronic diseases throughout the course of life.

The embryo epigenome has been proposed as a unique target for environmental pollutants.

The process of demethylation/remethylation that occurs during early embryonic development represents a window of susceptibility to environmental stressors. Methylation is one of the main mechanisms influencing the epigenetic status. While changes in DNA sequence induce alterations in the genotype (mutations), epigenetics affects the dynamic status of DNA, thus controlling the gene modulation and altering the phenotype. Environmental stressors and chemicals may affect the epigenetic status of cells, inducing alterations in cell homeostasis. The epigenetic machinery includes noncoding RNAs (ncRNAs), microRNAs (miRNAs) and chromatin histones. ncRNAs are small molecules performing regulation at transcriptional and posttranscriptional levels, which are transcribed from DNA but not translated into proteins. miRNAs regulate the degradation of RNA messengers. They also are not translated into proteins. Chromatin histones can be modified, without altering the DNA code. All of these molecules can undergo methylation as well as DNA itself. DNA methylation occurs by adding a methyl group to cytosine, usually at CpG sites.

A comprehensive review of the involvement of epigenetics in all stages of mammalian development has recently been published, showing that DNA methylation, histone modifications, and ncRNAs do not act separately, but are linked throughout mammalian development [19].

The placenta directly confers to the embryo the process of demethylation/remethylation. As it has recently been reported, placental miRNAs play an important role in development, differentiation, and homeostasis, and are able to send signals to maternal and fetal tissues, acting as the intermediates in maternal–fetal communication [72].

Epigenetic events can also occur in germ cells, during primordial germ cell development and after fertilization in all cells of the early embryo [19]. These changes at key stages of programming and reprogramming in the early embryo may have immediate effects on embryo viability or severe consequences on

future health in childhood and adult life [19,73]. Epigenetic changes affecting embryonic germ cells can have consequences on fertility and establish the basis for transgenerational effects. Therefore, a transgenerational change may represent epigenetic inheritance [19].

The list of toxins that can affect prenatal and postnatal health includes heavy metals, organophosphates pesticides, PCBs, PAHs, complex mixtures, such as particulate matter (PM), and other chemicals that can act as endocrine disruptors.

8.3.4 Environmental Endocrine Disruptors: The Steps Set Out to Recover Our Stolen Future

Since the book "Our stolen future" was first published in 1996, concern has been growing about the presence of endocrine disruptors in the environment and their contribution to children's health and human reproduction.

Until then what we had known about the adverse effects of hormonally active substances was the link with hormone responsive tumors and the possible cancer risk related to the use of estrogens in hormonal contraception [74].

Paradoxically, we knew better the effect of transplacental exposure to synthetic hormones, since Herbst *et al.* published in 1971 the report on the link between the development of cancer at puberty in daughters whose mothers had been treated with DES during pregnancy to prevent miscarriages [66]. In the following years we would have learned that the mother's exposure may trigger transgenerational effects extending through the second and third generation [75].

This was the first report on so-called environmental estrogens. It was also the first study to demonstrate the need of experimental protocols able to highlight adverse effects of exogenous hormones in the progeny.

Many years later, we are still facing that need as we became aware of the limitations of standard animal bioassays to correctly predict the risk of endocrine disruptors and support their classification.

The terms endocrine-disrupting chemicals and endocrine disruption were introduced for the first time in 1991 [75]. The first definition for endocrine disruptors was issued by the International Program of Chemical Safety (IPCS), where the endocrine disruptor was defined as "*an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) population*" [76].

Endocrine disruptors (EDs) represent a challenging issue in the field of regulatory toxicology. Even if a large bulk of scientific literature has been accumulated during the last 20 years improving our knowledge about the adverse effects elicited by EDs and expanding the list of possible EDs, it is difficult to translate this knowledge into regulatory criteria supporting decision policies. An update of the scientific knowledge on EDs was published in 2012 by

WHO including key concerns that needed to be addressed [76,77]. Among the key concerns highlighted by the WHO expert group, it was pointed out that only a small fraction of the 800 chemicals, which are known or suspected to elicit endocrine-disrupting effects, have been tested in appropriate assays to identify their toxicological properties. For most of them there is no sufficient data or no data at all. This concern poses the question of what amount of information is needed to highlight endocrine-disrupting properties and whether available assays, currently used in the regulatory toxicology, would be able to provide this information. A conceptual framework for testing and assessment of endocrine disruptors was provided in the same year by the Organization for Economic Cooperation and Development (OECD). The conceptual framework offers a guide to available tests, which can provide relevant information for the assessment of endocrine disruptors. Available tests are organized in five levels of information at different biological complexity [78]. Moreover, within the OECD Environment, Health and Safety Programme, great efforts have been made to improve existing *in vitro* tests or validate new ones to fulfill the 3Rs principles on reducing, refining, and replacing animal tests [79].

Following the publication of these reports, the European Commission undertook the review of the key scientific issues about testing and assessment of endocrine disruptors in order to support the European policies to protect human health and environment [80]. One of the key issues was related to the identification of thresholds for endocrine-disrupting activity.

As the principles of endocrinology can be applied to EDs, as exogenous hormones they can be expected to act at low and very low doses. Their binding to a specific receptor is considered one of the main initiating events in adverse outcome pathways [18].

Endogenous hormones bind the receptor with high affinity; their tissue-specific action can be easily detected; the receptor signals are amplified in the cells and lead to a definite biological response. EDs may compete for receptor occupancy, concurring to reach the level of occupancy required to generate the response. Thus, it is possible that even one molecule of a certain ED, acting as a receptor agonist, may activate the receptor and start the process leading to the adverse outcome [58]. This implies that EDs act at doses that are lower than NOAEL and that key events starting at these low doses are related to the adverse response. Even if there is no scientific consensus on the effects of EDs at low doses and their relevance to humans [81], there is a general agreement that low doses of EDs are sufficient to induce adverse responses in developing organisms during the window of sensitivity due to the immaturity of homeostatic mechanisms, immature metabolism, and absence of some endocrine axes [58]. For this reason, the threshold may be particularly low during fetal development and it is even possible that no threshold exists. It is also possible that thresholds would be identified with more appropriate testing approaches and strategies to capture effects of EDs even at very low doses [58].

Environmental EDs can be present in complex mixtures, where EDs sharing the same mode of action (MoA) are supposed to play additive effects [58]. The problem of environmental mixtures at low doses has recently been addressed, highlighting the role that the combination of chemicals, with different MoAs, targeting one or more cancer hallmarks, may have on onset and progression of tumors [20].

8.3.5 From Occupational to Environmental Exposures: Asbestos and Other Chemicals of Concern

8.3.5.1 Asbestos

As stated before, asbestos represents a paradigmatic example of human exposure for which epidemiological evidence from occupational exposures offers precious information to understand the possible risk for general population.

Asbestos is naturally present in the environment, especially in the geographical regions overlooking the Mediterranean Sea, with a large presence of asbestos-containing rocks. Indeed, mesothelioma cases from geological exposure to asbestos have been reported in Turkey [30].

Due to its widespread use for the last 100 years, asbestos fibers can be found in all environmental media.

The higher exposure of the general population is still related to the presence of operating plants for the production of asbestos and asbestos-containing products or the presence of nearby mines and caves. In these areas cases of nonoccupational mesothelioma have been registered [28].

However, even in countries where asbestos has been banned, dismissed plants and contaminated soil in the areas nearby plants represent a possible source of exposure.

It is debated whether the domestic exposure to asbestos would be responsible for asbestos-related diseases. Besides all the possible sources of asbestos exposure for the general population, which we discussed before and for which evidence of exposure-related pathologies exists, for example, fibers brought home on workers' clothing, domestic exposures would include also the use of asbestos-containing household materials [82]. Lung-burden studies and comparisons with workers at low level of exposure were used to highlight the risk for domestic exposures to asbestos. However, results from these studies did not give evidence for a significant association [82]. While other factors, including cigarette smoking, have been claimed to concur to induce lung cancer in asbestos-exposed workers [83], the role of smoking, including passive smoking on tumor incidence in nonoccupational exposures to asbestos, has never been adequately investigated.

Despite the large use of asbestos, the concentration of airborne fibers is limited. The concentration of asbestos in outdoor air ranges from 10 f/m^3

measured in rural areas to 100 f/m^3 registered in urban air. Higher concentrations are found only in the proximity of industrial areas, mines, factories, demolition sites, and unprotected waste sites [28]. However, in countries where asbestos has been banned, the commitment to reduce asbestos-related diseases has led to national programs and policies to further decrease the environmental exposures. In Italy, legal limits have been set to prevent indoor exposure in occupational and nonoccupational environments. The concentration of asbestos fibers at workplace should not exceed the limit of 0.1 f/cc and that in nonoccupational areas should be contained under 2 f/m^3. Even if the legal limit for asbestos fibers in outdoor air has not been set, the concentration of fibers at emission should not exceed 0.1 f/m^3.

Ingestion of drinking water contaminated by asbestos has fostered concerns about the possible impact on the health of the general population. Asbestos can enter the aquatic environment as the consequence of rock erosion, water pipe corrosion, industrial wastewater run-off, or from contaminated soil. The concentration of asbestos in drinking water under normal conditions is usually less than 1 f/ml. Increased concentration is detected following pipe ruptures and as a consequence of catastrophic events. However, in some areas the concentration of asbestos in drinking water has been reported to be much higher, up to 300 million f/l [28]. In 1998, US EPA, by using a linear model of cancer risk, established a threshold of 7 million f/l for asbestos in drinking water, a level at which no known or anticipated adverse effect would occur. This value has been considered as the Maximum Contaminant Level Goal (MCLG) by US EPA, in view of the increased risk of developing benign intestinal polyps. However, the MCLG is not a legal limit and is based only on human health considerations. No legal limit has been set for asbestos in drinking water in other countries.

8.3.5.2 Arsenic and Arsenic Compounds

Rather than asbestos, other chemicals pose higher risks for their presence in drinking water. Arsenic is a worldwide contaminant, whose toxicity is well known. Most arsenic in the environment and especially in groundwater comes from natural sources, since arsenic is largely present in rocks. Anthropogenic sources are represented by mining. The concentration of arsenic in water often exceeds the limit considered safe for humans, which has been set at 0.01 mg/l by WHO.

Drinking water is the most important but not the only source of exposure to arsenic. Arsenic can accumulate in seafood that contributes to increase the level of exposure through ingestion. Arsenic can be absorbed through the skin from contaminated water. Inhalation is considered of minor importance for general population exposure, while being the primary route for occupational exposures.

Arsenic is rapidly metabolized in the human body to As(III) and As(V), which then undergo further metabolism to As(III), the form that more easily

penetrates into the cell. Inorganic forms of arsenic (As(III)) show the highest toxicity and poisoning effects when the concentration in drinking water exceeds 10 mg/l [84].

Chronic exposures to low concentrations of arsenic in drinking water may cause arsenicosis, a chronic arsenic poisoning of skin, lung, liver, kidney, nervous system, and even more severe pathologies, such as the Blackfeet disease (BFD), a peripheral vascular disease, which is endemic in Taiwan, and cancer [84].

Metallic arsenic and arsenic inorganic forms have been classified as carcinogenic to humans based on sufficient evidence from epidemiological studies [28]. Other organic and inorganic forms have been found to induce cancer in experimental studies in rodents. These forms include dimethylarsinic acid, calcium arsenate, and sodium arsenite (sufficient evidence) and sodium arsenate, gallium arsenide, arsenic trioxide, and trimethylarsine oxide (limited evidence) [28]. However, some of the methylated products from the metabolism of inorganic arsenic in the human body are considered potent carcinogens [36].

Arsenic is thought to induce cancer through nongenotoxic mechanisms. Genotoxicity would play a secondary role along the adverse pathway. Two different initiating molecular events could be responsible for arsenic carcinogenicity. The first one is related to ROS-mediated oxidative stress. Free radicals are generated during arsenic metabolism that then disrupt cellular components including the mitochondrial membrane, which has been described as one of the main targets of arsenic-mediated oxidative stress [36]. Reaction of ROS-derived free radicals with DNA could be responsible for genotoxic effects. However, oxidative stress-derived inflammation is thought to play the main role in inducing cancer and noncancerous adverse effects from arsenic exposures [83,85,86].

The second mechanism is related to the ability of arsenic to disrupt the glucocorticoid receptor signal transduction pathway by inducing epigenetic modifications. Therefore, arsenic can be classified among EDs that can act at different levels of the epigenome, inducing transgenerational effects [19].

This mechanism is shared by cadmium, and it is thought to be responsible of the increased susceptibility to infections of children exposed to these chemicals *in utero*. Indeed, in umbilical cord blood of newborns exposed to arsenic *in utero*, the levels of miRNAs associated with the immune response were decreased, while the levels of miRNAs associated with signaling pathways related to cancer and diabetes were increased [87].

Epidemiological studies have shown that the exposure to arsenic in early stages of development is related to increased risk of cancer and cardiovascular and respiratory diseases in adult life [87]. Prenatal exposure to arsenic is also responsible for adverse pregnancy outcomes, such as the reduction of fetal growth, which appears to be gender-dependent, with an inverse association with fetal growth in boys [88].

8.3.5.3 Phthalates

Phthalates represent another example of widespread contaminants of concern. Phthalates are plasticizers used for manufacturing polyvinyl chloride products and are added to several other common plastic items, to which they confer flexibility and durability. They are also present in cosmetics, care products, and other consumer products, all of which represent a possible source of exposure for the general population. So far, only one occupational study has been available reporting that workers exposed to two phthalates, monoethylhexyl phthalate and monobutyl phthalate at concentrations at least one order of magnitude higher than the background level, show reduction of serum levels of testosterone [89]. This effect has never been confirmed in the adult general population exposed to lower concentrations.

The EU Commission has banned the use of phthalates in children's toys since 1999 and extended the ban to childcare articles in 2005 due to the risk to absorb concentrations of phthalates exceeding the acceptable daily intake calculated for noncarcinogenic endpoints. Recommendations on the use of phthalate-containing products and suggestions on imposing at least an interim ban of some phthalates for toys and childcare items have been provided by the Consumer Product Safety Commission in the United States [90].

Six phthalates are prohibited in children toys and articles: di-isononyl phthalate (DINP), di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP or DNBP), di-iso-decyl phthalate (DIDP), di-n-octyl phthalate (DNOP), and butylbenzyl phthalate (BBP).

Some but not all of them have been postulated to act as carcinogens and/or toxicants for reproduction by endocrine-disrupting mechanisms and for this reason, as well as for the high impact on the environment, they have been listed as substances of very high concern according to EC REACH regulation. In particular, DEHP is considered the most dangerous among phthalates and postulated to induce carcinogenesis through nongenotoxic mechanisms [18].

Due to their broad use, persistence, and ability to bioaccumulate, phtalates, including DEHP, are largely present in the environment where they represent a high risk of exposure through the food chain. DEHP is rapidly metabolized to an active metabolite that is 10 times more potent than the parent chemical. The most known mode of action for DEHP is represented by the activation of peroxisome proliferator-activated receptor (PPAR-alpha), as the main initiating event starting the signal leading to gene modulation [18]. The PPAR-alpha-dependent signaling pathway is still debated as to its relevance to humans. However, the effects related to its activation by DEHP at cell, tissue, and organ levels include adipocyte differentiation, glucose metabolism, and the storage of fatty acids and may account for the effects at the organism and population levels, such as obesity and diabetes, which have been described as possibly related to exposure to phthalates [91,92]. A different mechanism has also been hypothesized involving the activation of the constitutive androstane receptor (CAR) [18].

Phthalates are water-soluble and can easily reach the fetus and enter the amniotic fluid recycling system. Newborns and children, who require medical care in the hospital, may be exposed to phthalates through catheters and medical tubing [65].

In utero exposure to DEHP has been reported to decrease the levels of fetal testosterone through a mechanism involving Cyp11a1 and Cyp17a1, which support steroidogenesis. It has been suggested that this mechanism in the fetus is sustained by epigenetic effects induced by DEHP, thus ensuring the transgenerational effects seen in adults. Fetal exposure to DEHP can lead to altered Leydig cell differentiation and altered levels of testosterone in adult life.

The reaction with either PPAR-alpha or CAR is then the initiating event leading to epigenetic modifications, which may be responsible for reproductive impairment, cardiovascular disease, or cancer in adult life [18,93,94].

8.3.5.4 Pesticides

Pesticides represent a worldwide source of exposure.

At the beginning of the twenty-first century the global production of pesticides was estimated by WHO to be 2.26 million tons of active ingredients, with 25% of production from developing countries. Most of the pesticides are used in agriculture for crop protection and food preservation. However, a significant fraction of pesticides are used at home.

Pesticides are designed to be toxic at doses effective to kill, reduce, or repel insects, fungi, and rodents as well as unwanted plants.

Highly toxic pesticides have been replaced; however, the most persistent are still present in the environment. Dichlorodiphenyltrichloroethane (DDT) and other organochlorine pesticides, which have contributed extensively to the awareness of global contamination including the environmental persistence and the health effects from the exposure to pesticides, are still monitored in waterways, years after their use has been banned. DDT has also been one of the first chemicals to be recognized for its ability to induce endocrine-disrupting effects. It is still in use in several countries for malaria control [88].

Most of the industrialized countries have introduced strict regulations for marketing pesticides.

The registration of new pesticides requires the availability of complete information about the toxicological and ecotoxicological behavior of each active ingredient, the environmental fate and behavior of all the active ingredients in the formulation, the product performance, and the effects on nontarget species as well as residue chemistry.

Moreover, the product labeling should include all the information about the product handling and accurate instructions for its application.

The entire procedure to obtain the authorization for marketing new pesticides and the strict rules for the use of the products should ensure the highest level of protection of exposed workers, consumers, and the environment.

Occupational exposure to pesticides, including highly toxic pesticides, is still high in developing countries and in lower middle-income countries, where much of the constituent chemical production has been moved as a consequence of overall globalization and lower labor costs [55]. Occupational exposures may occur at manufacturing plants of active ingredients, during the preparation of formulations and packaging. Agricultural workers may be exposed during the application in open fields and greenhouses if proper regulations do not exist to provide advice to minimize the contact with the pesticides or if handling instructions are not followed [95].

The general population is exposed to pesticides mainly through food and beverages including drinking water, which may have been contaminated with pesticides. The exposure of the population resident nearby pesticide manufacturing plants as well as the exposure of family members and relatives of workers to hazardous substances brought home from the workplace, cannot be excluded [95].

While the presence and distribution of pesticides in environmental media, especially water, reflects the local agricultural production, the presence of pesticide residues in food is affected by the global market, even if major attention has been gradually paid to postmarket testing to avoid risk for the consumers.

Pesticides residues in food represent an example of both complex chemical mixtures and multiple exposures. Even when severe regulations, good agriculture practices, and pre- and postmarket testing ensure that individual pesticides are absent or at negligible concentrations, much below the legal limit and the safe threshold, it cannot be excluded that additive or synergistic effects at low concentrations, the presence of unknown metabolites, or traces of undetectable chemicals may contribute to trigger adverse effects. Moreover, the presence of pesticides in different kinds of food and beverages can contribute to elevate the daily and cumulative intake of hazardous chemicals.

For marketed pesticides acting as EDs, it is also possible that some adverse effects may be reached at doses much lower than legal limits, that is, the maximum residue level (MRL) set on the basis of results from standard tests. Penconazole, a systemic triazole fungicide mainly used on grapes, and whose residues are monitored in grapes and wine, has been found to modulate a set of genes involved in the thyroid cancer pathway at doses lower than that corresponding to MRL, thus confirming its activity as an ED and suggesting a possible role in thyroid carcinogenesis on the basis of early key events at the cell level [96].

Several pesticides have been found to be EDs, possibly inducing adverse effects in agricultural workers. Some epidemiological reports have suggested an association with breast and prostate cancer in workers but the data are largely heterogeneous [97].

IARC has evaluated 75 pesticides; only for arsenic-containing pesticides there is sufficient evidence to classify them as carcinogenic to humans. In 2014, IARC provided a list of pesticides that require evaluation or reevaluation including,

among others, glyphosate. Glyphosate is the highest volume herbicide worldwide. Its use in agriculture has been increased after the development of genetically modified crops, which are resistant to glyphosate. Glyphosate rapidly diffuses in air, water, and food after spraying. The general population is exposed if living nearby the sprayed areas or following its use at home. Exposure through contaminated food is also possible; however, the concentrations of glyphosate in food are generally very low.

After IARC reevaluation, malathion and glyphosate have been classified as probably carcinogenic to humans based on sufficient evidence from animal studies and *in vitro* evidence for genotoxic activity [98]. The IARC classification for glyphosate has been disputed by several regulatory agencies.

US EPA had first classified glyphosate as a carcinogen in 1985 and then reconsidered the classification in 1991, classifying glyphosate as noncarcinogenic to humans. EFSA has recently reviewed all available data for glyphosate including those that supported IARC classification, and has concluded that glyphosate is unlikely to be genotoxic or to induce carcinogenesis to humans.

So far, IARC has considered 12 pesticides as carcinogens acting through nongenotoxic mechanisms based on negative results in genotoxicity tests and positive results, that is, increased incidence of liver tumors, in rodent carcinogenicity bioassays [18]. The use of six of them has been banned in the European Union. For most of them, however, the mechanism of action leading to carcinogenesis is still unknown. Recently, it has been postulated that, based on the current regulatory testing paradigms, many of these pesticides would not be classified and that the rodent carcinogenesis bioassay, in the absence of other endpoints and information about the mechanism of action, would not be sufficient or sensitive enough to properly predict the carcinogenic activity of nongenotoxic pesticides [18].

However, it is often difficult to draw final conclusions on pesticide toxicity from a scientific point of view, since most of the data provided by industry for regulatory purposes are not publicly available.

The risk from exposure to pesticides is presumably higher for children due to the assumption that they will have higher sensitivity to lower concentrations.

In utero exposure to pesticides, especially household insecticides, has been claimed as responsible for infant leukemia [99]. Several mechanisms have been proposed to support the adverse pathway to the final outcome, and a chain of pathogenetic events linking exposure to pesticides with infant leukemia have been suggested [99]. The fetal exposure to pesticides, especially organochlorine pesticides, has also been described to play a role in adverse pregnancy outcomes, including low weight at birth. Other effects have been reported as consequences of exposure during fetal development, including hypertension (organochlorine pesticides) and low IQ (organophosphate pesticides) [53].

8.3.5.5 Mycotoxins

The diet represents an important source of unintentional exposure not only to manufactured chemicals but also to toxins of natural origin.

Mycotoxins are secondary metabolites of fungi that contaminate agricultural products, especially those rich in carbohydrates. Almost 300 mycotoxins have been recognized to affect human and animal health. Some of them induce severe adverse effects including cancer and neurological disorders. Several mycotoxins exert endocrine-disrupting activity.

Acute effects from mycotoxins have been known since the Middle Ages related to the contamination of rye with *Claviceps purpurea*, a fungi producing a toxin, which was later identified to be an analogous to lysergic acid, classified as an ergot alkaloid and called ergotamine. Acute effects (mycotoxicosis) from the ingestion of ergotamine-contaminated flour have been described in France, England, and other European countries during the seventeenth and eighteenth centuries and have been thought to be the cause of the symptoms registered in several young women from the town of Salem, New England, in February 1692, which started the Salem witchcraft affair.

In the modern era, acute poisoning from mycotoxins has been registered only in animals, especially livestock, such as the turkey X syndrome, which affected about 100,000 turkeys in England in 1960.

Chronic exposure to low concentrations of mycotoxins in contaminated food is a public health concern. Due to climate changes that are creating ideal conditions for fungi growth and crop contamination, the exposure to hazardous mycotoxins is now a worldwide problem. Several mycotoxins may aggregate in different kinds of food or accumulate in the same food matrix, leading to the exposure to multiple mycotoxins in the same meal. Mycotoxins are not degraded by temperature treatments as in the normal conditions of domestic cooking or freezing. They are not broken down by mammalian digestion. Only good agriculture practices and constant monitoring may reduce, but not completely eliminate, the risk of exposure.

Among mycotoxins, aflatoxins, fumonisins, ochratoxins, zearalenone, patulin, deoxynivalenol, and T-2 and HT-2 toxins are those considered to be of highest concern for their wide distribution, high probability of food contamination, and severe adverse effects as the consequence of chronic exposures. Aflatoxins, which are produced by *Aspergillus* species, ochratoxins by *Penicilium* sp. or *Aspergillus* sp., and fumonisins, secondary products of *Fusarium* sp., are all considered to be carcinogenic to humans.

The toxic effects and toxicological behavior of aflatoxins have been known for more than 50 years. Among the six predominant aflatoxins, aflatoxin B1 (AFB1) is considered the most potent known natural carcinogen and responsible for the endemic hepatic carcinoma in the subequatorial region. Epidemiological evidence has been supported by experimental studies [100]. Rats fed with a diet containing AFB1 developed liver tumors, whose incidence increased with

the increment of AFB1 concentrations [100,101]. AFB1 is considered a genotoxic chemical. It is metabolized in the liver to epoxides that are able to bind covalently to DNA forming adducts at the N7 position of guanine. AFB1 can form adducts to proteins too, providing a biomarker of exposure that can be easily detected in plasma and urine. The p53 tumor suppressor gene has been reported to be a target of AFB1-induced point mutations and this mechanism could be a key event in the onset of hepatocarcinomas [102,103]. Studies on liver cancer patients in Africa and China have confirmed the involvement of the p53 tumor suppressor gene mutated at codon 249 with a G-to-T transversion, which makes this specific mutation the first biomarker of exposure to carcinogens that remains fixed in tumor tissue [104]. Moreover, AFB1-DNA adducts can result in GC to TA transversions [105].

Epidemiological studies have been reported, showing that the occupational exposure to AFB1, at a plant processing peanuts, induced a significant increase of lung cancer in workers by inhalation of dust contaminated with AFB1 [104]. Aflatoxins also induced pulmonary carcinogenesis in animals [104].

AFB1 metabolites AFM1 and AFM2 are produced in the stomach of ruminants fed with feed contaminated with aflatoxins. These metabolites are considered to be stable contaminants of milk and dairy products and presumably pose a higher risk for children.

Aflatoxin exposure has been associated with childhood stunting, a condition affecting children's growth and inducing cognitive and language impairment in childhood as well as low stature, obesity, and reproductive impairment in adult life. Besides other causes related to the socioeconomic context, which affect the incidence of stunting in developing countries, studies performed in West Africa have demonstrated that the height and weight are lower in aflatoxin exposed children and the effect is dose related. Children exposed to high levels of aflatoxins also show immunomodulation with decreased levels of immune response modulators [102].

Fumonisins have been discovered more recently in 1988, and since then at least 28 toxins have been identified as belonging to this group of mycotoxins. Fumonisins are especially found in maize and maize-derived products. Crops are rarely contaminated by a single type of fumonisin. Fumonisins 1, 2, and 3 are often found together in the same maize sample. Due to their ceramide-like chemical structure, fumonisins have been postulated to disrupt the ceramide synthesis and impair sphingolipid metabolism in neuronal cells. This mechanism could be responsible for the neuronal tube disease, leading to defects of neuronal tube and spine cord, which has been described as the consequence of the exposure of pregnant women to high levels of fumonisins [102].

Fumonisin exposures may be a risk factor for esophageal cancer and the cause of the increased incidence of this type of tumor in the northeast of Italy and in other regions worldwide where the consumption of corn and cornmeal in the diet is high. A strict correlation between the fumonisin

exposure and esophageal cancer was found in the former Transkei region of South Africa. Similar associations have been described in some regions of China and in Iran related to the prevalent consumption of contaminated maize and rice [102,104].

Ochratoxins (OTAs) include three different metabolites (A, B and C) that contaminate a wide range of commodities due to the stability of OTAs at a wide range of temperatures, spanning from 0 to 37 °C. OTAs are potent renal carcinogens for animals and they are thought to induce cancer through genotoxic mechanisms. These findings have never been confirmed in humans, and for this reason, IARC classified OTA A as a possible carcinogen to human on the basis of sufficient evidence in animal bioassays. Kidney, however, represents the target organ for OTAs toxic activity in humans, where nephritic syndromes have been observed but only at very high levels of exposure, such as those detected in Egypt and Sierra Leone [102,103].

8.3.6 Air Pollution and Airborne Particulate Matter: The Paradigmatic Example of Environmental Mixtures

Air pollution is regarded as responsible for over a million premature deaths worldwide every year. It has been linked to acute and long-term adverse effects, including carcinogenicity, cardiovascular, and pulmonary diseases. WHO Air Quality Guidelines (2005) established limit values for several air pollutants, including nitrogen oxides, carbon monoxide, sulfur dioxide, lead, ozone, and fine particulates ($PM_{2.5}$) [106]. While exposure to several harmful pollutants such as sulfur dioxide, lead, nitrogen dioxide, carbon monoxide, and benzene, in ambient air, has decreased sensibly as a consequence of air quality guidelines, $PM_{2.5}$ and ozone, in particular, continue to pose significant health risks.

The respiratory system is the initial site of PM deposition and, as a consequence, the first target of PM health effects. Evidence from epidemiological studies as well as toxicological and controlled human exposure studies supports a causal relationship between short- and long-term exposures to PM and respiratory effects. PM exposure can induce airway inflammation [107,108] and can affect lung development [109], impairing lung function in both children and adults. In addition, PM exposure is related to the onset and exacerbation of obstructive lung diseases including chronic obstructive pulmonary disease (COPD) and asthma, which represent an important cause of morbidity and mortality worldwide [110–112]. A recent paper reporting a systematic review and meta-analysis of epidemiological studies supports a positive association between PM exposures and lung cancer incidence and mortality [113].

The role of PM on the onset of adverse outcomes in other organs and systems has been extensively explored. The results point out a causal relationship between $PM_{2.5}$ exposure and increased cardiovascular morbidity and mortality [50,114,115].

$PM_{2.5}$, measured at the urban background locations in large cities, is considered the best indicator for monitoring air quality and assessing the exposure of the general population. The EU Directive on ambient air quality and cleaner air sets the annual limit value at 25 μg/m^3 for $PM_{2.5}$ and 40 μg/m^3 for PM_{10}, in order to protect human health [116]. In 2013, the EU Commission adopted the Clean Air Policy Package [117] that set new air quality objectives to be reached by 2030.

Despite the efforts of regulatory and environmental agencies worldwide, the established air quality environmental standard values are often exceeded in industrialized countries due to local emissions sources, heating combustion, and vehicular traffic, and are strictly related to seasonal changes, meteorology, and photochemistry. The emission of primary PM and PM precursor gases decreased in recent years in the European Union. Despite the emissions decrease, 22–44% of the EU urban population has been exposed to concentrations of PM_{10} exceeding the limit values, in the period 2002–2011 [118]. According to the EEA report, 9% of the urban population from the 28 EU countries has been exposed to $PM_{2.5}$ levels above the EU target value, which became a limit value in 2015. Moreover, about 87% of the urban population has been exposed to $PM_{2.5}$ concentrations exceeding the stricter WHO AQG value in 2013 [119].

The current limit threshold is based mainly on the disease burden attributable to $PM_{2.5}$ exposure. It has been demonstrated that the exposure–response relationship between $PM_{2.5}$ and some adverse health outcomes, such as cardiovascular disease mortality, is not linear with a steep increase in risk at low exposure and flattening out at exposures above 50 μg/m^3 [120]. Conversely, the association of $PM_{2.5}$ exposure with lung cancer mortality appears to be nearly linear. This different behavior could be related to differences in the mechanisms of the pathogenesis of the diseases. Cardiovascular adverse effects may be mediated prevalently by particles themselves, whereas the onset of lung cancer has been mainly associated with the carcinogenicity of chemicals, such as PAHs, that are transported by the particles in the lung airways [121]. However, the persistence of adverse health effects at concentrations below the limit recommended by WHO is reported by recent studies [122]. Results from the epidemiological project ESCAPE (European Study of Cohorts for Air Pollution Effects) presented evidence that, even if the European population is generally exposed to $PM_{2.5}$ concentrations below the limits recommended in existing guidelines, the mortality rate of the population would increase by 7% for each additional exposure of 5 μg/m^3 $PM_{2.5}$ [123].

8.3.6.1 Characteristics of PM and PM Exposures

Atmospheric pollutants (particles and gases) are either emitted directly from sources (primary) or formed in the atmosphere (secondary) through chemical reactions. Primary pollutants are more lipid soluble and exhibit steep

concentration gradients close to sources, whereas secondary pollutants are more water-soluble and often homogeneously distributed at a regional scale.

The fate of inhaled pollutants depends on their water solubility [124]. Water-soluble gases (e.g., hydrogen peroxide) rapidly diffuse through the moistened surfaces of the mouth, nose, and upper airways and are then eliminated. Gas-phase PAHs and ozone are transported into the air-exchange regions of the lung. Particles penetrate into the air-exchange regions of the lung and deliver toxic substances into the respiratory tract. The aerodynamic diameter is the major determinant of the PM fate in the body. PM_{10} particles with an aerodynamic diameter ranging from 2.5 to 10 μm are deposited into the nasal cavities and the upper airways, whereas smaller particles, such as fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) particles, reach the bronchioles of the lung and then the alveoli. Fresh combustion-generated particles presumably retain their particle form after deposition, whereas secondary particles, including concentrated aqueous solutions, dissolve into the lung surfactant, delivering dissolved chemicals and sometimes releasing an ultrafine primary core. The release of chemicals is facilitated by the greater specific area of fine particles.

Fine and ultrafine particles may cross the endothelial barrier, enter the blood stream, and lead to adverse effects in the respiratory, cardiovascular, immune, and neural systems [125]. Moreover, a fraction of ultrafine particles (with a diameter less than 0.1 μm) may even enter the brain directly through the nose [126].

The timing of exposure, genetic factors, preexisting lung pathologies, or other exposures may influence the effects of the PM exposure on lung function. Even if not all the studies give concordant results, data suggest that the lung function could be negatively impacted by relatively low concentrations of PM. Therefore, even low PM concentrations may induce significant health outcomes, especially in highly susceptible populations, such as children and the elder population.

Health outcomes have been demonstrated to be deeply affected by the size of particles, but the associated hazard could be modified according to the chemical composition, which means the different pollutants present in the PM mixture [127,128]. The chemical composition of air samples of particulate matter varies according to both time and space, but it typically shows the same major components (sulfate, nitrate, ammonium, sodium and chloride, elemental carbon, organic carbon, mineral components, water, biological materials, and carbon component), although in considerably different proportions according to sampling location [129].

Adverse health effects have been related to the source category and with the PM-associated components, giving evidence that different health-related endpoints are associated with different sources of emissions. For instance, fossil-fuel combustion source categories have been consistently associated with both short- and long-term adverse effects of $PM_{2.5}$ exposures. The components that

originate from the Residual Oil Combustion and Traffic source categories were most closely associated with short-term effects, whereas long-term effects were associated with components from the Coal Combustion category [130].

Airborne particles also contain minor components such as trace metals and trace organic compounds. Among trace organic compounds, particular attention has been devoted to PAHs. PM is always contaminated by PAHs generated from the incomplete combustion of organic materials. The major source of PAHs is the combustion of biofuels, while other sources, such as combustion plants, various industrial and production processes, road transports, and the waste incineration, can contribute. Links between the human exposure to complex PAH mixtures and development of diseases, including cancer as well as respiratory and cardiovascular diseases, have been described [131,132]. Despite their structural similarities, PAHs vary greatly in their carcinogenic potency with both individual and complex mixtures of PAHs classified as possible or probable human carcinogens by IARC [131]. BaP is the most widely studied PAH and it is usually chosen as a marker for evaluating the toxicity of PAH mixtures, since the available toxicological data provide sufficient basis for conventional risk assessment, which assumes that the toxicity of each PAH can be calculated in terms of BaP equivalents. As a result of recent toxicological research, BaP has been classified as a known human carcinogen group 1, according to the IARC classification [133].

8.3.6.2 PM Exposures and Cancer

Epidemiological Evidence

The association between long-term exposure to PM and cancer, especially lung cancer, had been suggested a long time ago and recently supported by several epidemiological studies. As demonstrated by the ESCAPE project, a statistically significant association between the risk for lung cancer in the European population and PM_{10} exists with a hazard ratio (HR) 1.22 (95% confidence interval 1.03–1.45) per 10 μg/m^3. For $PM_{2.5}$ the HR is 1.18 (0.96–1.46) per 5 μg/m^3 [134]. These recent results confirm the findings from several studies carried out in nonsmokers in different geographical regions, linking long-term exposure to $PM_{2.5}$ with lung cancer mortality (or incidence) [132,135,136].

The association between exposure to air pollution or chemicals in polluted air with the formation of DNA adducts in exposed individuals has been reported in several studies that used DNA adducts as biomarkers of exposure and early effects [137]. The association has also been confirmed in the fetus using paired blood samples collected from mothers and newborns [138].

In 2013, both IARC and WHO classified outdoor air pollution and PM as group 1 carcinogens [139,140]. This evaluation is based on the evidence from epidemiological studies regarding the relationship between lifetime exposure to $PM_{2.5}$ and PM_{10} and lung cancer risk [113]. The lung is the target for the

carcinogenic effects from exposure to PM. Tumors arise as the consequence of a continued and long-lasting exposure.

Animal Studies

The effects of PM on tumor induction have also been explored in animal carcinogenicity studies. A dose-related increase in the incidence of lung tumors and accompanying DNA adducts has been demonstrated in Fischer 344 and Wistar rats after chronic inhalation exposure of $PM_{2.5}$ (2.2–7.0 mg/m^3). These results have not been confirmed in carcinogenicity inhalation studies performed in mice and Syrian hamsters. Rats differ from mice and nonhuman primates in both the pattern of particle retention in the lung and alveolar epithelial hyperplastic responses to the chronic particle exposure [141]. Therefore, the results obtained from rats exposed to PM by inhalation in carcinogenicity bioassays should be considered with caution, taking into account the rat-specific particle lung overload [142]. Moreover, the lowest concentration, at which a significant increase in the incidence of lung tumors is achieved in rats, is much higher than the environmental exposure concentrations, as assessed by studies on diesel emissions [143].

Genetically modified animal models, such as lung adenoma-prone mice, have been proposed to evaluate the carcinogenic hazard of air pollution. However, the exposure to cigarette smoke and diesel emissions from old technologies failed to elicit a clear dose-related increase in lung tumor incidence even when direct-acting mutagenic PM extracts were tested [144]. This result provides evidence for the limitation of the rodent carcinogenicity bioassay in detecting the carcinogenic properties of complex mixtures administered by inhalation.

However, in experimental studies, the direct rodent exposure to ambient air has given evidence for the clastogenic potential of air pollutants, especially the PM fraction [145].

In Vitro *Studies*

PM and PM extracts have been found to induce mutations in the Ames assay. The response is dose related and associated with direct-acting and promutagens as well as to the particles core [146]. $PM_{2.5}$ treatment causes the increase in the frequency of chromosomal aberrations and micronuclei in *in vitro* studies [147]. Also, PM-associated heavy metals elicit genotoxic damage that is counteracted by antioxidants and ROS scavengers [148].

Nongenotoxic mechanisms may also participate in the carcinogenicity of PM. The organic micropollutants associated with PM, like PAHs, may bind the aryl hydrocarbon receptor (AhR) and activate the expression of AhR-related genes [149]. The expression of Cyp1A1, which is regarded as a marker of the interaction of environmental pollutants with AhR, is enhanced in the BALB/c 3T3 cells treated with $PM_{2.5}$ [150].

The *in vitro* cell transformation assay (CTA), which relies on the ability of target cells to develop a transformed phenotype after the treatment with suspected carcinogens, is regarded as a test that can contribute to the weight-of-evidence approach to risk assessment. It also provides a phenotypic endpoint of oncotransformation to anchor early key events to the final adverse outcome. The combination of the BALB/c 3T3 cell transformation assay and transcriptomics was used to identify the toxicological profile of $PM_{2.5}$. Despite the absence of a clear cell transformation showing carcinogenic potential, the global gene modulation from the toxicogenomics analysis suggested the involvement of $PM_{2.5}$ in the carcinogenesis process. These early molecular events associated with carcinogenesis would be better anchored to the oncotransformation phenotypic outcome in CTA, after repetitive or prolonged exposures to $PM_{2.5}$ [150].

8.3.6.3 Possible Mechanisms of PM Toxicity

There are several common cellular as well as tissue events that underpin the onset of PM-induced adverse outcomes. Experimental and observational evidence indicates that the exposure to ambient air pollution, particularly to ultrafine particles, induces oxidative stress and consequently inflammation. In animal models, $PM_{2.5}$ triggers the activation of alveolar macrophages and endothelial cells leading to local and systemic inflammation [151]. *In vivo* studies demonstrate that the intratracheal instillation of aqueous PM extracts determines a transient self-resolving inflammatory response, which is induced mainly by metals in the PM extracts [152]. The response is characterized by increased cytokine levels, increased reactive oxygen species production, and the subsequent activation of defense mechanisms such as increased levels of catalase and glutathione peroxidase expression. An increase in proinflammatory mediators, including TNF-alpha, IL-6, and IL-1beta, has been observed in the hearts of rats exposed to $PM_{2.5}$. These findings are associated with pathological changes and ultrastructural damage in heart mitochondria; decreased activity of superoxide dismutase (SOD) and Na(+)K(+)-ATPase and Ca(2+)-ATPase; and increased levels of malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) [153]. It has been reported that mice, exposed to $PM_{2.5}$ by inhalation, displayed accumulation of collagen in liver tissues ultimately related to hepatic fibrosis [154]. The mechanisms that are implicated in air pollution-induced asthma exacerbation are not completely understood, but oxidative stress and immune dysregulation are involved [155,156].

These findings provide a scenario whereby exposure to PM sustains low-grade chronic inflammation of the lungs leading to the activation of normal phagocytes and epithelial cells that can contribute to local generation of ROS. These events lead to the onset of a vicious cycle, resulting in high levels of oxidative stress. Moreover, systemic inflammatory cytokines or oxidizing

molecules may be released from the lung into the general circulation, triggering systemic inflammation. Therefore, the PM exposure through the induction of oxidative stress and inflammation could trigger key events leading to the onset of fibrosis in the liver as well as in the lung and vascular oxidative stress [157].

Even if not completely explored, epigenome modifications play an important role in the carcinogenesis process [158]. Epigenetic events are now considered as key mechanisms in cancer development and should be carefully evaluated for carcinogen identification [159]. Gene-promoter hypermethylation, resulting in transcriptional inactivation and loss of expression of tumor suppressor genes, has been reported in human lung cancer, affecting CDKN2A (p16) gene and other genes that play a role in cellular growth and proliferation [160]. Increased methylation of p16 promoter has been observed in mouse lung after *in vivo* exposure to concentrated urban $PM_{2.5}$ by inhalation, as well as in primary murine alveolar epithelial cells treated *in vitro* [161].

8.3.6.4 The Role of PM Exposures in the Fetal Origin of the Disease

There is a growing amount of evidence linking urban air pollution and adverse pregnancy outcomes such as low birth weight [162,163], preterm birth [164], stillbirth [165,166], and infant mortality [167,168]. The epidemiological findings are supported by results from animal studies where the exposure of female mice to diesel exhaust or PM prior to or during pregnancy deeply affects placental morphology [169,170]. The changes in placenta functional morphology that affect maternal–fetal exchanges contribute to the induction of adverse pregnancy outcomes such as reduced fetal weight associated with exposure to air pollution [170].

Despite the adoption of different study designs and statistical evaluations and the presence of confounding variables (e.g., maternal smoking, gestational age, and socioeconomic factors), these investigations have suggested that the reported associations are causal.

Among all the adverse outcomes, preterm birth (PTB), that is, delivery at less than 37 weeks, is the leading cause of neonatal morbidity and mortality specifically as it accounts for 75% of perinatal mortality and more than 50% of the long-term morbidity. Surviving babies are at increased risk of neurodevelopmental impairments as well as respiratory and gastrointestinal complications. Common reasons for preterm births include preeclampsia or eclampsia and intrauterine growth restriction, both causes that can be affected by environmental pollutants. The frequency of preterm births has been estimated about 12–13% in the United States and 5–9% in Europe and other developed countries. However, the rate of preterm birth has increased in most industrialized countries. Besides the artificially conceived multiple pregnancies, which are considered to play an important role in the observed increase of preterm births, air pollution has been claimed as responsible for this adverse outcome. PM and

its components, especially PAHs and heavy metals, like lead or cadmium, have been associated with several adverse pregnancy outcomes including preterm births [171]. Indeed, exposure to lead is often revealed by the high blood lead levels in children, exceeding the limit of concern of 1 μg/dl. In 2012, a reference level of 5 μg/dl was set in the United States to identify children at risk for neurocognitive impairment, showing blood lead levels that are much higher than most children's levels. Lead exposure has also been associated with spontaneous abortions and other adverse birth outcomes, such as preterm deliveries and low birth weight. Recent reports have suggested an involvement of cadmium in preterm labor. Cadmium at low concentrations increases the response of calcium and oxytocin resulting in an increment of myometrial activity, whereas higher concentrations of this metal have an inhibitory action. This biphasic behavior seems to support the postulated hypothesis that cadmium can act as an ED. The increased responses to calcium and oxytocin in the presence of low amounts of cadmium support a role of cadmium in mechanisms of preterm labor [88].

The contribution of PAHs on pregnancy adverse outcomes has been explored and linked to low birth weight, reduced height, and low head circumferences [65]. These adverse effects have also been associated with exposure to PM_{10} [88]. It has been found that the adverse effects are related to the window of sensitivity during the first trimester of pregnancy. An increased risk for preterm birth was found associated with $PM_{2.5}$ exposure. The risk significantly increases by 6% per 10 $\mu g/m^3$ increase in $PM_{2.5}$ [88].

The proposed mechanism calls for an inflammatory response as a consequence of oxidative stress elicited in fetal tissue together with an inflammation-dependent immune response and reduction of heart rate variability, which can lead to the impairment of fetal growth and development [88]. Indeed, oxidative stress has been postulated to play a role in establishing a particular epigenetic pattern that can be the cause of early development of endothelial dysfunction in the offspring. This adverse effect, which has been observed in experimental studies, has been suggested to represent the fetal origin of cardiovascular diseases that become manifest later in adult life [172].

Binding to the AhR receptor represents another possible mechanism through which PAHs may affect the growth trajectory of children and induce predisposition to adult disease. This mechanism has been described in the case of voluntary exposure to PAHs (and other chemicals) through cigarette smoking. AhR-dependent mechanisms could be responsible of cancer onset in offspring of mother exposed to PAHs. Experimental studies showed that after the administration of dibenzo[a,l]pyrene to pregnant mice, increased mortality for T-cell lymphoblastic lymphomas was observed in the offspring and increased incidence of lung tumors and, to a lesser extent, liver tumors in survivors at later age [87].

8.4 Conclusions and Future Perspectives

Despite prodigious efforts to make the workplace and the outdoor environments free from dangerous toxins, environmental pathologies are still among the major causes of death. While occupational exposures affect only part of the adult life, exposure to environmental pollution could be responsible for prenatal impairments, which may influence the growth trajectory of children and increase the incidence of noncommunicable diseases, including cancer, in adult life.

The dawn of the twenty-first century brought expanded knowledge, experience, and achievements from the previous years as well as new technologies to investigate the still unexplored field of environmental toxicology. After 15 years of research, new insights have been provided, which have fostered new approaches to deal with the environmental complexity, explore the real risk from environmental exposure, and improve the prevention of the risks for human health.

The incidence of occupational disease has been reduced in long-term industrialized countries, following the introduction of new regulations aimed at decreasing exposure in the workplace and improving the protection of workers, by adopting proper procedures and protective equipment. Occupational exposures to known carcinogens, however, still represent a risk for workers in newly industrialized countries, emerging economies, and less-developed countries.

The study of several occupational exposures has improved the knowledge of the causal relationships with adverse effects from hundreds of chemicals that turned out to be environmental pollutants. The increasing attention to the impact of industrial toxins on the environment has promoted the introduction of new regulations to decrease the emissions from industrial sources, has improved the monitoring of pollutants in environmental media, and has reduced the exposure of the general population and, particularly, children to known carcinogens. This also highlighted the gaps in knowledge of the complexity of environmental exposures, the lack of sufficient information about the toxicological profile of chemicals, and limitations in the current approaches to hazard and risk assessment.

The advent of toxicogenomics technology has allowed the identification of genes and gene pathways whose modulation plays the key role in the onset and progression of diseases. Toxicogenomics is regarded as the tool to bridge both genotoxicity and nongenotoxicity events to carcinogenesis [173]. The application of toxicogenomics to the study of the cancer process has identified the molecular events sustaining the pathway to adversity, to highlight the mode of action of chemicals affecting biological traits fundamental in maintaining cell homeostasis, and to improve the comprehension of the mechanisms leading to disruption of homeostasis. This has led to a new approach to the description of relationships that link exposure to the final outcome.

In 2012, OECD launched the Adverse Outcome Pathway Programme based on the use of effective tools to describe the key events leading to the final adverse outcome that affect the organism and the population as the consequence of a molecular initiating event closely related to the exposure. AOPs are merely based on the chemicals MoAs.

The OECD AOP Programme is a further step into the next generation safety assessment that looks at the integration of data from chemoinformatics, cell culture-based *in vitro* tests, 3D tissue models, genomics, and exposure models to predict safety. AOPs can provide the framework for developing an integrated approach to testing and assessment (IATA). IATA can be defined as a "*structured approach to hazard identification (potential), hazard characterization (potency) and/or safety assessment (potential/potency and exposure), which strategically integrates and weights all relevant data to inform regulatory decisions regarding potential hazards and/or risk and/or the need for further targeted and therefore minimal testing*".

IATA supports the new vision to hazard and risk assessment by weighting all the existing literature information to support the decision, by recognizing the gaps in the available information, and by planning additional experiments, which should be consistent with the 3Rs Principles for a more ethical use of animal testing and the promotion of alternative methods.

In 2015, OECD recognized the need of developing an IATA for nongenotoxic carcinogenesis that is the most likely mechanism through which environmental carcinogens drive cancer. The twentieth century has been characterized by the genotoxicity paradigm, which conceives the cancer process as a sequence of mutations affecting key genes with nongenotoxic chemicals acting as supportive of the proliferation of initiated cells. From the nongenotoxic carcinogenesis point of view, cancer is still a sequence of mutations established in a tissue damaged by a prolonged exposure to stressors that are responsible for the earlier initiating events. It is possible that nongenotoxic mechanisms supported by the induction of tissue inflammation triggered by the oxidative stress are strictly related to the exposure to low doses of environmental mixtures [18].

With the discovery of the epigenome, the role of epigenetic changes has become clear. Such changes do not affect the DNA code but induce genomic perturbations and instability, thus initiating and sustaining several chronic-degenerative diseases including cancer. The modulation of the epigenome is the driver of cell differentiation and organ development during the early stages of life. Any perturbations during this delicate period, which represents a window of vulnerability, result in changes that may affect the embryo viability, the pregnancy outcome, or the health in adult life. Genomic instability triggered by epigenetic modifications is one important cancer hallmark and one of the mechanisms postulated for chemicals such as endocrine disruptors, which are active at very low doses.

The response to low-dose exposure is one of the most challenging issues in environmental risk assessment. Several aspects have become clear during

recent years due to the studies on EDs. The widespread presence of EDs in the environment has prompted research on the identification of thresholds for environmental mixtures and has pointed out the need for a different testing strategy to inform hazard assessment, which can overcome the limitations of current assays, including the 2-year carcinogenicity rodent bioassay, to correctly identify EDs and other chemicals acting through nongenotoxic mechanisms.

However, even if ED research has provided new insights into the possibility to measure mixture effects, when mixture components are present at ineffective levels leading to the description of the so-called "something from nothing" phenomenon [174], the majority of the studies on environmental pollutants are still focusing on single chemicals, single emission sources, and single exposure pathways. The identification of a direct cause–effect relationship between the adverse health outcome and the exposure to a mixture of pollutants from ingestion of contaminated food or water and/or from inhalation of pollutants in air is still a challenging issue. The association is even more difficult to establish when it deals with exposures that have occurred many years or decades before the onset of long latency diseases or during prenatal and early life.

The examples that have been chosen to discuss the complexity of the modern approach to occupational and environmental exposures represent some of the EU priorities and are currently addressed in several member countries, including Italy, where they are included in the National Plan for Health Prevention 2014–2018, issued by the Italian Ministry of Health. They are representative of emerging exposures to low doses of substances and chemicals, continuously monitored in the environment media, whose single concentrations are below the legal limits but could represent a risk from their bioaccumulation in mixtures. The examples also include exposures to chemicals and substances already banned but persistent in the environment and being the cause of cancer that may become manifest many years after the occupational exposure. A particular case has been discussed regarding the exposure to airborne PM, a complex mixture whose composition varies according to emission sources, climate conditions, and geographical location, making difficult the application of common policies to reduce the concentrations to safe levels.

This picture depicts situations common to all developed countries where attention to environmental impact and human health risk has been growing, leading to the adoption of environmental acts and occupational regulations.

Very different situations have been found in many developing nations and particularly in newly industrialized countries, which have faced spectacular and rapid progress in industrialization and export of manufactured goods and products worldwide. Here, the level of occupational exposures and environmental pollution has increased accordingly and the incidence of exposure-related cancers increased dramatically, even in the general population, as

industrialization, urbanization, and the use of automobiles spread, leading to a decline in environmental quality.

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Part Three

Gene–Environment Interactions

9

Ethnicity, Geographic Location, and Cancer

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9.1 Introduction

Cancer, also known as a malignant tumor or neoplasm, is a group of related diseases that originate in many different parts of the body (National Cancer Institute, 2015). Cancers have common features of abnormal cell growth, capacity to invade other parts of the body spreading to distant organs via blood vessels or lymphatic systems. Cancer, if untreated, can lead to serious illness and death. Normally cells grow, divide to make new cells, and die in an orderly way. During the early years of an individual's life, normal cells divide faster to allow the person to grow; in an adult, most cells divide only to replace dying cells or to repair injuries. Cancer develops when cells start to grow out of control or to invade other tissues in another part of the body.

Cancer is a group of diseases caused by alterations to the genes that control the way our cells function, especially how cells grow and divide. When DNA is damaged, normally the cell either repairs the damage or dies. In cancer cells, the damaged DNA is not repaired, and the cell does not die. Instead, the cell continues making new cells that the body does not need, and that have the same damaged DNA as the original cell does. Individuals can inherit abnormal DNA, but most of DNA damage is caused by environmental factors such as cigarette smoking or sunlight exposure, or mistakes that happen while a normal cell is reproducing.

While the exact cause of cancer is not known, gene and environment are believed to play an important role in the development of cancer. A previous large twin study of the Finnish population has indicated that environments (lifestyle, physical and psychosocial) could account for up to 75% of liability in all cancers together [1]. In the United States, cigarette smoking or tobacco use accounts for a significant portion of cancer death [2]; other factors such as

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occupational exposure or environmental pollution may contribute to the risk of cancer. For example, benzene, a common pollutant in vehicle exhaust, is a known cause of human leukemia; radon, a natural radioactive gas, raises the risk of lung cancer; and high levels of arsenic in drinking water may be associated with risk of skin, liver, bladder, and lung cancer. These risk factors explain a limited amount of liability, but may not account for much of cancer risk clearly associated with geographic variation, due to exposure to sunlight, the lack of certain trace elements in the soil [3], or other ecological factors.

The level and pattern of cancer risk differ greatly by ethnicity in the United States. This is because ethnicity measures not only the difference in genetic makeup that is believed to contribute to the risk of cancer but also its social aspects such as lifestyle, dietary behaviors, social environment, and social interaction that affect the risk of developing cancer [4,5]. Individuals within the same ethnic group may share or be exposed to common environmental risk factors at the community level. Individual factors may interact with the macroenvironment at the geographic level to affect the development of cancer. Therefore, integrating the ethnicity and geographic location may reveal how genetic and cultural factors at the individual level, interacting with ecological factors at a geographic location or community level, affect the risk of developing cancer.

9.2 Classification of Cancer

9.2.1 Classification by Histology

Classification of cancer is important in describing the pattern of cancer types by ethnicity or geographic location. In histology, the type of tissue in which cancer originates classifies cancers. The international standard for the classification and nomenclature of histology is the *International Classification of Diseases for Oncology*, 3rd Edition (ICD-O-3).[1] Overall, hundreds of different cancers can be largely grouped into five major categories: carcinoma, leukemia, lymphoma, myeloma, and sarcoma (Table 9.1).

The most common cancer is carcinoma, a malignant neoplasm of epithelial origin in the internal major subtypes of carcinoma: adenocarcinoma and squamous cell carcinoma. Adenocarcinoma forms in an organ or a gland, and generally occurs in mucus membranes; it is most prevalent in lung, prostate, pancreatic, esophageal, and colorectal cancer. Squamous cell carcinoma originates in the squamous epithelium and occurs in many areas of the body. In the United States, most carcinomas affect organs or glands with the capability of secretion, such as the lungs, the breasts, colon, prostate, or bladder.

1 http://training.seer.cancer.gov/icdo3/.

Table 9.1 Classification of cancer by histology.

Category	Subtype	Type of tissue	Type of organ
Carcinoma	Adenocarcinoma, squamous cell	Epithelial tissue	Skin, gastrointestinal, lungs, breast, colon, prostate, bladder
Sarcoma		Supportive connective tissues	Bone, tendons, cartilage, muscle, fat
Leukemia	Myelogenous lymphocytic, erythremia	White, lymphocyte, red blood, various blood cells	Bone marrow
Myeloma	Multiple myeloma	Plasma cells	Bone marrow
Lymphoma	Non-Hodgkin and Hodgkin lymphoma	Lymphocytes	Lymphocytes

Source: http://ratecalc.cancer.gov/.

Leukemias are cancers of the bone marrow, where blood cells are produced, and featured by overproduction of immature blood cells. Immature white blood cells do not perform the functions of normal mature white blood cells; therefore, the patient with leukemia is prone to infection. Immature red blood cells can cause poor blood clotting and fatigue due to anemia. Common leukemias include myelogenous leukemia, lymphocytic leukemia, and erythema. By how quickly the disease develops (chronic or acute) as well as by the type of blood cell that is affected (lymphocytes or myelocytes), leukemias are grouped into four main types: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelocytic leukemia (AML), and chronic myelocytic leukemia (CML). In addition, multiple myeloma is a malignancy or cancer of the plasma cells in bone marrow. Plasma cells are a type of white blood cells that normally secrete antibodies, which recognize and attack germs (e.g., bacteria, viruses, fungi, and protozoa). In multiple myeloma, increased numbers of plasma cells in bone marrow produce abnormal numbers of antibody-immunoglobulin proteins in the blood, which causes a variety of symptoms in the human body.

Lymphomas develop in the glands or nodes of the lymphatic system, a network of vessels, nodes, and organs including the spleen, tonsils, and thymus that purify body fluids and produce infection-fighting white blood cells, or lymphocytes. Lymphomas may also occur in specific organs such as the stomach, breast, or brain. These lymphomas are referred to as extranodal lymphomas. There are two subtypes of lymphoma: Hodgkin lymphoma and Non-Hodgkin lymphoma. Diagnostically, the presence of Reed–Sternberg cells in Hodgkin lymphoma distinguishes Hodgkin lymphoma from non-Hodgkin lymphoma. In addition, sarcoma is a rare cancer originating in supportive and connective tissues.

9.2.2 Classification by Primary Location

Cancers are also classified by primary location in the body where cancer first developed. This classification is more familiar to the public. In the United States, the most common cancers include skin, lungs, female breasts, prostate, colon and rectum, and cervix and uterus.

Skin cancer is one of the common cancers in the United States. It includes three primary types: basal cell, squamous cell, and melanoma. Basal and squamous cell cancers are derived from the epidermal layers; whereas melanomas are from the melanocytes, or pigment cells, in the deepest level of the epidermis. Basal cell and squamous cell cancers usually occur on the face, ears, or parts of the body exposed to the sunlight. These cancers are curable, especially if detected and treated early. In contrast, melanomas, which form dark moles that spread over the surface of the skin, are more lethal because they metastasize very quickly.

Lung cancer is the leading cause of cancer death in both the United States and most of the populations in the world. Generally, there are two major categories of lung cancer: non-small cell lung cancer and small cell lung cancer (www.cancer.gov). Non-small cell lung cancer accounts for about 85–90% of the diagnosis of lung cancer; it can be further divided into several subtypes such as squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, which are named for the type of cells in which cancer develops. Small cell lung cancer accounts for 10–15% of diagnosis of lung cancer, which occurs in women more often. Lung cancer is more lethal with both high incidence and mortality, and it is very difficult to detect at an early stage because the symptoms often do not appear until the disease is advanced.

Breast cancer is one of the most common cancers in women in the United States. It is estimated that about one in eight women will eventually develop breast cancer in their lifetime in the United States [6]. Risk factors associated with breast cancer include age over 50 years, familial history of breast cancer, never had children, or had their first child late. Other risk factors include psychosocial stressors, socioeconomic status, education, obesity, a high-fat diet, early menarche, and late menopause [7]. Routine breast examination is recommended as a way to have early detection of breast cancer. Women over the age of 40 years should have periodic mammograms.

Prostate cancer is one of the most prevalent cancers in older men. As men age, the prostate may enlarge and block the urethra or bladder, which may cause difficulty in urination or interfere with sexual functions. This condition is called as benign prostatic hypertrophy (BPH) [8]. While BPH is not cancerous, the symptoms are similar to those for prostate cancer. The common symptoms may include weak or interrupted flow of urine, urinating often especially at night or with some difficulty, and pain or burning during urination. Although people with family history of prostate cancer tend to have more risk of developing the

disease, environmental factors including acute prostatitis significantly increase the likelihood of having prostate cancer [9].

Colon and rectal cancers are the most common ones that affect the large intestine in both men and women. Symptoms include blood in the stool, or a change in bowel habits, such as severe constipation or diarrhea. In addition to family history [10], Western lifestyle and physical inactivity have been associated with colon cancer [11]. Cancer of the uterus is also the most common gynecologic malignancy, which usually presents with abnormal uterine bleeding. This cancer occurs often after the age of 60. Currently, the exact causes for uterine cancer are not known, but women who received pelvic radiation are more likely to develop the disease. Risk factors associated with uterine cancer may also include some health conditions such as diabetes, hypertension, obesity, and improper estrogen levels.

Because of recent advances in the genetic and molecular study of cancer, a molecular classification of cancer is under development. While cancers are normally classified by histology and primary location, molecular biology research over the past decades has revealed that diagnoses based on clinical presentation and pathology evaluation are not always accurate. A new classification of cancers, based on their underlying genetic defects, is becoming increasingly urgent [12]. These classifications may have important implications for the future discovery of biomarkers [13], which can be used for early diagnosis, targeted treatment, and evaluation of treatment efficacy.

9.3 Ethnicity and Cancer

Ethnicity refers to a population group whose members identify with each other based on common nationality or shared cultural traditions. Unlike race that refers to the concept of dividing people into populations or groups based on various sets of physical characteristics, ethnic groups share not only genetic or biological traits but also social or cultural traits. Ethnicity may have important implications for the etiological study of human diseases, such as cancers, which develop because of changes in genes that control the way cells grow and divide. A small number of cancers are caused by inherited genetic changes, but most causal and functional genetic changes occur during the course of the individual's life. Therefore, exposure to environmental risk factors may be the major cause of cancer.

9.3.1 Cancer Death and Incidence

According to the National Vital Statistics Reports by the US Center for Disease Control and Prevention in 2012, the top 10 leading causes of death are quite different across ethnic groups. Heart diseases and cancers were the first two leading causes of death in all ethnic groups. Malignant neoplasms have been the

Table 9.2 Top 10 causes of deaths by race in the United States, 2012.

	White		Black		AI/AN		API	
	Deaths	%	Deaths	%	Deaths	%	Deaths	%
All cause	2,175,178	100	295,222	100	16,527	100	56,352	100
Heart disease	514,272	23.64	7,0123	23.75	2,950	17.85	12,266	21.77
Malignant neoplasm	496,885	22.84	67,379	22.82	3,019	18.27	15,340	27.22
Chronic lower respiratory disease	131,782	6.06	9,375	3.18	708	4.28	1,624	2.88
Accident (unintentional injuries)	110,789	5.09	12,709	4.30	1,922	11.63	2,372	4.21
Cerebrovascular disease	107,964	4.96	15,894	5.38	580	3.51	4,108	7.29
Alzheimer's disease	76,590	3.52	5,451	1.85	217	1.31	1,379	2.45
Diabetes	57,806	2.66	12,983	4.40	985	5.96	2,158	3.83
Influenza and pneumonia	43,585	2.00	5,000	1.69	306	1.85	1,745	3.10
Intentional self-harm (suicide)	36,606	1.68	2,357	0.80	485	2.93	1,152	2.04
Nephritis, nephritic syndrome	36,066	1.66	8,207	2.78	295	1.78	1,054	1.87
Chronic liver disease and cirrhosis	30,747	1.41	2,791	0.95	918	5.55	523	0.93
Septicemia	28,945	1.33	5,983	2.03	255	1.54	659	1.17
Assaults(homicide)	7,838	0.36	8,241	2.79	263	1.59	348	0.62

Source: Data was extracted from the National Vital Statistics Report, Vol. 64, No. 10, 2015.

second leading cause of death in both white and black populations behind heart disease, but are the first cause of death in American Indians or Alaska natives (AIAN) and Asian or Pacific Islander (API). In 2012, cancer accounted for 27.2% of all deaths in the API population, 22.8% of the black and white populations, and 18.3% in the AIAN population (Table 9.2).

That the API population in the United States has a higher risk of death caused by cancers than any other disease is also supported by the people living in their native countries, such as in China and Japan. According to the State Statistics Bureau of China, malignant tumors are the first cause of death in China. The mortality of cancers was about 160 per 100,000 individuals in 2009, which was consistent between rural and urban areas.[2] A similar pattern is also observed in Japan. In 2012, the number of reported deaths from cancers was more than 350,000 cases,

2 Source: Earth Policy Institute from the National Bureau of Statistics of China, 2009.

75% higher than 200,000 cases reported for heart diseases as the second leading cause of death. It is likely that genetic factors, or combined with lifestyle and environmental risk, together contribute to the high risk of cancer deaths.

Cancer site-specific death is quite different as related types of cancers in the United States. According to the American Cancer Society, the total estimated number of deaths due to common cancers was 412,950 in 2015 (Table 9.3). The most common type of cancer is breast cancer, with more than 234,000 new cases expected. The next most common cancers are prostate cancer (220,800 cases) and lung cancer (221,200 cases). The estimated number of deaths is 158,040; 49,700; 40,560; 40,290, and 27,540 cases for lung, colorectal, pancreatic, breast and prostate cancer, respectively. It is estimated that lung, colorectal, breast, and prostate together account for 61.54% of new cases and 66.74% of deaths in the United States in 2015.

While incidence and mortality measure the risk of developing cancer and dying from cancer, respectively, the mortality to incidence ratio (MIR) is used to

Table 9.3 Estimated new cases and deaths of common cancers in the United States in 2015.

Cancer type	New cases	%	Deaths	%	MIR (%)
Bladder	74,000	5.65	16,000	3.87	21.62
Breast					
Female	231,840	17.69	40,290	9.76	17.38
Male	2,350	0.18	440	0.11	18.72
Colon and rectal (Combined)	132,700	10.12	49,700	12.04	37.45
Endometrial	54,870	4.19	10,170	2.46	18.53
Kidney (renal cell, renal pelvis)	61,560	4.70	14,080	3.41	22.87
Leukemia (all types)	54,270	4.14	24,450	5.92	45.05
Lung (including bronchus)	221,200	16.88	158,040	38.27	71.45
Melanoma	73,870	5.64	9,940	2.41	13.46
Non-Hodgkin lymphoma	71,850	5.48	19,790	4.79	27.54
Pancreatic	48,960	3.74	40,560	9.82	82.84
Prostate	220,800	16.85	27,540	6.67	12.47
Thyroid	62,450	4.76	1,950	0.47	3.12
Total	1,310,720	100	412,950	100	31.51

Note: Calculations were based on data sourced from *Cancer Facts and Figures 2015*, American Cancer Society, Atlanta, GA, 2015. To qualify as a common cancer for the list, the estimated annual incidence for 2015 had to be 40,000 cases or more; %, stands for the proportion of specific cancers in all cancer together.

measure severity or relative survivorship of cancer for most site-specific cancer. According to the estimate in 2015, while the incidence rate is not high, the MIR, calculated by number of deaths to new cases, is highest for pancreatic cancer (82.84%); the next is lung cancer (71.45%) and leukemia (45.05%), suggesting the relative severity of these three cancers. For some cancers, 1 minus MIR is used as a proxy of 5-year survival rate [14]. For example, 5-year survivorship of pancreatic cancer is 17.16% (i.e., 1 minus 82.84%); lung cancer is 18.55%. The incidence of lung cancer is the highest along with prostate and breast cancer, and lung cancer deaths account for 38% of the total number of common cancer deaths in the United States.

Variation in cancer incidence by race and sex is evident in the United States. Thus, from 1999 to 2012, considering all cancers combined, men were more likely to develop cancer than women, largely due to the prevalence of smoking (http://www.cdc.gov/cancer/dcpc/data/race.htm). However, the incidence of cancer in men has evidently declined during that period. This pattern is quite different when ethnicity is considered. In men, the black population has the highest incidence rate; next are the white and Hispanic populations, and the API and AI/AN populations have the lowest incidence rates of cancer. While incidence rates have a slower decline in women than men during the same time period, the racial or ethnic differences were still evident. In contrast, white women tend to have a higher risk of developing cancer than any other ethnicity. The evident decline of cancer incidence in men could be due to effective control of tobacco usage [15].

9.3.2 Site-Specific Cancer Incidence

The site-specific cancer incidence is different by ethnicity in the United States. Based on calculations using the combined data from 2008 to 2012, the risk of developing prostate cancer is highest in the US population. The incidence of prostate cancer was 131.8 cases per 100,000 individuals for all races, and 204.9 cases for the black population (Table 9.4), which has two- to threefold high risk as the API and AI/AN population. The next highest risk was for breast, lung, and colorectal. Although there is a slightly different pattern of cancer incidence by ethnicity, the four types of cancer consistently present with a high cancer risk for all ethnic groups in the United States.

While the incidences of other cancers such as stomach and liver cancer are moderately high, there are large disparities by ethnicity in the United States. Stomach cancer is more prevalent in non-white populations. The incidences of stomach cancer are 10.7, 11.1, and 10.2 cases per 100,000 individuals in the black, API, and Hispanic populations, respectively, all of which are almost doubled compared to 5.9 cases per 100,000 individuals in the white population. This is consistent with the prevalence of *Helicobacter pylori* infection that is believed to cause the high risk of stomach cancer. *H. pylori* is a type of bacteria that may cause stomach cancer and lymphoma of the stomach lining. It can also cause stomach

Table 9.4 Age-adjusted cancer incidence by primary site and ethnicity in the United States, 2008–2012.

Cancer sites	All races	White	Black	API	AI/AN	Hispanics
All cancer sites combined	462.0	462.3	472.5	297.5	287.7	361.4
All cancer (comparable to ICD-O-2)	454.4	454.6	466.5	292.1	283.2	355.2
Oral cavity and pharynx	11.3	11.5	9.4	7.6	6.8	7.2
Esophagus	4.7	4.8	4.7	2.2	3.1	3.0
Stomach	6.6	5.9	10.7	11.1	6.1	10.2
Liver and intrahepatic bile duct	7.4	6.6	9.8	13.6	9.3	12.8
Colon and rectum	41.9	40.9	49.7	33.5	31.0	36.8
Pancreas	12.3	12.0	15.4	9.2	7.6	11.1
Lung and bronchus	63.7	64.4	66.8	36.3	44.8	33.4
Melanomas of the skin	19.9	22.6	1.0	1.3	4.6	4.2
Male and female breast	66.3	66.3	69.6	48.5	36.4	49.7
Cervix	7.7	7.5	9.8	6.3	6.7	10.2
Corpus and uterus, NOS	25.4	25.8	24.2	17.6	16.2	21.2
Corpus	24.5	25.0	22.6	17.1	15.5	20.3
Ovary	11.8	12.3	9.4	9.0	8.7	10.6
Prostate	131.8	121.7	204.9	67.7	70.7	112.3
Testis	5.5	6.4	1.4	1.8	3.2	4.4
Urinary bladder	20.8	22.1	11.7	8.7	8.6	11.5
Kidney and renal pelvis	16.0	16.1	17.3	7.5	15.8	15.8
Brain	6.2	6.7	3.7	3.3	3.1	4.8
Thyroid	13.6	14.2	8.5	13.8	6.4	12.3
Lymphomas	22.0	22.7	16.7	14.2	12.4	19.8
Non-Hodgkin lymphoma	19.2	19.8	14.0	12.9	11.3	17.4
Myeloma	6.2	5.6	12.4	3.6	4.1	6.1
Leukemias	13.3	13.7	10.3	7.7	7.6	10.7
Miscellaneous	17.3	17.3	16.7	10.9	13.2	14.9

Note: Combined data from 2008–2012 per year, rates are per 100,000 persons and are age-adjusted to the 2000 US standard population. https://nccd.cdc.gov/uscs/cancersbyraceandethnicity.aspx.

ulcers. In the United States, *H. pylori* infection is more prevalent in Hispanics, African Americans, and the elderly. The prevalence is 60% in Hispanics, 54% in African Americans, but only 20% in the white [16]. This pattern of *H. pylori* infection by ethnicity is similar between men and women. The infection of

H. pylori is also prevalent in the developing countries such as China. For instance, in some areas of Jiangsu province with a high risk of gastric cancer, *H pylori* infection reaches 62% among adult people [17]. The *H pylori* infection has a strong link to stomach cancer in other countries such as Japan. In contrast, the high prevalence of *H. pylori* infection has been reported in countries such as India and Bangladesh, but low gastric cancer rates are observed [18]. *H. pylori* infection, commonly seen in patients with gastric cancer, is likely interacting with concomitant environmental factors such as high salt, preservatives, and smoked foods that may contribute to gastric cancer [19–21].

Liver cancer is more common in the API and Hispanic populations than other ethnic groups. In the United States, the incidences of liver and intrahepatic bile duct cancer are 13.6 and 12.8 per 100,000 people in the API and Hispanics, respectively. About 50% of liver cancer is hepatitis B virus related, and Asian Americans have the highest rate of hepatitis B virus infection [22]. Hepatitis B virus infection increased the risk of developing liver cancer more in men than women [23]. Studies have shown that hepatitis C virus infection also causes an increased risk of liver cancer as well as non-Hodgkin lymphoma [24], and its association with hepatocellular carcinoma (HCC)-related mortality was observed in the Hispanic population [25].

Myeloma and melanoma are also more likely associated with specific ethnic groups. The black population has a higher risk of myeloma, and the incidence of 12.4 cases per 100,000 individuals is two- to threefold high as those in all other ethnic groups. The white population has the highest risk of melanomas of the skin. The incidence of skin melanomas in the white was about 22.6 cases per 100,000 individuals, which is moderately high among all cancers in the white population, but fourfold higher than the AI/AN and Hispanic populations, and around 20-fold higher than black and API populations. This difference in skin cancer incidence by ethnicity is probably attributable to genetic factors, or specific gene–environment interactions.

9.3.3 Site-Specific Cancer Incidence between the United States and China

Comparing the incidence of site-specific cancer by ethnicity with that in people of their native origins is an important approach in immigrant epidemiology to assess the role of genetic and environmental factors in the development of cancer at the population level. Among the four most common cancers (e.g., prostate, breast, lung, and colorectal) for all races in the US population, prostate and breast cancer were also common in African countries, based on the estimated new cases in 2008.[3] However, lung cancer is the fifth most common in men, but not in the top 10 cancers in women in Africa, suggesting environmental factors may play some roles in the development of lung cancer.

3 American Cancer Society (2011) *Cancer in Africa*, American Cancer Society, Atlanta.

The pattern of common cancers in the Asian countries is quite different from that in the United States. For example, except for lung cancer, gastrointestinal cancers such as stomach, liver, and esophagus are major forms of cancer in men of China [26]. According to the estimates in 2012, the age-standardized incidences by world population (ASRW) were 52.9, 33.7, 32.8, and 18.6 cases per 100,000 individuals for lung, liver, stomach, and esophagus cancer, respectively, in China (Table 9.5). The incidences of all three gastrointestinal cancers

Table 9.5 Estimated cancer incidence (2012): China and the United States, male, all ages (crude rate and age standardized rate of incidence by world population (ASRW), per 100,000 individuals).

	China			The United States			
Cancer	**Numbers**	**Crude**	**ASRW**	**Numbers**	**Crude**	**ASRW**	**China/USA**
Male							
All cancers	1,822,769	258	211.2	824,698	528.6	347	0.61
Lung	459,495	65.0	52.8	112,054	71.8	44.2	1.19
Liver	293,318	41.5	33.7	22,541	14.4	9.8	3.44
Stomach	283,487	40.1	32.8	13,149	8.4	5.3	6.19
Esophagus	160,436	22.7	18.6	13,467	8.6	5.5	3.38
Colorectum	146,528	20.7	16.9	69,045	44.3	28.5	0.59
Prostate	46,745	6.6	5.3	33,159	149.5	98.2	0.05
Kidney	44,372	6.3	5.1	36,345	23.3	15.9	0.32
Bladder	41,993	5.9	4.8	52,099	33.4	19.6	0.24
Pancreas	39,299	5.6	4.5	21,713	13.9	8.6	0.52
Leukemia	38,394	5.4	5	22,433	14.4	10.3	0.49
Brain, nervous system	34,611	4.9	4.2	11,897	7.6	6.1	0.69
Non-Hodgkin lymphoma	26,097	3.7	3.1	34,286	22	14.7	0.21
Gallbladder	23,764	3.4	2.7	4,181	2.7	1.6	1.69
Nasopharynx	23,581	3.3	2.7	1,431	0.9	0.7	3.86
Larynx	18,308	2.6	2.1	9,803	6.3	4.1	0.51
Lip, oral cavity	13,656	1.9	1.6	17,325	11.1	7.5	0.21
Thyroid	11,269	1.6	1.3	13,142	8.4	6.4	0.20
Multiple myeloma	6,794	1.0	0.8	10,780	6.9	4.3	0.19
Other pharynx	5,444	0.8	0.6	9,584	6.1	4.3	0.14
Melanoma of skin	5,312	0.8	0.6	40,078	25.7	16.8	0.04
Testis	2,627	0.4	0.3	8,073	5.2	5.0	0.06

(*continued*)

Table 9.5 (*Continued*)

Cancer	China			The United States			China/USA
	Numbers	Crude	ASRW	Numbers	Crude	ASRW	
Hodgkin lymphoma	1,243	0.2	0.2	4,804	3.1	2.8	0.07
Kaposi sarcoma	155	0	0	973	0.6	0.5	0.00
Female							
All cancers	1,242,669	189.8	139.9	778,888	487.4	297.4	0.47
Lung	193,347	29.5	20.4	102,172	63.9	33.7	0.61
Breast	187,213	28.6	22.1	232,714	145.6	92.9	0.24
Stomach	121,509	18.6	13.1	8,006	5	2.7	4.85
Colorectum	106,899	16.3	11.6	65,304	40.9	22.0	0.53
Liver	101,452	15.5	10.9	7,908	4.9	2.8	3.89
Corpus uteri	73,188	11.2	8.6	49,645	31.1	19.5	0.44
Esophagus	62,870	9.6	6.7	3,501	2.2	1.1	6.09
Cervix uteri	61,691	9.4	7.5	12,966	8.1	6.6	1.14
Thyroid	35,092	5.4	4.4	38,984	24.4	20	0.22
Ovary	34,575	5.3	4.1	20,874	13.1	8	0.51
Brain, nervous system	31,016	4.7	3.7	9,714	6.1	4.6	0.80
Gallbladder	27,699	4.2	2.9	5,250	3.3	1.6	1.81
Leukemia	27,384	4.2	3.7	17,225	10.8	7.1	0.52
Pancreas	26,428	4.0	2.8	21,172	13.3	6.5	0.43
Kidney	22,094	3.4	2.5	21,877	13.7	8.5	0.29
Non-Hodgkin lymphoma	16,502	2.5	2	28,780	18	10.2	0.20
Bladder	13,493	2.1	1.4	16,540	10.4	5.1	0.27
Nasopharynx	9,617	1.5	1.1	599	0.4	0.3	3.67
Lip, oral cavity	7,757	1.2	0.9	8,739	5.5	3.2	0.28
Melanoma of skin	4,502	0.7	0.5	29,031	18.2	12.6	0.04
Multiple myeloma	4,101	0.6	0.5	8,846	5.5	3.0	0.17
Larynx	1,706	0.3	0.2	2,570	1.6	1.0	0.20
Other pharynx	1,080	0.2	0.1	2,515	1.6	1.0	0.10
Hodgkin lymphoma	858	0.1	0.1	3,797	2.4	2.2	0.05
Kaposi sarcoma	84	0	0	154	0.1	0.1	0.00

Source: Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2013) Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11, GLOBOCAN 2012 v1.0, International Agency for Research on Cancer, Lyon, France. Available at http://globocan.iarc.fr (accessed July 20, 2016).

in men in China were three- to sixfold as high as that in the United States, and even more than twofold higher than in the Asian Americans of the United States. This suggests that the development of gastrointestinal tract cancer in China is more likely due to exposure to risk factors in the local living environment. It is interesting to note that ASRW of lung cancer in men were very similar between the United States (44.2/100,000) and China (52.9/100,000). In addition, there were huge differences in ASRWs for several other cancers such as melanoma, multiple myeloma, testis, and lymphoma, for which the ASRWs were notably lower in China.

Breast and lung cancer are also common in women of China, but the ASRWs are much lower than that in the United States. The ASRWs are 22.1 and 20.4 cases per 100,000 individuals, respectively, in China, but 92.9 and 37.2 cases per 100,000 individuals for breast and lung cancer, respectively, in the United States. The ASRW of breast cancer in the United States is fourfold that seen in China. This may be partly due to the higher smoking rate in women in the United States, 1 of 6 women smoke, which is much higher than 1 of 25 women in China [27]. The next most common are gastrointestinal cancers such as stomach, liver, and colorectal in China, where the incidences are lower than the United States except for colorectal cancer, which is twofold as common as in China. In contrast, melanoma, lymphoma, and thyroid are the next most common cancers in women in the United States. This different pattern of incidence in women between China and the United States may provide an insight into the etiology of site-specific cancer.

9.4 Geographic Location and Cancer

9.4.1 Mapping Human Diseases to Geographic Location

The development of human disease is mostly an outcome of host–environment interactions. Hosts are individuals with a variety of genetic makeups that determine how they respond to different environmental exposures, which may include biological agents, the physical environment, psychosocial stress, social support, and social network. For example, biological agents include bacterial and viral infections that are transmitted through person to person contact or via other vectors; the physical environment may be involved with exposures to specific factors such as cigarette smoking, alcohol use, or even physical exercise, dietary intake, and behaviors, or due to water or air pollution or radiation. All these factors can be measured at the individual level. However, some unobserved or unobservable factors in geographic location or even in community or neighborhood may affect the risk of cancer or through interaction with individual risk factors or genetic variants.

Human populations tend to live aggregated as a community by geographic location. Individuals who live in the same geographic area or community may

share some common exposures to the ecological or contextual environment. Some of those may be associated with the risk of human disease, but may not easily be measured or even observable. Mapping human disease to geographic location can help to reveal obscured relationships between disease and possibly environmental factors that are hardly presented in individual data. The use of geographic mapping to human diseases dates back to more than 200 years ago, with the terrifying epidemic diseases of the eighteenth and nineteenth centuries. This approach is regarded as the founding event of modern epidemiology [28]. One of the first and most famous disease maps was created by Dr. John Snow, an English physician (March 15, 1813–June 16, 1858), who mapped the death of cholera to the water pumps in London. Although chemical and microscope examination did not find specific hazardous agents in the water sample from the Broad Street well pump, his studies of the pattern of the disease were convincing enough to persuade the local government to disable the well pump. This action led to the ending of the cholera outbreak in London.

Mapping the distribution of disease and health problems to a broad geographic location has been considered a part of medical geology, an emerging interdisciplinary subject that studies the relationship between natural geological factors and their effects on human and animal health [29,30]. It is widely known that our environment affects people in many ways, and it is sometimes said that you are what you eat and drink. Approximately one-third of cancer deaths that occur in the United States each year are due to poor nutrition and physical inactivity, including excess weight (www.cancer.org). For example, one of the most prevalent geochemical diseases is iodine deficiency. More than one billion of people around the world are exposed to iodine deficiency [31], and insufficient intake is the most common cause of mental retardation and brain damage. One of the main risk factors for cardiovascular diseases has been linked to water hardness, which means that magnesium and calcium in the water may play a role. Other factors such as radiation, imbalance of intake trace elements in dietary, inefficiency or excess of fluoride, and an excess amount of potassium, calcium, and phosphorus may also affect the development of human disorders [32].

9.4.2 Geographic Variation and Cancer in the United States

Modern researchers have successfully used the examination of geographic patterns in identifying etiological factors for cancer. Mapping of cancer mortality in the United States from 1950 to 1969 found very high rates of lung cancer along the eastern seaboard, particularly in parts of Georgia, Florida, and Louisiana [33]. These patterns had led to further investigation and several studies found that occupational exposure to asbestos accounted for a significant part of the excess mortality from lung cancer [34,35]. Similarly, based on the data from the National Survey on Cause of Death from 1973–1975, Li *et al.* mapped the mortality of common cancers such as stomach, esophagus, liver,

cervix, lung, colon and rectum, breast, nasopharynx, and leukemia as well as five less common cancers – brain tumors, malignant lymphoma, and so on in 2392 counties across China. The map suggested that lung cancer mortality in Xuanwei County, Yunnan province, China, was "exceptionally" high in non-smoker females [36]. This led to the discovery that the use of smoky coal for cooking in the region is a risk factor for lung cancer in women, and necessary intervention has further confirmed the use of smoky coal as the etiologic factor of lung cancer.

Geographic variations in a variety of cancers have been observed in the United States. Cancer mortality varies greatly by state (Figure 9.1). In all cancers

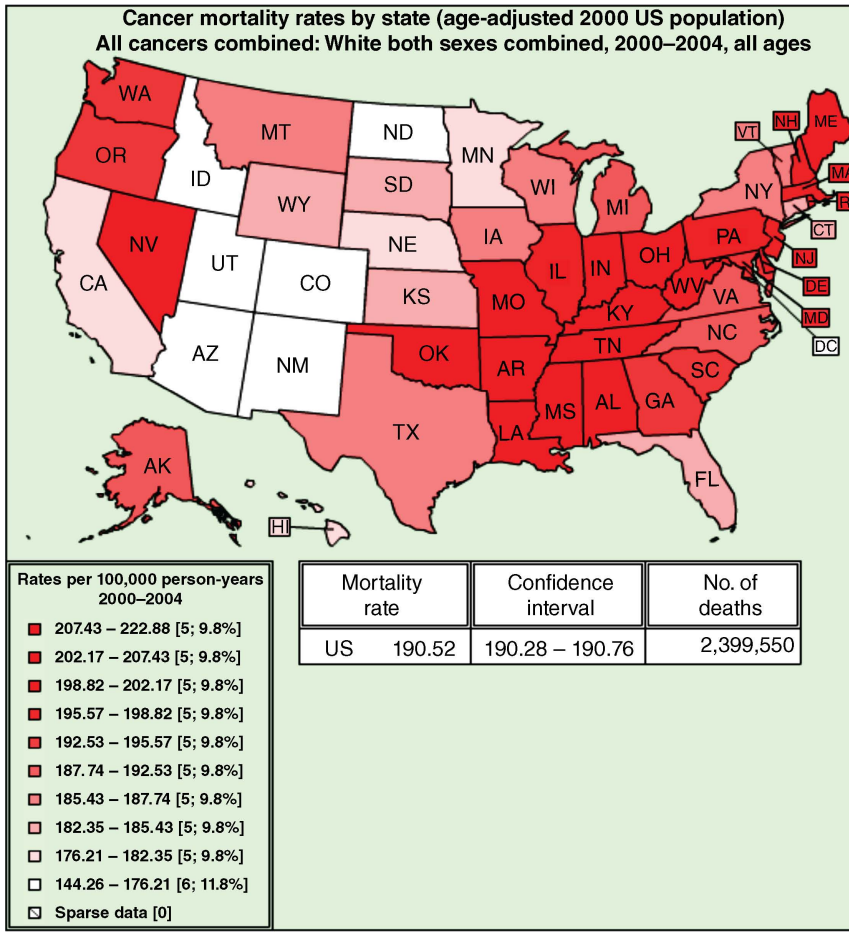

Figure 9.1 Geographic variation in all cancer mortality in the United States from 1970 to 2004.

combined from 2000 to 2004, the white population in the Appalachian Mountains, the northeast region, and the state of Nevada tended to have an elevated risk of cancer mortality, which reaches 200 deaths per 100,000 individuals. In contrast, the Rocky Mountain States and the southwest region of the United States, including Arizona, Utah, Colorado, New Mexico, and Idaho, have the lowest mortality rates. A similar pattern of distribution was observed in the black population, but with an even higher mortality rate. Examination of geographic variation in cancer mortality allows us to discover associations of ecological factors with cancer mortality at a geographic level. Using multivariate ecological analysis at a state level, one study found that solar ultraviolet-B was associated with reduced risk of cancer, which provides further support for that photosynthesis of vitamin D is inversely associated with cancer mortality [37]. Unfortunately, there were no data available on vitamin D supplementation in this study.

9.5 Ethnicity, Geographic Location, and Lung Cancer

9.5.1 Ethnic Differences

Lung cancer is the second-most commonly diagnosed cancer and the leading cause of cancer-related death in both men and women in the United States. Lung cancer affects some races more than others. Blacks have higher incidence and mortality rates than do whites; blacks and whites have much higher incidence and mortality than do other races. According to the US Center for Disease Control and Prevention, the year 2000 US population age-adjusted incidence of lung cancer was 60.4 per 100,000 individuals in 2012. The highest incidence is observed among blacks (63.3), followed by whites (61.0), AI/ANs (40.1), and APIs (35), and the lowest in Hispanics (31.2). Hispanics had much lower lung cancer incidence (37.3) than non-Hispanics (71.9). This trend was consistent in men among all age groups. It is noted that black women have a lower incidence of lung cancer than do the white women after the age of 60 years old.

The incidence of lung cancer has consistently declined over the past decade in the United States. From 2003 to 2012 (http://www.cdc.gov/cancer/lung/statistics/trends.htm), the incidence decreased significantly by 2.5% per year in men, but by 2.8%, 3.1%, 2.6%, and 1.8% in men of the black, Hispanic, AI/AN, and API, respectively. The incidence of lung cancer decreased slower in women than in men, but still significantly by 0.9% per year, 1.0 and 1.3% per year in black and Hispanic women, respectively. Smoking has been proved as the most important risk factor for lung cancer. The decline in the incidence of lung cancer is more likely due to antismoking over past decades in the United States [38,39], and is consistent with the decline of smoking rate.

9.5.2 Geographic Variation

Geographic variation in the incidence of lung cancer is evident in the United States. According to the combined data from 2009 to 2013, the higher incidence was observed in white men in the state of Kentucky (118), Mississippi (103), West Virginia (101), Arkansas (99.6), Alabama (95), and Tennessee (97), all of which are located in or near the Appalachian Mountains (Table 9.6); the incidence of lung cancer in white women has similar geographic variation. In black men, almost all these states except West Virginia in or near the Appalachian Mountains, along with Mid-Western states such as Wisconsin (118), Nebraska (106), Indiana, Iowa, Michigan, Pennsylvania, Kansas, and Ohio, have a higher incidence of lung cancer. However, black women appear to have a higher incidence of lung cancer in the states of the Mid-West region than the Appalachian Mountains.

People who live near or close to the area of the Rocky Mountains appear to have a lower risk of lung cancer. Among the 50 states of the United States, the lowest incidence of lung cancer in white men was observed in Utah (34.3), New Mexico (51.4), Colorado (52.6), Wyoming (56.0), Idaho (59.1), and Arizona (60.1). In addition, there was a lower incidence rate in California (55.7), where the mountain range of Sierra Nevada crosses the state of California. These states have lower incidence consistently in both men and women, and in both white and black populations. These might be due to more exposure to sunlight or other ecological factors associated with high altitude, which might also result from the difference in the geographic distribution of air and water pollution.

Tobacco consumption may contribute to geographic variation in lung cancer. According to Behavioral Risk Factor Data: Tobacco Use in 2011 or later in the United States, five states (Kentucky, Mississippi, West Virginia, Arkansas, and Tennessee) with the highest incidence of lung cancer, also have the highest smoking rates, which are about 24–27.3% among adults. Studies have found that living at high altitude, a factor second to cigarette smoking, is strongly associated with a lower incidence of lung cancer, even after controlling for exposure to sunlight and fine particulate matter [40,41]. It is likely that altitude may be a measure for some other ecological factors that could explain the geographic variation in the incidence of lung cancer in the United States.

9.5.3 Individual Risk Factors

Smoking is a known major cause of lung cancer. It accounts for about 79% of lung cancer deaths in men and 25% of deaths in women in the less developed countries, but 93% of lung cancer deaths in men and 71% in women in the industrialized countries [42]. Tobacco use also explains the global pattern and trends of lung cancer. In several western countries such as the United Kingdom, Canada, and Australia, tobacco use reached a peak in the middle of the

Table 9.6 Geographic variation in lung cancer incidence by race and sex in 2009–2013 in the United States (age-adjusted to the 2000 US standard population).

	Men				Women			
Geographic area	**All**	**White**	**Black**	**Hispanic**	**All**	**White**	**Black**	**Hispanic**
United States	74.6	74.2	88.6	42.3	53.3	54.9	50.0	25.8
Northeast	73.9	74.7	75.5	49.3	56.6	59.0	48.3	30.8
Connecticut	70.9	70.7	75.7	63.8	57.7	59.3	46.8	42.4
Maine	86	86.7	—	—	66.1	66.2	—	—
Massachusetts	72.7	73.4	66.6	—	61.9	64.2	39.9	—
New Hampshire	73.5	73.9	—	61.3	64.4	64.8	—	73.6
Rhode Island	78.3	79.6	65.1	44.2	64.0	65.2	49.6	23.6
Vermont	74.2	74.0	—	—	61.2	61.1	—	—
New Jersey	67.7	69.0	74.4	46.8	53.1	56.1	47.6	29.2
New York	72.0	74.1	67.2	48.3	54.7	59.5	41.3	29.6
Pennsylvania	80.0	78.6	101.7	—	56.5	55.6	70.6	—
Midwest	80.0	78.6	100.6	41.3	57.8	57.4	64.5	28.9
Illinois	81.0	80.0	99.7	37.3	58.5	59.1	64.2	26.8
Indiana	91.1	90.9	102.9	41.9	61.7	62.0	63	30.1
Michigan	78.9	76.4	100.3	57.6	59.1	58.4	63.2	36.6
Ohio	85.6	84.6	98.4	38.3	59.7	59.7	63.9	24.1
Wisconsin	70.3	67.7	118.5	41.9	54.5	53.0	73.9	35.1
Iowa	80.2	80.0	102.3	34.8	52.7	52.7	74.1	27.7
Kansas	73.6	72.1	100.0	42.6	53.5	52.8	65.2	30.8
Minnesota	62.9	62.0	78.6	54.2	50.1	49.9	49.5	25.4
Missouri	90.8	89.6	107.1	43.7	64.7	64.5	69.3	32.2
Nebraska	70.4	69.5	106.5	36.2	49.9	49.5	64.2	33.3
North Dakota	69.8	68.3	—	—	47.5	45.7	—	—
South Dakota	67.4	65.7	—	—	50.9	49.5	—	—
South	82.7	81.9	92.0	46.4	54.5	56.6	46.2	24.5
Delaware	83.6	84.4	82.3	—	62.3	64.5	56.0	—
District of Columbia	72.0	38.6	95.3	—	49.7	32.7	58.3	21.6
Florida	73.6	74.4	68.3	55.3	54.4	57.1	35.8	27.5
Georgia	86.7	88.2	86.5	43.1	53.2	58.2	41.7	25.9
Maryland	67.9	67.8	74.2	29.0	52.9	56.4	48.0	21.7
North Carolina	90.5	88.6	100.7	32.9	55.9	58.3	47.0	29.7

Table 9.6 (*Continued*)

Geographic area	Men				Women			
	All	White	Black	Hispanic	All	White	Black	Hispanic
South Carolina	87.8	86.7	92.6	34.2	54.3	58.1	43.2	33.7
Virginia	75.2	73.9	91.2	—	52.2	54.4	48.8	—
West Virginia	101.0	101.4	93.1	—	65.9	66.5	46.6	—
Alabama	95.3	94.5	100.5	29.4	53.4	57.7	38.5	19.1
Kentucky	118.3	118.7	123.9	—	80.2	80.7	81.8	—
Mississippi	103.1	98.9	114.6	28.3	56.5	60.6	48.1	22.9
Tennessee	97.6	96.8	108.0	34.7	61.2	62.9	52.7	22.2
Arkansas	99.6	97.7	114.2	93.8	59.4	61.0	47.6	65.6
Louisiana	92.2	86.9	110.6	46.6	55.5	57.8	50.8	31.1
Oklahoma	87.7	84.7	101.7	46.4	58.5	56.0	60.5	37.7
Texas	70.1	68.8	93.7	42.8	45.5	45.6	50.4	21.9
West								
Arizona	58.0	59.0	61.5	39.6	46.4	47.5	46.5	26.2
Colorado	49.8	49.5	59.5	43.1	42.4	42.6	46.8	35.1
Idaho	56.4	56.2	—	44.3	46.9	47.0	—	25.9
Montana	62.6	60.6	—	73.6	54.9	53.2	—	60.5
New Mexico	48.4	50.0	70.9	40.7	36.8	39.1	28.7	29.9
Utah	34.4	34.1	54.1	34.5	24.2	24.0	—	34.2
Wyoming	52.6	53.1	—	39.9	43.1	43.7	—	27.9
Alaska	71.6	63.5	102.5	53.8	55.6	53.7	—	—
California	53.6	53.4	72.9	33.2	41.2	43.3	48.9	22.2
Hawaii	58.0	53.6	59.6	78.6	38.7	44.8	—	49.4
Oregon	65.2	64.5	84.5	39.6	54.9	55.1	58.6	43.9
Washington	67.1	67.5	75.7	40.6	54.7	55.9	51.7	30.1

Source: US Cancer Statistics Working Group (2016) *United States Cancer Statistics: 1999–2013* Incidence and Mortality Web-Based Report, US Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute, Atlanta, GA. Available at www.cdc.gov/uscs.

twentieth century, lung cancer has declined in men and reached a plateau in women [43]. In the less developed countries such as China, however, tobacco use has just reached a peak or continues to rise. It is likely that lung cancer rates will continue to rise over the next several decades in China. Tobacco use may be

a consensus factor that explains the high rate of lung cancer across the continents, and in both the more developed and the less developed countries, although other factors may also contribute to the regional variation.

Physical environmental factors have been indicated in assessing risk for lung cancer. Low selenium in the serum is detected in patients with lung cancer [44,45]. Some environmental substances or exposures such as radon can increase the risk of lung cancer. Radon is an odorless gas released by some soils and rocks that contain uranium; some houses may have high levels of radon, especially on the lower levels, when they are built on soil that naturally contains radon. Residential exposure to radioactive radon and its decay products has been estimated to account for 10–12% of all lung cancer deaths in the United States, and even higher in the nonsmoking population [46]. Industrial substances, including arsenic, uranium, beryllium, vinyl chloride, nickel chromates, chromium, formaldehyde, coal products, gasoline, and diesel exhaust, may increase the risk of lung cancer [47]. In addition, air pollution such as particulate matter has been evidenced as a risk factor for lung cancer in several different countries [48,49].

Socioeconomic status and lifestyle have been associated with risk of lung cancer. A large cohort study in Canada has indicated an inverse risk between lung cancer incidence and educational attainment, income and occupation in both men and women [47]. Neighborhood deprivation, which is measured by the variables education, income, unemployment, and welfare assistance, is associated with lung cancer incidence and mortality in Sweden even after controlling for individual characteristics such as age, marital status, family income, education, immigration status, urban or rural residential status, mobility, and comorbidities [50]. Dietary and other lifestyle factors have also been associated with lung cancer [51].

9.6 Common Cancers in China

China has a different pattern of cancer from Western countries such as the United States. The overall difference in the incidence of cancers between the United States and China has been described above. In addition to ethnicity, the difference in the pattern of incidence and mortality is probably due to its vast variation in geography, high population density, and diverse culture involving both dietary behaviors and lifestyle. According to the national estimates of mortality in 2011, several gastrointestinal cancers such as liver (39.27/100,000), stomach (22.06/100,000), and esophagus (16.25/100,000) cancers, individually just rank behind lung cancer (48.32/100,000) as major causes of death in China [52]. While mortality and incidence of major cancers such as lung, prostate, female breast cancer, and colorectal cancer have started declining in the United States, they are increasing in China or even surpassing the

United States due to a fast economic transition and the adoption of a Western-like lifestyle involving high calorie-food intake and inactivity.

High incidences and mortality from gastrointestinal cancers are also common in other less developed countries. Gastrointestinal cancer is a term for cancers that affect the digestive system; it includes cancers of the esophagus, gallbladder, liver, pancreas, stomach, small intestine, bowel (large intestine or colon and rectum), and anus.

Three common gastrointestinal cancers show a great geographic variation in incidence and mortality in China. According to the 1973–1975 National Survey on Causes of Death, the mortality by esophageal cancer is high in Henan and Hebei provinces along the Taihang Mountains, located in the central northern part of China. Gastric cancer is prevalent in the regions from the less developed northwest (Gansu and Qinghai provinces) to the most developed east of China (Jiangsu province and Shanghai). Liver cancer is mostly prevalent in the areas from the more developed east and southeast coast (Jiangsu, Zhejiang, Fujian, Guangdong), to the northeast (Jilin province) and to the less developed Guangxi province in China. This suggests that from ecological perspectives, there are various risk factors associated with these common cancers in China.

Ecological factors have been associated with risks of multiple types of cancers. An epidemiological study in 24 regions of eight provinces has revealed that higher selenium levels in blood are significantly associated with lower level mortality of stomach and esophagus cancer in both men and women [53]. A study conducted in Linxian County, an area with high risk of esophageal cancer in China [54], confirmed this finding. An inverse correlation between regional liver cancer incidence and selenium contents in both blood and grains was observed in Qidong County, an area having the highest risk of hepatoma in China [55].

9.6.1 Liver Cancer

9.6.1.1 Geographic Variation

Liver cancer is highly clustered by geographic location in China. It is prevalent in the areas from the northeast of China (Jilin province) to the more developed east coast (Jiangsu, Zhejiang, Fujian, and Guangdong province) and to the less developed Guangxi autonomous region in the south of China. Most of them are along the east coast of China. While the specific hazardous agent is not clear, drinking water from polluted sources is one of the suspected risks for developing liver cancer in a study conducted in the high-risk Qidong County in Jiangsu province, the most industrially developed province in China [56]. Intervention through changing the drinking water supply from river to deep-well sources greatly reduced the incidence of liver cancer in Qidong. Long-term inorganic arsenic (iAs) exposure through drinking water may also be associated with the mortality risk of liver cancer [57].

The fact that liver cancer incidence among Asian Americans is lower than the residents of Asia is compatible with the substantial variation in the prevalence of etiologic factors such as hepatitis-B infection. In China, however, the incidence of hepatitis B virus (HBV) infection varies with geographic location. According to the reported estimates from 2005 to 2010, the lowest incidence of HBV infection was observed in Tibet (14.6 per 100,000) and Jiangsu (21.3 per 100,000); the highest incidence of HBV infection was observed in Qinghai (283.0 per 100,000) and Gansu (225.7 per 100,000). The fact that Jiangsu along with other provinces such as Jilin and Zhejiang have the lowest incidence of HBV infection [58], but with a high incidence of liver cancer, suggests ecologically that HBV infection may not account much for the geographic variation in liver cancer in China, at least in the east coast of China.

9.6.1.2 Urban Residence and Sex

Liver cancer is observed with distinct disparities of residence and sex in China. The incidence of liver cancer in rural areas is higher [59], although overall cancer incidence is higher in the urban areas [29]. This might be associated with water supply and socioeconomic status. One study conducted in 24 townships in Haimen City of Zhejiang province, the highest risk area in China, found that lower income areas tend to have a higher incidence of liver cancer [60]. However, one should note that liver cancer is more prevalent in the east coast where the more developed areas in China are located. The incidence of liver cancer is 38.32 cases per 100,000 individuals in men, which is almost threefold high as women (13.85/100,000). This is probably due to prevalent alcohol use and cigarette smoking in men.

9.6.1.3 Hepatitis B Virus Infection

Multiple studies have found that HBV infection, eating raw fish, alcohol consumption, and their interaction affect the development of primary liver cancer [61,62]. High HBV and liver cancer tend to coexist in the Asian countries, including China. In one of the high-risk areas, Qidong County also has a high prevalence of hepatitis B infection. HBV infection is regarded as one of high-risk factors for liver cancer. Dietary supplement with selenium reduces not only the HBV infection but also the development of liver cancer in patients with HBV infection [63]. This might suggest that HBV and liver cancer are comorbidities. Men who are infected with HBV are more likely associated with high mortality due to liver cancer [23]. In general population, hepatitis B surface antigen (HBsAg) prevalence in China was 9.75% in 1992, but it declined to 7.18% in 2006 due to the administration of universal HBV vaccination in infants in China. The major HBV genotypes B and C are more prevalent in the southern and northern parts of China [64].

While ecological factors such as culture and water supply and HBV infection have been associated with risk of liver cancer, some individual lifestyle factors

may affect the risk of liver cancer. Aflatoxin consumption [65], alcohol use, and cigarette smoking [66] are generally believed to be risk factors for liver cancer. In contrast, green tea consumption and vitamin E intake reduce the risk of liver cancer in China [67]. Poor socioeconomic status is associated with high mortality of liver cancer in Korean men [68]. While liver cancer is more prevalent in the most developed provinces along the east coast of China, lower income communities are associated with higher risk of liver cancer within the high-risk areas [60].

9.6.1.4 Familial Aggregation and Genetic Variants

Hepatocellular carcinomas have familial forms in different populations, including the Chinese [69]. Family history is associated with risk of liver cancer. A large prospective cohort study showed that individuals whose mother had liver cancer are more likely to develop liver cancer in women (RR = 7.42) than men (RR = 4.48). This is opposite to the sex bias in the general population, that is both mortality and incidence of liver cancer in men is threefold higher than in women. A meta-analysis of 26 case–control or cohort studies also showed consistent evidence for family history associated with increased risk of liver cancer [70].

Genome-wide association studies have identified common genetic variants associated with HCC. Using patients with both HBV infections-related HCC as cases and individuals with HBV infections only as controls, a genome-wide association study found an intronic SNP rs17401966 at *KIF1B* on 1p36.22 is associated with HCC, and suggested associations of *UBE4B* and *PGD* with HCC [71]. The association of *KIF1B* with HCC was not consistently replicated [72], and a meta-analysis showed that rs17401966 at *KIF1B* was not associated with HCC in females. Loci 6p21.3 and 21q21.3 are also indicated for HCC in the Han Chinese population [73]; common genetic variants at *MICA* and *DEPDC5* are associated with hepatitis C virus-related HCC in other populations [74,75]. In addition, candidate gene association studies found that common genetic variants, *CYP1B1* rs1056836 [76], 1p34.2 rs621559 and 14q21 rs398652 [77], *ADIPOQ* rs1501299 [78], *EZH2* rs6950683 and rs3757441 [79], and c.1161G>A and c.1779C>G variants at *XRCC1* [80] were associated with HCC, but these findings need to be further validated in additional independent studies.

Whole genome analysis indicated a high number of mutated genes in HCC. These include *TP53*, *CTNNB1*, *AXIN1*, and *CDKN2A* but most of them have much lower allele frequencies and loss of function. Therefore, it is hard to develop novel therapeutic target. Global DNA hypomethylation, promoter methylation, aberrant expression of noncoding RNAs, and dysregulated expression of other epigenetic regulatory genes such as *EZH2* are the best-known epigenetic abnormalities [81]. *EZH2* inhibition has been proposed as a therapeutic strategy for lymphoma with EZH2-activating mutations [82].

Genetic and molecular studies of HCC have implicated multiple pathways that have uncovered potential therapeutic targets. These pathways include growth factor signaling, WNT signaling, the NFE2L2-mediated oxidative stress pathway, chromatin regulation, TERT pathway, Notch pathway, and viral factors such as HBV integration and HCV core protein [83]. These may serve as critical information for new molecular classification of liver cancer, and then for the precision medicine.

9.6.2 Gastric Cancer

Unlike esophageal cancer that is more prevalent in the central northern part of China, the etiology of gastric cancer appears to be heterogeneous – as it is highly prevalent in both less developed Gansu and Qinghai provinces and the most developed Jiangsu and Shanghai areas. Some ecological factors may explain a portion of the geographic variation in the risk of gastric cancer. Based on data from 46 rural countries, gastric cancer mortality is moderately correlated with the prevalence of *H. pylori* infection ($r = 0.4$) in China [84]. Using the county-level data collected in 65 counties from the same time period, a study showed mortality of stomach cancer is correlated with aridity ($r = 0.27$), heat zone ($r = -0.56$), latitude degree north ($r = 0.53$), and population employed in agricultural sectors ($r = 0.28$), in both men and women [85]. These correlations appear stronger than dietary factors at the aggregate level. Although one should be careful in interpreting these correlations at the county level to the cancer risk at individual level due to ecological fallacy, this at least provides some evidence for integrating these ecological factors into the study of individual risk factors for gastric cancer.

9.6.2.1 *H. pylori*

Infectious agents might contribute to the risk of gastric cancer, but the causal relationship has not been established yet. Infection of *H. pylori* is more prevalent in Shanghai, one of the regions with high risk of gastric cancer in China [86]. A randomized-controlled clinical trial with 7.5 years of follow-up shows that overall no reduction in incidence of gastric cancer was observed in participants who received *H. pylori* eradication treatment compared with those who did not in Fujian, China. However, eradication of *H. pylori* significantly decreased the development of gastric cancer only in individuals who are *H. pylori* carriers but without precancerous lesions [87]. Recently, a study suggested that HBV infection is also associated with gastric cancer [88].

Individual lifestyle affects the risk of gastric cancer. However, lifestyles are highly heterogeneous and varied with the geographic location. A large prospective study of 18,244 men with a 20-year follow-up showed that smoking and alcohol consumption were major risk factors associated with gastric cancer in

Shanghai [89]. In Jiangsu province that has the highest risk of gastric cancer in China, a case–control study found that consumption of pickled foods, fast eating, irregular dietary behaviors, and lack of fresh fruits intake are risk factors [90]. In the less developed Linxian County in Henan province, formal education, using tap water at home, increased consumption of eggs, and fresh fruits and alcohol consumption are protective factors for gastric cancer, suggesting that poor socioeconomic status may play an important role in the development of gastric cancer in the less developed areas.

9.6.2.2 Familial Aggregation

Gastric cancer has a familial aggregation. It is estimated that 20% of gastric cancers are familial, that is, at least one first-degree relative has gastric cancer [91,92]. A large prospective study in Linxian County, where esophageal cancer incidence and mortality are of the highest in the world, found that increased age and a positive family history of esophageal cancer were significantly associated with risk of both esophageal cancer and gastric cancer [93]; and after 15 years of follow-up, socioeconomic status was found associated with risk of upper gastrointestinal tract cancer. Family history of tumors is also associated with gastric cancer in Jiangsu province [90].

9.6.2.3 Genetic Susceptibility Factors

Multiple common genetic variants are associated with gastric cancer. Genetic variants such as rs2274223 in *PLCE1* that has been associated with esophageal cancer also affects the development of cardia gastric cancer in Henan [94]; these associations are replicated in a study on the Korean population [95]. rs2274223 at *PLCE1* is also associated with the survivorship of patients with gastric cancer in the Han Chinese populations [96]. A study conducted in Jiangshu province found that common genetic variants at *CYP2E1*, *NAT2*, *XRCC1*, *MTHFR*, and *IL-1B* are associated with risk of gastric cancer [90], of which *CYP2E1*, *IL-1B*, and *MTHFR* have been associated with risk of esophageal cancer in the high-risk area. *XRCC1* is involved in DNA repair where it complexes with DNA ligase III. In addition, polymorphisms in the DNA mismatch repair gene, *MSH2*, were associated with risk of gastric cancer in a population-based study in three counties of Jiangsu province [97]; genetic variants at *PRKAA1* (rs13361707) are associated with gastric cancer risk in the eastern China [98].

A histone methyltransferase enhancer of the zeste homologue 2 (EZH2) gene has been implicated in multiple cancers. Genetic variants in *EZH2* are associated with risk of gastric cancer [99]. EZH2-mediated histone H3 lysine27 (H3K27me3) is frequently deregulated in a wide range of human cancers. *EZH2* is primarily responsible for *ANXA6* inhibition in gastric cancer cell [100], an important transcription factor involved in the proliferation and metastasis of tumor cells, and as direct target of multiple microRNAs such as miR124 [101],

and miR217 [102]. While miR217 is frequently dysregulated in cancer, miR124, a pivotal member of the p53 network, is downregulated in multiple types of tumors and reported as a tumor suppressor.

9.6.3 Esophageal Cancer

9.6.3.1 Geographic Variation

Esophageal cancer has a great geographic variation and is more prevalent in central northern China. Mapping the mortality from 1973 to 1975 to geographic locations has revealed that mortality from esophageal cancer has more distinct clustering at the county level. Geographic mapping has identified Yangcheng County in Shanxi province (135.2 per 100,000 individuals) and Hebi County in Henan province (139.8 per 100,000 individuals) with the highest mortality of esophageal cancer, and two others with the lowest mortality: Hunyuan County (1.4 per 100,000 individuals) and Datong County (2.8 per 100,000 individuals) in Shanxi province [103]. The huge variation in mortality is consistent with the prevalence of gullet cancer in domestic fowls in these areas. These led to the discovery that ecological factors such as higher nitrogenous compounds in food and water and a deficiency of molybdenum in soil are likely the casual factors for esophageal cancer in these areas [103].

Environmental exposures and individual lifestyle are associated with the risk of esophageal cancer in China. However, the etiologic factors are quite different between the United States and China [104]. In the Western countries, tobacco use and alcohol drinking are the major risk factors associated with esophageal cancer [104–106]. As discussed earlier for gastric cancer, multiple factors such as tobacco use, nutrition deficiency, dietary habits such as having hot foods and drinks, fast eating, and having pickled vegetables or lack of fresh vegetables in diet are major risk factors of esophageal cancer in the high-risk areas in China [107,108].

9.6.3.2 Viral Infections

Human papillomavirus (HPV) is one of the viruses that cause cancer in humans. In addition to cancer of the cervix, the association of HPV infection with esophageal cancer has been suggested. Using a 1: 3 matched case–control design, a study in Anyang County, one of the areas with the highest incidence of esophageal cancer in the world, found that HPV infection is strongly associated with the risk of esophageal cancer [109]. Another study suggests that HPV infection may only be associated with risk of esophageal cancer in cigarette smokers and alcohol drinkers [110]. A study in Africa found that it is human immunodeficiency virus (HIV) rather than HPV infection that increases the risk of esophageal cancer in Africa [111]. These case–control studies provide statistical evidence, but are more susceptible to confounding effects. One may have to rule out that the high prevalence of

HPV or other virus infection is not a result of the cancer development, or comorbidity with gastric cancer.

A large cohort study of the Chinese population did not find the association of HPV (subtypes 16, 18, and 73) infection with the development of esophageal cancer or gastric cancer. The study was conducted in Linxian County in Henan, one of the areas with a high risk of esophageal cancer in China [112]. All cases and controls were from a clinical trial study with 29,584 participants in the general population of Linxian County. Therefore, the study is less likely to have selection bias or confounding effect.

9.6.3.3 Familial Aggregation

Familial aggregation is distinct in cancer of the esophagus [113]. Family history in first-degree relatives may modify the effect of lifestyle factors on esophageal cancer [107], suggesting that common genetic variant and lifestyle together play a role in the development of this cancer. In Mei County and Chaoshan City of Guangdong province, and Min Nan, the Southern Fujian province, where most people are of the Hakka ethnic group, much higher incidence of esophageal cancer was observed, even after they have migrated for more than 20 generations from Henan, a location known for its high prevalence of esophageal cancer. In fact, a study has shown that these three areas with high prevalence of esophageal cancer are genetically related. They share a similar matrilineal genetic background, in which haplogroups D4a and D5a are associated with esophageal cancer [114]. In addition, patrilineal genetic markers on the Y chromosome also have been associated with the risk of esophageal cancer in Chaoshan city [115].

9.6.3.4 Genetic Susceptibility Factors

Several common genetic variants have been associated with esophageal cancer. A nonsynonymous missense SNP rs2274223 in *PLCE1* has consistently been associated with the risk of esophageal cancer in multiple populations [116]. Multiple regression analysis has demonstrated that a model with rs2274223, rs2274224, family history, and smoking can best predict the disease status [117], suggesting the effect of rs2274223 on esophageal squamous cell cancer (ESCC) may be independent of family history and smoking status. Upregulation of *PLCE1* is highly correlated with increased expression of NF-κB-related proteins in tumor tissues compared with normal esophageal tissues [118], indicating the involvement of inflammation in esophageal cancer. So far at least three independent studies have consistently found that rs671 at *ALDH2* and rs1229984 at *ADH1B* are associated with ESCC [108].

A recent study using whole genome sequencing or exome sequencing of esophageal cancers found eight mutations, of which *TP53*, *RB1*, *CDKN2A*, *PIK3CA*, *NOTCH1*, and *NFE2L2* are known as tumor-associated genes, and *ADAM29* and *FAM135B* are novel loci. In addition, MIR548K, a microRNA

encoded at 11q13.3-13.4 region enhanced malignant phenotypes of ESCC cells, and several important histone regulator genes such as *MLL2* (also called KMT2D), *ASH1L*, *MLL3 (KMT2C)*, *SETD1B*, *CREBBP*, and *EP300* are frequently altered in ESCC. Pathway analysis reveals that the somatic aberrations are mainly involved in the Wnt, cell cycle, and Notch pathways [119].

Alcohol drinking interacting with *ALDH2* and/or *ADH1B* may increase the risk of ESCC [120]. While the interaction between alcohol drinking and variants in *ALDH2* was replicated [121] in an independent study, genetic variants at *ADH1B* (rs1229984) are found associated with ESCC, which is independent of alcohol drinking and smoking status. In addition, genetic variants *CYP1A1/CYP2E1* at 15q24.1, *MTHFR*, *FAT4*, interleukin 1B, *CHRNA5-A3-A4* (rs667282) gene cluster at 15q25 [108], genes in DNA repair/EGFR signaling pathway [122], sex hormone, and metabolic genes are associated with esophageal cancer [123]. Another genome-wide association study found that rs7447927 at 5q31.2, rs1642764 at 17p13.1, rs7447927 at *TMEM173*, rs1642764 at *ATP1B2*, near *TP53*, and *HLA* class II at 6p21.32 are associated with the risk of esophageal cancer [124]. Genetic variants at *SLC39A6* are also associated with the survivorship of patients with ESCC in the Han Chinese population [125].

The genes identified implicate possible biological pathways through which ecological and individual environmental factors may act. One should note that these genetic studies were mostly carried out in samples collected from the areas with the highest risk of ESCC in China, and that genes identified appear to be involved with metabolism. For example, *ALDH2* is the second enzyme of the major oxidative pathway of alcohol metabolism. In humans, *ADH1B* encodes an enzyme, which is a member of an enzyme family that metabolizes a wide variety of substrates, including ethanol, retinol, hydroxysteroids, and lipid peroxidation products. *CYP1A1* encodes a member of the cytochrome P450 superfamily of enzymes, monooxygenases, which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. What is more, the two SNPs at *ALDH2* and *ADH1B* associated with risk of esophageal cancer are only polymorphic in Asian populations according to the 1000 Genomes data. This fact might partially explain the difference in the risk of esophageal cancer between Asians and other populations.

9.6.4 Lung Cancer

Lung cancer is the leading cause of cancer death in China consistent with other populations such as in the United States. The mortality of lung cancer was 52.76 per 100,000 in men and 25.08 per 100,000 in women according to the estimates of the 2011 National Cancer Registration. Although smoking may account for the majority of cancer death, a geographic variation in cancer is marked. Lung cancer is more prevalent in the northeast and in Yunnan province in the south

of China. The northeast of China is more developed in heavy industry such as coal mining, iron and steel, and petrochemical industry; industrial air pollution may be the important risk factor for lung cancer. In contrast, Yunnan is more developed in mining. In particular, tin mining, occupational exposure to radon, and arsenic may account for the elevated risk of lung cancer in Yunnan.

Risk factors associated with lung cancer in the northeast of China may be quite different. A large study conducted in Shenyang, one of the largest industrial cities in the northeast of China, found that smoking accounts for 55% in men and 37% in women of lung cancer mortality; indoor air pollution is also a major factor associated with lung cancer in the northeast [126,127]. This is because the northeast of China tends to have a longer winter period and uses coal for heating.

9.6.5 Genetic Susceptibility Factors

While mostly caused by individual smoking or air pollution, lung cancer has a genetic component. A genetic epidemiologic study of 370 nuclear families in Xuanwei county in Yunnan shows that the segregation ratio of lung cancer was 0.15, and the genetic model is polygenic. The heritability of lung cancer was at 0.246, 0.146 for men, and 0.378 for women [128]. Genetic variants may modify environmental risk factors associated with lung cancer. A genome-wide association study of lung cancer in the Han Chinese population found two novel loci *TP63* at 3q28 and *TERT-CLPM1L* at 5p15.33 [129], and confirmed two other loci at *MIPEP-TNFRSF19* at 13q12.12 and *MTMR3-HORMAD2-LIF* at 22q12.2 that were reported for lung cancer in other populations. Using samples from China, South Korea, and Japan, Lan and colleagues found that rs7086803 at 10q25.2, rs9387478 at 6q22.2, and rs2395185 at 6p21.32 were associated with lung cancer in women who never smoked [130]; and the same study also successfully validated the loci at 5q15.33, 3q28, and 17q24.3 that were found associated with lung cancer in the Han Chinese population.

Genetic variants associated with lung cancer are different by ethnic group. Loci 5q15.33 and 15q25 were consistently associated with lung cancer in multiple genome-wide association studies in European ancestry populations [131–133]. The locus 15q25 contains several genes, including three that encode nicotinic acetylcholine receptor subunits (*CHRNA5, CHRNA3*, and *CHRNB4*), which are independently associated with both nicotine dependence and lung cancer. The genes at 15q25 most likely play a direct causal role in lung cancer by interfering with nicotine acetylcholine receptors and stimulating tumor growth, because in nonsmokers there was still statistically significant association between variation in 15q25 and lung cancer [133]. However, the association with 15q25 was not replicated in multiple studies of lung cancer in the Han Chinese populations. This is very interesting and may suggest that the lung cancer is heterogeneous; some of the lung cancer may be caused by air pollution, not cigarette smoking in China.

9.6.6 Cervical Cancer

Cervical cancer is the second primary cancer behind breast cancer that affects women only. The incidence of cancer of the cervix in China is 13.40 per 100,000 individuals, which is similar to liver cancer (13.85 per 100,000) and esophageal cancer (13.05 per 100,000) in women, according to the National Cancer Registration in 2011 [52]. It is more prevalent in Inner Mongolia, Shanxi, Shaanxi, Hubei, Hunan, and Jiangxi, all of which are located in the inner central north to south of China, and they are less developed areas. Human papillomavirus infection is the major risk factor for genital carcinoma [134]; however, there is still a great geographic variation in HPV subtypes that cause the development of cervical cancer from Liaoning in the north [135] to Hunan in the south [136]. HPV vaccination and early detection of cervical cancer may be an effective way to reduce the incidence of cervical cancer in China.

9.7 Cancer Risk Factors and Prevention

While it is not easy to determine why an individual person develops cancer, research has shown that some factors may increase a person's risk of developing cancer, and some others may have protective effect on the development of cancer. Aging is a consensus factor that increases the likelihood of developing cancer. According to the National Cancer Institute of the United States, almost 80% of cancer occurs in age of 55 years and above. Several other cancers such as bone and leukemia are more frequently diagnosed under age of 20 years. Lifestyle such as alcohol and tobacco use, diet behaviors, physical activities, or level of psychosocial stress that an individual may experience affect the likelihood of developing cancer. Different patterns of cancer by geography and ethnicity between the United States and China may imply different etiological factors for cancer that may facilitate the understanding of cancer causation and provide effective interventions.

9.7.1 Environmental Chemical Exposure

Cancer is caused by genetic changes that alter the way the cells function. While some of these genetic changes occur when DNA is replicated during the process of cell division, a large proportion of them result from environmental exposures [137]. These exposures may include substances such as the constituents of tobacco smoke, radiation such as ultraviolet rays from the sun, or chemical exposures such as heavy metals, and so on. People can reduce cancer risk by quitting smoking, and by avoiding exposure to these chemicals in the water, air, or in their occupations. It is estimated that 24% of diseases are caused by environmental exposures that can be averted [138].

However, some of these exposures are difficult to avoid. For example, China has experienced a rapid economic transition as well as industrialization and urbanization over recent decades. Air pollution, especially particulate matter (PM) in the outdoor air in Chinese cities, has been observed at the highest levels in the world [139]. PM has been known to be carcinogenic to humans, not only causing a rise in incidence of cancer but also affecting population health in the next generation. Fine particle (PM2.5) has been associated with increased incidence of lung cancer in China [140]. It is very important to evaluate individual exposure by finding potential biomarkers and disease status [139], so that necessary effective prevention can be developed. In addition, according to the National Cancer Institute, there have been a number of cancer-causing chemicals, including aflatoxins, arsenic, benzene, coal tar, radon, and vinyl chloride; thus, monitoring the levels of these chemicals in the air and water is important in order to avoid environmental exposure. Chemical exposures are in principle preventable causes of cancer [141].

9.7.2 Infectious Agents

While most of lethal infectious diseases have been under control in the more developed countries, infections of infectious agents such as viruses, bacteria, and parasites are still health concern, especially in the less developed countries with high prevalence of infections with these agents. While some of the infectious agents may directly increase the risk of developing cancer, others may cause chronic inflammation that lead to evaluated risk of chronic disorders including cancer. It is indicated that increased mortality from cancer is related to viral infection acquired in early years of life [142]. Infections with high-risk types of HPVs cause nearly all cervical cancers. Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) may cause increased risk of liver cancer; and infection with Epstein–Barr virus (EBV) has been linked to an increased risk of lymphoma and cancers of the stomach and nasopharynx. Some other infections of viruses can also cause cancer. For example, human T-cell leukemia/lymphoma virus type 1 (HTLV-1) can cause a type of leukemia and lymphoma. Human immunodeficiency virus (HIV) infection can cause increased risk of Kaposi sarcoma, lymphoma, and cancers of the cervix, liver, lung, and anus; and human herpes virus 8 (HHV8) can cause Kaposi sarcoma.

9.7.3 Psychosocial Stress and Social Network

Psychosocial stress plays a very important role in the development of chronic diseases. Studies have shown that stress-related psychosocial factors have adverse effects on cancer incidence and survival for breast, lung, and several other cancers [143,144]. Individuals suffering from prolonged stress may result in stress-induced immune dysregulation that may have significant health

consequences for immune-related disorders, including viral infections, chronic autoimmune disease, and tumor growth and metastasis [145]. Studies in both humans and animals have shown that the sympathetic and neuroendocrine response to psychosocial stress significantly impacts cancer, partly through regulation of inflammatory mediators. Psychosocial stressors stimulate neuroendocrine, sympathetic, and immune responses that result in the activation of the hypothalamic–pituitary–adrenal (HPA) axis, sympathetic nervous system (SNS), and the subsequent regulation of inflammatory responses by immune cells.

However, a quasi-prospective study indicates that certain types of coping strategies and personality dispositions predispose some women to an increased risk of developing breast cancer after experiencing a major stressful life event [146]. Experiencing a major stressful life event was found to be potentially much more damaging, particularly if the individual was unable to externalize her emotions and obtain appropriate help and counseling. Social network and the size of the network may also affect the outcome of cancer; social networks affect mortality and morbidity of cancer through immunologic pathways [147].

9.7.4 The Developmental Origin of Adult-Onset Cancer

Cancers like other chronic diseases may have a developmental origin. The emerging concept of developmental origin of diseases opens an interesting view on chemical carcinogenesis. People are exposed to chemicals during the fetal period and the disturbances in human development caused by chemical compounds that lead to cancer later in life have been exemplified by ionizing radiation and diethylstilbestrol [148]. Most of the compounds to which the mother is exposed during pregnancy are also transported more or less at the same concentrations to fetal circulation. The worldwide increases concurrent in both cancer incidence and the number and quantity of chemicals in the environment raise concerns about a link between chemical exposure of the developing fetus and cancer. The developmental origins and related mechanisms in chemically induced human cancer are important to pursue [141].

Embryonic development is controlled by functionality of genes in which the existing networks can act on both transcriptional and translational regulation. Genetic variants and the environment also affect transcriptional and translational regulation through epigenetic mechanisms. The disturbance of epigenetic regulation may affect embryonic germ cell development, and thus result in delayed or blocked maturation, and potentially leads to germ cell cancer (GCC). Studies of patients with disorders of sexual development (DSD) have raised our knowledge; that genetic, epigenetic, and environmental factors play essential role in normal gonadal development; and the disturbance of epigenetic regulation that is affected by gene and environment may affect embryonic germ cell development and lead to delayed or blocked germ cell maturation that determines the risk for GCC formation [149]. Identification of epigenetic alterations could lead to better

understanding these processes and development of specific markers for early detection, eventually leading to development of targeted treatment.

Epigenetic regulation is believed to be an important mechanism of cancer development. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. Recent advancements in the field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs, and microRNAs. Epigenomics provides a powerful tool in understanding of both mechanistic and clinical aspects of cancer, including chemical carcinogenesis [150]. Of various epigenetic modifications, methylation patterns of genes have been shown as an important feature of clinical cancers. In those cancers where environmental carcinogens are known to play a role, promoter hypermethylation of tumor-suppressor genes, which lead to transcriptional silencing of tumor-suppressor genes, and methylation aberrations in other genes relevant to carcinogenesis are often observed [151]. In addition, histone modifications by methylation, acetylation, and other changes play an important role in the regulation of gene expression, notably in the context of developmental decisions and cell fate. Histone modification defects have also been implicated for cancer as well as developmental disorders [152].

9.7.5 Cancer Prevention and Intervention

Nearly half of cancer can be prevented if current knowledge about risk factors were translated into effective public health strategies [153]. Determining and reducing specific exposure to risk factors should be the first step to prevent the cancer. This approach has demonstrated some success, for example, tobacco control, vaccination against oncogenic viruses, reduced exposure to environmental and occupational carcinogens, and screening among high-risk individuals. Weight control, increased physical activity, and avoidance of psychosocial stress [154] may contribute to more effective prevention of cancer. Cancer prevention should also be carried out with knowledge of precision medicine. While some known exposures to carcinogens can be avoided or reduced, some others may be largely unavoidable, for example, exposure to particulate matter. Using genomic or molecular tools, potential biomarkers associated with both environmental exposures and disease status can be detected. These potential biomarkers could largely result from aberrant epigenetic regulation that is caused by environmental exposure. This knowledge can facilitate early detection, diagnosis, and prevention of cancer.

Because epigenetic regulation is reversible, it provides an opportunity to reduce the development of cancer by reversing the epigenetic changes that may cause cancer. Diet and DNA methylation interaction may affect cancer development [155]. Some bioactive food components have been shown to have

cancer inhibitory activities by reducing DNA hypermethylation of key cancer-causing genes through their DNA methyltransferase (DNMT) inhibition properties. Reversal of hypermethylation-induced inactivation of key tumor suppression genes by dietary DNMT inhibitors could be an effective approach to cancer prevention and therapy [156]. Targeting the epigenome, including the use of histone deacetylase (HDAC) inhibitors, is a novel strategy for cancer chemoprevention. Sulforaphane (SFN), a compound found in cruciferous vegetables, inhibits HDAC activity in human colorectal and prostate cancer cells. In the prostate it acts as an HDAC inhibitor, which causes enhanced histone acetylation and disrupts the cell cycle and induce apoptosis via derepression of genes such as *P21* and *Bax*, leading to cancer prevention [157,158]. Phytochemicals have been shown to have major potential for cancer prevention [159].

In addition, bioactive dietary components can interfere with various epigenetic targets to result in cancer prevention and therapy. These agents include curcumin (turmeric), genistein (soybean), tea polyphenols (green tea), resveratrol (grapes), and sulforaphane (cruciferous vegetables), as previously mentioned. These bioactive components alter the DNA methylation and histone modifications required for gene activation or silencing and mediate epigenetic modifications associated with the induction of tumor-suppressor genes and inhibition of tumor-promoting genes, such as human telomerase reverse transcriptase. Remarkable advances in our understanding of basic epigenetic mechanisms as well as the rapid progress that is being made in developing powerful new technologies are enabling sensitive and quantitative detection of epigenetic and epigenomic changes in cancer biology that ultimately may hold great promise for novel epigenetic approaches to cancer prevention and therapy.

Finally, fighting against infectious agents that may cause chronic inflammation throughout life span may help reduce the risk of developing cancer. In the United States, children aged 11 and 12 are recommended to receive routine HPV vaccination that prevents infection with the types of HPV that cause most HPV-associated cancers. Children as young as age 9 and adults as old as 26 can be vaccinated. HPV infections in the cervix can be found with specific tests. Although HPV infections themselves cannot be treated, the cervical cell changes resulting from these infections can be treated. Since the 1980s, infants in the United States and most other countries have been routinely vaccinated against HBV infection. It is recommended that individuals who have not been vaccinated against HBV and have an increased risk of HBV infections such as health care workers or professionals, who come into contact with human blood, get vaccinated as soon as possible. In addition, infection with *H. pylori* can be detected and treated. A clinical study has shown that broccoli extract sulforaphane can prevent lipid peroxidation in the gastric mucosa and may play a cytoprotective role in *H. pylori*-induced gastritis [160]. All these public health care actions may contribute to the prevention of cancer risk.

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10

Dietary/Supplemental Interventions and Personal Dietary Preferences for Cancer: Translational Toxicology Therapeutic Portfolio for Cancer Risk Reduction

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10.1 Introduction

Diet-related and lifestyle-related chronic diseases have reached global levels. During the second half of the twentieth century noncommunicable diseases, such as cardiovascular disease (CVD), cancer, and diabetes, have been on the rise in developed and now developing countries [1]. These chronic diseases are the leading cause of the global health burden and are linked to adaptable lifestyle factors such as nutrition, eating habits, body weight status, physical activity level, and alcohol/tobacco intake [2–4].

Diet is a cause of approximately one-third of cancers. A landmark report in 1981 by Doll and Peto reported that 35% (range of 10–70%) of cancer deaths were related to diet [5]. In 2007, the World Cancer Research Fund/American Institute of Cancer Research (WCRF/AICR) published one of the most comprehensive nutrition and cancer reports to date looking at all the evidence related to nutrition, physical activity, and cancer prevention. This report validated the nutrition and cancer association and found that 35% of cancer is caused by diet while 30% from smoking and 35% from other factors [6]. The Continuous Update Project (CUP) reports from WCRF/AICR provide on-going evidence analysis on nutrition and physical activity for various cancers [7–10].

The full sequencing and publishing of the human genome in 2003 brought special attention to the interaction of diet, nutrients, and health outcomes [11]. An individual's genetic background may determine who will respond to the thousands of different bioactive food components (BFCs). BFCs play a role at the molecular level in inflammation and metabolism and are influential regulators in gene expression patterns given that their ability to turn genes on and off influences phenotype expression [1,12]. Diets' effect on the

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expression of genes has indicated certain biological responses to nutrients may prevent or potentially reverse carcinogenesis depending on individuals' unique genotypic profile [13]. In addition to the development of genomics and nutrition, research indicates diet and lifestyle factors that can be modified via behavior modification and mind–body practices are beginning to be used in managing chronic diseases such as cancer and cancer comorbidities [4].

10.2 Gene Expression and Epigenetics

Cancer, the malfunction of genes that control cell growth and division, is influenced by diet, physical activity, and stress, all of which potentially play a role in altering genes and gene expression [11]. The variation in response in nutrition clinical trials has provided the insight that all individuals do not respond identically to diet or dietary components [12]. Nutritional genomics offers an explanation to these individual differences. Nutritional genomics is a broad term that encompasses nutrigenetics, nutrigenomics, and nutritional epigenomics. Nutrigenomics includes the interactions between dietary components and the genome along with the resulting changes in protein and other metabolites that affect gene expression. Nutrigenetics refers to how an individual's genetic makeup influences and affects the utilization and response to dietary components. In essence, nutrigenetics is the genetic variability between individuals accounting for the variations in health status and disease risk despite similarities in dietary intake [11].

For example, genetic information may assist in identifying individuals at risk from high intake of red meat. Red meat is associated with increased risk of colon cancer. Heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) are carcinogens formed when red meat is cooked at high temperatures; HCAs and PAHs may contribute to the increased colon cancer risk from red meat. DNA damage from HCAs and PAHs can be repaired by the nucleotide excision repair (NER) pathway [14]. In a study of colon cancer patients and healthy controls that looked at NER gene single-nucleotide polymorphisms (SNPs), red meat intake, and colon cancer risk, Steck *et al.* found an interaction between the *XPC 939* genotype, well-done red meat intake, and colon cancer risk [15]. Of interest, those individuals with a particular genotype, the *KK* genotype, who ate more well-done meat compared to those with the genotype who ate less well-done meat were at higher risk of colon cancer [15].

Genetic information may also assist in identifying those individuals who will benefit from increased fish intake. The evidence from epidemiological studies has been inconclusive as to whether dietary fish intake may protect against prostate cancer. Hedelin *et al.* studied fatty fish intake and genetic variation in cyclooxygenase (COX)-2, a key enzyme in fatty acid metabolism and inflammation, in prostate cancer patients, and population controls. They found that

frequent consumption of fatty fish reduced prostate cancer risk and there was a strong inverse association with increasing intake of salmon-type fish among carriers of the variant allele [16]. This modified association of fish intake among those with a genetic variation of *COX-2* may explain the variation in results from epidemiological studies of fish intake and prostate cancer risk.

Nutritional epigenetics refers to the influence of diet and other heritable changes on gene expression without changing the DNA sequence [17]. Unlike genetic variants, which are inherited and involve alterations in DNA sequence, epigenetic changes are heritable but are potentially reversible modifications in DNA that affect gene expression and function without altering the nucleotide sequence [12]. Nutrients can modify epigenetic events and offer another explanation for how environmental factors, such as diet, can influence biological processes and phenotypes. Methylation, acetylation, or regulation of microRNAs are major epigenetic processes that alter proteins that control gene expression and can influence the adverse or beneficial effects of lifestyle exposures [11,13].

One of the most extensively studied epigenetic mechanisms is DNA methylation, which refers to the degree to which methyl groups are present or absent from certain regions of genes and impacts patterns of gene expression, typically resulting in gene silencing [1,11]. Animal studies in the agouti mouse model have provided the strongest evidence that diet plays a role in epigenetics through DNA methylation [11,18]. Maternal mice diets supplemented with folic acid, vitamin B_{12}, betaine, and choline to increase methylation resulted in offsprings born with a pseudoagouti or brown coat color and were leaner versus the characteristic yellow agouti coat color and obese mice. The leaner phenotype is associated with less risk for obesity, diabetes, and cancer [18]. Furthermore, supplementation with the soy compound, genistein, to maternal agouti mice also led to increased methylation of the *Agouti* gene of the offspring, which persisted into adulthood [19].

The Dutch hunger winter famine (1944–1945) is an example of early-life environmental conditions causing epigenetic changes in humans that persist over the life span. Despite fetuses that seemed healthy at birth, individuals who were conceived during the famine whose mothers received less food during their first trimester had different health outcomes compared to those born to mothers who were in their second or third trimester at the time of the famine [20,21]. Researchers found that those prenatally exposed to the famine had less DNA methylation of the *insulin-like growth factor* (*IGF-2)* gene compared to their siblings who were not affected by famine [22]. Fifty years later, these same individuals who had been conceived during the famine and fed less during the first trimester were heavier and weighed an average of fourteen or more pounds, had an average of 1½ inch larger waist, and were three times as more likely to have coronary heart disease than those whose mothers were in their second or third trimester at the time of the famine [23]. The changes during the first trimester during the Dutch famine may have

altered the fetuses' metabolism and resulted in "a thrifty metabolism" during limited food supplies. However, once more food was available, the epigenetic changes in the "thrifty metabolism" most likely never reversed and the children were more likely to weigh more as adults.

Fathers also may play a role in the epigenetic changes of a fetus. In a study of newborns, the gene for IGF-2 was less methylated in those born to obese fathers than normal-weight fathers, which might increase the risk of obesity in adulthood. Dietary and feeding patterns of newborns may also influence epigenetic markers [24]. In an animal study, mice who were overfed the first few weeks of their lives showed subtle changes in the methylation of genes in the hypothalamus, which helps regulate body weight. The overfed mice were more overweight as adults [25].

Nutrients and BFCs are known to modify methylation of DNA in a manner that can increase or decrease carcinogenesis [3]. Folate inhibits the DNA methylation that is observed in number of cancers [3]. Folate, vitamin B_{12}, and vitamin B_6 are involved in one-carbon metabolism and play a critical role in DNA methylation [11]. Too much or too little of these nutrients has the potential to disrupt DNA and histone methylation patterns [11]. *In vitro* studies indicate that certain BFCs can change epigenetic markers in cancer cells, such as curcumin in turmeric, epigallocatechin gallate in green tea, genistein in soybeans, resveratrol in red grapes, and sulforaphane in cruciferous vegetables [26].

Although genetic backgrounds may help us predict who is at risk for cancer and other diseases, most chronic diseases are multifactorial and factors such as life stage, environment, and diet contribute to the process. Nutritional genomics provides insight into how manipulating the diet affects phenotype and potentially offers clinicians an approach to customizing dietary recommendations. Nutritional epigenetics gives us insight into how diet at critical periods in the life span may affect phenotype for generations.

10.3 Environmental Lifestyle Factors Affecting Cancer Prevention and Risk

10.3.1 Obesity

The US obesity rates are among the highest in the world; about two-thirds of Americans are overweight or obese. Obesity is a significant risk factor for several cancers and some cancer reoccurrences [27]. About 14–20% of all cancer deaths are related to overweight and obesity in the United States – approximately 100,000 cancer cases per year are obesity related. [27,28] A predictive analysis of obesity in 2030 indicates that if every adult reduced their body mass index (BMI) by 1% (approximately 1 kg or 2.2 lbs for an adult of average weight), 100,000 new cancer cases could potentially be avoided [29].

In addition, approximately three-fourths of all cancers are diagnosed in persons aged 55 years and above and 64% survive for at least 5 years after their initial diagnosis resulting in an aging cancer population [30]. In 2022, the number of cancer survivors is projected to increase by 31%, leading to more than 4 million survivors who are at a greater risk for developing comorbidities and increased mortality from noncancer causes than the general population [31].

Numerous epidemiological studies confirm that obesity is associated with an increased risk for several cancers and has been linked to 20% of cancer deaths in women and 14% in men [28]. The WCRF/AICR concluded that there is convincing evidence that excess body fatness, evaluated by BMI, waist circumference, and waist–hip ratio is associated with at least the following cancers: esophageal; liver; pancreatic; colorectal; postmenopausal breast; endometrial; kidney; and possibly others [6–8,32–34]. Excess body fatness also probably increases the risk of gallbladder and stomach (cardia) cancers [9,10]. On the other hand, there appears to be an inverse relationship between BMI and body fatness with lung and premenopausal breast cancers [6,27,31].

Overweight and obese women have an increased risk for postmenopausal breast cancer of 1.13 and 1.25, respectively, compared to women of normal weight [35]. In fact, obese women with a BMI > 35 are at 1.58 times greater breast cancer risk compared to normal weight women [36]. Estrogen levels are higher in overweight and obese postmenopausal women; the more adipose tissue present the more circulating hormones and fat cells become a critical source of endogenous estrogen production. Aromatase, an enzyme found in adipose tissue, converts precursor androgens into estrogens such as estradiol. Data from two large cohorts found that postmenopausal breast cancer risk increased with higher levels of circulating estrogen [27,31,37].

The connection between inflammation and tumorigenesis has been established over the last decade. Inflammatory bowel disease as a risk factor for colon cancer is an example of inflammation in the gastrointestinal tract promoting colon cancer development [38]. Although not all chronic inflammatory diseases increase the risk of cancer, low subacute inflammation has been associated with obesity and increased cancer risk [39,40]. Visceral fat, which lies deep in the abdomen and surrounds organs, secretes inflammatory cytokines and hormones, including estrogen, insulin and leptin [41]. Abnormal cytokine production from interleukin IL-1, IL-6, and tumor necrosis factor (TNF), for example, results in proinflammatory markers such as systemic C-reactive protein (CRP) and activation of macrophages [41]. CRP is a marker for low-level inflammation [42]; elevated CRP has been associated with increased cancer risk (metastatic prostate, colorectal, lung), poor survival, and mortality [42].

Elevated levels of insulin, insulin-like growth factor (IGF) -1, and other growth factors are linked to cancer and are thought to inhibit apoptosis and promote cell proliferation. Insulin levels are higher in obese individuals [43]. Furthermore, insulin levels have been associated with breast cancer outcomes five years

postdiagnosis and higher levels have been associated with more advanced stages and/or poor outcomes in prostate and colorectal cancers [43–45].

Obesity is associated with glucose intolerance. Higher levels of fasting glucose have been associated with increased overall cancer risk and mortality [46–48]. The Warburg effect is an enhanced utilization of glucose by rapidly proliferating tissues, including cancer cells, and manifests as a shift in glucose metabolism from primarily oxidative phosphorylation to aerobic glycolysis [46]. This effect often stimulates the idea that sugar in the diet could directly fuel cancer growth. Even without sugar or carbohydrate intake in the diet, the body will derive glucose from other sources in the body, including muscle and fat. Insulin levels and other related growth factors are likely more of an influence on cancer cell growth than dietary sugar intake.

Adipokines, hormones that are produced by fat cells, can either inhibit cell growth or cause proliferation – they are considered crucial mediators linking obesity and chronic inflammation [41]. Leptin is an adipokine that is proinflammatory and is in higher amounts in obese individuals. On the other hand, adiponectin, an adipokine that is less abundant in obese people, is anti-inflammatory and has an antiproliferative effect in addition to being an essential insulin-sensitizing agent [29,41,49]. Decreased levels of circulating adiponectin have been associated with increased incidence of malignant transformation, including endometrial, breast, prostate, and colorectal cancers [41]. A high BMI and low levels of adiponectin may lead to increased insulin resistance, type 2 diabetes, and other possible chronic diseases [41].

It has also been hypothesized that increased cancer risk is related to mechanisms that may change immune response, affect the nuclear factor kappa beta system and oxidative stress [29,39]. Oxidative stress may be associated with cancer recurrence [50]; oxidative stress can arise from nutritional risk factors such as increased consumption of meat, and fatty foods and weight gain [51]. Over the last decade, there is mounting evidence of how inflammation promotes cancer growth. However, the molecular mechanisms and processes of how whole foods and dietary constituents affects specific biomarkers, including IL-1B, IL-4, IL6, IL-10, TNF-alpha, and CRP are not clearly understood [38,52].

10.3.2 Weight Loss

Although there is limited evidence regarding the cancer survival benefits related to weight loss, intentional weight loss after a cancer diagnosis is possible and survivors can be successful with a weight loss regimen [53]. In the Exercise and Nutrition to Enhance Recovery and Good Health for You (ENERGY) trial, the intervention group received a group-based behavioral intervention that was supplemented with customized newsletters and personal contact via phone counseling to encourage a 500–1000 calorie deficiency and 60 min/day of purposeful moderate intensity physical activity with a goal of 7% weight loss in 2

years [54]. Results indicated a modest effect of 6% weight loss from initial weight in the intervention group compared to 1.5% weight loss in the control. However, the study showed that increased physical activity was possible for the breast cancer population. The challenge remains to avoid regaining weight once that personal contact and support is reduced. In a secondary analysis using data from the ENERGY trial, 692 overweight/obese women, treated for early stage breast cancer, were randomized to either a 1-year group-based behavioral intervention or a less intensive control intervention. Sedjo *et al*. found that the women randomized to the intervention group had modest weight loss and also had fewer medical conditions compared to the control group (19.6 versus 32.2%, $p < 0.001$) at 12 months; however by 24 months there was no longer a difference [55]. Weight regain at 1-year postintervention follow-up most likely contributed to this change and underscores the need for continued support for weight loss sustainability. Weight regain is a common phenomenon after weight loss [55].

Although the biological mechanism of how weight loss potentially affects carcinogenesis is unclear, weight loss mechanisms may lead to an improved understanding of obesity-related cancers and potential preventative treatments [27]. Weight loss through diet can reduce levels of inflammation, insulin, and bioavailable estrogen, which may reduce breast cancer risk. Studies indicate that there is a great amount of variability in how people lose weight. Genetic variation may account for differences in body weight loss. A number of heritability studies indicate that 40–70% of body weight is heritable and genetic variability can account for both weight gain and weight loss [56].

Cornelis *et al.* [57] evaluated the relationship between genetic susceptibility to obesity and cognitive restraint by looking at two US cohorts, the Nurses Health Study and the Health Professionals Follow-up Study. The combined effect was 32 SNPs, which was assessed for individuals using an obesity genetic risk score (GRS). SNPs associated with BMI were also associated with higher emotional and uncontrolled binge eating scores [57]. Their findings suggest eating behaviors may play a significant role in the link between genetic variation and the development of obesity. Cornelis *et al.* also reported that the obesity GRS was positively associated with BMI, emotional eating, and uncontrolled eating indicating a genetic component to eating behaviors [57].

10.3.3 Physical Activity

Leisure-time physical activity of moderate to vigorous intensity has been associated with lower risk of several cancers such as liver, lung, and kidney, irrespective of smoking history or body size [58]. Exercise as a lifestyle intervention has a positive impact on cancer survivors by influencing long-term side effects of cancer treatment such as cognitive dysfunction (chemo brain), cancer-related fatigue, and pain [59]. In a systematic review and

meta-analysis, a variety of exercises (yoga, aerobics, strength-training) were shown to have an overall positive effect on health-related quality of life (QOL) factors such as anxiety, emotional well-being, and fatigue [59].

Exercise is recommended during primary cancer treatment for its therapeutic benefits and is considered safe [60]. In a meta-analyses of aerobic and/or resistance exercise in cancer patients, cancer-related fatigue improved both during and following treatment [61]. Exercise interventions during treatment had a palliative effect while exercise after treatment had a recuperative influence [61]. Regular aerobic activity may also improve sleep and prolong survival of breast cancer patients by reducing obesity and helping to alleviate fatigue and depression [62].

There is substantial evidence supporting the role of physical activity in decreasing circulating levels of inflammatory biomarkers, such as, CRP, IL-6, and TNF-alpha [63,64]. Physical activity has been shown to reduce CRP concentrations among overweight and obese women, suggesting physical activity may be an intervention to decrease inflammation in this population and potentially reduce cancer risk [65,66]. In a study that evaluated the impact of higher amounts of aerobic exercise (300 min/week versus 150 min/week) on inflammatory markers in postmenopausal women, as exercise time increased there was a stronger but not significant reduction of CRP and IL-6 [63]. Further research is needed to determine the optimal exercise regimen to decrease proinflammatory biomarkers for cancer patients and survivors [59,60].

The American Cancer Society's (ACS's) recommendations for maintaining a healthy weight focus on active living with an emphasis on regular physical activity [67]. For cancer survivors, it is recommended to engage in at least 150 min of moderate intensity activity or 75 min of vigorous intensity activity each week – or a combination – preferably spread out throughout the week. Strength training should be included at least twice a week [67].

10.4 Dietary Patterns

There is considerable interest in the role of diet and lifestyle changes in those with cancer [68]. Studying dietary constituents apart from lifestyle factors, such as physical activity, stress, or obesity, can be challenging. The majority of nutrition research has focused on BFCs, individual nutrients, and/or specific foods rather than dietary patterns and diets [60]. Despite these challenges, research indicates that a plant-based diet, including whole grains, fruits, vegetables, beans, legumes, and other whole foods, may prevent certain cancers [6,60,69,70]. Dietary patterns that have a high consumption of processed foods, red meats, and high-fat dairy products are associated with higher mortality in individuals compared to individuals eating a diet high in fruits, vegetables, whole grains, poultry, and fish and a diet that followed the ACS, WCRF/AICR, and 2015–2020 Dietary Guidelines for

Americans (DGAs) [70,71]. In the Dietary Patterns Methods Project, a collaboration of four research groups and three large cohort studies collected data on the quality of four different dietary indices and death from cancer. The four indices captured the key components of a healthy diet and further, a more consistently higher diet quality was significantly associated with greater survival and an 11–28% decrease in mortality [72].

Whole foods are an ideal source of a variety of nutrients that may be important for cancer prevention, including fiber, phytonutrients, prebiotics, antioxidants, and anti-inflammatory compounds [69,73]. The guidelines from the WCRF/AICR, ACS, and 2015–2020 DGAs recommend individuals achieve a healthy weight while consuming nutrients from whole food rather than dietary supplements. The WCRF/AICR advises that at least two-thirds of the diet is plant-based and should include a variety of fruits, vegetables, whole grains, and legumes [6]. Based on information from the Behavioral Risk Factor Surveillance System, Americans do not meet those guidelines. During 2007–2010, half of the total US population consumed less than one cup of fruit and less than 1.5 cups of vegetables daily. Only 13.1% of respondents met fruit recommendations of 1.5–2 cups a day and 8.9% met vegetable intake recommendations of 2–3 cups a day [74]. The gap in the consumption of plant-based foods in the United States led to the determination in the DGAs that fiber, potassium, calcium, and vitamin D are nutrients of concern [70].

Although high dietary sugar intake has not directly been shown to increase cancer progression, simple sugars, including honey, raw sugar, brown sugar, high-fructose corn syrup, and sugary drinks typical in the American diet provide empty calories, which lack nutrients. The dietary intake of foods high in refined sugar often replaces foods that are nutrient dense, which may be important for the prevention of cancer and other chronic disease. The WCRF/AICR, ACS, and the 2015–2020 DGAs recommend limiting added sugars and sugar sweetened beverages [6,67,75].

Cancer patients and survivors are at higher risk of comorbidities, such as CVD and diabetes. The recommended range of macronutrients for cancer survivors for the prevention of comorbidities is 20–35% fat, 45–65% carbohydrate, and 10–35% protein (a minimum of 0.8 g/kg) [60,76]. In regards to fat intake, some studies have indicated that omega-3 fatty acids from fish and plant sources, such as walnuts, may potentially enhance some forms of cancer treatment, improve QOL, and lower the risk of chronic disease, including CVD. Fatigue may be provoked by altered cytokines and stress hormones and be related, at least in part, to inflammation. Previous observational data indicate that proinflammatory biomarkers, such as IL- 6, IL-receptor antagonist, and TNF, are associated with higher levels of omega-6 fatty acid intake [77–79]. Protein foods should be low in saturated fat and should include lean meats, fish, seeds, nuts, legumes, eggs, and low-fat dairy products. Healthy carbohydrate options include whole grains, which provide fiber and BFC, such as phenolic

Table 10.1 Selected bioactive food components for cancer prevention and their dietary sources.

Bioactive food component	Dietary source
Allyl sulfur compounds	Garlic, onions, leeks, chives
Beta-carotene	Green leafy vegetables, carrots, pumpkin, sweet potatoes, squash, spinach, apricots, cantaloupe, pink grapefruit, green peppers
Beta-glucans	Oats, barley, mushrooms, yeasts, seaweed, algae
Curcumin	Spice turmeric
Lignans	Flax seed, sesame seed, rye, wheat, oar, barley, soybeans, cruciferous vegetables, apricots, strawberries
Lutein	Green leafy vegetables, gazpachos, egg yolk, kiwi fruit, grapes, oranges, zucchini, squash, pistachio nuts, corn
Lycopene	Tomatoes, tomato products, guava, watermelon, pink grapefruit, apricots
Omega-3 fatty acids	Fish
Quercetin	Onions, red wine, red grapes, green tea, apples, berries, broccoli
Soy isoflavones (genistein and daidzein)	Soybeans and soy products

acids, flavonoids, tocopherol, and lignans and may decrease cancer and CVD risk [80]. See Table 10.1 for selected bioactive food components for cancer prevention and their dietary sources.

Although vegetarian dietary patterns are being studied in the cancer population, it is unclear if a vegetarian diet protects against cancer or cancer recurrence. In a prospective cohort of 96,001 American women of the Adventist Health Study-2, [81] women who were consuming vegan, lacto-ovo vegetarian, pesco-vegetarian, semi-vegetarian, and nonvegetarian dietary patterns did not have a significantly lower risk of breast cancer [82]. Although not statistically significant, the women following a vegan diet had consistently lower breast cancer risk [81].

In a prospective study pooling data from two British cohorts, vegetarians had a 63% lower risk of stomach cancer compared with meat eaters [83]. Colorectal cancer did not differ between meat eaters, vegetarians, or vegans; however, there was a 34% lower risk of colorectal cancer in fish eaters compared to meat eaters [83]. From one of the British cohorts, Schmidt *et al.* [84] looked at the metabolic profiles of meat eaters, fish eaters, vegetarians, and vegans and found all four groups had distinct metabolite profiles with 79% of metabolites differing between the different habitual diet groups [84]. Vegans clearly differed in that

they had lower concentrations of glycerophospholipids and spingolipids. However, it is unclear if these metabolic differences in profiles translate into different cancer risks. These studies underscore the need for more studies looking at whole diets, including vegetarian and vegan dietary patterns diets, the risk of cancer recurrence, and whether observed differences in metabolic profiles between different diet groups play a role in lowering disease risk in some individuals who follow vegetarian and vegan dietary patterns [81].

10.5 Complementary and Integrative Oncology Interventions/Restorative Therapeutics

The National Institutes of Health's (NIH) National Center for Complementary and Integrative Health has categorized complementary and alternative medicine (CAM) into two categories. The first category is biological products, which includes diets, dietary supplements, and nonvitamin, nonmineral dietary supplements. The second category is mind–body interventions (MBIs) and includes modalities such as meditation, yoga, deep breathing, tai chi, and qigong [85]. CAM is not typically considered part of allopathic traditional medicine. CAM modalities that are used together with conventional medicine are considered to be "complementary" while modalities that are in place of conventional medicine are considered to be "alternative" medicine. CAM use has increased over the decades, from 25% use in the 1970 and 1980s to greater than 32% in the 1990s to 49% after the year 2000 [86].

There are many motivators for the increasingly frequent CAM use among cancer patients. In a survey of breast and prostate cancer patients undergoing treatment, the most common reason for CAM use was to prevent cancer recurrence (96%) followed by playing a more active role in recovery (92%), boosting the immune system (89%), helping to manage stress (84%), and giving hope (82%) [87]. A systematic review indicated that 14–32% of cancer survivors initiate supplement use after receiving a cancer diagnosis [88]. In a genetic testing program for breast and ovarian cancer predisposition, CAM use was significantly more prevalent among women who had cancer compared with unaffected women [89].

Many studies have indicated that younger, more educated women with cancer are more likely to use CAM, in particular vitamins and herbal products [90]. CAM use is independently associated with tertiary education level, greater physical activity, greater anxiety, and lower breast cancer risk [91]. Gross *et al.* [92] found that 90% of women with metastatic breast cancer used one form of CAM modality for at least 6 months and 68% of the women used two or more CAM modalities and bought herbal and vitamin supplements [92]. In 2002, Ashinkaga *et al.* [93] reported that the most common CAM treatments in women with female-specific cancers were herbal treatments (21%), meditation (21%), and traditional massage

(20%) [93]; the use of these treatments are still popular in the general United States and the cancer population [85,94].

In the United States, cancer patients and long-term survivors' top and most often used CAM modalities are biological methods that include vitamin or mineral supplements (64–81%, respectively) and multivitamin/mineral supplements (26–77%, respectively). Dietary supplement and multivitamin/mineral supplement use in the general US adult population is less than the cancer population and is estimated to be 50 and 33%, respectively [88]. Unlike in the United States where the biologic modalities are most often used, the top CAM modalities used in European cancer centers and hospitals are acupuncture (55%), homeopathy (40%), herbal medicine (36%), and traditional Chinese medicine (36%) [95].

Some dietary supplement clinical trials using single micronutrients, such as beta-carotene, selenium, and vitamin E have shown mixed results and also some have shown increased morbidity and mortality. In the Selenium and Vitamin E Cancer Prevention Trial (SELECT), men who took supplemental selenium or vitamin E had a higher incidence of diabetes and prostate cancer, respectively [96]. In another trial, individuals who smoked, consumed alcohol, or both while receiving beta-carotene supplements had an increased risk of colorectal adenoma recurrence [97]. In the Women's Health Initiative study of over 35,000 postmenopausal women who were randomized to calcium (1000 mg elemental calcium carbonate daily) and vitamin D (400 IUs daily) or a placebo, the women who received the calcium and vitamin D did not have a lower risk for colorectal cancer than those taking placebo [98]. Additional randomized control trials (RCTs), such as the Carotene and Retinol Efficacy Trial observed an increased risk of lung cancer in the treatment group receiving 30 mg daily of beta-carotene per day and 25,000 IUs of retinol (vitamin A in the form of retinyl palmitate). The investigators stopped the trial 21 months early concluding that after 4 years of supplementation this combination of beta-carotene and vitamin A did not improve the incidence of lung cancer, CVD and have may even worsened the incidence of lung cancer in smokers [99]. In another study, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, investigators looked at daily supplementation of either beta-carotene (20 mg/day) and alpha-tocopherol (50 mg/day) or both in smokers. Also, there was an increased risk of lung cancer with the beta-carotene supplement and a decreased risk of prostate cancer with vitamin E (50 mg) supplementation [100].

These studies highlight the need to be cautious in dietary supplement use since there can be both beneficial as well as harmful outcomes. Clinically, dietary supplements may be considered if less than two-thirds of a patient's food intake is consumed [60]. Although dietary supplements are indicated for nutrient deficiencies and may be beneficial for overall health and for managing some health conditions, excess levels of dietary supplements do not improve overall cancer survival rates and may be harmful. Even multivitamin/minerals,

which are often used as *nutritional insurance* may not be helpful and possibly increase mortality rates in healthy individuals [60,101]. While the US Food and Drug Administration (FDA) oversees dietary supplements, the regulations for dietary supplements are different from those of medications. Dietary supplements do not require premarket review or approval by the FDA that medications require. The supplement companies are responsible for having evidence that their products are safe and that the label claims are truthful and not misleading; supplement companies do not need to provide evidence to the FDA before the product is marketed.

Another area of CAM used in the cancer population is MBIs, which have exponentially increased over the last decade in the US population. In particular, yoga, tai chi, and qigong have linearly increased from 2002, 2007, and 2012, respectively, based on data from the National Health Interview Survey (NHIS); yoga accounts for approximately 80% of the prevalence of these MBI approaches [85]. Yoga, tai chi, qigong, and acupuncture are based on Eastern philosophy and have been researched in the cancer population along with other relaxing techniques such as mindfulness-based stress reduction (MBSR), biofeedback, hypnosis, guided imagery, art therapy, and music therapy [102,103]. Data suggest that 32% of cancer survivors report current or past use of MBIs. Due to the distressing and pervasive side effects of cancer treatments, cancer patients and survivors use MBIs to promote physical and emotional well-being [104,105].

Research indicates that physical activities with a mind–body component also serve to reduce stress above and beyond the effect of exercise itself. Practices such as yoga, tai chi, and qigong are associated with improved QOL measures in cancer patients and survivors [103]. Conventional exercise regimens can be challenging for some cancer patients who have undergone surgery, chemotherapy, and/or radiation treatment and additionally are experiencing stress and anxiety about the possibility of a reoccurrence [102]. MBIs may not fit the paradigm that physical activity equals energy expenditure. However, MBIs have been shown to simultaneously address the physical and psychological needs of cancer patients and survivors.

Cancer-related fatigue is one of the most common side effects reported by cancer patients. Additionally, depression and anxiety are the most common psychological distress symptoms reported by cancer patients and survivors [106]. Insomnia is also widespread; 60% of cancer survivors report sleep disturbances, which is two to three times higher than individuals without a cancer diagnosis. Disrupted sleep can lead to increased inflammation, overproduction of epinephrine and cortisol, and potentially immunosuppression [107,108].

Acupuncture, the stimulation of different anatomical points on the body typically with needles, has been studied in relation to managing treatment side effects such as cancer-related fatigue. In a meta-analysis that included RCTs looking at acupuncture and its impact on reduced cancer-related fatigue,

acupuncture and education intervention versus usual care did reduce cancer-related fatigue, however, it was unclear if the effect was due to the education or acupuncture [109]. Qigong and tai chi are MBIs originating from China. There are a variety of either traditional or modern forms of qigong, with the purpose of gentle movements and breath to train the mind and body. Tai chi, known as moving meditation, is multidimensional in that it incorporates slow and gentle movement and deep breathing [85]. There is evidence that stress response pathways are targeted with tai chi practice [110].

In a Cochrane review of aerobic exercise, such as walking, cycling, strength training, and resistance training compared to either qigong or tai chi for 12 weeks, tai chi and qigong had positive effects on fatigue and cancer-specific QOL while the other aerobic exercises had no impact on these parameters [111]. Tai chi and qigong have not been found to reduce depression and anxiety. As with other MBIs, more well-designed RCTs are needed to determine the therapeutic benefits [110].

MBSR is an MBI that was first developed by Kabat-Zinn in the late 1970s to help relieve pain and suffering among individuals with chronic pain, anxiety, and other unexplained symptoms [104]. MBSR is an 8-week program that consists of sitting and walking meditations, yoga practices, and a body scan. Studies with MBSR and the cancer population have shown positive psychological outcomes, including fewer symptoms of stress, anxiety, and depressed mood [104,112,113]. In a meta-analysis of MBSR's impact on psychological distress, breast cancer survivors had a significant improvement in depression, anxiety, stress, and overall improvement of QOL [114].

Yoga is a MBI that has different components of movement, mental focus, and breath techniques. Additionally, different yoga lineages encompass varying degrees of meditation, which may have its own positive effects on psychological wellness of cancer patients [112,115]. A systematic review found yoga to be generally helpful in terms of depression, anxiety, sleep, chronic pain, and stress [116]. A meta-analysis found that yoga had a positive effect on anxiety when it was practiced for longer than 3 months [117]. In a systematic review and meta-analysis of a total of 930 subjects (16 RCTs) comparing yoga groups/interventions to control groups and looking at different treatment-related side effects, anxiety, depression and gastrointestinal symptoms were significantly improved while other side effects such as fatigue, sleeping quality, and pain were not significantly affected by yoga [117]. In a RCT of 200 breast cancer survivors who were assigned to either 12 weeks of a hatha-based yoga class (90 min) twice a week or a control wait-listed group, the yoga group had positive inflammatory changes, including a significant decrease in cytokine production (IL-6, TNF-alpha, and IL-1B) [118]. In this study, the more frequent the yoga practice, the greater the improvements in fatigue, vitality, and inflammation [118]. Currently, no conclusive recommendations can be made due to the need for more rigorous studies with more diverse populations [116]. Additionally, results need to be

validated with RCTs that are designed to decrease bias and evaluate long-term effects of yoga in cancer patients and survivors [106,119].

Additional studies with MBIs are looking at the role of hypothalamic–pituitary–adrenal axis and the autonomic nervous system activity in increasing parasympathetic tone and immune function [104]. Although there are variations in the side effect outcomes with MBIs, research indicates that there is likely a common physiological response stimulated by different types of MBIs called the relaxation response (RR). RR is characterized by decreased oxygen consumption, increased exhaled nitric oxide, and general reduced psychological distress. In a study done by Dusek *et al.* [120] of blood transcriptional profiles of long-time MBI practitioners, they found that the RR elicits specific gene expression changes in short-term and long-term practitioners of MBIs [120]. Another study by Bhasin *et al.* [121] found genomic changes in both short-term and long-term MBI practitioners; there were significant changes in genes associated with energy metabolism, mitochondrial function, insulin secretion, telomere length maintenance, and a decreased expression of genes associated with inflammatory and stress-related pathways, even after just one session of an MBI [121]. These genomic changes associated with MBIs are preliminary and need to be further researched with the cancer population.

MBI studies showing a small effect on physical health parameters may be an important part of understanding the impact of MBIs on the physical and psychological health of cancer patients and survivors [122]. Despite the inconsistencies seen between different MBIs, evidence suggests that MBIs may play a role in the cancer population by reducing psychological distress, sleep disturbances and fatigue, improving QOL, impacting biomarkers, and potential genomic changes [104].

10.6 Special and Alternative Diets

According to the NHIS data, the seventh most popular complementary health approach in the United States for adults is special diets [85]. Alternative diets are appealing to cancer patients in part due to the vulnerability patients feel with a cancer diagnosis; patients often seek control over their condition and search for options to manage symptoms such as fatigue, anxiety, and stress [73,103]. The stress of a cancer diagnosis along with the possibility of cancer reoccurrence, and possible death increases individuals' willingness to try new and potentially unproven options [104]. Often cancer survivors seek a CAM diet in hopes of improving their QOL, boosting their immune system, decreasing inflammation, and reducing body fat [123,124]. On the other hand, cancer patients and survivors feel overwhelmed by the abundance of dietary information and often misinformation that is available on the Internet and offered by family and friends.

For some individuals, alternative diets provide hope of a potential cure. Certain diets, such as the Gerson and Gonazales diets, became popular as individuals shared anecdotal accounts of remission [125]. Unfortunately, there is limited or little to no evidence to support most of the common special diets used by cancer patients. The lack of evidence and clinical data around specific diets make it challenging for health professionals to confirm the use of particular diets [126].

As a result, some individuals are willing to make changes in their diet and lifestyle in order to slow down or prevent the progression of cancer despite the lack of sufficient evidence of RTCs to guide those decisions [68]. In extreme cases, patients may choose to use special cancer diets as an alternative rather than a complementary modality for the treatment and prevention of cancer. A recent Medline search by Huebner and colleagues found that the top cancer diets are the alkaline, fasting, Gerson, ketogenic (carbohydrate restricted), macrobiotic, raw food, and vegan [126]. Additional popular diets are Budwig, Gonzalez regimen, no sugar diet, and others [125] (see Table 10.2 for selected popular special diets used in the cancer population).

10.7 Popular Anticancer Diets

10.7.1 Macrobiotic Diet

The Japanese philosopher George Ohsawa popularized the macrobiotic diet (MBD) in the United States in the 1960s under the name "Zen macrobiotic diet." [127] In the 1970s, Ohsawa's student, Michio Kushi, modified the diet to avoid extreme dietary practices while promoting locally grown and in-season foods [73,85,131]. Earlier versions of the MBD, which emphasized mainly whole grains and limited the diet to specific vegetables and few fruit, were linked to nutrition deficiencies and in some cases death [131].

There has been research on the MBD in regards to nutrient composition. Harmon *et al.* [123] compared standard sample MBD menus to a nationally representative sample from the 2009–2010 National Health and Nutrition Examination Surveys (NHANES) *What We Eat in America* [123]. Compared to intakes from the NHANES, the MBD had a lower percentage of energy from fat and higher total dietary fiber [123,132]. The MBD met or exceeded the Recommended Dietary Allowance for nutrients with the exception of vitamin D, vitamin B_{12}, and calcium [123]. These same three nutrients were also deemed shortfall nutrients by the 2015–2020 DGAs in the general American public. They are nutrients of public health concern given their under consumption has been linked to adverse health outcomes [70]. Sodium and saturated fat were found to be low in the MBD and are reported by the DGAs as being over-consumed by the US public [70].

Table 10.2 Selected popular cancer diets [41,57,120,121,127–130].

Diets	Premise	Nutrient composition	Nutritional concerns	Evidence	Comments
Alkaline	An acidic environment increases cancer risk	80% of diet from plant-based foods and low sugar fruits; limit 20% of diet to acid forming foods such as grains, meat, eggs, dairy, coffee, sugar, and alcohol	May be low in calories, protein, calcium, and vitamin D	None	Requires water intake of > 2 quarts/day, avoidance of certain food combinations or eating at specific times customized for the individual
Budwig	Abundance of trans fats and deficiency of omega-3 and 6 fats causes cancer	Mostly vegetarian or vegan; avoids hydrogenated oils, trans fat, animal fat, and dairy; emphasizes unrefined foods	May be limited in protein, calcium, and calories	None	Diet may be combined with specific exercises and enemas. Cottage cheese is often mixed with flaxseed oil
Fasting	Supports the immune system by starving tumors and stimulating cellular repair	Strict fasting allows only water for several days at a time; dry fasts avoid all liquids; juice fasts allow juice and water for several days, may include tea and broth; some variations include 200–500 calories/day	Extended fasting can lead to nutritional deficiencies and/or malnutrition. Extended dry fasts can lead to dehydration. Side effects include headaches, dizziness, fatigue, and low blood pressure	Preclinical only	Due to bioavailability of some drugs with food, need to monitor medication dosing
Gerson	Imbalance between potassium and sodium causes cancer. Goal is to increase potassium in the cells and decrease sodium via dietary intake with the goal of detoxing the body and building up the immune system	Typically three vegetarian meals and snacks with 15–20 lbs juice/day to be consumed as 1 cup ideally every hour for 13 h and limit whole grains for 6 weeks	Severe nutritional deficiencies and malnutrition; dehydration; colitis. The excessive use of coffee enemas can lead to sepsis, electrolyte deficiencies, dehydration, colitis, and possibly death	None	An organic, vegetarian diet in which food preparation is specific – a particular juicer to press the fruits and vegetables, recommends cast-iron pans. Includes biological supplements and pancreatic enzymes; coffee or other enemas regularly

(*continued*)

Table 10.2 (*Continued*)

Diets	Premise	Nutrient composition	Nutritional concerns	Evidence	Comments
Gonzales therapy	Cancer is related to environmental toxins and processed foods and pancreatic enzymes help eliminate toxins and help normal cells repair damaged cells	Emphasizes organic foods; range from vegetarian to diets high in meat and fat; 10 basic diets and 90 variations based on individual metabolic profiles	Flu-like symptoms, low-grade fever, muscle aches, skin rashes, misbalances of electrolytes	Limited with conflicting results	Daily coffee enemas, vitamin/mineral supplements, extracts of animal organs
Ketogenic	Based on Warburg effect, which describes cancer cells as gaining energy preferably by anaerobe glycolysis. Low carbohydrates will stop growth of cancerous cells	Diet energy distribution is 90% fat, 8% protein, 2% carbohydrates; 4:1 ratio of fat to carbohydrates and protein	Deficiency in micronutrients, loss of appetite, nausea, constipation, weight loss, hypoglycaemia, hyperlipidemia, dehydration, metabolic acidosis, fatigue, sedation	Limited	Variations include a 3:1 ratio of fat to carbohydrate and protein, the Atkins diet, and the LGIT. Growing body of evidence that KDs may be beneficial for other neurodegenerative diseases such as epilepsy, Alzheimer's, and Parkinson's disease
Macrobiotic	Based on the philosophy of decreasing toxins via diet by consuming an individualized primarily plant-based diet that focuses on eating organic and locally grown foods and in-season fruits and vegetables recommended	40–60% from whole grains, 20–30% from vegetables, 5–10% from beans, including soy products. Typically avoids meat, dairy, refined sugars, artificial sweeteners, caffeine, and processed foods	Weight loss, anemia, potential protein inadequacies, and possible deficiencies in vitamins (B_{12},D), zinc, calcium, and iron	Lower plasma estradiol levels in women; limited clinical studies	Includes sea vegetables; occasional fruit intake; seeds, nuts, and fish (primarily white fish) monthly; limited to no microwave use; and focus on chewing the food well

Raw food diet	Cooked food leads to cancer; less processed foods and fewer added ingredients preserve enzymes in food and provide health benefits	Mostly uncooked and unprocessed foods; no meat, dairy foods and eggs. Vitamins D and B_{12}, and calcium supplements recommended	Weight loss, deficiencies in protein, vitamin B_{12}, D, calcium, iron, and zinc	None	Low tolerance to raw foods in patients with mucositis
Vegan	Excludes dairy, eggs, and all animal products with a strict adherence to plant products	A plant-based diet that typically meets protein requirements if seeds, nuts, legumes, and cereal grain products are consumed in appropriate amounts	Weight loss, deficiency of vitamin B_{12}, D, Ca, zinc. Typically vitamin D may need to be supplemented if there is inadequate exposure to ultraviolet light from the sun or if insufficient vitamin D in the form of fortified foods is consumed	Limited	Routine evaluation for nutrient deficiencies, such as plasma vitamin B_{12}, vitamin D and bone density evaluation, should be considered for individuals following vegetarian diets

Limited studies have investigated the MBD in regard to cancer. Some research suggests that sea vegetables and soy intake promoted in the MBD may decrease the risk of breast cancer [124,133]. Urinary phytoestrogen metabolites and lignans have been found to be 10–20 times higher in women on a MBD than women eating an omnivorous diet [128]. The higher phytoestrogen excretion levels of women consuming a MBD is likely due to the higher intake of lignans from whole grains, seeds, soy, and other plant-based foods [124,128]. Furthermore, women eating a MBD had substantially higher fecal excretion and lower urinary excretion of estrogens [134], which has been suggested to put women eating a MBD to be at lower risk of breast cancer, however, studies have not confirmed an association [124].

10.7.2 The Ketogenic Diet

The ketogenic diet (KD) was first described in 1921 by Dr. R.M. Wilder for patients with epilepsy. A variation of a fasting diet, the KD consists of a high fat, moderate to low protein, and very low carbohydrate dietary intake. Interest in a KD for adjuvant cancer treatment was spurred in 1987 when decreased tumor weight and improved cachexia were observed in mice with colon adenocarcinoma xenografts eating a KD [135,136]. The KD mimics the biochemical changes of fasting and induces the body to burn fat instead of glucose for adenosine triphosphate synthesis [137]. Therefore, fat metabolism occurs via oxidation of fatty acids in the liver producing the ketone bodies acetoacetate, beta-hydroxybutyrate, and acetone [135,137]. Since cancer cells are thought to have increased levels of mitochondrial-derived reactive oxygen species (ROS) and cancer cells increase glucose and hydroperoxide metabolism to compensate for the increased ROS, it is theorized that diets low in glucose and other carbohydrates and high in fat selectively cause metabolic oxidative stress in cancer cells [137]. In animal models, KDs reduce tumor growth and improve survival of malignant glioma, colon cancer, gastric cancer, and prostate cancer [137]. In humans, case reports demonstrated decreased glucose uptake in two pediatric patients with advanced stage malignant astrocytoma [138] and an improvement in glioblastoma multiforme in an adult female [139]. In a quality of life study in patients with advanced cancer, the KD had no severe adverse side effects and improved emotional functioning and reduced insomnia [140].

A modified version of the KD, the low glycemic index treatment (LGIT), aims to maintain low, stable insulin levels by eating foods with a low glycemic index. Examples of low glycemic index foods include most fruits, green vegetables, beans, legumes, and peas; food that have a high glycemic index that would not be included on the LGIT include refined grains, juice, sugar sweetened beverages, and some fruits. The Atkins diet is another version of the KD. The Atkins diet was made popular by Dr. Robert Atkins as a treatment for obesity, and this modified variation of a KD that provides a 3:1 ratio of fat to carbohydrates.

10.7.3 Fasting Diet

For centuries, the practice of fasting has been done for cultural, religious, or spiritual purposes [141]. Although the effects of calorie restrictions, tumor growth, and normal cell metabolism dates back to 1914 when Payton Rous suggested restricted food intake could decrease tumor growth, it was not until recently that there is a renewed interest in fasting as a treatment option for cancer [135,142]. Animal studies suggest that fasting may make cancer cells more susceptible to cancer treatment while protecting noncancerous cells. In preclinical trials, fasting induces a state of ketosis and has been shown to enhance responsiveness to chemotherapy by sensitizing a range of cancer cell types to chemotherapy [129,143].

Preclinically, starvation increases the ability of commonly administered tyrosine kinase inhibitors to block cancer cell growth, inhibit the mitogen-activated protein kinase-signaling pathway, and to strengthen E2F-dependent transcription inhibition [144]. Intermittent or short-term fasting may be beneficial if done around or during chemotherapy [129]. Although results from human clinical trials are not available, several trials using various forms of fasting are underway, including fasting 24-h before and after the administration of chemotherapy in breast cancer patients; no-calorie fasting for 1, 2, or 3 days prior to chemotherapy, or a 48-h fast in patients with solid tumor malignancies; combination of fasting and the KD and reirradiation in recurrent glioblastoma; 2 days per week modified fasting with 25% energy intake and 5 days per week at 100% energy intake for cancer prevention; alternate day fasting versus calorie restriction daily for 3 months for cancer prevention; and others (www.clinicaltrials.gov). These trials and others may clarify if fasting as an adjuvant cancer treatment reduces the cancer burden, the type of fasting that is most effective, and the cancer types that are most responsive.

The Alkaline Diet's premise that an acidic diet increases cancer risk is not consistent with how the human body tightly controls blood pH and how kidneys normally maintain pH balance regardless of the acid or alkaline content of the diet [145]. The pH of urine is used to assess the efficacy of the diet and an alkaline diet typically results in more alkaline urine. An alkaline diet can also potentially result in lower urinary calcium, however, there is no evidence for protection from osteoporosis [145]. Despite the popularity of this diet, there is no scientific research indicating an alkaline diet is efficacious for the prevention of cancer.

Gerson Therapy was developed by Max Gerson, a German physician, and is based on his empirical observations and knowledge of research in cell biology in the 1930–1950s [130,146]. The supplement recommendations include potassium, Lugol's solution (potassium iodine, iodine, water), vitamins A, C, B_3 (niacin), flaxseed oil, pancreatic enzymes, and pepsin. Because of the 1989 FDA ban on injectable crude liver extract due to contamination with *Campylobacter*,

this supplement that had been used in the Gerson Therapy was replaced with desiccated liver capsules, and more recently with coenzyme Q10 and vitamin B_{12} [146,147]. There have been a few poorly designed clinical trials, which do not support the use of Gerson therapy for cancer treatment [130,146,147].

The Gonzales Regimen, which is similar to the Gerson diet, includes dietary modifications, dietary supplements, pancreatic supplements, and detoxification routines [148]. In a controlled observational study trial, patients with inoperable pancreatic adenocarcinoma were allowed to choose treatment with either gemcitabine-based chemotherapy or a pancreatic enzyme treatment known as Gonzales Regimen. Those patients who chose chemotherapy over the Gonzales Regimen survived three times longer and had improved QOL [149].

Raw Food Plan also known as "The Living Foods Diet" allows only raw foods or foods heated to 105 °F (40.5 °C). About 75% of foods consumed are fruits and vegetables but proponents also eat seaweed, sprouts, seeds, beans, whole grains, and nuts. Techniques such as sprouting and dehydration are common. Because all foods are eaten raw, some BFCs that are absorbed better when cooked, such as lycopene in tomatoes, may not be well absorbed with this diet plan. There is no evidence to support the raw food plan for cancer patients.

10.8 Conclusion

Healthy eating and active living are essential for decreasing cancer risk and for those who are at a greater risk for recurrence and developing secondary cancers due to the effects of treatments, unhealthy lifestyle behaviors, underlying genetics, and risk factors that contribute to cancer [60]. Research is providing insights into how diet, exercise, stress, and eating behaviors affect carcinogenesis. Nutritional genomics offers an explanation on how people's genetic background may affect their response to BFCs. Although certain BFCs, nutrients, foods, and dietary patterns may influence overall survival, cancer progression, and decrease risk of recurrence, not much research has focused on special diets that are popular among cancer patients and survivors [123,125,137,150]. In fact, many of these diets lack scientific evidence supporting their use. For a variety of reasons, CAM use, including biologics and MBIs, is increasing, especially in the cancer population. Although more research is needed to elucidate the role of CAM modalities in the cancer population, the evidence suggests that many of these modalities are improving psychological health of cancer patients and survivors.

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11

Social Determinants of Health and the Environmental Exposures: A Promising Partnership

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11.1 Introduction

Inequalities in cancer incidence, prevalence, and mortality are well documented. In many cases, these inequalities are associated with factors such as poverty, race, sexual behavior, gender, ethnicity, age, residence location, type of housing, and education. Together these factors have been described as "social determinants of health" (SDOH). Extensive public health research indicates that such factors are proxies for a variety of specific health outcomes, including discrimination, differential access to care, prevalence of risky health behaviors, and differential exposures to environmental and occupational hazards.

In recent decades, substantial progress has been made in the cataloging and tracking of cancer outcomes, the measurement of potential social determinants of health, and diverse observational studies examining the relationship between cancer and SDOH. Social, structural, and organizational factors can be conceived of as exposures and these exposures can influence cancer prevention, treatment, and survivorship both directly and indirectly. An example of a direct physiological effect is elevated levels of stress related to living in a dangerous neighborhood. However, individuals can also be at increased risk for poor health due to the indirect effects that arise as a consequence of discrimination, unequal access to health care, and other social processes. Despite repeated calls for the integration of biological and social sciences for health research (e.g., Ref. [1]), research and funding in health continues to focus on biological and biomedical approaches to disease and less on the comprehensive understanding that arises from the intersection of biology with behavioral, social, and economic factors.

Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer, First Edition. Edited by Michael D. Waters and Claude L. Hughes.

The biological/medical model of cancer includes examining cancer at the molecular level, at the organ systems level, and at the level of the individual body. But this perspective can be broadened to the social level as well, taking into account the concept that bodies form a part of a social collective, as neighborhoods, social classes, and communities [2,3]. One of the more complex aspects of untangling social determinants of health more broadly, and the causal relationships between inequities and social factors, more specifically, is that health outcomes depend on multiple levels of influence. These can range from individual factors such as debt and unemployment influencing access to health care; interpersonal factors such as family expectations and responsibilities about health care decisions; neighborhood and community factors such as safe places to exercise and access to healthy foods; and larger social forces such as racism and discrimination affecting experiences with health care.

The major aim of this chapter is to contend that the inclusion of an SDOH perspective could accelerate the "translation" in "translational toxicology." Specifically, as toxicologists identify new harmful exposures and new methods for preventing or treating such exposures, translation requires implementing cost-effective measures to reduce exposures and deliver treatment equitably across the population. An SDOH perspective should help identify populations at risk more effectively and should help researchers and policy makers design and deliver more appropriate interventions. In addition, attention to the SDOH framework could help generate novel hypotheses concerning windows of susceptibility to toxic exposures. For example, the environmental justice movement highlighted the link between asthma and air pollution among poor and minority communities that led to significant new epidemiological research exploring links between air pollution, asthma, and obesity [4,5]. By "windows of susceptibility" we refer to the broad notion that exposures at a particular point in the life course could have an immediate and significant effect compared with the same exposure at a different stage in the life course, but also to the possibility that the consequences of such exposures might manifest themselves much later in life [6,7]. Past literature has referred to these possibilities somewhat inconsistently as periods or windows of vulnerability and susceptibility as well as more generically in the context of discussion of environmental effects on developmental origins of risk (e.g., Ref. [8]).

An SDOH perspective also helps emphasize that translation requires consideration of population-level approaches as well as individual approaches to reducing toxic exposures and their outcomes [9]. For example, reduction in childhood pesticide exposures in farm children might require consideration of immigration policy, living conditions for migrant workers, or labor laws that could have a more pervasive and sustained effect on childhood pesticide exposure than a focus on the control of pesticide exposures merely at the level of individual applicators [10–12].

The intent of this chapter is not to suggest specific solutions to the instances of health outcomes or exposures linked to windows of susceptibility, but to encourage readers of this chapter to think critically about proximate and ultimate (or "downstream" and "upstream") causes of adverse exposures and their consequences. In the remainder of the chapter, we introduce a conceptual model for integrating a socioecological framework with ideas based on an SDOH perspective into a life course approach to translational toxicology. The socioecological framework posits that multiple levels of influence ranging from biological to social, community and societal, factors influence health behaviors and health outcomes [13,14]. Progress in understanding the causes of health outcomes requires attention across these levels.

In this chapter, we discuss race/ethnicity as exemplar of the benefits and some of the challenges of the SDOH perspective. Lastly, we illustrate two key aspects of SDOH thinking via a discussion of geographic/spatial variation in cancer incidence, mortality, and health issues related to gender/sexuality. In the next sections of this chapter, we discuss a conceptual model for linkages between social determinants of health and windows of susceptibility over the life course. Thereafter we define health disparities and then turn to a discussion of specific social determinants.

11.1.1 Conceptual Model

Figure 11.1 illustrates the conceptual model that forms the basis of our current thinking. As illustrated, SDOH results in differential susceptibility to environmental exposures, differences in health behaviors, and differences in exposures to social stressors. Various stages of the life course may also display differential susceptibility to such exposures resulting in differential health outcomes [15,16]. To the extent that environmental and social stressors are unequally and unfairly distributed, these processes can result in inequitable health outcomes [17]. Grandparental, maternal, and paternal effects (at the biological, genetic, behavioral, and social levels) are included because of extensive evidence from animal models that such influences matter for health. Examples related to cancer and metabolism includes maternal diet effects on mammary tumorigenesis [18] and consequences of paternal fasting for offspring metabolism [19]. More work could be done to link studies of maternal and paternal effects on biological traits [20] and on epidemiological studies of energy balance and its consequences in humans [21]. As illustrated, cross-generational effects may be due to genetic, epigenetic, behavioral, or cultural processes. Better interventions to improve health and reduce health disparities might arise from efforts to tease apart the relative importance of these different causal pathways. This is a topic of intense interest among public health researchers at present [22].

Windows of susceptibility represent specific stages in the life course where people are more vulnerable to exposures and their consequences. In addition, it

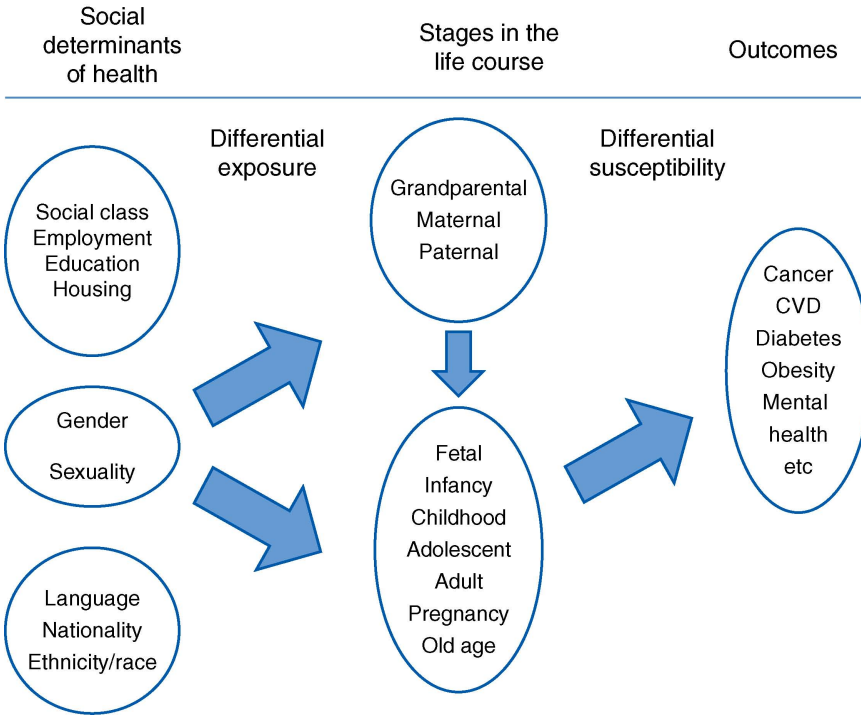

Figure 11.1 Social determinants of health result in differential exposures to environmental toxins, poor health behaviors, and social stressors. Various stages of the life course may display differential susceptibility to such exposures. Together these processes result in health outcomes.

could also refer to periods of increased vulnerability to effects of exposure of which some may not be realized until much later in life. Importantly, they may also provide leverage points that could be used to develop policies with potential to address disparities in exposures and outcomes in a more efficient manner. Classic examples of toxic exposures associated with windows of susceptibility include lead exposure in childhood [23], fetal alcohol exposure [24], tobacco uptake in adolescents [25], and diverse teratogens. A presumption of this chapter is that research has not yet fully addressed these toxicological challenges to health and health equity, so we highlight these diverse pathways to morbidity, mortality, and health disparities.

11.1.2 Difference versus Disparity

A key challenge in discussions of SDOH involves distinguishing between "differences" and "disparities" in health and health outcomes. This topic has

been discussed extensively in the public health literature [26]. A simple example involves gender-specific risk of cancer at different sites. Only men can be at risk for prostate cancer and only women can be at risk for cervical cancer. This is not a disparity in cancer risk but a difference. On the other hand, differences in breast cancer mortality between non-Hispanic whites and African-Americans could be due to a mix of social factors influencing exposure, access, and treatment choices as well as biological factors related to the incidence of different subtypes of breast cancer [27]. An overwhelming body of evidence has documented the existence of health disparities in the United Sates related to race/ethnicity, economic factors, gender/sexuality, and other demographic constructs (e.g., Refs. [28–30]). Nevertheless, appropriate interventions and guidance for policy depend critically on careful empirical work teasing apart the relative contributions of biological differences between individuals or groups and social factors in influencing differences in health and health outcomes. The fact that differences in vital rates arise from these two causes makes it so that any assessment of a "difference" or a "disparity" is an interim judgment, pending more complete understanding of the causal web influencing the outcome of interest. An important element of the concept of disparity involves inequity. This is the idea that if differences in health outcomes are the result of inequity, then they are unjust and should be addressed via policy change. Thus, Krieger *et al.* [27] have argued that health differences become disparities when they are linked with inequities.

Box 11.1: Health Disparities

The National Cancer Institute (NCI) defines "cancer health disparities" as "adverse differences in cancer incidence (new cases), cancer prevalence (all existing cases), cancer death (mortality), cancer survivorship, and burden of cancer or related health conditions that exist among specific population groups in the United States." These population groups may be characterized by gender, age, race, ethnicity, education, income, social class, disability, geographic location, or sexual identity. People who are poor, lack health insurance, and are medically underserved (have limited or no access to effective health care) – regardless of ethnic and racial background – often bear a greater burden of disease than the general population [31].

11.2 Social Determinants of Health

11.2.1 Race/Ethnicity

One of the earliest means that health disparities were identified and tracked in the United States was through racial differences in health outcomes. Although

racial categories are grounded in visible physical differences (such as skin color), sociological and anthropological evidence has demonstrated that the epidemiological category of "race" is in large part a social category [32]. Thus, the utility of "race" as a measure of biological differences in genetic predisposition is much smaller than commonly believed even among biomedical researchers. Reinforcing the concept that "race" or "race/ethnicity"' may be better conceived of as an important social category, particularly in the United States, due to the historically legacy of colonialism and slavery. Kaufman and Cooper [33] argue:

> "[E]ven for those who embrace the view that the biologic content of racial/ethnic categories is limited, a rationale for continued focus on these quantities is that they encode important variations in environment because of the central role they play in social stratification." (p. 291)

Box 11.2: Changing Race/Ethnic Categories in the US Federal Statistical System

Measurement of race/ethnicity in the US Federal Statistical system has undergone several changes in recent decades (Appendix 1 of Ref. [34]). Current groupings [35] are based on mixed criteria, including geographic origin (Asian-American), language (Hispanic), and a mix of racial identity and geographic origin (African-American/black). For example, the category of African-American is complicated; over the last 30 years, the composition of the "African-American" population in the United States has changed from one consisting largely of the descendants of people brought to the United States as slaves to an increasingly diverse mix that includes not only people of different African origins but also more recent immigrants from the continent of Africa, the Caribbean, and Latin America who may identify (or be categorized) as African-American but have potentially experienced very different exposures and social milieus. In addition, as these populations intermarry and move across the world and the United States, the challenge for research is how we can better understand the intersection of these ever-changing racial and ethnic identities with social, economic, biological, and environmental factors.

A classic example of the intersections of the life course perspective and SDOH is the case of racial and ethnic differences in birth outcomes in the United States. Infant mortality rates are recognized as an important indicator of the health of a nation because they are associated with outcomes related to maternal health, access to quality health care, socioeconomic conditions, and the public health practices of the population [36]. Racial and ethnic disparities in infant mortality have been evident for as long as vital statistics have been collected in the United States, and have in fact increased for some racial and ethnic groups [9,37,38]. This suggests that not all infants born in the United

States have benefited from social and medical advances [36]. For example, in 2013 (the most recent data available) rates vary greatly among different racial and ethnic groups. Infant mortality rates are highest for non-Hispanic black women (11.11 deaths per 1000 live births), while for American Indian and Alaskan Native women and Puerto Rican women the infant mortality rates are 7.61 per 1000 and 5.93 per 1000, respectively. Comparatively, the rate among non-Hispanic white women is 5.06 deaths per 1000 live births, and more troubling, the disparity in infant mortality rates between non-Hispanic black women and non-Hispanic white women has more than doubled in the last decade [38]. Therefore, explanations for these disparities must be sought in social variables and may be attributed to rates of preterm and low birth weight deliveries, socioeconomic status, discrimination, and access to quality medical care among other social factors.

Analyses of cancer incidence and mortality have proved to be rich sources of examples concerning differences and disparities and the subtleties of distinguishing between them. For instance, until the 1950s deaths from cancer were lower among blacks than whites. But by 2000, the rate of death for whites had remained relatively stable; however, the rate among blacks had increased 40% over the 50 years. In particular, deaths from lung and ovarian cancer declined for both blacks and whites, but mortality from colorectal, breast, and prostate cancer increased significantly for blacks while remaining relatively stable for whites [9,39]. For breast cancer, recent research has demonstrated that women of African descent are more likely to be diagnosed beyond stage I breast cancers [40]. Incidence of breast cancer is lower among blacks (among women over 50 years of age), but mortality is higher. In comparison to white women, black women diagnosed with breast cancer are twice as likely to die from the disease within 5 years. When disentangling race from socioeconomic status, a meta-analysis found black race to be a statistically significant predictor of cancer mortality, even when various measures of socioeconomic status (SES) were taken into account [41]. Known biological differences between these two race/ethnic groups do not account for these mortality differences, and considerable evidence suggests that at least part of them are due to disparities in treatment and other social factors [9,39]. Hence, a number of researchers are working toward examining disparities as interactions among social factors, broadening analysis beyond simple variables of race and ethnicity, or SES. Kish *et al.* [42] used the Surveillance, Epidemiology, and End Results (SEER) Registries to examine census tract-level SES index to estimate the survival disparities for a number of cancer outcomes. This project was noteworthy in that it looked at both SES and racial categories, which allows for a finer analysis of the ways in which both SES and race/ethnicity affect cancer survival rates.

As noted by Williams and Jackson [39], race generally is a "marker for differential exposure to multiple disease-producing social factors. Thus, racial disparities in health should be understood not only in terms of individual

characteristics but also in light of patterned racial inequalities in exposure to societal risks and resources" (p. 325). Therefore, it is important to examine racial disparities in cancer diagnosis and mortality as part of a larger system of interactions including both social and biological factors.

11.2.2 Social Determinants of Health: "Place" and Its Correlates

Place and analyses of differences between places have played a critical role in identifying and understanding the causes of health disparities including those related to cancer [43–45]. The idea of "place," including the environments where people are born, grow, live, and die, has a relevant impact on their everyday health, and it can include aspects of the built, natural, economic, transport, and social environments [46,47]. Thus, aspects of geography and the built environment are key to place, but diverse social factors such as class, access to education, job opportunities and advancement, health care services, transportation options, quality schools, and opportunities for economic stability that promote and support a healthy lifestyle and housing all combine to define "place" [48]. In addition, locality and place are fundamental to experiences of health and health outcomes overall.

There is a rich literature linking toxic exposures and increased risks for cancer, but it is less widely understood the ways in which neighborhoods and socioeconomic status can interact to increase risk for poor health and result in higher rates of disease [49,50]. These neighborhood and socioeconomic factors can be understood as being environmental exposures that can be protective or toxic and determine health outcomes. Such exposures are in addition to the more traditional focus on place as a proxy for specific toxicological exposures. Boscoe *et al.* [51] used the North American Association of Central Cancer Registries (NAACCR) and the National Cancer Institute's SEER data to examine the relationships between area poverty rate and site-specific cancer incidence in the United States. Using the poverty-level of census tract, Boscoe *et al.* aimed to bring SES into the mainstream of US cancer surveillance. By coding the data based on the poverty categories (areas with people less than 5% below poverty, 5–10%, $10 \leq 20\%$, and >20%), they found that poverty is related to a majority of cancer sites. Those most associated with higher poverty include Kaposi sarcoma, larynx, cervical, and penile cancers. The sites most associated with lower poverty rate included melanoma, thyroid, and other nonepithelial skin. These findings indicate that over and above the effect of race/ethnic identity, poverty is an independent correlate of cancer incidence [51]. Careful analyses are needed to tease apart the independent effects of behaviors associated with cancer incidence and environmental exposures to toxins as both are known to be correlated with neighborhood deprivation [52,53].

Other projects investigating deaths from colorectal cancer (CRC) have examined the links among racial/ethnic, socioeconomic, and geographic

inequalities [54,55]. Historically, deaths from colorectal cancer were higher in communities of high socioeconomic status and in northern versus southern states. However, in the past few decades, particularly since screening shifts in the 1980s, this trend has reversed. Using 2008–2010 mortality data from colorectal cancer as collected from the National Vital Statistics System, Jemal *et al.* [54] demonstrate that death rates decreased with increasing educational attainment within each racial/ethnic group. They conclude that "approximately 50% of premature deaths resulting from CRC that occurred nationwide from 2008 through 2010 – the equivalent of 7,690 deaths annually – could have been avoided by eliminating racial, socioeconomic, and geographic inequalities in CRC mortality rates" (p. 2). Such geographic inequalities include higher rates of death among those living in southern states that Jemal *et al.* link to an overall higher prevalence of unavoidable risk factors and more limited access to CRC screening and treatment. Wang *et al.* [55] argue that much of inequalities in colorectal mortality relate to socioeconomic status, particularly as it relates to the diffusion of preventive practices and recommendations.

In addition to the links between socioeconomic inequalities and cancer mortality, a number of researchers are examining the associations between chronic stress (a correlate of place) and cancer. Biologically, chronic psychosocial stress, similar to that experienced in poor neighborhoods and under financial stress, results in the activation of specific pathways in cancer cells and the microenvironment of tumors. Epidemiological evidence suggests that depression is related to increased mortality [56], and Moreno-Smith *et al.* [57] describe in some detail biological pathways that may link stress and its correlates with cancer progression. Animal models are also being used to examine these pathways. For example, solitary housing versus group housing (5/cage) from puberty onward of Sprague-Dawley rats increased relative risk of malignancy and mammary tumor burden [58]. This increased risk was associated with increased stress responses in the socially isolated rats, and perhaps such experiments are relevant to housing and social environments in humans. Related results have also been obtained in studies of mice [59]. Transgenic mice (FVB/N Tag) housed alone had greater tumor burden than group housed mice along with changes in behavior and gene expression in the mammary gland. Mouse models are also being used to explore potential windows of susceptibility to social stress and its consequences for tumorigenesis. Schuler and Auger [60] examined social stress initiated during the peripubertal period (3–6 weeks of age) and its link to tumorigenesis within the mammary glands. This work illustrates how early-life stress experiences can trigger and modulate development within the mammary glands. These early-life stress and their biological correlates can arise both as a consequence of neighborhood or "place" characteristics, but might also be indicators of personal history. For example, a number of researchers have examined allostatic load, an index of the biological consequences of stress in relation to immigration history. Allostatic load is

associated with region of origin and nativity history as well as with stress conditions over the life course within a country [61,62]. Further research is needed to develop an understanding of the mechanistic pathways [63] that account for epidemiological evidence linking social factors and health [64], but research on allostatic load and related topics, especially research on the developmental origins of health and disease as well as growing interest in life course epidemiology [15,16], has provided strong evidence that biological pathways underlie these associations. NIH investment in this topic is also growing, for example, via the Breast Cancer and Environment Research Centers [65].

As social determinants of health compound to create disparities in both incidence and survival of cancer, researchers have argued that risk for poor outcomes goes beyond income. In addition to neighborhoods that contribute to chronic levels of stress, a related concept is economic deprivation/disparities that are more severely experienced in economically deprived and low resource neighborhoods. For example, wealth may play a significant role in resilience after job loss or illness. In the United States, race/ethnic groups such as African-Americans have significantly lower assets than non-Hispanic whites and this may account for health and economic disparities over the life course [66–69]. Recent research has linked chronic financial stress with increased inflammatory factors in African-American women [70]. There is some emerging evidence about the "financial toxicity" of cancer care and how low-income and not well-insured people are making decisions on whether to undergo or complete their cancer treatment because of the high cost of care. Covering the high costs associated with cancer treatment out-of-pocket appears to be linked to decreased treatment adherence and poorer quality of life [71–73].

Residential segregation may compound many of the risk factors detailed above by creating neighborhoods characterized by poverty, poor educational opportunities, lack of health care services, and higher rates of violence and homicide [39]. Researchers hypothesize that one of the mechanisms linking low socioeconomic status and poor cancer outcomes involves a higher incidence of behavioral risk factors. Types of cancer most associated with behavioral risk factors, such as alcohol, tobacco, intravenous drug use, sexual transmission, and poor diet, tend to be most associated with higher poverty [51,74]. Such patterns in the United States have also been observed in other countries with quite different social systems and class structure. For example, in the United Kingdom, a long series of cohort studies have documented relationships between social status and health outcomes, despite access to nationalized health care (e.g., Ref. [75]).

Overall in the United Kingdom, the cancer survival rate for affluent patients was between 5 and 15%, and they were more likely to survive after 5 years [76]. As noted by Michel Coleman, a researcher at the London School of Hygiene and Tropical Medicine, "This shows that cancer survival is not even a lottery

because a lottery is fair. A lottery ticket buys you the same chance of winning as everyone else but this is not true for cancer survival. Your chances depend on the area in which you live, and if the survival rates of all patients were as good as those achieved in affluent areas we would avoid many deaths" (p. 318 of Ref. [77]). As described in this section, "place" and its correlates have important implications for the social determinants of health and windows of susceptibility. Place becomes an important tool to integrate perspectives on neighborhood (including environmental exposures) with socioeconomic measures and racial/ethnic identities. Overall, we conceptualize "place" as both a proxy for toxicological exposures and a set of descriptions of a geographic "space" with a variety of social and environmental characteristics that can have direct impacts on health, positive and negative.

Other important considerations in examining identity include gender and sexuality. The following section illustrates how a life course perspective on the social determinants of health includes critically examining how gender and sexual identity influences both incidences and experiences of cancer.

11.2.3 Gender and Sexuality

Our final example of an SDOH and its consequences for health over the life course involves gender and sexuality. The terms "sex" and "gender" are often used interchangeably, although social scientists have designated them as two distinct categories. "Sex" refers to the biological characteristics associated with "male" and "female," most often related to reproductive functions and anatomy. "Gender" refers to the socially constructed characteristics associated with masculinity and femininity ascribed by a particular culture. Gender is created in the daily social interactions within dynamic and cultural contexts. Social scientists have advocated for the use of "gender" to reinforce the idea that not all differences between men and women are explained simply by biology, or sex differences [78]. In fact, as illustrated later, sexual variation in cancer incidences and rates can be further exacerbated by gender differences in screening and treatment protocols.

Colorectal cancer is among the most common cancers throughout the world, and one of the most common causes of cancer mortality among women. However, the incidence and mortality among populations over 65 show clear sex differences, whereby the mortality is higher and the 5-year survival rate is lower among women versus men [79]. Evidence from recent research has demonstrated a higher proportion of women present with right-sided colon cancer, which is a more aggressive type tumor and therefore more commonly at an advanced stage at diagnosis [80]. Differences in colorectal incidences between men and women could be related to both sex- and gender-related characteristics. Women often have a longer transverse colon that could potentially lead to more cases of incomplete colonoscopy and decreased

detection of tumors [81]. On the other hand, incidence and mortality from colorectal cancer remain greater in men than women (http://seer.cancer.gov/statfacts/html/colorect.html), supporting the consensus that multiple factors influence sex and gender differences in colorectal cancer incidence including dietary fiber consumption among women [79,82] and various genetic and behavioral risk factors.

Gender-specific screening behavior also affects cancer outcomes for men and women. Previous work has shown that men are less likely to access routine health care screenings than women [83–86]. However, it is also important to recognize that gender-specific health care behaviors are not homogenous and can also be influenced by racial/ethnic identity and socioeconomic status [83]. The above discussion of sex and gender differences in colorectal cancer risk just touches on a very complex epidemiological and biological literature. A key take-home message is that distinguishing between differences and disparities in health outcomes depends critically on understanding the causes of such differences. For example, differences in colorectal cancer risk and in the benefits of colonoscopy between men and women appear to depend on a mix of factors including colon length, genetic and chromosomal factors related to colon cancer progression, and the distribution of risk factors [81,87].

In addition to gender- and sex-based differences in cancer incidence and experiences, sexuality is another important aspect of social determinants of health. Sexuality and sexual identity can impact access to care, experiences in health care, and rates of cancer incidence related to behavioral risk practices. However, in some cases lesbians are more at risk for certain types of cancer because they are more likely to be nulliparous and have a lower contraceptive use, which can include protective factors for breast cancer and cervical cancer [88]. Data from the Women's Health Initiative demonstrated higher rates of breast cancer among lesbian and bisexual women, despite similar mammography screening rates [89].

Lesbian, gay, bisexual, transgender/transsexual, and intersex (LGBTI) individuals are often underrepresented in research. Many of the databases predominantly used in cancer research do not collect information on the sexual identity of cancer patients and survivors [90]. Although the National Institutes of Health is committed to integrate data collection of sexual identity within electronic health records [91], there remain a number of challenges ahead in developing valid and reliable methods for asking individuals to classify their sexual identity [92]. To this end, the Centers for Disease Control and Prevention's National Center for Health Statistics added questions about sexual identity to the National Health Interview Survey (NHIS) beginning in 2013. Health disparities for the LGBTI community are often related to stigma and discrimination experienced in society at large and within health care settings. Many individuals do not feel comfortable disclosing their sexual orientation, which can put them at higher risk for particular cancers because their providers are unaware of their risk status. This social stigma can also

have mental health repercussions, leading to feelings of shame or self-loathing related to their sexual identity [93]. Kamen *et al.* [94] used the LIVESTRONG dataset to investigate psychosocial distress impacting lesbian, gay, bisexual, and transgendered cancer survivors. Their analysis demonstrated that LGBTI identity was associated with a significant increase in depression and social/relationship concerns compared with heterosexual cancer survivors. In many cases, support for cancer survivors is often geared toward heterosexuals, with discussions around sexual relationships and functions assuming heterosexual relationships [93].

Health providers' assumptions about sexual identity and risk factors can also influence cancer risks and outcomes. For instance, the risk for cervical cancer among lesbians is often underestimated and underdetected, since the majority of cervical cancer cases are associated with human papillomavirus infections, and much of this risk is assumed to affect women who have sex with men [95]. However, research has demonstrated that both positive HPV tests and squamous epithelial lesions have been detected in women who exclusively have sex with women [96]. Because of the assumptions that women who have sex with women are at lower risk for sexually transmitted infections and therefore cervical cancer, lesbians are less likely to get regular pap screening [95].

This section demonstrated the importance of including gender and sexuality as a critical component of social determinants of health. Gender and sexual identity can impact both the incidence and sites of cancer, as well as experiences in cancer treatment. Health researchers, including funding agencies such as the National Institutes of Health, are committed to integrating sexual identity as an important aspect of research on cancer incidence and treatment. However, given the difficulties related to studying identity, as well as the stigma attached to disclosing sexual orientation, health disparities related to gender and sexuality continue to impact people across their life course.

11.3 Conclusions: Social Determinants of Health and Windows of Susceptibility

Throughout this chapter we discuss specific "windows of susceptibility" and their underlying biological properties describing a variety of social determinants of health that can affect people throughout their life and across the whole cancer continuum from prevention to survivorship (http://cancercontrol.cancer.gov/od/continuum.html). Successful translation of discoveries in toxicology to interventions aimed at reducing adverse health effects of toxic exposures and health disparities continue to require an SDOH perspective. So far race and poverty have been a primary focus of such work. New developments in geospatial thinking and new perspectives on gender and sexuality suggest that more work on these topics will have much more to contribute to public health in the United States and beyond.

We tend to parse effects in our studies because it is difficult and sometimes impossible to do multilevel and multifactorial studies that capture the biology, behavioral, social and economic factors, and their interactions. Thus, we are unable to talk about the impact of these factors as they interact with each other simultaneously to create disparate health outcomes. However, as we juxtapose these studies we can see how these factors affect health outcomes for people living in deteriorating neighborhoods, facing limited choices for health care, with limited financial options and lower levels of education, and facing discrimination and prejudice resulting in inequitable treatment choices and outcomes. This necessitates the conceptualization, understanding, and incorporation of SDOH as environmental factors that have varying effects on prevention, treatment, survival for cancer, and other health outcomes across the life course.

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Part Four

Categorical and Pleiotropic Nonmutagenic Modes of Action of Toxicants: Causality

12

Bisphenol A and Nongenotoxic Drivers of Cancer

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12.1 Introduction

Carcinogen exposure induces a wide variety of cellular responses that have been classified into genotoxic and nongenotoxic mechanisms. The direct alteration of a cell's genetic material by genotoxic carcinogens often provides clear biomarkers and mutation hallmarks for the initiation of cancer. Nongenotoxic carcinogens are chemicals that promote carcinogenesis without directly reacting with DNA. Despite the lack of mutagenicity, nongenotoxic carcinogens can also profoundly influence the development and progression of cancer, through a number of indirect mechanisms that may increase the rate of cellular proliferation, disrupt cellular structures, generate reactive oxygen species (ROS), induce receptor mediated signaling, and alter gene expression patterns or epigenetic programming of cells. These complicated and varied secondary mechanisms by which nongenotoxic carcinogens induce neoplasia are largely tissue and species specific, and rarely follow the low-dose linearity often observed with genotoxic agents, such as ionizing radiation. These characteristics present significant challenges to assessing the human health risk of these agents, and also pose difficulties for researchers and regulatory agencies. To illustrate the complexity in evaluating the mechanisms of tumor promotion by a nongenotoxic carcinogen, we will examine the toxicant and candidate carcinogen bisphenol A (BPA), a ubiquitous and well-known estrogenic chemical used in consumer goods.

BPA is released into the environment through the manufacturing of polycarbonate plastics, epoxy resins, and thermal paper. Polycarbonates are used in a large variety of consumer products, including plastic storage containers and medical devices. Epoxy resins are also used in a wide variety of consumer

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products, including the linings of food and beverage containers as well as in dental composites and sealants [1]. Human exposure occurs through inhalation, ingestion, and skin absorption and has been measured in serum in the nanomolar range [2,3]. Initial examination of the metabolism and toxicokinetics of BPA revealed that after ingestion BPA was quickly metabolized in the liver into bisphenol A glucuronide or bisphenol A sulfate, then eliminated through urination [4]. These findings contributed to the early assessment that very little free BPA would circulate through the body and harmful estrogenic effects would be minimized by fast metabolism. However, there is now growing evidence that free BPA circulates throughout the body [5,6], and even at low doses can act as a potent endocrine disrupting chemical (EDC), significantly impacting human health and reproduction (for reviews see Refs [7–11]). There is also growing evidence that BPA promotes the generation of ROS [12–16], alters a number of important tumor suppressing cell signaling pathways [17–20], induces epigenetic reprogramming [21–23], and stimulates inflammatory responses [24–27]. Together these data indicate that BPA initiates a number of cellular responses that can promote or contribute to carcinogenesis and represents a potent nongenotoxic carcinogen, whose mechanisms are poorly understood (Table 12.1). Given the fast pace at which new BPA studies are emerging and the complexity of the responses observed, here

Table 12.1 Overlapping mechanisms of action of bisphenol A contribute to its carcinogenic potential.

Cellular and molecular events	Examples	Developmental outcomes	Carcinogenic potential
Changes in receptor-mediated events	ERβ-mediated induction of MAPK family, ERK1/2 and JNK ERRγ/GPER30-mediated ERK1/2 activation Increases in ERα: ERβ ratio Signaling cascade changes (p53, mTOR, MAPK, etc.), not conclusively ER or mERs linked		Increase in inflammatory factors, microglial activation [25] Increased cell proliferation of breast and prostate cells [28–31], altered DNA damage response and apoptotic signaling [32–35] ERα increases are predictive for progression of breast hyperplasia to malignancy [17] Dose-dependent increases in cell proliferation proteins and evasion of apoptosis in breast cell lines [17,18]; increases in ovarian cell migration [20,36] and oncogenes [37,38].

Table 12.1 (*Continued*)

Cellular and molecular events	Examples	Developmental outcomes	Carcinogenic potential
Changes in transcription, translation, or epigenetic programming	Global gene expression changes after exposure in breast cell lines, ovarian cell lines, and mouse embryonic cell lines Alteration in methylation status miRNA expression levels changes	Alteration in Oct4 and Nanog proteins during mammary development [39] Hypomethylation and hypermethylation phenotypes affecting mammary development and fertility [40–42] Alteration in gene expression for reproduction and development [43]	Increases in oncogenes (MYC, STAT3, BCL-2), loss of proapoptotic factors (p53, BAX), changes in DNA repair proteins (BRCA1, BARD1), and hormone signaling [19,22,23,37,44–46] Increase risk of prostate cancer Pde4d4 silencing [47] and breast cancer through LAMP3 silencing [21] Alteration in gene expression for cell cycle, DNA repair, response to DNA damage [43]
Oxidative stress	Induction of ROS Alteration in antioxidant balance	Underdevelopment of testis, brain, and kidney [14,16,48]	Direct and indirect DNA damage [12,13,44,49–52]
Inflammation	Global gene expression changes after exposure in numerous model systems Increases in inflammatory proteins		Upregulation of inflammatory response genes, some partially mediated by ERα [25,53] Increase in CRP and/or IL-6 in polycystic ovary syndrome and postmenopausal women [24,54]; increases in TNF-α and IL-6 in males [27]
Immune modulation	miRNA expression Activation of microglial cells Cytokine production	Alterations in gene expression govern innate immune response Decreases in T regulatory $CD4^+CD25^+$ cells [55]; dysregulation of mast cells [56]	Production of proinflammatory factors, TNF-α, and ROS

we will review the current literature supporting BPA as a nongenotoxic carcinogen to help further research evaluating the health risks of exposure and to provide a framework from which all potential contributing effects of this toxicant to carcinogenesis may be considered.

12.2 Dosing

A significant impediment to the evaluation of BPA as a nongenotoxic carcinogen was inconsistent literature findings based on dosing. BPA, like other estrogenic compounds and hormones, shows a nonmonotonic dose response [57]. A mixture of doses, durations, and endpoints are used in BPA studies making the evaluation of carcinogenicity difficult. In 2007, the NTP-CERHR (National Toxicology Program Center for the Evaluation of Risks to Human Reproduction) monograph found significant inconsistencies in the literature as a result of incomplete data on the dose–response effects of BPA, which made evaluating the human health consequence of persistent BPA exposure more difficult [58]. Since this evaluation, research efforts, including the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on Toxicity of BPA), have focused on developing an improved understanding of the toxicokinetics of BPA exposure, including a better understanding of the free circulating BPA in human serum [6,59].

The development of reference dosing levels for BPA exposure has been significantly impeded by our lack of understanding of the mechanisms by which BPA conjugation–deconjugation cycling occurs. Furthermore, while the conversion of BPA into glucuronide (BPAG) and sulfate (BPAS) metabolites may inactivate its estrogenic properties, few studies have evaluated our exposures to these compounds. A recent report examining BPAG exposure *in vitro* demonstrated that it is not inactive but induces adipogenesis [60]. Deconjugation of BPAG and BPAS increases the circulating free BPA that we are exposed to in a tissue- and organ-specific manner and can be especially detrimental to fetuses, where increased concentrations of BPAG and BPAS have been observed due to the placental barrier [61]. Deconjugation of these increased BPAG and BPAS levels have been attributed to β-glucuronidase or arylsulfatase C enzymes present during fetal growth and the downregulation of key BPA metabolizing enzymes, such as Ugt2b1 [62–64]. It has also been posited that tumor microenvironment might contribute to BPA conjugation–deconjugation cycling through increased β-glucuronidase due to induced inflammation and lysosomal release [65,66].

Despite the lack of definitive human exposure ranges, near human exposure level BPA studies have continued to emerge showing that chronic low-dose exposure to BPA can have significant effects on cell proliferation, gene expression, signaling, and survival [21,22,67–69]. At the same time, a number of higher dose studies have emerged showing consistent effects in altered signaling pathways, gene expression changes, and epigenetic changes, though there are inconsistencies in cell proliferation changes, stimulation of calcium release, and body weight changes [12,17–19,25,68,70]. With nonmonotonic dosing, endpoints and durations must be carefully evaluated since the potential for a dramatic difference between doses is only a magnitude apart.

Overall BPA dosing is still highly controversial, yet there are consistent threads throughout the current BPA literature that indicate a wide variety of BPA doses can significantly alter gene expression and signaling in cells. This clearly indicates that active, unconjugated BPA and its numerous metabolites have more significant exposure effects than originally considered, and research efforts that explore broad dose ranges and a number of cellular outcomes are needed to better understand the health consequences of exposure.

12.3 Receptor-mediated Signaling

A common mode of action for nongenotoxic carcinogens is receptor-mediated effects. Steroids and xenoestrogens can cause cancer through hormone receptor-mediated interactions, which can perturb hormone balance, increase cell proliferation, and alter gene expression patterns. For example, estrogenic ligands like 17β-estradiol bind estrogen receptors (ERs) and induce carcinogenic effects by altering genomic and nongenomic regulation of transcription. Binding of estrogenic ligands to estrogen receptors α and β (ERα and ERβ), members of a nuclear receptor superfamily, activates these complexes to bind estrogen-responsive elements (ERE) in the promoter regions of target genes, regulating their transcription [71]. Gene expression changes can also be induced independent of ERE elements through the interaction of ERα and ERβ with DNA-bound transcription factors [72,73]. Nongenomic signaling can also be induced by estrogenic ligand binding to nonclassical membrane estrogen receptors (mERs) or other estrogen binding proteins that induce kinase signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathway [72,73]. Together these alterations induce changes in cell growth, differentiation, motility, and DNA damage response and repair that can contribute to the development and progression of breast, ovarian, and endometrial cancers [72,74].

Given the estrogenic nature of BPA, numerous studies have examined the effects of BPA exposure on ERα and ERβ. BPA has a significantly lower affinity, ~1000-fold lower than estradiol for ERα and ERβ [75,76]. This low affinity, coupled with the expected inactivation by glucuronidation, limited the study of BPA as a potent EDC [4,6]. More recent BPA literature has identified a number of cell membrane and nuclear targets for BPA binding, and the affinity of these interactions falls well within the low-dose exposures estimated for the population [8,11,67,77–84]. BPA has been shown to activate estrogen-related receptor (ERR) γ altering the ERK1/2 signaling pathway in cells and stimulating proliferation in breast and prostate cells [28–31]. BPA has also been shown to bind mERs G-protein-coupled receptor 30 [32–35]. These interactions induce rapid nongenomic effects that alter critical cell signaling pathways that govern cell proliferation, DNA damage response, and apoptotic signaling.

Alterations in p53 signaling and activation of MAPK, PI3K, and AKT have all been noted in recent studies, and gene expression changes or interactions in several important oncogenes, such as MYC and STAT3, have also been reported, though these effects have not been conclusively linked to any one estrogen-related receptor [17,18,25,36–38].

One final receptor interaction of interest is the report that BPA can bind K-Ras and Rheb and compete with nucleotide exchange [85]. The RAS family of proteins are oncogenes and are often mutated in human cancers [86]. The interactions of this protein family with cell signaling have made them attractive drug targets for chemotherapeutics for pancreatic, colon, and lung carcinomas [87]. Though the cellular consequences of these interactions need to be more fully investigated, BPA's direct interaction with these proteins suggest that we have only scratched the surface on the complexity of BPA's receptor-mediated effects.

Taken together, BPA can bind a large number of estrogen-related receptors at the low doses typically experienced by the population, and influence a number of critical cell signaling pathways promoting cell proliferation, apoptosis evasion, and transformation. In addition to effects already described, BPA has also been implicated in androgen and progesterone receptor interactions [62,84,88,89] and found to alter thyroid hormone signaling [81]. This evidence indicates that BPA can induce a number of receptor-mediated effects, with and without endocrine modulation, that can contribute to carcinogenesis and further study is required to better understand the complexity of these receptor interactions and tumor promotion.

12.4 Epigenetic Reprogramming

In addition to the rapid, genomic and nongenomic signaling induced by BPA interaction with receptors, there is also substantial evidence that low-dose BPA exposure can influence the epigenetic programming of cells. Epigenetics examine the modulation in gene expression induced by DNA methylation, histone modification, and noncoding RNAs. Methylation or demethylation of cytosine–guanine (CpG) dinucleotides in promoter regions of the DNA can act to silence or promote gene expression. A common feature in human cancers is global genomic hypomethylation and tumor suppressor gene hypermethylation [90]. Other changes include histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation that can change the nucleosome structure and reduce the dynamic behavior of nucleosomes, which is essential for transcription. These changes are unique in that the DNA sequence fidelity is retained, but expression of genes can be altered or silenced, inducing changes in differentiation, development, and responses to external stimuli, including DNA damage response and repair.

These types of alterations can be induced by a number of environmental exposures. For example, exposure to tamoxifen has been associated with nongenotoxic epigenetic changes that contribute to hepatocarcinogenesis. These changes include global changes in methylation patterns, reduction in expression of methyltransferases, demethylation of histone H4, and alteration of microRNAs (miRNAs) [91].

Epigenetic programming also plays a critical role during the development and growth of a fetus, and certain epigenetic modifications from environmental exposures can be retained and passed down to the developing fetus [92,93]. Environmental exposures during pregnancy can also influence the epigenetic programming, and EDCs have been found to alter DNA methylation and produce heritable epigenetic marks [93–95]. BPA exposure through maternal diet has been associated with hypomethylation phenotypes in offspring [40], and alterations in the global methylation state and gene expression profiles of mammary tissues in offspring [23]. Key methylation changes in cell signaling genes induced by neonatal BPA exposure have been associated with an increased likelihood of prostate cancer through the hypomethylation of phosphodiesterase type 4 variant 4 (Pde4d4) [47].

In addition to developmental effects, BPA exposure has been demonstrated to alter the DNA methylation state in a number of cell culture disease models and in adult human and animal studies [21,23,41,42,96–100]. BPA exposure in a human breast cancer cell line showed silencing of lysosomal-associated membrane protein 3 (LAMP3), increasing the risk of developing breast cancer later in life [21]. Chronic exposure of estrogen-dependent breast cancer cell lines to BPA demonstrated global epigenetic reprogramming with permanent alterations in hormone signaling [22].

Additionally, BPA exposure was shown to alter miRNA expression [97,101,102]. Genome-wide profiling of miRNA levels in placental tissues from BPA-exposed pregnant women revealed increased expression of miR-146a [103]. This is consistent with previous reports from human placental cell lines showing BPA-induced increases in miR-146a, slowed cell proliferation, and induced higher sensitivity to bleomycin [97]. Exposure of BPA to a mouse Sertoli cell line TM4 altered the expression of over 37 miRNAs, with most being downregulated over the course of exposure [43].

BPA has been shown to affect histone modification and chromatin structure. BPA exposure was shown to increase expression of the histone methyltransferase EZH2 in a human breast cancer cell line and in mammary glands of *in utero* BPA-exposed offspring [42]. Increases in H3K27 were also observed to be consistent with the regulatory role of EZH2 [42,102]. Our recent work also demonstrated that BPA exposure induces transient compaction of chromatin, reducing the access of key DNA repair proteins and downregulating the expression of these genes [44]. Though there have been limited investigations into BPA's ability to modulate chromatin structure and histone modification, it

is clear that BPA is altering the structure in addition to methylation status, and more work is required to determine the underlying mechanism.

Together these epigenetic changes significantly affect the gene regulation and stimuli response capabilities of cells exposed to BPA. As shown in our recent work, these changes can also compromise a cell's ability to maintain genomic fidelity and repair DNA lesions [44], and tightly regulate oncogenes, which may make them susceptible to transformation. Further study is required to understand the complexity of these changes; however, this substantial groundwork demonstrates the variety of mechanism by which BPA exposure can influence development and disease.

12.5 Oxidative stress

Induction of oxidative stress in cells exposed to nongenotoxic carcinogens has also been proposed as a mode of action for the development of carcinogenesis. Two common ways nongenotoxic carcinogens induce oxidative stress are by generating ROS during their metabolism in the cell and/or by depletion of the antioxidant defense mechanisms in the cell that counterbalance both endogenous and exogenous ROS. BPA primarily induces ROS through the enzymatic (H_2O_2/peroxidase and NADPH/CYP450) and nonenzymatic (peroxynitrite/CO_2 and ^{-}OCl/HOCl) formation of BPA phenoxyl radicals [13]. These phenoxyl radicals can then be further converted by NADPH or intracellular glutathione to form superoxide, hydroxyl radicals, and H_2O_2 [13]. Generated ROS can then damage cellular macromolecules and induce DNA strand breaks, purine and pyrimidine lesions, and DNA proteins cross-links. In addition to producing ROS, the enzymatic processing of BPA by cytochrome P450 also generates the DNA reactive quinone form of BPA [49]. While BPA–DNA adducts have been observed *in vivo* and *in vitro* after high dose exposure of BPA [50–52]; there is not sufficient evidence that these forms are genotoxic at this time. Therefore, we consider these adducts as a minor mechanism of tumor promotion compared to the other mechanisms presented in this chapter.

Several studies using a wide dose range of BPA, in a number of model systems, have verified the induction of ROS and demonstrated the induction of DNA damage through measurement of oxidatively induced DNA lesions, like 8-oxo-guanine [12–14,16,24,31,35,104–106]. In addition to generating ROS, BPA has also been shown to alter the antioxidant balance of cells depleting intracellular glutathione and altering the expression of catalase and superoxide dismutase [12,14,15,107,108]. Additionally, exposure of mice to BPA during pregnancy and continued exposure of the offspring during infancy has been shown to cause oxidative stress by decreasing antioxidant enzymes and increasing lipid peroxidation, leading to underdevelopment of the testis, brain, and kidneys of the offspring [14,16,48].

Recent work by our laboratory has also demonstrated that the induction of oxidative stress by BPA induces a number of cellular changes that, when challenged by additional oxidative stress, induce an adaptive response, promoting cell survival [44]. This adaptive response was characterized by an initial compaction of cellular chromatin that prevents the excision of oxidatively induced DNA lesions followed by an upregulation of DNA repair proteins that increases the repair of oxidatively induced DNA lesions [44].

These results demonstrate that induction of oxidative stress by BPA contributes significantly to its toxicity. Given the importance of oxidative stress in a large number of disease pathologies, these mechanisms need to be evaluated more thoroughly to understand the role they play in addition to the endocrine disrupting properties of BPA.

12.6 Inflammation and Immune Response

In addition to the induction of oxidative stress, inflammation and modulation of the immune response are also important mechanisms of action for some nongenotoxic carcinogens. As discussed in previous sections, exposure to nongenotoxic carcinogen can increase cell proliferation, induce injury oxidative stress, adapt the cellular microenvironment, and evade apoptosis. The combination of these events can lead to increases in the expression of growth factors and cytokines that ensure survival, while inducing inflammation and altering the immune response. Chronic inflammation is associated with an increased risk of cancer, and impairment of immune response, whether through immunosuppression or impaired surveillance, can contribute to tumor promotion [109,110].

Several studies have linked BPA exposure with inflammatory responses. Examination of a large number of published gene expression studies by Roy et al. [53] identified a consistent upregulation in inflammatory response genes, including *AHR, CSF2, HMOX1, IFNG, IL1B, IL6, LEP, MIF, MMP9, NOS2, NOS3, PARP1, PTGS2, SOD2*, and *TNF*, after BPA exposure [53]. Expression changes observed for IL-1, IL-6, and TNF-α were also correlated with the phosphorylation of ERK1/2 and JNK [25,111], which have been reported in a number of other studies not focused on inflammation [28,31,32,34,36]. Activation of the mitogen-activated protein kinase (MAPKs) and NF-κB pathways have also been implicated in BPA induction of oxidative stress and inflammation response and are proposed to be mediated through the nuclear or membrane ER signaling mechanisms [25,32,112]. There are some inconsistencies about the dose dependence in these signaling events, and there are some tissue or cell-specific findings that may depend on the expression level and presence of specific estrogen-related receptors. As argued by the authors in these studies, the nonmonotonic dose response of BPA, as well as the duration

of the measurement, can dramatically alter the observed outcome, which may explain some of these inconsistencies.

Population studies support a role for BPA-induced inflammation, with an increase in C-reactive protein (CRP) levels observed in postmenopausal women [24], increases in IL-6 and CRP observed in premenopausal women with polycystic ovary syndrome [54], and increased levels of IL-6 and TNF-α observed in males [27].

Coupled with the induction of proinflammatory genes, several studies have demonstrated that BPA exposure induces changes in the immune response. Prenatal exposure of mice to BPA promoted the production of T_H2 cytokines and was associated with a decrease in T regulatory $CD4^+CD25^+$ cells [55]. Perinatal exposure to BPA also promoted the production of proinflammatory mediators through the dysregulation of mast cells [56]. Other links to mast cell degranulation, lymphocyte proliferation, and antibody response have also been reported [64,113–115]. Whether these inflammation and immune changes directly influence the progression and development of cancer has not been examined, and the effects of these changes on allergic responses and asthma have not been conclusively verified [56,115]. Given the association of inflammation with cancer and the importance of immune surveillance in the removal of precancerous cells, more work is necessary to determine how BPA is influencing these responses. However, the robust responses of IL-6 and TNFα observed in a number of studies indicate that it may play an important role.

12.7 BPA-Induced Carcinogenesis

The current regulatory view of BPA is that it is not a mutagen or a robust carcinogen. However, the emerging literature studying BPA has begun to more effectively demonstrate that population exposure relevant doses of BPA induce carcinogenesis. These studies also suggest that the status of BPA should be reviewed again with the updated weight of evidence. A comprehensive review of the evidence of BPA as a carcinogen in mammalian model systems was recently published [116], so here we will just highlight some of the recent findings linking BPA exposure and carcinogenesis.

A large body of work has focused on examining BPA exposure in estrogen responsive tissues. For mammary carcinogenesis, both rat and mouse models have shown that BPA exposure induces changes in the mammary glands, including increased proliferation and decreased apoptosis, and increase in the number of terminal end buds [116]. Perinatal low-dose exposures were also linked to carcinogenesis in mammary glands of rat dams and their female offspring with even the male offspring showing morphological changes as a result of exposure [117,118]. Interestingly, coexposure of BPA with

carcinogenic insult by *N*-nitroso-*N*-methylurea (NMU) or 7,12-dimethylbenz (a) anthracene (DMBA) resulted in an increase in tumor formation [119–122], which is especially relevant for human exposures since chronic BPA exposure is coupled with exposure to environmental carcinogens and therapeutic carcinogens. Human evidence for mammary carcinogenesis is not as clear, though studies of a number of animal models have indicated that early life exposure to BPA increase the risk of breast cancer [116].

There is also a limited amount of evidence that BPA exposure contributes to ovarian pathologies, though clear links to carcinogenesis have not yet been made [116]. The most compelling evidence for a potential link between BPA exposure and ovarian cancer is that circulating levels of BPA were higher in women with polycystic ovarian syndrome [123], and as already discussed, this group also has higher levels of inflammation markers [11,54] and an increased risk of developing ovarian cancer [124].

BPA exposure has also been linked to prostate cancer by a number of studies in both rat model systems and human prostate epithelium [125–128]. BPA exposure induced increase in proliferation and migration in the prostate [129,130], and the proinflammatory and immune disruption effects of BPA were found to aggravate pre-existing benign prostate hyperplasia [131]. In addition to inflammatory modes of action, an epigenetic mechanism for the induction of prostate cancer after BPA exposure was recently proposed to explain the increase in prostate stem–progenitor cell self-renewal and the increase in expression of stem cell-related genes [126]. These findings are also supported by the correlation of elevated BPA levels in human prostate cancers [132].

Finally, there is evidence that perinatal BPA exposure induces hepatic tumors in mice in a dose-dependent manner [100]. The mechanism for this induction has not been identified, though another recent report found that BPA exposure induces lysophosphatidic acid (LPA) *G* protein-coupled receptors, which influence the cell proliferation and motility of cells [133]. Though these early studies indicate that BPA exposure may influence the development of hepatic cancer, further study is required to confirm this effect and determine the underlying mechanisms.

While the tissue specificity and mechanisms of tumor promotion still require further investigation, it is clear that BPA exposure, especially during development, can influence the risk of carcinogenesis. These risks seem unsurprising when one considers the emerging evidence in cell model systems that BPA induces prosurvival effects when coexposed with chemotherapeutic agents [65,134–136] and oxidative stress [12].

The adaptive response observed in our results indicated that BPA induces oxidative stress and genotoxicity, while inducing a robust adaptive gene expression response to coexposure [44]. If this coexposure was coupled with carcinogenic insults, which could overwhelm the DNA repair capacity of

tissues, then the robust adaptive response may improve cell survival. However, the long-term consequences of this improved cell survival are not yet known. The adaptive response may blunt the genotoxicity of BPA and the damaging event, or conversely, it may promote the survival of damaged cells and lead to tumor promotion.

It is also unclear how BPA exposure affects individuals with DNA repair deficiencies, due to an inherited condition or gene variations. Our adaptive response was observed in cells deficient in nonhomologous end-joining, while DNA repair competent cells lacked a robust adaptive response. It is possible that BPA exposure in individuals with intrinsic deficiencies in DNA repair capacity could result in more susceptibility to the carcinogenic effects of BPA. This possibility was raised after the observation that human breast cancer cell lines exposed to low dose BPA for 2 weeks revealed an increase in DNA repair proteins, including *BRCA1* and *BRCA2* [45]. The authors of that study conclude that women with mutations in these genes may be particularly susceptible to the negative effects of BPA exposure [45].

There are significant gaps in our knowledge of the carcinogenic potential of BPA. The complex modes of action by which BPA has been shown to induce carcinogenesis are still largely undefined, and there remains a need for improved cell, animal, and human studies examining exposure. However, the increasing evidence of BPA-induced carcinogenesis and the growing evidence that BPA coexposure induces a wide spectrum of cellular effects, argues that regulatory agencies should revisit the classification of BPA and determine new guidelines for dosing. This is also true for the numerous bisphenol derivatives that have emerged over the past 10 years to replace bisphenol A.

12.8 Fresh Opportunities in BPA Research

The emergence of a new analytical toolbox over the past decade offers fresh opportunities for BPA research. As we have already illustrated, profiling of whole genome epigenetic marks and gene expression indicators can now be combined with precise quantifications of DNA alterations and metabolic response molecules (e.g., GSH) to better understand the effects of BPA. In addition, the use of model systems where the genetic background can be altered, so as to tease apart toxicant responses that otherwise would be confounded, is an especially important tool. These advances will enable development of more precise and practical biomarkers of BPA exposure and the attendant metabolic responses. The possibility of understanding patterns of genetic susceptibility to BPA in the human population is especially important to consider, as is the need to understand the topic of developmental stage susceptibility. Transfer of the laboratory-based tools reviewed here to population-based research is within

reach over the next decade, and this represents an important opportunity for the future toxicogenomics research.

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13

Toxicoepigenetics and Effects on Life Course Disease Susceptibility

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13.1 Introduction to the Field of Toxicoepigenetics

In 2003, the Human Genome Project officially concluded, but the dream of "cracking" the genetic code and gaining the ability to predict disease risk and onset was not fully realized. Instead, sequencing the human genome exposed another layer – a biological control. Literally meaning "above the genome," the epigenome encompasses a cast of heritable modifications, which can affect gene expression without altering the underlying DNA sequence. This additional layer of biological complexity is helping to explain how less than 20,000 human genes can propagate such complex and phenotypically diverse human characteristics, as well as chronic disease risk.

Environmental exposures, including toxicants, diet, stress, and other social factors, can lead to altered gene expression and phenotypes not only through genetic mutations but also by modifications to the epigenome [1]. The epigenome is particularly susceptible to environmental deregulation during gestation, neonatal development, and puberty. Nevertheless, it is most vulnerable to the environmental factors during embryogenesis because the DNA synthesis rate is high, and the elaborate DNA methylation patterning required for normal tissue development is established during early stages of development. In addition, as the human life span is extended, the potential for chronic environmental exposure to toxins and toxicants, such as synthetic chemicals, dietary constituents, and lifestyle factors, increases. Thus, the field of environmental epigenetics, also referred to as toxicoepigenetics, investigates the molecular biological processes that potentially link the environment to its impact on disease risk and outcome. Epigenetic changes may also be

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transmitted across multiple generations [2]. For example, in mammals, when a pregnant female is exposed to an epigenotoxicant, it may directly impact not only her epigenome, but also the epigenome of her offspring and grand offspring, commonly referred to as intergenerational effects [3].

13.1.1 The Epigenome

There are (a) variety of metaphors used to help describe the relationship between the epigenome and the genome. The most extensively referenced one, however, is the computer metaphor in which a computer's hard drive represents the genome and a computer's various software programs represent the epigenome. A computer's hard drive contains a lot of data, but all of that data cannot operate without its numerous software programs, and vice versa. In this scenario, the epigenetic software directs the genomic hardware on when, where, and how to operate (i.e., express genes). It is the epigenome that allows two cells in the human body, each with the exact same genetic information, to be phenotypically different cells with very different jobs; a liver cell, a heart cell, or a white blood cell, for example.

13.1.2 Epigenetic Marks are Heritable and Reversible

Epigenetic marks are modifications that can be added to the genome resulting in changes in gene expression. These marks do not alter the genetic code and are heritable through cell division, exclusive of genetic factors [4]. Consistent with the computer metaphor introduced above, the regiment and location of epigenetic marks and resulting gene expression in the liver cell compared to the white blood cell is different. Most of the epigenetic alterations that occur throughout the life of an organism are natural and necessary; however, some aberrant epigenetic marks can occur, either stochastically or through exposure to environmental factors. Unlike the inherited genetic code, which remains static and is nonmodifiable, epigenetic marks are plastic, dynamic, and potentially modifiable [5]. This understanding has led to a surge in interest and funding in the field of toxicoepigenetics. For example, epigenetic marks have the potential to serve as biomarkers of disease or exposure as well as potential targets for therapy.

Several different types of epigenetic alterations have been identified, but the most commonly studied are DNA methylation and histone modifications. The influence of regulatory noncoding RNA (ncRNA), including small interfering RNA (siRNA) and piwi-interacting RNA (piRNA), as well as long ncRNA, on gene transcription is another field of epigenetic gene regulation that is now emerging. For example, recent findings suggest that micro-RNAs may be important regulators of cytokines involved in T-cell polarization and the allergic response [6]. Histone modifications and histone variants are epigenetic

modifications that serve a wide range of purposes from nucleosome stability to chromatin dynamics and play a critical role in gene expression [7]. The most commonly studied epigenetic mechanism is DNA methylation, which in the field of toxicoepigenetics has received more attention than histone modifications or micro-RNAs [5]. This may be due to the difficulty and scalability of working with RNA and proteins at a population level. DNA is more stable, which makes storage and handling easier. The availability of high-throughput methods for DNA methylation analysis that require small amounts of sample at a relatively low cost is also a driving factor for the discrepancy [8].

13.1.3 DNA Methylation

DNA methylation (5mC) is a phenomenon where a methyl group is covalently bonded to the carbon-5 position of the cytosine ring in a cytosine–guanine dinucleotide, also called a CpG. The distribution of CpG sequences in mammalian genomes is nonrandom [9]. CpG dinucleotides are greatly underrepresented in the mammalian genome due to evolutionary spontaneous deamination of 5mC to thymine. The majority of unmethylated sites occur in CpG islands, defined as discrete regions containing more than 50% CpG content. Normally, they are located within or near the gene promoters or first exons of the housekeeping genes. In contrast, the promoter and regulatory regions of transposable elements are methylated, thereby inhibiting the parasitic transposable and repetitive elements from replicating. It is becoming increasingly clear that in addition to the CpG islands, CpGs a short distance from the island, called CpG shores, may also be important for gene regulation [10]. While methylation in promoter regions can silence gene expression and recruit additional repressive epigenetic modifications, methylation within gene bodies may actually promote transcription and play a role in the regulation of splicing [11,12]. 5-hydroxymethylcytosine (5hmC), a stable intermediate that follows oxidation of 5mC by ten-eleven translocation (TET) enzymes, has recently been considered an epigenetic signal in its own right that may further refine the regulatory role of 5mC. 5hmC is enriched in brain tissue, and associated with euchromatin and actively transcribed genes [13]. It may also play a role in splicing and cell lineage commitment during embryonic development [14].

Methylation of the CpG requires an enzyme called DNA methyltransferase, which is able to transfer a methyl group from *S*-adenosylmethionine (SAM) to the fifth carbon of cytosine. SAM is enzymatically made available through a process called one-carbon metabolism in the presence of micronutrients such as folate or choline. Thus, dietary intake of such nutrients has become one of the focal points of epigenetic research, and disruption of the one-carbon metabolism pathway is one mechanism through which toxicants can impact 5mC levels.

Aberrant Global DNA Methylation

Aberrant methylation can lead to altered phenotypes [15]. Data from cancer-related research have shown that genomic reductions in methylation, also called global hypomethylation, are a trademark of this altered cellular phenotype [16]. In animal models, hypomethylation is associated with increased mutation rates and genomic instability [17,18]. Similarly, global hypomethylation is also associated with genomic instability in human cancer tissue [19]. It has been established that genomic instability in the context of cancer is principally the result of demethylation in intergenic and intronic regions where repeated sequences and transposable elements are located [20]. After initial observations in the cancer field, it was found that global changes in methylation could impact noncancer diseases as well. For example, immunodeficiency–centromeric instability–facial abnormality (ICF) syndrome is a rare human disease where DNA methyltransferase 3B (*DNMT3B*) is mutated resulting in global hypomethylation [21]. Loss of DNMT3B function leads to immune dysfunction, compromised lymphocyte function, and chromosome rearrangement in these patients.

Aberrant Gene-Specific DNA Methylation

Aberrant gene-specific methylation can result in upregulation of genes that are typically suppressed and suppression of genes that are typically upregulated. Epigenetic modification within the CpG island of the promoter region is thought to interfere with binding of transcription factors and increases affinity for other epigenetic modifiers and corepressors [22,23]. Adding to the complexity of gene-specific epigenetic dysregulation is the novel research that is describing the role of the molecular machinery that "reads," "writes," and "erases" epigenetic modifications [24]. Removal of epigenetic marks is thought to make transcriptional binding sites more accessible and prime the system for increased protein production. In this way, researchers are now being challenged to provide functional validation of the epigenetic changes by examining their effects on RNA or protein expression [5].

13.1.4 Histone Modifications and Chromatin Packaging

Epigenetic manipulation of the cellular phenotype is also driven by alteration of the chromatin structure through covalent histone modifications and incorporation of histone variants into the nucleosome [25]. Chromatin is a nucleoprotein complex that packages linear genomic DNA through an array of nucleosomes. Each nucleosome consists of 147 base pairs of DNA coiled around an octamer of histone proteins. Each octamer contains two copies each of the four core histones H2A, H2B, H3, and H4. Chromatin may be further modified by the association with linker histones, histone variants, and nonhistone proteins as well as a myriad of posttranslational modifications of

histone proteins, including histone acetylation, methylation, ubiquitinylation, phosphorylation, sumoylation, and ADP-ribosylation [26,27].

Histone acetylation is usually associated with transcriptional activation because the affinity of histone proteins for DNA is reduced and chromatin packaging is relaxed. Histone methylation results in various transcriptional consequences depending on histone number and the lysine residue modified [28]. Each lysine residue may be methylated in the form of mono-, di-, or trimethylation, adding enormous complexity to the histone code [29]. Furthermore, histone modifications interact with DNA methylation patterns to recruit multisubunit chromatin–protein complexes, such as the repressive PcG proteins or the activating SWI–SNF proteins, adding yet another layer of complexity to the epigenetic gene regulation. Cross talk between DNA methylation and histone modifications influences the patterning of both forms of epigenetic regulation and is partially mediated by interaction between SET domain histone methyltransferases and DNA methyltransferases [30].

13.1.5 Noncoding RNAs

Complementary RNA has been long known to induce gene silencing, but the exact mechanisms are being elucidated. Short antisense RNA transcripts are produced within the nucleus by the action of the enzyme Dicer, which cleaves double-stranded RNA precursors into 21–26-nucleotide long RNA species [31,32]. These then associate with silencing–effector complexes, such as RNA-induced silencing complex (RISC), which directs cleavage of cognate mRNA or causes translational repression and RNA-induced initiation of transcriptional silencing (RITS), which mediates heterochromatin formation at target loci and abrogates gene expression [32]. Thus, regulation mediated by small ncRNAs occurs both at the posttranscriptional and transcriptional levels. The latter transcriptional regulation is referred to as "epigenetic silencing," and is mediated either by covalent modifications of chromatin (such as H3 methylation at Lys9) or by DNA methylation [33].

13.1.6 Key Windows for Exposure-Related Epigenetic Changes

It is suggested that there are key developmental periods, or windows, that are important for epigenetic programming and vulnerable to environmental insults. Thus, epigenetic modifications represent a potential mechanism of the "developmental origins of health and disease" (DOHaD) paradigm, which posits that early life environmental exposures can alter disease risk in adulthood. Within the scope of the DOHaD hypothesis are adult chronic diseases such as metabolic syndrome, obesity, cancer, and neurodegenerative disorders [34]. It is important, therefore, for researchers to make perinatal exposures and their effects on the epigenome a key area of investigation. For example, the

Dutch Famine of 1944 was an unfortunate incident but also a remarkable natural experiment. Children born to mothers who experienced the famine in their first trimester of pregnancy compared to the third trimester, have differential methylation patterns at an imprinted gene related to insulin production [35].

13.1.7 Evaluation of Environmentally Induced Epigenetic Changes in Animal Models and Humans

Toxicoepigenetic studies are aimed at identifying biomarkers of effect that can provide insight for disease detection and prevention. To this end, researchers investigate the molecular effects of the environment by employing epigenetic methodologies, which often utilize biologically available DNA (e.g., blood, buccal cells, stool, etc.). The methylation profiles of these peripheral matrices are not always correlated with the target tissue most relevant to the exposure or disease of interest. However, successful associations have been shown between peripheral DNA-related biomarkers and cancer, disease states, and environmental exposures [36,37].

One solution for the knowledge gaps that can be caused by the ethical and logistic limitations of human epidemiologic research is to compliment these studies with an appropriate animal model. Surrogate models have many experimental advantages, including direct control over diet, stress, and genetic variation as well as access to both target and biologically available tissues. By utilizing this compliment strategy, scientists in the toxicoepigenetics field are equipped with the tools needed to investigate the complex relationships between environmental exposures, epigenetic tissue specificity, and time-dependent epigenetic drift. It is important, however, that the data generated by these animal models are interpreted with care. While these studies are necessary, some of the results will nevertheless be limited in their applicability to human health by genetic, metabolic, and other differences.

13.2 Exposures that Influence the Epigenome

Epigenetic adaptations in response to environmental factors play an important role in developmental plasticity and disease susceptibility [34]. Environmental factors, including nutrition, xenobiotics, and even low-dose radiation, can directly and indirectly affect methylation and chromatin remodeling factors to alter the epigenome and subsequent gene expression patterns. Here, we describe a representative selection of well-characterized environmental exposures, including toxicants (air pollution, metals, endocrine disrupting chemicals (EDCs)), diet, and stress and the epigenetic alterations associated with these exposures that may mediate observed health effects.

13.2.1 Air Pollution

Exposure to air pollution is ubiquitous but highly variable depending on geographic location, time of year, environmental conditions, and human behavior. Ambient levels of air pollution are associated with mortality rate and death from lung cancer and cardiopulmonary disease in the United States and, therefore, a major health concern [38]. The potential health impacts associated with air pollution are determined by several factors including but not limited to the mechanism of formation, size fraction, and environmental conditions.

Particulate matter (PM) air pollution is a small subdivision of solid matter that is suspended in gas or liquid. PM is categorized into three main types: (1) PM_{10} where all particles are 10 μm or smaller in aerodynamic diameter, (2) $PM_{2.5}$ where all particles are 2.5 μm or smaller in aerodynamic diameter, and lastly, (3) Coarse fraction where the particles are between PM_{10} and $PM_{2.5}$. The surface of these particles can be contaminated with polycyclic aromatic hydrocarbons (PAHs), sulfates, black carbon, and other substances.

$PM_{2.5}$, also known as the fine fraction, is especially important in the context of lung health because this subset of particles can readily bypass the body's natural inhalation pathway defenses (e.g., nose hair and mucociliary escalator) and deposit in the alveolar spaces. Furthermore, fine fraction exposure can have systemic effects, including neurological effects, such as stroke and diseases of the central nervous system, cardiovascular effects, such as changes in heart rate variability and changes in blood pressure, and reproductive effects, such as premature birth and decreased birth rate [39].

Recent studies suggest that coarse fraction may play a larger role in the exacerbation of lung health perturbations than originally thought. The ratio of PM size fractions and the chemicals found on the surface vary by season [40,41]. The coarse fraction can contain a large amount of biogenic material (e.g., endotoxin, beta-glucans, mold spores) that has been linked to a strong proinflammatory response in the human airway [42]. The unique and variable characteristics of this mixture make the study of PM challenging.

While air pollution can impact health at all life stages, early life exposures have been linked to the risk of developing asthma and allergic diseases in adolescence [43], and epigenetic changes may underlie this association. In a Belgium birth cohort study, $PM_{2.5}$ exposure during pregnancy was associated with placental global methylation [44]. Maternal exposure to polycyclic aromatic hydrocarbons (PAHs), another type of PM contaminant, has been linked to changes in methylation patterns in umbilical cord blood and placental tissue [45]. The adult epigenome is also influenced by air pollution exposures. A Belgium study of nonsmoking adults found that several measures of air pollution, including $PM_{2.5}$, were associated with global methylation, which was analyzed by HPLC [46]. In this study, only summer time $PM_{2.5}$ was significantly associated with global methylation and men exhibited more hypomethylation

compared to women. A study of elderly subjects found that exposure to traffic-derived $PM_{2.5}$ and black carbon was associated with decreased LINE-1, but not Alu methylation [47,48]. This effect of LINE-1 hypomethylation was observed for $PM_{2.5}$ and black carbon exposures on a subchronic scale.

Global methylation can be informative in the context of respiratory health, but to elucidate a mechanism, gene-specific analysis is also key to understanding which immunological pathways are affected. In a population of elderly men within the Normative Aging Study, subchronic exposure to air pollutants, including nontraffic $PM_{2.5}$, were evaluated for associations with DNA methylation changes in genes related to lung inflammation and immunity [49]. The researchers found that slope estimates for air pollutants were higher for participants with below median levels of methylation at several CpG locations for two out of nine airway inflammation-related genes assessed.

While most studies of PM investigate the effect of ambient pollution, it is important to note that indoor sources may be variable, which is especially important in the United States because people spend about 87% of their day indoors [50]. For example, indoor sources of biomass-generated PM have been linked to poor health outcomes in developed and developing countries [51,52]. In the United States, it is estimated that the number of susceptible individuals (elderly and children were considered susceptible) who are exposed to residential indoor wood smoke is approximately 5 million people [53], and the numbers are much higher worldwide.

When studying environmental exposures, randomized controlled trials are the gold standard; however, they are rare because of cost and ethical considerations. Although the sample size was small, Bellavia *et al.* [54] used a randomized short-term cross over study design to show that controlled doses of fine and coarse fraction could induce both global and gene-specific hypomethylation, which was associated with changes in blood pressure [54].

Epigenome-wide association studies (EWAS) can generate hypotheses by revealing disease-related loci and are especially important tools for studying complex diseases. Panni *et al.* [55] conducted the first EWAS that focused on fine particulate matter and DNA methylation. Their analysis revealed 10 CpG sites that were associated with 2-, 7-, or 28-day average PM concentrations. The authors noted that the CpG sites were found in genomic regions previously found to be related to oxidative stress, carcinogenesis, systemic metabolic conditions, or inflammation pathways.

Animal models are especially important for air pollution studies, allowing researchers to finely tune the timing, volume, concentration, and size fraction ratio of the PM exposure. In this way, researchers have focused on different anthropogenic sources of PM such as traffic-related PM and wood smoke PM, which have also been termed urban and rural sources, respectively. Ding *et al.* [56] found in a rat model that prolonged exposure (28 day) to traffic-related air pollution was associated with DNA methylation of LINE-1, inducible

nitric oxide synthase (iNOS) gene, and adenomatous polyposis coli (APC) gene, but these results were only seen in DNA from the target tissue – lung and not from blood [56].

13.2.2 Metals

Exposure to heavy metals is detrimental to the health of the general public and also certain occupational groups worldwide. In the United States, environmental regulatory bodies rank pollutants of concern based on known toxicity and likelihood of exposure. Four of the top seven ranked pollutants are heavy metals – arsenic, lead, mercury, and cadmium (As, Pb, Hg, Cd) and chromium (Cr) is seventeenth [57]. Here, we focus on lead and mercury as representative metals exhibiting toxicoepigenetic properties.

Lead (Pb)

In the United States, regulations have contributed to dramatically reduced environmental lead exposures; however, as recently revealed in Flint, Michigan, acute and chronic exposures to toxic levels of lead are still possible especially in susceptible populations (e.g., young children, individuals living in aging housing stock). Lead exposure can negatively affect normal neurological function and is particularly toxic in children during early development [58]. According to the Centers for Disease Control and Prevention (CDC), environmental lead exposures also disproportionately affect low-income and minority populations.

After removing lead from the industrial production processes of gasoline and paint in the United States, the incidence of toxic exposure events decreased [59]. However, in some locations the potential for lead exposures has increased. Large urban demolition projects can generate lead exposures from housing materials [60] and the usefulness of the vacant land produced by the demolition is also problematic due to lead accumulation in the soil [61]. Ingestion of lead due to failing infrastructure in the water supply system can also occur, as was the case in the Flint, MI water crisis [62]. Children are especially susceptible to lead contamination in water as they can absorb approximately 50% percent of an oral dose compared to 10% in adults [63].

Early-life lead exposure has been linked to altered adolescent neurodevelopment [64] and adult Alzheimer disease-like pathology in a primate model [65], which suggests a role for lead-related epigenetic alterations. Rodent studies corroborate this hypothesis by exploiting metastable epialleles, which are gene regions that are variably expressed in genetically identical individuals due to epigenetic alterations during early windows of development [66]. For example, viable yellow agouti mice, which are used widely as an epigenetic model of environmental exposure [67], have been used to show that maternal exposure to lead can alter DNA methylation at two metastable loci in a dose-dependent fashion [68]. Several cross-sectional or short-term longitudinal studies have

found data that supports the role of epigenetic mechanisms in the lasting impact of Pb following exposure at vulnerable time periods. For example, patella lead levels, a biomarker for chronic lead exposure, were associated with LINE-1 repetitive element hypomethylation in elderly men recruited through the Normative Aging Study [37]. Among newborns exposed to lead *in utero*, DNA methylation perturbation has been observed at specific genes using a candidate gene approach [69] or at thousands of loci throughout the genome using an epigenome-wide approach [70]. Interestingly, many of the lead–DNA methylation associations were sex specific, much like the health impacts of lead exposure often are.

Mercury (Hg)

Mercury exists as elemental mercury, inorganic mercury, and organic mercury. Mercury is naturally occurring in the environment and, therefore, everyone is subject to a level of background exposure. According to the Agency for Toxic Substances and Disease Registry (ATSDR), elemental mercury (e.g., dental amalgam fillings) and organic mercury in the form of methylmercury (e.g., contaminated fish) are two of the most common human exposures. The health impacts associated with these exposures are largely dependent on the dose and duration of exposure [71]. Toxic levels of mercury are known to impact the neurological system, cardiovascular system, and kidneys.

Methylmercury has recently been linked to epigenetic alterations and may impact the epigenome throughout the life course of humans as well as animals. For example, prenatally exposed mouse pups showed depression-like symptoms along with epigenetic changes at the brain-derived neurotrophic factor (*Bdnf*) promoter IV when compared to control mice [72]. The authors noted that the chromatin structure at the *Bdnf* promoter of the pups in the exposed group was in a repressive state, and this was consistent with mRNA levels of *Bdnf* that were measured in the hippocampus.

While randomized control trials of mercury exposure in humans are not ethical and longitudinal studies focusing on mercury and the epigenome are limited, cross-sectional studies have been conducted that support an epigenetic mechanism in mercury exposure-related human health outcomes. For example, in a study of reproductive-aged women, promoter methylation of an antioxidant gene was associated with blood levels of mercury [73]. Similarly, mercury measured in hair was associated with methylation of an antioxidant gene among dental professionals [74].

13.2.3 Endocrine Disrupting Chemicals (EDCs)

Bisphenol A

Bisphenol A (BPA) is a commercially produced monomer used in manufacturing processes for polymers (e.g., polycarbonate and epoxy resins), polyvinyl

chloride polymerization, and flame retardants [75]. Consumer products such as beverage containers, baby bottles, medical devices, and dental materials often contain BPA [76]. Therefore, BPA exposure is widespread and detectable levels have been found in urine for the majority of study populations in countries such as the United States, China, and Korea [77,78]. The integrity of the BPA monomer is subject to temperature and pH changes, and BPA can be liberated from these product materials and migrate to food [79], air [80], and saliva [81]. It is estimated that ingestion accounts for more than 90% of all human BPA exposure [82]. BPA can act as a synthetic estrogen, and its deleterious effect on reproduction has been known since the 1930s [76]. Studies have found that BPA can disrupt normal endocrine function by weakly binding to a number of steroid receptors, including estrogen receptors and the thyroid hormone receptor [83–85]. BPA may play role in gene regulation through activation of transcription factors [86], which suggests BPA could have an effect on early development by perturbing normal cell function.

Using animal models, several research groups have investigated the effect of BPA exposure during early development on epigenetic programming. After gestational exposure to BPA at a concentration of 10 μg/(kg day), the adult male rat was observed to have changes in DNA methylation at a gene that encodes for an enzyme involved in the cyclic AMP pathway in prostate tissue, which may increase susceptibility to carcinogenesis in these cells [87]. Perinatal exposure to BPA shifts the distribution of the offspring's coat color in the viable yellow agouti mouse model, which corresponds with a change in DNA methylation at the A^{vy} locus [88,89]. Further, this shift was attenuated by supplementation of methyl donors that feed into the one-carbon metabolism pathway or genistein. The effects of BPA have been shown to also reach the brain. Maternal exposure in mice was found to alter expression of two enzymes (DNA methyltransferases 1 and 3) that are responsible for methylating CpGs in the cortex and hypothalamus of the offspring [90]. Wolstenholme *et al.* [91] suggest that BPA exposure may have sexually dimorphic effects on social interactions that are linked to sex-specific developmental epigenetic programming events in the brain.

The number of human epidemiological studies investigating the impact of BPA exposure on the epigenome is increasing. BPA measured in healthy fetal liver tissue was associated with altered DNA methylation and corresponding expression levels of a number of xenobiotic metabolizing enzymes, including glutathione *S*-transferase [92]. In a study of 60 girls aged 10–13 years, DNA methylation profiles (measured in saliva) were altered in select genes involved in immune function and metabolism with increasing urinary BPA concentrations [93].

Interestingly, recent publications have noted a potential role for epigenetic mechanisms involving early life BPA exposure. For example, Wang *et al*. [94] found evidence that BPA may increase the risk of atopic disorders in adolescent children. Children with high urinary concentrations of BPA at age 3 exhibited a

higher risk of asthma at age 6 [94]. Another interesting area of research into the impact of BPA is effect modification by dietary constituents. For example, soy intake appears to modify the relationship between urinary BPA and pregnancy outcomes. Chavarro *et al.* [95] found that soy may be protective against the deleterious reproductive effects of BPA. The evaluation of epigenetic effects in these populations will be important for the elucidation of the mechanisms associated with exposures, effect modifications, and outcomes.

Phthalates

Phthalates are dialkyl or alkyl aryl esters of phthalic acid and are used in the manufacturing processes of plastics to modulate flexibility and opacity [96]. They are used to manufacture a wide variety of goods, including toys, clothing, building materials, cosmetics, food packaging, and medical appliances [97]. Because phthalates are not physically bonded to polymers, they can diffuse out of plastics and enter the environment [96]. High molecular weight phthalates (e.g., di-(2-ethylhexyl) phthalate (DEHP)) are used in polyvinyl chloride (PVC), while low molecular weight phthalates (e.g., dibutyl phthalate (DBP)) are used in cosmetics and pharmaceuticals [98]. Phthalates from plastic products can leach into the air [99] and food [100], and human exposure routes include ingestion, inhalation, and dermal absorption [100–102]. In the body, phthalates are metabolized to a monoester form through a variety of enzymatic conversions and excreted in the urine [103]. Data from the National Health and Nutrition Examination Survey (NHANES) show that metabolites of DEHP and diethyl phthalate (DEP) have been identified in the urine of the majority of the population sampled [104]. As is the case with many toxicants, children appear to be disproportionately affected by phthalates compared to adults, with higher measured levels of metabolites in the urine [104–106].

High doses of phthalates affect reproductive endpoints (e.g., prolonged estrus cycle) in the rat model [107,108]. Similarly, human epidemiological studies suggest phthalate exposure is associated with developmental and reproductive endpoints [109]. For example, both high- and low-molecular weight phthalates have been associated with the timing of breast development and pubic hair development [110]. Results from recent birth cohort studies bolster a hypothesis that phthalates impact reproductive outcomes through epigenetic modifications. For example, LaRocca *et al.* [111] determined that exposure to phthalates in the first trimester was associated with hypomethylation of *H19*, an imprinted gene [111].

EDCs, including phthalates, may also play a role in the development and exacerbation of asthma and other allergic diseases, recently reviewed by Robinson *et al.* [112]. Animal models suggest that phthalates, such as DEHP, may act as an adjuvant and enhance immune responses to antigens through the expression of proinflammatory factors [113,114]. While an epigenetic mechanism has not been fully characterized, DNA methylation is one

possible way phthalate exposure modulates the expression of immunoregulatory genes. Epidemiological studies have determined that urinary metabolites of phthalates are associated with markers of airway inflammation in children [115] and markers of oxidative stress in pregnant women [116]. Several prospective studies have identified associations between early life exposures to phthalates and incidence of asthma and asthma-related symptoms in adolescence [117–120], which is suggestive of an epigenetic mechanism.

More research is needed but evidence from *in vitro* models show that phthalate exposure could modify the progression rates of some cancers by activating oncogenes and oncogenic pathways. For example, phthalate metabolites from a common plasticizer in hospital plastics have been shown to activate the hedgehog pathway, which plays a critical role in prostate cancer [121]. Similarly, phthalate metabolites were also found to upregulate genes implicated in colorectal cancer [122]. Thus, it is possible that phthalates affect cancer pathways through epigenetic modifications.

13.2.4 Diet

Humans are exposed to a myriad of chemicals and dietary factors through the consumption of foodstuffs daily; thus diet may be one of the most important exposures occurring in the twenty-first century [123]. From the time of the industrial revolution, human lifestyle has become urbanized [124] and this has led to changes in behavior and dietary intake [125]. Evidence from epidemiological studies suggest that overnutrition and gene–diet interactions may be driving a persistent systemic inflammatory state and contribute to diseases such as cardiovascular disease (CVD), diabetes, asthma, allergies, cancer, skin and digestive disorders, and neurological diseases [126–132].

Epidemiological findings support the hypothesis that maternal and early-life exposure to dietary patterns or particular nutrients can influence epigenetic programming, and thus affect disease risk later in life [125] or even in the following generations [133]. Furthermore, it is possible that acute exposure to some nutrients can invoke more transient epigenetic effects, which may be important for disease severity or control.

Dietary Patterns and Human Health

Studying the epigenetic effects of single nutrients is necessary in determining the overall biological impact diet has on human health and it is possible that specific nutrients are especially beneficial or deleterious. However, we must be aware that humans do not consume nutrients in a vacuum but rather in concert with other nutrients coming from multiple foodstuffs that contribute to a dietary pattern. The health benefits of the Mediterranean diet (MD) pattern have been known for half a century. The Seven Countries Study, which began in the 1940s, was the first study to systematically examine the effect of diet

(including MD) and lifestyle on heart disease. The MD is consistent with the traditional diet of southern Italy, Greece, and Spain, which includes high intake of olive oil and unrefined grains [134]. MD typically contains very low saturated fats and cholesterol and very high monounsaturated fats, fiber, and carbohydrates [135]. In addition to heart health benefits, the MD may also reduce the risk of developing certain types of cancer. New evidence from a single-blind randomized trial found that compared to a control diet the women who ate a MD were less likely to get breast cancer [136].

In stark contrast, the Western diet (WD) pattern, consumed in much of the developed world (i.e., Europe, United States, etc.) is characterized by convenience and highly processed foods high in saturated and trans fats. Adoption of the WD is associated with a rise in obesity and inflammatory diseases, and exposure to these dietary factors affects certain populations disproportionately. Observationally, it has been shown that immigrants who move to developed countries tend to adopt the host country's dietary habits [137] and these ethnic minority groups are at risk for developing obesity, type 2 diabetes, and heart disease [138–141]. Although human studies are limited in the types of tissue samples that can be accessed, epigenetic biomarkers in proxy tissues have been explored. A recent cross-sectional study in Italy was conducted on a group of nonpregnant cancer free women ($n = 177$) and found that individuals who were not adhering to the traditional MD diet were more likely to have LINE-1 hypomethylation sampled from blood leukocyte DNA [142].

Changes in methylation have been linked to high fat diet patterns in the mouse model. Ding *et al.* [143] showed that a high fat diet fed over multiple generations could influence methylation levels in the promoter regions of key inflammatory genes measured in adipose tissue. The observed methylation changes corresponded to alterations in gene expression, which provides a possible mechanism explaining how dietary patterns can influence inflammatory related diseases.

Intake of Dietary Factors Involved in One-Carbon Metabolism

Dietary methyl donors play an important role in one-carbon metabolism and the production of *S*-adenosylmethionine, which along with a methyltransferase is required for DNA methylation. Thus, intake of methyl donors can influence patterns of methylation resulting in differences in gene transcription. Landmark animal studies exploiting the phenotypic characteristics of the agouti mouse with the viable yellow agouti (A^{vy}) allele have demonstrated the impact that nutrition can have on early development [144]. Waterland *et al.* [145] showed that maternal supplementation of methyl donating nutrients, such as folate and choline, during early development was associated with the production of leaner, dark-coated offspring with hypermethylation at the A^{vy} locus.

Waterland *et al.* also showed that maternal environment affects metastable epialleles in human offspring. The researchers took advantage of seasonal

differences in food consumption in rural Gambia and found that intake of methyl donor nutrients during pregnancy was associated with altered DNA methylation of metastable epialleles measured in hair and blood in the offspring [145]. Joubert *et al.* [146] recently conducted an epigenome-wide meta-analysis of 1988 newborns to determine the effect of maternal plasma folate on the offspring epigenome. In addition to genes related to neural tube defects and neurological function, the authors found 48 differentially methylated CpGs that were significantly associated with folate levels, highlighting the potential biological importance of this methyl donor [146]. A group of Korean scientists published data from a case–control study providing evidence that both the intake of dietary folate and enzymatic activity (specifically methionine synthase, which is involved in a precursor step to the production of SAM) were related to gastric cancer risk [147]. Although more research will be needed, these data indicate that dietary folate intake may be required for epigenetic regulation of genes related to the gastric cancer phenotype.

Dietary Fiber and Immunoregulation

Dietary fiber has recently received a rapid increase in attention for its potential role in immunoregulation. Dietary habits can influence the microbial diversity of the gut, which can contribute to low-grade inflammation through immune dysregulation. Dietary fiber is at the interface between the gut microbiota and normal immune function [148]. It is estimated that there are more than 100 trillion resident microbes in the human gut and as much as 150-fold more genes in the "microbiome" compared to the host genome [149]. The diversity and health of the gut microbiota is dependent on the nutritional intake of the host and fiber appears to play a key role in ensuring that nutrients reach the large intestine where the majority of the microbes reside [148]. Therefore, it is possible that the recent trends toward more processed foods in the Western diet could be compromising normal host immune function by undernourishing the gut microbiota. In this way, fiber intake could indirectly affect epigenomic modification by facilitating absorption of the nutrients involved in DNA methylation.

13.2.5 Stress

Increasingly, scientists are evaluating the link between social and behavioral factors and epigenetic regulation. The molecular mechanisms underlying gene expression following social and behavioral factors are not well understood [5]. Weaver *et al.* [150] were among the first to investigate epigenetic mechanisms explaining how maternal behavior during early development impacts behavioral response later in life for the offspring. This research group found that pups of attentive mother rats grew up to have less stress compared to pups of neglectful mothers and determined that these behavioral differences had to do

with the levels of a receptor for glucocorticoid, a stress hormone [150]. Interestingly, a series of cross-fostering studies confirmed that the changes in gene expression and resulting stress response were most likely due to an epigenetic mechanism rather than a genetic mechanism because the offspring's response was defined by the foster mother's care [151]. It was determined that contact between the mother and pup could result in long lasting epigenetic alterations in the hippocampus, including demethylation at the gene that coded for the stress receptor [152].

Although human epidemiological studies are limited by tissue availability, some very interesting evidence does exist to partially corroborate animal findings. For example, prenatal exposure to maternal depression was found to be associated with the methylation status of the human glucocorticoid receptor gene measured in infant cord blood DNA and stress reactivity in the offspring at 3 months of age [153]. A group of Canadian researchers have found that children born to mothers who were exposed to intense distress (e.g., a natural disaster) during the third trimester have more incidences of eating disorders as well as altered DNA methylation patterns when compared to unexposed children [154]. Suicide victims with a history of child abuse show altered methylation patterns in the promoter region of the glucocorticoid receptor gene, and these patterns were associated with decreased levels of mRNA expression in the brain [155]. Similarly, women who were exposed to sexual abuse as children have altered methylation patterns in the promoter region of a serotonin transporter gene and have an increased risk of antisocial behavior as adults [156]. Early life socioeconomic position is associated with increased adult mortality and morbidity, and emerging evidence suggests that this early-life stressor also has the potential to modify DNA methylation patterns [157,158].

13.3 Intergenerational Exposures and Epigenetic Effects

In mammals, the mother, G0, hosts the development of the offspring, F1, from zygote stage to birth. During F1 offspring development, a separate lineage of cells, called the primordial germ cells (PGCs), migrate and differentiate into gamete precursor cells that will eventually become the F2 "grand-offspring" generation. Thus, when a pregnant woman is exposed to an epigenotoxicant, it may directly impact not only her epigenome, but also the epigenome of her offspring and grand-offspring, commonly referred to as "intergenerational effects." In the field of intergenerational environmental exposures (see Figure 13.1), much attention has been given to G0 exposure and F1 effects. For example, developmental exposures and intergenerational epigenetic effects in offspring following maternal exposure to BPA were linked to changes in coat color and obesity risk in mouse F1 offspring via decrease in DNA methylation [159].

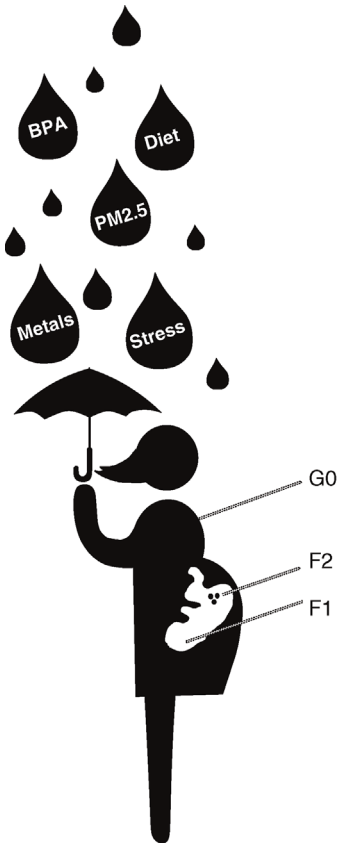

Figure 13.1 Intergenerational environmental exposures. Among mammals, maternal exposures during gestation, whether harmful or beneficial, may directly influence not only the mother (G0) and child (F1) but also future grandchildren (F2) via exposure of their primordial germ cells (PGCs) during gestation. The epigenetic impacts of such exposures on the F2 generation are a rapidly growing area of study.

Much less attention, however, has been given to direct effects of exposures on the germ line, the eventual F2 (grand-offspring) generation. This may be due to the intense focus over the last decade on the potential for exposures to influence transgenerational effects (discussed in detail later). By convention, the "first wave" of epigenetic resetting refers to the reprogramming of the epigenome within these PGCs, and the second wave refers to the reprogramming that happens shortly after zygote formation. In females, the PGC remain largely unmethylated until maturation in the F1 adult during each estrous cycle. During fertilization, the F2 gametes combine and undergo the second, more complete, wave of demethylation in preparation for establishment of somatic tissue-specific methylation patterns. Thus, any environmental influences on the pregnant G0 female can affect epigenetic patterning and subsequent adult disease susceptibility intergenerationally – in both the F1 and F2. The Escher Fund for Autism research, hosts a website, www.germlineexposures.org, that details the need for more research on direct environmental effects on the germ

line and eventual F2 generation. There has been some recent speculation that the increase in prescription drug use by pregnant mothers in the 1950–1960s or the increase in the percentage of women of child bearing age smoking following World War II, may have impacted germ line epigenetic program, with effects on the F2 generations, for example, increased autism or obesity rates seen recently.

In addition, environmental exposures have been shown to influence transgenerational effects on the F3 (great grandchildren) generation, who were never directly exposed. Nilsson and Skinner [160] reviewed evidence from animal studies of transgenerational inheritance of diseases via epigenetic changes that were elicited by environmental contaminants. They proposed that one key mechanism of transgenerational transmission of susceptibility to cancer, obesity, and other physical changes is incomplete and/or inaccurate reprogramming of DNA methylation of germ cells (sperm and egg) following exposure to contaminants that become fixed or "imprinted-like." Conducting studies of environmental contaminants and epigenetic changes across generations in humans pose a unique set of challenges, due to larger life spans and access to biological samples across generations.

13.4 Special Considerations and Future Directions for the Field of Toxicoepigenetics

13.4.1 Tissue Specificity

Possibly the greatest limitation to environmental epigenetic studies in human cohorts is access to the target tissue of interest. Researchers rely on more accessible proxy tissues like blood leukocytes, saliva, buccal cells, or placenta. To help assess differences between target and proxy tissue analyses, the National Institute of Environmental Health Sciences (NIEHS) has recently launched the TaRGET II: Environmental Epigenomic Analysis in Tissue Surrogates Program. The goal of TaRGET II is to explore the conservation of environmentally induced perturbations of epigenetic marks across target tissues and proxy tissues using animal models of environmentally relevant diseases. Ultimately, this consortium intends to provide insights into the design and interpretation of epigenetic epidemiological studies in which target tissues may often be inaccessible. Additionally, adding epigenetic inquiries to ongoing human cohort studies is potentially complicated by the historical collection and storage methods of tissues, which may have not been implemented with DNA, RNA, or protein isolation in mind. When designing new animal and human environmental epigenetic studies, researchers should take care to consider sample collection and storage, exposure and outcome assessment, and appropriateness of the proxy tissue to maximize the potential for meaningful and relevant discoveries.

Several groups have sought to justify the use of proxy tissues by investigating how and to what degree epigenetic patterns in accessible tissues relate to those in the target tissue. To illustrate the difficulty of this task, a series of studies, which collectively aim to determine if gene expression modulated by DNA methylation is linked to major psychosis, will be used as an example. First, to evaluate if DNA methylation is related to psychiatric disorders such as schizophrenia, Dempster *et al.* [161] used peripheral blood as a proxy to compare biomarkers in 22 pairs of monozygotic twins who were discordant for psychosis and observed disease-associated methylation differences. An obvious limitation of this study was the lack of corresponding brain tissue for each subject, which ultimately has the greatest relevance for psychiatric diseases. In an effort to resolve this issue, researchers evaluated the correlation of methylation patterns of between-postmortem samples of brain and proxy tissues, including blood, using a between-subject approach [162,163]; however the interpretation of these results are limited first by the between rather than within subject approach and second by evidence suggesting that several postmortem factors can affect DNA methylation. Therefore, Walton *et al.* [164] later obtained paired blood and temporal lobe biopsy samples simultaneously from 12 epilepsy patients. The authors found that only 4.1% of all CpG sites that are associated with genes expressed in the brain were variable and also significantly correlated between blood and brain tissue [164]. Thus, the authors concluded that if future studies aim to use blood methylation as a proxy biomarker for brain methylation, only these CpG sites are justified. It should be noted, however, that without healthy controls the results of this study might not be entirely translatable because the methylome of these diseased subjects may not be representative of the general population. Second, using a whole genome approach, Ziller *et al.* [165] investigated 30 different cell and tissue types and observed that about 20% of autosomal CpGs were dynamically regulated. Gu *et al.* [166] used two complementary methods to estimate 54 high-resolution methylomes and found that about 20% of the 26 million autosomal CpGs were variably methylated. Interestingly, the authors found that regardless of the cell type, approximately 11% of the CpGs were unmethylated, although these CpGs were cell specific [166]. While these studies broaden our understanding of epigenetic landscape, they are limited because they are comparing the methylomes of cells and tissues from multiple subjects and are naive to any developmental factors or exposures that could have contributed to the observed methylation patterns.

Studies that are able to consider within and between subject correlations for several tissues and cell types of healthy and diseased individuals at several time points during the life course accompanied by a complete exposure history would be most informative, however, this is often impractical due to budget, collection, and time constraints. Therefore, any strategy to evaluate environmental epigenetics in humans must be at least two-pronged. Moving forward

epidemiological studies should strive to include two or more proxy tissues for each individual and conduct appropriate epigenetic inquiries with respect to the nature of the study and disease phenotype with a goal of identifying environmentally induced epigenetic changes that correlate across tissue types. Additionally, animal studies, including the TaRGET II consortium, are poised to help bridge the knowledge gaps left by the complexities of epidemiologic research and should be used to complement human studies. As such, care should be taken to properly collect and store tissue suitable for DNA, RNA, and protein isolation from as many organs as possible even if current research does not aim to specifically utilize the full suite of tissues.

13.4.2 The Dynamic Nature of DNA Methylation

Transient epigenetic changes in response to acute environmental exposure have been demonstrated in the adult human population. For example, workers exposed to air pollutants from a steel factory as well as elderly participants exposed to urban traffic-related pollutants had exposure-associated gene-specific and global methylation measurements [48,167]. Similar observations have been made in animal models. For instance, levels of 5hmC measured in the hippocampus of mice were influenced by acute stress using an epigenome-wide technique [168]. While these transient epigenetic effects can occur in short windows of time at later life stages, the broader understanding is that environmental exposures have the greatest propensity to invoke considerable and lasting epigenetic change when the insult occurs chronically and encompasses early life stages. Therefore, studies that aim to investigate the impact of chronic exposure to an environmental toxicant on an epigenomic biomarker should consider the possibility of a background level of epigenetic change due to age. This phenomenon has been inconsistently termed either "age-related methylation changes" or "epigenetic drift" [169–171].

Dynamic methylation is another special consideration to be aware of especially when designing longitudinal studies. Early studies in epigenetics, which observed that global and region-specific methylation changes occurred as the result of age, led to the hypothesis that DNA methylation may be vulnerable to variability due to incomplete maintenance during cell divisions over the life span [172]. The gradual loss of DNA methylation over time is in fact a key difference between a normal cell that ages and one that is immortal [173]. In addition to the general epigenomic loss of methylation, repetitive elements such as LINE-1 and Alu also exhibit decreased methylation levels and increased variability with age [174]. Interestingly, in conjunction with global hypomethylation, CpGs in specific regions (e.g., gene promoters) become hypermethylated with age, and this is a major risk factor for neoplasia [175]. Recent studies in the murine model suggest that the age-related methylation changes, which have been investigated in homologous regions to the human genome, are

conserved across tissues and potentially between mammalian species [170]. Thus, when it is possible, for animal and human studies, which aim to evaluate exposure-related methylation changes over time, the background level of change that occurs as the result of aging alone should be measured.

13.5 Future Directions

Programs for Identifying Population and Individuals at Risk for Environmentally Induced Changes to the Epigenome

Over the past several years, large consortia efforts, such as the NIH Roadmap Epigenomics Mapping Consortium, have begun to analyze and curate data on DNA methylation, mRNA expression, and changes in histones and in chromatin accessibility, annotating these data across a sweeping array of human cell types and creating genome-wide annotation maps. These efforts can lead to novel studies of epigenomic changes in development and disease, as well as of the relations among genomic and epigenomic variations [176]. It is important to now extend these mapping efforts to include epigenomic responses to environmental exposures, as well as systematic measurement of matched samples over time – in order to better understand epigenetic drift with age.

New efforts to support analyses of environmental exposure data, including epigenetic and epigenomic data from children's health studies are recently underway. The NIEHS-led Children's Health Exposure Analysis Resource (CHEAR) network launched in 2015 provides selected children's health researchers access to laboratory and data analysis services to add or expand environmental exposures and biological responses, such as epigenetics, as a component of their research. A related National Institutes of Health (NIH) program slated to launch in 2016–2017, the Environmental Influences On Child Health Outcomes (ECHO) program, will combine extant birth cohorts to support longer term follow up of children, and to provide the resources and support infrastructure to generate extensive data on these cohorts, including genomic and epigenomic data using a large number of experimental techniques. Both CHEAR and ECHO contain infrastructure for overall program coordination, as well as biostatistical and high-dimensional data analyses.

Locus-Specific Epigenome Editing

Once large scale operations, such as the Epigenome Roadmap and CHEAR, described already, identify individuals within populations at risk for environmental influences on the epigenome, it will be important to develop strategies for intervention and therapy to prevent or reduce negative health outcomes mediated by epigenetic mechanisms. Currently, the development of technologies for locus-specific epigenome editing remains a central challenge in

therapeutic epigenomic approaches. For example, many current technologies act globally and cannot target individual loci. Pharmaceutical agents, such as azacytidine, are widely used to inhibit DNA methyltransferases, resulting in global hypomethylation in dividing cells [177]. An advantage of global approaches lies in their well-characterized use as human therapeutics and for basic research in cell lines and animals. Disadvantages, however, include their pleiotropic effects caused by indiscriminate epigenomic activity and propensity to affect biochemical pathways separate from the epigenome. Recently, new methods of locus-specific epigenetic editing have been developed that rely upon transgenic technologies. For example, fusions of epigenome-modifying enzymes to programmable DNA-binding proteins hold promise for targeting DNA methylation [178] as well as histone acetylation [179] and epiproteomes [180] at specific loci, but have drawbacks. For example, every zinc-finger domain must be custom evolved to target a specific sequence, and target motifs are size limited. One recent innovation in the field of target-specific DNA methylation is the development of a suite of tools, based on the Piwi-interacting RNA (piRNA) system, to accurately induce DNA methylation of targeted loci in adult tissues (NIH Grant ES026877). The major strength in the piRNA approach is that induced changes in DNA methylation will be propagated through mitosis by endogenous epigenetic maintenance pathways. Thus, piRNA treatment for both laboratory and clinical use will be acute and systemic, rather than chronic with potentially decreasing effectiveness.

13.6 Conclusions

The field of toxicoepigenetics addresses three areas that are critical to our understanding of environmental impact on the epigenome and its ultimate implication for human health and disease: (1) mechanisms linking toxicants to epigenetic modification, (2) biological significance of results, and (3) best practices for study design. Environmental factors have the ability to alter the epigenome at various life stages from conception to old age and across generations. In this chapter, we detailed classes of environmental chemicals (e.g., air pollution and metals) as well as diets and other stressors that human populations are commonly exposed to and how they perturb the epigenome in epidemiological cohorts, and animal models. Environmental factors have the ability to change mitotic inheritance of epigenetic marks and to exert lasting multi- and transgenerational impacts. In the foreseeable future, new NIH programs expanding the assessment of exposures and biological responses will enhance the field of environmental epigenetics to identify individuals and populations at risk for epigenotoxicant exposures. The development of precise tools to target the epigenome will be crucial to develop therapeutic strategies for altered epigenome responses.

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14

Tumor-Promoting/Associated Inflammation and the Microenvironment: A State of the Science and New Horizons

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14.1 Introduction

Historically, the evaluation of the carcinogenic chemical risk has initially relied on *in vitro* genotoxicity assays and then, finally, on the assessment of tumor formation in animal models. This approach, while experimentally satisfying, is highly restrictive, emphasizing the "tumor initiation" properties of single compounds. Thereby it neglects the complexity of tumorigenesis that occurs over time, and that has been synthesized into multiple programs or "cancer hallmarks" [1]. These hallmarks occur over time at "multiscale" levels that include genetic and epigenetic changes, metabolism, secreted cytokines, host tumor microenvironment, and host immune system. Hence, the cancer hallmarks represent the different aspects of carcinogenesis, including many host-related perturbations and the remarkable antitumor defences. Incorporating this intricate etiology into studies about the link to the environment and cancer development has been an imposing challenge to the field. How could one address this experimentally in a way that incorporates this complex biologic understanding? In this chapter, we describe the role of inflammation and the microenvironment in tumor evolution, how carcinogenesis may occur through these programs, and provide one possible unifying way of thinking about how the tumor controls the microenvironment and how this control may be an Achilles' heel for the treatment and cancer.

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Over the last few decades, there is a growing number of chemicals in the human environment with ever-increasing exposure of people to low-dose mixtures of man-made chemicals. This is occurring in the absence of much needed attention and resources to innovate within the field of chemical carcinogenesis, including expanding beyond genotoxicity and single agent research to the study of mixtures in biological systems as targets of chemicals in cancer pathogenesis.

The inflammatory microenvironment influences multiple cancer hallmarks, including cell proliferation/death, angiogenesis, invasion, and metastasis [2], and increasing data suggest the link between the majority of cancers with the chronic inflammation that can contribute to cancer development from initiation and throughout malignant progression. In addition, the characteristics of chronic inflammation, that is, infiltration of immune cells, influence of inflammatory mediators, tissue remodeling, and angiogenesis, can, however, be found in tumors in which a causal relationship to inflammation has not been found [3].

Inflammation is mediated by immune cells as a direct defense versus infections or injury by dangerous stimuli. Innate immune cells (e.g., neutrophils, mast cells, and especially macrophages) have receptors that signal the secretion of biologically active proteins and defense molecules in response to extraneous substances or altered self-molecules [4]. The infiltration of cancer of immune cells first described by Virchow was thought to be a failed effort of the immune system to counteract cancer development. Although this has remained, in part, true, the role of the immune system and inflammation in tumor evolution is much more nuanced.

The presence of inflammatory cells commonly precedes tumor development [5]. Inflammation was first observed to be associated with tumorigenesis in experimental models of acute and chronic inflammatory ocular diseases. These and other studies have led to the detection of at least three distinct inflammation response phases. During the acute phase, there is an initial response to an irritant or infectious organism that mimics the healing response to a wound or during an infectious process. Subsequently, there is an intermediate phase that, in a healthy state, serves to downregulate or dampen the acute response to resolve inflammation. Finally, there is a chronic response phase that, if unresolved, can have potent pathologic properties. As a consequence of persistence, a “proinflammatory” state sustains the release of cytokines and chemokines, able to promote progressive alterations in the cellular and molecular microenvironment composition. This leads to elevated levels of promutagenic reactive oxygen (ROS) and reactive nitrogen species (RNS), alterations in the vasculature (e.g., vascular hyperpermeability, neovascularization, and angiogenesis), disturbances in mitochondrial function, and, importantly, the disruption of normal cell–cell signaling/cross talk such as recruitment of macrophages with suppressive function to disable T-cell-mediated tumor immunity. The chronically inflamed state or “failed wound healing” response

or localized "system" response has been identified as a common feature in tumor development and metastasis. In this scenario, the immune system, it seems, recites an important contribution to tumorigenesis.

Many basic and clinical studies illustrate a important role for the immune system in cancer. Many types of cancer are more frequent in human patients who undergo immunosuppressive treatment [6,7]. Conversely, heightened antitumor activity of the immune system has been suggested in many reports of spontaneous cancer regression [8,9]. Active tumor immunity is provided through the pleiotropy or duality (polarity) of the immune system via the self-terminating and protective properties of acute inflammation or maintenance of balance in tumoricidal (yin) and tumorigenic (yang) properties of immune surveillance.

The exposition to extraneous elements promotes innate or adaptive immune responses that by tissue and/or systemic factors defend the host [4]. The category of immune-stimulating factors is very wide, including as host defective cells (cancerous or senescent), pathogens, and biological, chemical, or environmental hazards (pollen, dust, pesticides, asbestos, paints, detergents, cosmetics, and food additives). Much less is understood how man-made environmental chemicals influence the immune response. Chronic and mixed exposures to specific chemicals may disrupt the regulatory mechanisms of the immune system to deal with xenobiotics, altered-self, and other exposures. Although many chemicals have been recognized as potentially contributing to altered inflammation and host response contributing to both immune and neuronal clinical disorders, in contrast, only modest consideration has been given to the role of environmental carcinogens effects on inflammatory response and resolution mechanisms. Nevertheless, the immune response to the tumor through both innate and adaptive immunity plays a complex important role [10].

14.2 The Immune System

14.2.1 Innate Immune Response

Innate immune cells, including macrophages (MΦs), neutrophils, dendritic cells (DCs), and innate lymphoid cells (ILCs), are implicated in the early response to tissue perturbation and can have a double-sided role: preventing/contrasting the cancer initiation/progression or supporting the malignant transformation. Innate immune cells are able to favor a mutagenic microenvironment, for example, the epidermis Langerhans cells and active chemical mutagenic carcinogens, promoting epithelial DNA damage and provoking squamous cell carcinoma [11]. The macrophages, instead, are able to directly produce mutagenic mediators.

There are two opposing subsets of macrophages (the proinflammatory M1 and the anti-inflammatory M2), but with functional plasticity and great ability

to change in the different phenotypes [12,13]. Usually, the M1 MΦs exhibit a tumoricidal effect on established tumors, but in chronic inflammation, they also account for the mutagenic microenvironment, supporting the tumor formation [14], as reported in colon and pancreatic cancer.

The MΦs ablation of the anti-inflammatory transcription factor Stat3 induces a great boost of colonic proinflammatory MΦs, which ultimately induce colon cancer [15]. The infiltration of MΦs evokes tumor-initiating characteristics in pancreatic cells and elevates the frequency of cancer stem cells in a pancreatic cancer model [16,17].

Finally, accumulating evidence suggests an essential link between control of cell proliferation and the aptitude of innate immune cells to detect incipient cellular transformation [18]. For example, the natural killer (NK) cells, a subset of the ILC population, contrast the tumor outgrowth by inducing senescence in tumor cells, but they can also eliminate senescent tumor cells expressing p53 [19,20]. Thus, the single cell population of innate immune response exercises a specific and sometimes contrasting role in the cancer battle.

Innate lymphoid cells include NK cells and lymphoid tissue-inducer (LTi) cells. NK cells recognize a vast array of tumor cells, which they help to eliminate through cytotoxicity and the production of cytokines, especially IFN-γ. The other ILCs are found mostly in the mucosa and mucosal-associated lymphoid tissues, where they rapidly initiate immune responses to pathogens without the need for specific sensitization [21]. The role of ILCs in starting and cancer development is unknown. NK cells are an important subset of ILCs and have a key anticancer role. Nevertheless, the cancer cells adopt different strategies to contrast the effective NK activity, especially obstructing their recruitment to the tumor [22]. NK cells can target tumor-starting cells in melanoma and colon cancer [23,24]. After IL-15 stimulation, NK cells can eradicate different established tumors by a perforin-dependent cytotoxicity, while the DCs become able to induce apoptotic cell death in tumor cells [25,26]. The significant anticancer role of IL-15 is confirmed by the observation that colorectal cancer patients with deletion of the IL-15 locus have an elevated risk of recurrence [27].

DCs can play a critical anticancer role at the interface between the innate and adaptive arms of the immune system. DCs induce primary immune responses, potentiate the effector functions of previously primed T cells, and orchestrate the communication between other innate and adaptive immune cells [28]. DCs are able to elicit a strong tumor antigen-specific immune responses ($CD8^+$ and $CD4^+$ T cells) [29] and to enhance the antitumor activity of NK cells by increasing their cytolytic abilities and IFN-γ production [30].

Also, DCs can control the malignant development of colitis-associated cancer through the production of IL-22BP, which neutralizes the effect of IL-22 [31]. This can stimulate the proliferation of intestinal epithelial cells and induce chronic inflammation [32]. In a mouse model, the depletion of $IL\text{-}22^+$ cells blocks the development of colon cancer [33]. In addition to their known

tumoricidal activity, DCs also can have protumorigenic effects [34,35]. This can be caused by impaired IFN-α secretion or upregulation of Foxo3 (transcription factor Forkhead box O3) [36,37]. Tissue hypoxia favors the loss of the antitumor function of DCs [38]. This can also reduce the tumoricidal ability of NK cells by promoting the recruitment of T-cell-suppressive myeloid cells [39].

The myeloid-derived suppressor cells represent a heterogeneous population of myeloid cells at different stages of differentiation [40]. Accumulation of pathologically activated immature myeloid cells with potent immune-suppressive activity is one of the major immunological hallmarks of cancer. In addition to their immune-suppressive activity, myeloid-derived suppressor cells (MDSCs) influence the initiation of cancer [41]. They can promote tumor metastases by promoting angiogenesis and tumor cell invasion [42]. MDSC accumulation in the tumor microenvironment of patients is correlated with both response to therapy and survival in many solid cancers [43–46].

MDSCs can exert potent immune suppression [47] by inducing ROS, arg-1, and nitric oxide (NO). Peroxynitrite (PNT), the product of interaction of superoxide and NO, could cause nitration of T-cell receptors (TCRs)–CD8 complex. This can reduce binding to the peptide MHC class I complex and render T cells unresponsive to antigen-specific stimulation [48]. PNT also hampers the recognition of cancer cells by cytotoxic T cells [49]. Finally, the depletion of L-arginine and cysteine in the tumor microenvironment caused by MDSCs results in decreased CD3ζ chain expression, diminished production of IL-2 and IFN-γ, and inhibited T-cell proliferation [50–52].

Tumor-associated macrophages (TAMs) contribute to the innate leukocyte infiltration in a tumor. TAMs have similar characteristics of the M2 subset and share the M1 and M2 signature polarization [53]. TAM can promote tumor progression through the induction of angiogenesis, stroma remodeling, and immune suppression. In addition, TAMs secrete various enzymes such as plasmin and MMP that can promote tumor cell invasiveness and metastasis [54,55]. Cancer infiltration by TAMs is associated with a poor clinical prognosis [56,57]. Although TAM resembles M2 polarization such as elevated IL-10 secretion, these cells also coexpress IFN-inducible chemokines. They display anti- and proinflammatory activities, suggesting as their key role in the epithelial–mesenchymal transition favoring tumor formation and metastasis [58].

M2-like TAMs can be reeducated into a tumoricidal phenotype. Thus, NK cells are capable to reprogram M2 macrophages by IFN-γ secretion and miR-155 overexpression [59,60]. In lung cancer, the activation of TLR-3 (Toll-like receptor 3) coupled with TICAM-1 (Toll–IL-1 receptor domain-containing adaptor molecule) promotes the TNF-α production, and so converts M2-like TAMs into the tumoricidal M1 subset [61]. In a glioblastoma model, the shift M2 to M1 was obtained after the treatment with an inhibitor of CSF-1R (colony-stimulating factor-1 receptor) [62].

Importantly, TAM consists of a heterogeneous group of macrophages with functionally distinct proprieties [63]. For example, the Tie-2+ MΦs support the tumor angiogenesis binding to the ANGPT2 (angiopoietin-2) displayed by endothelial cells [64]. For this purpose, CSF1 can lead to the recruitment of monocytes from the bone marrow, which then differentiate into proangiogenic macrophages, thereby further expanding the subset of Tie2+ MΦs [65]. Another TAM population is positive for CCR2 (C–C chemokine receptor type 2) that recruits to a metastatic site by CCL2 (secreted by tumor or stromal cells) [66–68]. The interplay between cancer, endothelial, and myeloid cells contributes to a niche that can promote tumor growth [69]. This has been particularly documented in human breast cancer [70].

The role of tumor-associated neutrophils (TANs) in cancer is poorly defined. They were thought to have a negligible role because of their short life span and fully differentiated phenotype [71]. However, there was a report from decades ago that peripheral blood neutrophils have been associated with short 5-year survival in humans [72]. Then, more recently, TANs were shown to be an independent poor prognostic factor [73].

Furthermore, an increase in the presence of TANs correlates with advanced disease and poor outcome in patients in many cancer types, including renal [74], colorectal [75], hepatocellular [76], gastric [77], pancreatic [78], and melanoma [79]. However, other studies have seen no relationship between TAN and prognosis [80–83].

TANs have been shown to induce the angiogenic switch during early cancer progression [84], and continue to support cancer cell growth and invasion by remodeling the extracellular matrix and modulating tumor cell biology in later stages [84–87]. In addition, neutrophils release nitric oxide derivatives and reactive oxygen species (ROS) [88,89]. Accordingly, neutrophil-derived ROS, such as the MPO-mediated formation of HOCl, has been associated with DNA damage [88].

Neutrophils, similar to macrophages, polarize in the tumor microenvironment to a protumor (N2) phenotype [90]. Neutrophil-derived MMP-9, oncostatin M, the small cytokine CXCL8 (IL-8), and Bv8 have been shown to promote angiogenesis [91], and are associated with the N2 phenotype of TANs [85]. Neutrophils seem to play a mostly protumor role. The N2 phenotype of TANs can be reversed to an antitumor N1 phenotype with TGF-β blockade [90]. IFN-β can instruct neutrophils to have an antitumor phenotype [92]. Hence, TANS are likely to have both pro- and antitumor effects depending on the microenvironment.

14.2.2 Adaptive Immune Response

The adaptive immune response is key to the anticancer immune response that can be divided into two phases: activation and effector. In the activation phase, DCs

process antigens from the cancer cells and present them to naive T cells. Before this, DCs must receive an immunogenic maturation stimulus (e.g., proinflammatory cytokines) that activates them upon capture and antigen presentation. Without such a stimulus, an opposite reaction will induce tolerance by T-cell deletion and/or the production of regulatory T cells (Tregs) [93,94].

Subsequent to the stimulation, the DCs will process the captured antigens and present them on MHC class II molecules, at which point they are transported to the draining lymph node, interact with T cells, inducing an immune response. After MHC and processed antigens bind together, TCRs will interact with them, and a costimulatory signal in the form of either plasma membrane ligands on the DCs that interacts with stimulatory or inhibitory receptors on the T cell, or in the form of secreted cytokines, takes place for the purpose of mounting an effective immune response to tumor cells [95].

Following these costimulatory events, the T cells will become activated. A specific immune response then will result in the effector phase. The activated T cells travel to the tumor site where they recognize tumor antigens and eliminate the cancer cells. However, the tumor adopts different defense mechanisms. During the immune response, the immune system prevents attacking "self" cells under the help of immunological checkpoints. Tumor cells can use these immune checkpoints as mechanism to inhibit a tumor immune response. Thus, immune checkpoints can facilitate tumor tolerance, resulting in tumor "escape" from the immune system.

Although cytotoxic CD8+ T cells have been considered to be the main protagonists in the production of immune antitumor effects, increasingly, several aspects of CD4+ T-cell biology suggest that this T-cell population has a key role in the cancer-specific immune response. CD4+ T cells can coordinate diversity of immune reactions that can be fitted to maintain immune response against cancer antigens. Originally CD4+ T cells were defined as Th1 and Th2 subpopulations. Now, the Th $CD4^+$ T cells have been further divided into other subsets, such as the suppressive Tregs and proinflammatory Th17, and more recently the Th9, Th22, and follicular helper T cells [96–101]. Although Th1 and Th2 subsets were thought to be mutually exclusive lineages, it is now evident that this depends upon their differentiation state [102]. Likewise, Th17 and Treg subsets do not represent permanent states, but rather these subsets retain their plasticity allowing them to adapt to different environments [103].

The Th1 subset produces IFN-γ, TNF-α, and interleukin-2 (IL-2), and has a strong antitumor role by orchestrating cell-mediated immunity against cancer cells [104]. Mouse studies demonstrate that the initiation of an effective antitumor $CD8^+$ T cell response is subject to the presence of $CD4^+$ T cells [105]. The DC stimulation is the major helper mechanism used by Th1 cells to support the antigen presentation and to provide costimulatory signals such as CD40-CD40L to effector $CD8^+$ T cells [106,107]. Significantly, it has also been documented that Th1 cells boost the $CD8^+$ T-cell infiltration into the

cancer [108,109], by the production of IFN-γ-dependent chemokines (e.g., CXCL9 and CXCL10) [110].

In addition, $CD4^+$ Th1 cells exhibit $CD8^+$ T cells' independent antitumor activity. In fact, the IFN-γ exerts an antiproliferative, proapoptotic activity, and inhibits angiogenesis in tumor cells [111]. Furthermore, Th1 cells also recruit and activate inflammatory cells (MΦs, granulocytes, eosinophils, and NK cells) in and around the tumor [104]. IFN-γ can induce the upregulation of MHC molecules on tumor cells, leading to enhanced effector T-cell recognition [111]. This mechanism enables MHC class II restricted killing independently of B, NK, or other T cells [112]. Indeed, some $CD4^+$ Th1 cells also have direct tumor-recognizing ability [113]. They are able to kill MHC-II^+ tumors by cytotoxic activity, using perforine and granzyme, and by TNF-related apoptosis, inducing ligand (TRAIL) receptor and Fas/Fas ligand (FasL) pathways [104,114].

$CD4^+$ T cells also can provide help for themselves. A Th–Th interaction enables the activation of $CD4^+$ T cells specific for a poorly immunogenic epitope [115]. In cancer patients, spontaneous $CD4^+$ T-cell responses against tumor antigens have been documented in different studies (as reviewed by Niccolai *et al.* [116] and Galaine *et al.* [117]). Accordingly, a high density of tumor-infiltrating Th1 cells has been identified as a useful prognostic marker in various human cancers [118,119]. On the other hand, subsets such as Th2, Tregs, or, under some conditions, Th17 cells may have tumor-promoting activity, which may need to be reduced to achieve a most favorable anticancer response [120]. Th1 immune response has been shown to mediate effective anticancer effects in human patients. Many investigators are trying to develop antitumor Th1 immunity-stimulating immunotherapy.

The $CD8^+$ T cells seem to be the principal effector population of the cancer-specific immune response. The antigen-specific $CD8^+$ T cells have the ability to recognize and destroy infected or malignant cells [121], but before obtaining the effective functionality, the naïve $CD8^+$ T cells need to be activated owing to the detection of related antigen presented on MHC-I by antigen-presenting cells (APCs), which usually occurs in secondary lymphoid organs. The activated $CD8^+$ T cells must then locate and efficaciously enter into the injured tissues to support the host defense. Because newly activated $CD8^+$ T cells will next differentiate into a long-lived memory population, the process regulating $CD8^+$ T-cell trafficking and localization is critical for the optimization of vaccine strategies [122,123]. In addition, different experimental models and ever-increasing clinical data have suggested that both activation and localization of $CD8^+$ T cells are essential for the achievement of tumor immunotherapy [124]. In fact, the migration and following infiltration of $CD8^+$ T cells into tumors are key factors that predict clinical outcome of patients [125,126].

In the majority of cancer types, $CD8^+$ T-cell infiltration is associated with a Th1 functional nature and correlates with better progression-free survival and overall survival, especially for colorectal, lung, breast, bladder, ovarian, pancreatic,

prostatic, and hepatocellular cancer, as well as melanoma [120]. Colorectal cancer has been extensively studied and represents a paradigm of these findings. In these patients, not only the overall density of memory Th1/$CD8^+$ T cells was important, but also the localization of the immune microenvironment [127]. Thus, both the concentration of these immune cells in the cancer and the invasive margin are prognostic factors [126]. These observations led to the delineation of an immunoscore, established by the density evaluation of infiltrating T $CD8^+$/$CD45RO^+$ or $CD3^+$/$CD8^+$ in the central and marginal cancer site. The score (from 0 to 4) substantially predicts the progression-free survival and overall survival in patients with colorectal cancers (up to stage III) [128,129].

However, many exceptions to the immunoscore have been reported, including in clear cell–renal cell cancer [130], Hodgkin lymphoma [131], and ocular melanoma [132]. In these patients, an elevated cancer infiltration by $CD3^+$ and/or $CD8^+$ T cells correlates with lower patient survival. These seemingly opposing data have to be interpreted in view of the great intricacy of the cancer microenvironment. In addition to the adaptive immune response, evoked by the infiltration of Th1/cytotoxic T cells, various cells can inhibit protective T-cell responses, such as immune cells (especially MDSC and Tregs) or the cancer cells themselves. In addition, different cells of the cancer microenvironment support tumor outgrowth and spread by producing growth and angiogenic factors, such as VEGF, that increase tumor vascularization, resistance to apoptosis, and, together with suppressor cells, inhibition of T-cell responses [133]. Thus, the role of CD8+ T cells in tumorigenesis is likely to depend on the context of the tumor microenvironment that dictates their functional status that, in turn, would contribute to the clinical outcome [134].

Finally, B cells also contribute to the adaptive immune response in the cancer. Although, experimentally, B cells have been suggested to contribute to tumorigenesis [135], many clinical studies suggest that their presence can be associated with a favorable prognosis in patients with colorectal cancer [127], lung cancer, melanoma, and breast carcinoma [136–138]. There remains much to understand about their function in antigen presentation, cytokine production, and the identification of the antitumor antibodies they may produce.

A number of cellular mechanisms involved in inflammation-induced tumor initiation, promotion, and progression have been reported [4]. These include genomic instability events not directly involving DNA mutations like chromatin remodeling, epigenetic changes, and altered gene and microRNA (miRNA) expression. Over the last two decades, inflammation has emerged as an important contributor to carcinogenesis. For this reason, the identification of molecules acting on immune cells and molecular targets linked to tumor-promoting or associated inflammation is significant [4,139–141].

For the remarkable hallmark inflammation and cancer, we selected as prioritized chemicals from the environment bisphenol A (BPA), polybrominated diphenyl ether (PBDE), nonylphenol (NP), phthalates, and atrazine.

These ubiquitous environmental chemicals are not actually classified as carcinogens, and in addition they are not considered genotoxic; they act on immune cells and molecular targets mechanistically linked to cancer-associated inflammation. The goal, as suggested by the Halifax Project, was to investigate if these chemicals, alone or in combination with other exposures, influence cancer risk in humans [141,142].

14.3 Prioritized Chemicals

14.3.1 Bisphenol A

Perhaps the most abundant and well-studied environmental endrocrine disrupter is the synthetic xenoestrogen BPA. While the role of BPA as an endocrine disrupter has been extensively reviewed elsewhere, the impact of BPA on the immune system and as an immune disrupter is less recognized [4,139,140]. BPA is present in the environment as a result of everyday exposures from food packaging, plastic bottles, water pipes, electronic equipment, paper, and toys. The recent review by Thompson *et al.* [4] highlights the evidence for both immune-activating and immune-inhibiting consequences of exposure to BPA, and suggests that the inconsistency in reported effects reflects a more generalized disruption in the innate immune balance as opposed to more easily defined and specific effects on antigen-driven immune or adaptive immune responses.

In rats, it has been shown that early-life exposure to BPA mimics estrogen-induced prostate intraepithelial neoplasia (a prostate cancer precursor lesion), which includes BPA-dependent epigenetic reprogramming of DNA along with the development of lateral prostate inflammation in the adult animal, reported earlier to reflect BPA effects on prolactin levels [4]. Because inflammation of the prostate is "insufficient" for the development of prostate cancer in animal models and since the role of inflammation in human prostate cancer is unclear, it has been argued that the effects of BPA in rodents may not be relevant to humans. An alternative explanation is that in the presence of genotoxic or other cofactors, the immune-deregulating effects of BPA on the prostate act to enhance or accelerate tumor development in the rat and while not sufficient are necessary exposures for carcinogenesis [4].

In addition to the work in prostate, evidence for a BFA effect on the immune system is present in others studies, particularly about the T-cell compartment. BPA seems to act largely on the immune system by promoting "immune" cell proliferation, though the exact nature of the effect on specific cells of the immune system and, thus, the consequences are complex and poorly delineated [4]. CD4+ T lymphocytes, for example, comprise the Th1 and Th17 helper T cells that produce proinflammatory cytokines, whereas the Th2 or Treg cells produce anti-inflammatory or regulatory cytokines. A number

of studies have been conducted on BPA effects on CD4+ T-cell polarization toward one or the other subtype with highly mixed results. There are results indicating BPA activation of Th1 and Th2, often with the dominance of one type over the other, effects that vary depending on the dose, duration, and timing (adult or early life) of the exposure; there are no reported effects on Th17 cell differentiation. Currently, it is unclear why BPA-exposed CD4+ cells polarize to either a pro- or anti-inflammatory state, but there is sufficient evidence to support an effect of BPA on CD4+ T cells at exposure levels comparable to those in humans. Much like the BPA-exposed T cells, results from studies on macrophages and B cells are also conflicting [4,140].

The immunomodulatory effect of BPA on cells has been linked to estrogenic activity [4]. In estrogen-responsive ovarian cancer cells, BPA upregulated SnoN, a negative regulator of TGF-β signaling, and downregulated pSmad3, a transcription factor in the downstream pathway of TGF-β signaling pathway, suggesting that BPA exposure, simultaneously, leads to the denigration of TGF-β signaling in the process of induction of EMT and migration of BG-1 cells via estrogen receptor (ER) signaling. Cotreatment of dietary GEN reversed the downregulation of TGF-β signaling by estrogenic chemicals, in a chemopreventive fashion [143]. A recent study also indicated that low levels of BPA exposure alone could significantly disturb the immune response of fish primary macrophages *in vitro*, and for the first time revealed the synergistic action of ERα and nuclear factor-κB (NF-κB) transcription factors in the BPA effect [144].

In addition to the ER, there is growing interest on the effects of BPA and BPA analogs on members of the PPAR nuclear receptor family members α, β/δ, and γ. Various studies implicate a role for the three isoforms of PPAR in the pathogenesis of inflammatory diseases in combination with the effect of other nuclear receptor-independent pathways [145–147].

Exposure to plasticizers leads to the activation of peroxisome proliferator-activated receptors, the increase of fatty acid oxidation, and the reduction in the ability to cope with the augmented oxidative stress leading to reproductive organ malformations, reproductive defects, and decreased fertility [148].

The role of PPARs as BPA targets (PPARγ isoform is present on macrophages, dendritic cells, T cells, and B cells) is further suggested by observations that other BPA analogs (e.g., tetrabromobisphenol A, a brominated BPA found in flame retardants) antagonize PPARs in direct relation to the bulkiness of the brominated BPA analogs [4].

14.3.2 Polybrominated Diphenyl Ethers

Like flame retardants, PBDEs are ubiquitous in the environment in a number of consumer products from textiles to electronic parts. Leaching of PBDEs from treated products results in air, food, water, and soil contamination, where exposure through ingestion and inhalation is associated with an estimated

half-life of the common congeners in human adipose tissues of 1–3 years [4]. Body burdens of PBDE have increased over the past few decades, raising concerns about long-term health effects. The review by Thompson *et al.* [4] reported that for women living near electronic waste sites, the placental burden of PBDEs is approximately 20-fold higher than for women residing in a referent site. These results support very early-life exposures for which the long-term health effects are unknown, including the cancer risk. There is currently little experimental evidence that the PBDEs act as direct mutagens. The activity and chemical structure of PBDEs are similar to TCDD. Pro- and anti-inflammatory factors play a critical role in the placenta during fetal development and at parturition, wherein the proinflammatory cytokines induce PGs that promote uterine contraction and cervical ripening. Thus, during pregnancy, potent anti-inflammatory cytokines, in particular IL-10, are elevated as a defense against preterm birth induced by bacterial infections. Polybrominated diphenyl ethers (congeners 47, 99, and 100) enhance the production of proinflammatory cytokines by the placenta. This may increase the risk of infection-mediated preterm birth by lowering the threshold for bacteria to stimulate a proinflammatory response(s) [149].

Recent studies found that placental explants treated with a mixture of the cogeners BDE-47, BDE-99, and BDE-100 and then exposed to *Escherichia coli* were "reprogrammed" toward a proinflammatory response (increased IL-1β and TNFα) and away from the expected anti-inflammatory response (decreased IL-10) compared with untreated placenta [4]. Thus, chronic PBDE exposure may "lower the threshold for bacteria to stimulate a proinflammatory response." This study is noted here given the established link between bacteria and cancers, such as *Helicobacter pylori* and gastric cancer, where tumor development is dependent on inflammation. Emerging evidence also shows that many other human cancers may have a bacterial component, with cancers of the gastrointestinal tract (esophagus, liver, stomach, pancreas, colon, and rectum) strongly believed to involve a disturbance in the interaction between normal flora and the immune system that promotes chronic, low-grade inflammation (i.e., dysbiosis) [4].

Penta- and octa-BDE, but not deca-BDE, might promote the expression of proinflammatory proteins in bronchial epithelial cells, possibly by activating protein kinases and/or stimulating nuclear receptors related to subsequent activation of transcriptional factors (e.g., AHR) [150].

Bioaccumulation, biotransformation, and toxicity of BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47 were studied using in early life stages of the zebrafish (*Danio rerio*) [151]. This model is very useful to investigate toxic effects induced by PBDEs as a product of the combination of multiple independent mechanisms. For example, BDE-209 activated human aryl hydrocarbon receptor, peroxisome proliferator-activating receptors, CF/b-cat, activator protein 1, Oct-MLP, and the estrogen receptor-related alpha (ERRα) receptor in cell-based assays. The risk of the potential impact on human and environmental

health of bioavailable BDE-209 was assessed combining human *in vitro* cell assays and zebrafish embryos. The study showed that BDE-209 has the potential to cause impacts on both human and environmental health [152].

To our knowledge, there has been no consideration of the role of environmental immune disrupters, such as PBDEs, as contributors to these cancers, where incidence rates have increased in parallel to industrialization.

14.3.3 4-Nonylphenol

A ubiquitous environmental chemical implicated recently in inflammation is 4-nonylphenol (4-NP). Human exposure to 4-NP occurs through ingestion of contaminated food and water from liquid detergents, cosmetics, paints, pesticides, and other common products, where NP ethoxylates are used as nonionic surfactants [4]. As an endocrine disrupter, 4-NP is recognized for its potent reproductive effects. Thompson *et al.* [4] reported some of the effects induced by 4-NP prenatal exposure. 4-NP effects on adipogenesis in the perinatal period confer transgenerational inheritance of the obesogenic effects observable in F2 offspring, consistent with genome reprogramming through an epigenetic process [4]. With the recognized overlap in signaling molecules between the endocrine and the immune system, 4-NP may be acting as an immune disrupter. 4-NP induced COX-2 protein and gene expression in the murine macrophage cell line RAW264.7 and significantly increased PGE2 production. 4-NP was further shown to activate the Akt/MAP kinases/CRE signaling response elements involved in the activation of COX-2 expression. This observation is the first insight on a potential mechanism for the observed lung inflammation and asthma in mice exposed to 4-NP. In addition, Maradonna *et al.* [145] reported that 4-NP, through COX-2 gene transcription significant upregulation, can also give rise to metabolic disorders in fish and highlight the potential for their vertical transfer through the trophic levels and ultimately to humans.

As we previously reported for PBDEs, there is interest in studying the link between the inflammatory response upon activation of the aryl hydrocarbon receptor (AHR). Studies suggested that 4-NP may disturb physiologic function of DCs through, in part, AhR-dependent mechanisms, supporting the importance of 4-NP exposure on the regulation of DC functions and allergic inflammation [153].

Given the iniquitousness of 4-NP and evidence favoring transgenerational transmission of exposure effects, there is sufficient evidence to recommend the investigation of cancer risks associated with 4-NP exposures.

14.3.4 Atrazine

The triazine herbicide atrazine is widely used in agricultural to control the unwanted growth of grasses and broadleaf weeds. Being one of the most

commonly used pesticides in the world, atrazine is widespread in the environment and a frequently detected contaminant in waterways [4,140]. Like BPA and other chemicals, there are scientific indications that atrazine has endocrine-disrupting potential, causing mammary gland tumors in rodents and altering male reproduction [4]. In a recent study highlighted in Thompson *et al.*, both atrazine and its major metabolite diaminochlorotriazine induced changes in the antioxidant capacity of the liver and decreased the transcription of genes involved in testosterone production, supporting that oxidative stress may contribute to alterations in reproductive capacity. Indeed, *in vitro* experiments using interstitial Leydig cells support that suppression of oxidative stress by the flavonoid quercetin prevents atrazine-induced toxicity by attenuating oxidative stress partially by modulating the NFκB pathway [4]. One of the reputed actions of atrazine is the regulation of NO production, an important bioactive molecule that can have a weighty impact on cancer development by contributing to angiogenesis, suppressing apoptosis, and limiting the host immune response to the tumor itself [4]. Whether changes in NO levels are reflective of induction/inhibition of iNOS expression in mammalian systems is not known. Atrazine also significantly decreased cytokine production (e.g., TNFα, IFN-γ) as well as impaired lymphocyte proliferation and natural killer cell function [4].

Recent studies also showed neurotoxic effects of atrazine in SH-SY5Y human dopaminergic neuroblastoma cells via microglial activation [154].

Long-term individual, or in combination, exposure of atrazine can induce the dysregulation of pro-/anti-inflammatory cytokine expression (e.g., TNF-α). The information regarding the effects of atrazine in combination with cytokine mRNA expression generated in this study will be important information for pesticides toxicology evaluation [142,155]

14.3.5 Phthalates

Humans are exposed to phthalates through multiple routes that include food and drink, inhalation, skin absorption, and even medical procedures such as blood transfusions [4,140]. Body burden studies suggest that diethylhexyl phthalate (DEHP), a high-molecular-weight species used in plastic wrapping of foods, is a major source of exposure for humans as a result of contamination from the packaging, an effect made greater with microwave heating [4]. As with other environmental exposures, there is a particular concern for early-life prenatal exposures. Thompson *et al.* [4] warned of the concerns of high levels of pthalates in children from toy products as well as exposure to breakdown products of the smaller molecular weight diethyl phthalate in personal skin care items such as lotion and soap.

So far, little evidence has been shown in the associations between maternal biomarkers of phthalate exposure and inflammation using repeated measurements across pregnancy. To investigate inflammation as a mechanism of

phthalate effects in humans, biomarkers from target tissues or fluids, though difficult to measure in large-scale studies, may be necessary to detect effects. Ferguson revealed significant associations of urinary phthalate metabolite with biomarkers of inflammation and oxidative stress across pregnancy in Puerto Rico [156,157].

More recently, an interest in the effects of phthalates and related metabolites on inflammation has emerged where the focus has been the risk of asthma [4]. This and other population studies have suggested phthalates act as immune disrupters [4]. While the findings across *in vitro* and *in vivo* studies confirm the effects of phthalates on macrophages, lymphocytes, eosinophils, and neutrophils, no consistent effects have emerged, and the actual consequence of exposure seems to be contextually dependent. For example, chronic exposure to airborne DEHP increased the numbers of eosinophils, lymphocytes, and neutrophils in the lung and lavage fluid, but only at very high (not human exposure-related) concentrations [4]. In a separate study of the major metabolite of DEHP (MEHP), exposure at much lower doses showed similar proinflammatory effects, indicating the importance of metabolism in effective dose [158]. Both human innate and adaptive immunity are influenced *in vitro* by phthalates (secretion of IL-6), and that phthalates therefore may affect cell differentiation and regenerative and inflammatory *in vivo* processes [159].

Similar to BPA, the endocrine disruptive effects of phthalates could be generated by multiple mechanisms involving the AHR and other NRs [147]. Consistent with the evidence observed for endocrine disrupters, phthalates disrupt gene expression in a pattern very similar to that of BPA, where the compounds exhibit a high degree of sharing of effects on interacting genes and proteins in an immune-disrupting signature. The latter has been suggested as a potential tool for future research efforts to characterize the inflammatory potential of a compound.

In general, phthalates exhibit immune-disrupting activity depending on the conditions of exposure (dose, duration, tissue type, and development). These complex and often paradoxical observations have made a translation to humans a challenge, but do not dismiss the potential relevance of these exposures in human diseases [4].

14.4 Experimental Models of Carcinogenesis through Inflammation and Immune System Deregulation

Our understanding of the associations between low-dose exposure to ubiquitous chemicals and cancer-specific outcomes in humans originates mainly from the comparison of cancer burden among exposed and unexposed individuals in observational epidemiologic studies. While this approach has successfully contributed to the identification of some important carcinogens and the extent

of their impact, the multifactorial nature of cancer onset hampers the definition of a causal link between chemical exposures and cancer risk. This is also particularly true when the carcinogenic potential of chemical exposition is influenced by unmeasured or immeasurable factors such as the dose, duration, or precise timing of the exposure or by population heterogeneity. For these reasons, knowledge that has been gained about the etiopathogenesis of cancer in the study of environmental chemical effects must be integrated with details on cellular and molecular processes characterized in experimental models.

The importance of inflammation as both promoting and associated factor in cancer encouraged an increasing number of *in vitro* and *in vivo* studies on chemicals involved in proinflammatory events.

The effect of BPA on viability, and substrate adherence capacity of macrophages, has been investigated *in vitro* [160]. Experimental results demonstrate that BPA does not significantly alter macrophage viability at any of the assessed concentrations, but it inhibits substrate adherence of rat peritoneal macrophages in a dose-dependent manner. Since adhesion is the first step in the phagocytic process and antigen presentation, its inhibition is likely to modulate both immune and inflammatory responses in the presence of BPA. Moreover, mechanisms of BPA-induced modulation have been evaluated on primary macrophages from head kidney of *Cyprinus carpio* [144]. Results obtained in fish indicate a potential involvement of both the ER and NF-κB pathways in the observed dynamic response of BPA-treated macrophages. BPA exposure significantly enhanced antibacterial activity of macrophages at 0.1, 1, or 10 μg/l of BPA, but induced apoptosis at 100, 1000, and 10,000 μg/l. The potential proinflammatory effect is suggested by the increased production of nitrite oxide and reactive oxygen species and the induction of interleukin-1β, NF-κB, and other related genes. ERα and NF-κB pathways interplay has also been demonstrated antagonizing those in presence of BPA. It has been shown that inhibiting ER or NF-κB pathways the production of ROS is significantly reduced even in the presence of BPA, thus suggesting that BPA exposure can significantly affect the immune response in an ERα and NF-κB-dependent manner.

BPA is also able to suppress the immune response influencing the activity of T helper type 1 blood cells [161]. The effect of BPA on interferon-γ-induced tryptophan breakdown and neopterin production, in fact, has been evaluated *in vitro* on human peripheral blood mononuclear cells. This proinflammatory cytokine is known to induce the conversion of tryptophan into kynurenine thorough the enzyme IDO-1. During the process, neopterin, a marker of immune activation, is produced. Data indicate that cells treated with BPA are subject to a significant dose-dependent suppression of tryptophan breakdown and neopterin formation coupled with IFN-γ levels decrease.

On the contrary, a different study indicates that BPA enhances the inflammatory response to allergen challenge [162]. The study aimed to assess whether short-term exposure to BPA can promote the release of histamine and cysteinyl

leukotrienes in mast cells *in vitro*. Primary murine bone marrow-derived mast cells were used and exposed to BPA or estradiol added to culture media. It has been shown that while both BPA and estradiol are able to increase histamine release, the use of an estrogen receptor antagonist partially blocked the ability of estradiol, but not BPA, to promote its release. Moreover, estrogen receptor inhibition did not abrogate BPA-induced release of cysteinyl leukotrienes. On the contrary, BPA is unable to enhance histamine and cysteinyl leukotrienes release when the ERK pathway is blocked. These data suggest that acute BPA exposure enhances the proinflammatory response *in vitro* even without the involvement of the estrogen receptor, but it requires, at least in part, the activation of ERK pathway. That being so BPA may cause acute inflammatory response via enhanced mast cell activation.

Since BPA is also considered a potential obesogen compound, it has been studied for its estrogen mimetic activity and metabolic regulation interference. BPA effects on insulin action and inflammation in human subcutaneous adipocites and 3t3-l1 (mouse embryonic fibroblasts) cells have been studied [163]. Subcutaneous adipose tissue-derived adipocytes and differentiated 3T3-L1 incubated with BPA have been evaluated to assess its effect on glucose utilization, insulin sensitivity, and inflammation-related cytokine secretion. It has been proved that BPA at nanomolar concentrations is able to induce an inflammatory response in human adipocytes increasing the release of factors like IL-6 and IFN-c.

The pollutant activation of JNK, JAK/STAT, and NF-kB pathways in treated adipocytes and their direct stimulation could also be accompanied, *in vivo*, by an effect on inflammatory infiltrates in the adipose tissue. The anthrapyrazolone-mediated inhibition of JNK activity almost completely rescues insulin receptor signaling and restores insulin-stimulated glucose utilization in BPA-treated adipocytes, suggesting a primary involvement of inflammatory factors. It has been hypothesized that BPA can trigger JNK via Toll-like or estrogen receptors directly counteracting insulin action [164,165], or the release of IL-6 and IFN-c may, in turn, promote JNK activation adipose tissue and impair glucose uptake. On the therapeutic side, it has been observed that the pharmacological activation of autophagy can mitigate the BPA-induced inflammatory effects [166]. This was suggested by the observation that BPA exposure during the early postnatal period increases the expression of autophagy-related proteins, such as the phosphatidylethanolamine bound form of the microtubule-associated protein 1A/1B-light chain 3 (LC3-II) and the sequestosome 1 (SQSTM1), together with an increase in oxidative stress and apoptosis. This BPA-induced generation of reactive oxygen species and apoptosis is significantly mitigated by rapamycin administration. These evidences provide a functional link between autophagy activation and inflammation and encourage the research for therapeutic interventions that may act through AMPK and mTOR pathways.

The inflammation involvement in BPA-mediated toxicity inspired a number of studies that explore the protherapeutical potential of both natural products and synthetic substances. It has been suggested [167,168] that natural antioxidants like quercetin, green or black tea that possess strong free radical scavenging activity, are able to counteract BPA-induced oxidative stress at low doses. For example, results indicate that the addition of green tea extract to erythrocytes suspensions containing high doses of BPA caused an amelioration in pollutant-induced hemolysis in a concentration-dependent manner.

Since DNA hypomethylation induced by BPA exposure has also been suggested to be associated with cancer onset, also in a prenatal exposure setting, the effects of maternal nutrient supplementation during early development have also been investigated [169]. *In vivo* investigations demonstrated that while the epigenetic patterning during early mouse development is sensitive to BPA exposure, an appropriate maternal dietary supplementation, which included either methyl donors like folic acid or the phytoestrogen genistein, significantly abrogates the hypomethylating effect of BPA.

It is also important to note that BPA is able to influence the efficacy of therapies influencing molecular pathways targeted by some anticancer agents. It has been demonstrated that it may impair the efficacy of androgen deprivation therapy (ADT) used in prostate cancer treatment [170,171]. While ADT is often effective at first with the majority of patients undergoing a period of remission, sometimes patients develop ADT-resistant tumors with unfavorable prognosis. While the reasons for therapeutic resistance are still mostly unknown, a number of somatic mutations arising from the selective pressure of prostate cancer treatments, which facilitate sensitivity to environmental contaminants, have been identified. It has been demonstrated that some of these mutations that affect the function of the androgen receptor allow BPA to trigger the receptor, producing increased proliferation and tumor growth when ADT is administered, thus suggesting that environmental exposure can dramatically influence the outcome of specific therapeutic regimens.

DEHP has also been investigated for its ability to interfere with immune system response and inflammation. *In vitro* studies [172,173] demonstrated that DEHP and other endocrine disrupter could affect the production of inflammatory cytokines by human macrophages. It has been shown that when differentiated macrophage-like THP-1 cells are exposed to DEHP, the concentrations of TNF-α, IL-1β, IL-8, and IL-6 in the media increase. Gene expression analysis indicates increased levels of IL-8, CXCL1, CXCL2, CXCL3, CXCL6, CCL3, MMP3, MMP10, MMP14, and CSF2 mRNA in DEHP-treated THP-1 cells. DEHP also induces p65 NF-κB translocation into the nucleus.

In vivo effects of DEHP exposure have also been evaluated in rodent models. The *in utero* exposure to the chemical has been shown to alter the expression of immune response and inflammation markers in the epididymal whole-adipose tissue and isolated stromal vascular fraction. C-reactive protein and TNF serum

levels were increased, with the latter also increased in adipose tissue homogenates at concentration as low as 50 mg DEHP/kg. Immunofluorescence studies also revealed focal macrophage infiltration in whole-adipose tissue confirmed by the increased CD163 tissue content. These findings suggest how DEHP exposure promotes local adipose tissue inflammation and chronic low-grade systemic inflammation.

Human and rodent neutrophils exposed to DEHP have been shown to increase the expression of integrin alpha M by more than 200% [174]. This molecule is known to mediate inflammation by regulating adhesion and migration of leucocytes (including macrophages, monocytes, granulocytes, and NK cells), and these results indicate how DEHP itself is proinflammatory in human and rat blood. Since DEHP is used as plasticizer for polyvinyl chloride, DEHP-plasticized PVC has also been assessed on neutrophyls. *In vitro* models indicate that DEHP–PVC exposure produces a similar increment in integrin alpha M as soon as after 20 min of incubation in both human and rodent blood. Methanol washing of the PVC before treatment produced a significant reduction in integrin upregulation in both blood types, but particularly in humans.

DEHP has also been shown to assume a detrimental role in cancer treatment inhibiting the apoptotic effect of chemotherapeutic agents. In particular, this environmental pollutant has been implicated in the suppression of tamoxifen-induced apoptosis [175]. Cytotoxicity assays demonstrate that the reduced cell viability of tamoxifen-treated GH3 cells can be reversed when cells are incubated with DEHP for 4 days. Results indicate that the enhanced PARP cleavage exerted by tamoxifen exposure is significantly inhibited by DEHP, thus suggesting a possible molecular mechanism for its antiapoptotic and protective effect on treated cells.

On the preventive and therapeutic side, recent evidences suggest that the pro-oncogenetic phenotype induced by DEHP exposure may be counteracted through the inhibition of the aryl hydrocarbon receptor/ERK/SK1/S1P3 signaling pathway [176]. Researchers demonstrated that this pollutant is able to promote the metastatic process through the increase of migration, invasion, and epithelial–mesenchymal transition of hepatocellular carcinoma cells. DEHP exposure also increases the number of cancer cells that exhibit a cancer stem cell-like phenotype. Results indicate that in the same cellular model, curcumin administration suppressed phthalate-induced metastatic phenotype and decreased the proportion of CSC-like cells *in vitro* and inhibited tumor growth and metastasis *in vivo*.

A study of atrazine immunomodulatory properties was performed on B6C3F1 mice using a panel of immune assays and host resistance models designed to evaluate cell- and antibody-mediated immunity [177]. Atrazine administration significantly increased the number of splenic CD8+ and cytotoxic T cells together with mixed leukocyte responses, and reduced host resistance to B16F10 melanoma. Spleen functions are also influenced with

total spleen cell numbers and fixed macrophage function reduced in exposed mice. In the thymus, atrazine induces a decrease in the number of CD4+/CD8+ cells, and at the higher doses the decreases in the thymic T-cell populations were still present 1 week after the last administration [178]. In the spleen, the increase in CD8+ cells is accompanied by a decrease in MHC-II+ and CD19+ 1 day after atrazine discontinuation. Moreover, the proportion of mature splenic dendritic cells is also decreased and persists for at least 1 week after the last atrazine dose, thus suggesting that the molecule can inhibit dendritic cell maturation. *In vivo* observations coherently support the hypothesis that atrazine exposure is detrimental to the immune system by decreasing cell number and affecting lymphocyte distribution even after exposure cessation.

PBDEs also seem to act through the promotion of oxidative stress and inflammation. It has been recently demonstrated that the nuclear factor E2-related factor 2 (Nrf2), an antioxidative transcription factor, may exert a protective effect in BDE-47-induced inflammatory responses [179]. In particular, a pretreatment with *tert*-butyl hydroquinone (tBHQ) or sulforaphane, known Nrf2 inducers, reduces BDE-47-promoted IL-6 release by augmentation of cellular antioxidative system via Nrf2-positive regulation. While researchers speculate on the potential role of Nrf2 as a therapeutic target to reduce adverse pregnancy outcomes, it may be possible to extend those conclusions to tumor-related outcomes in which the toxicant-induced oxidative stress and inflammation is a contributory cause.

4-NP has been functionally implicated recently in inflammation. As an endocrine disrupter it has been studied for its potent reproductive effects, but recently it has also been shown to increase progenitor white adipose levels, body weight, and overall body size in rodents exposed prenatally [180]. An *in vivo* study demonstrated that its perinatal exposure affects the adipogenesis in both male and female F1 offspring, and this effect can be progressed to the F2 offspring through the maternal line through an epigenetic process of genome reprogramming. This proadipogenic effect has been associated with a decrease in ERα activity adipose tissue in line with its endocrine disrupting activity. These results suggest that 4-NP may also interfere with immune system response due to the recognized interplay between the endocrine and the immune systems.

Hung *et al.* [181] assessed the effects of alkylphenols on plasmacytoid dendritic cells in the presence of immunostimulants. Their results show that NP treatment is able to significantly increase TNF-αexpression and suppress IL-10 production in the range of physiological doses. This modulation is concurrent to the activation of the MKK3/6-p38 signaling pathway to the acetylation of histone 3 and 4 at the TNFA gene locus. Moreover, in the immunostimulated cells, NP reduces the production of type I IFNs production, thus negatively regulating IRF-7 and MKK1/2-ERK-Elk-1 pathways. NP showed similar effects *in vivo* when administered to mice showing similar cytokine

changes upon immune system stimulation. It has also been observed that NP increases allergic lung inflammation in animal models.

It is interesting to note that oxidative stress triggering is often an intrinsic and maybe required step of the malignant transformation process. While many different sources of reactive oxygen species exist, they can be collectively lead back to inflammation, aberrantly activated oncogenic signaling, dysfunctional mitochondrial metabolism, or the promotion of motility and invasion. Oxidative stress is a multifaceted phenomenon in cancer. On one side, it has been extensively demonstrated that increased ROS production can promote transformation. Conversely, cellular systems that exert a protective function against the cytostatic or proapoptotic activity exerted by ROS are needed for the optimal malignancy of transformed cancer cells. Recently, the identification of NUDT1 as an endogenous tumor-promoting gene in RAS-driven tumor cells provides additional evidences toward the importance of oxidative state control in cancer [182]. The protein encoded by this gene degrades oxidized purine nucleotides counteracting the oxidative stress-derived resistance to transformation arising from RAS activation. In particular, since high level of oncogenic ROS impairs the transformation process by promoting DNA damage and oncogene-induced senescence or cell death, oncogenic oxidative stress mitigation exerted by NUDT1 promotes cancer survival and growth in RAS-driven cells. This is even clearer in cancer therapy: Radiotherapy and some forms of chemotherapy are actually based on the production of oxidizing free radicals. A quite obvious consequence, then, is that a concern that antioxidants might reduce the effectiveness of the therapy actually exists. Some evidences seem to suggest that the use of exogenous antioxidants produces favorable effects in cancer-affected patients, and except for a few specific cases, animal and human studies demonstrate no reduction of efficacy of chemotherapy or radiation when given with antioxidants [183]. On the contrary, some studies suggest that many cancer therapeutic agents increase their potential while decreasing adverse effects, when given concurrently with antioxidants. Like many other human diseases, cancer is represented by an altered homeostasis in tightly regulated cellular framework: Each piece of the system can assume opposite roles, depending on how far it is from equilibrium.

14.5 Antioxidants and Translational Opportunities

Recent studies in which mice on a high-fat diet (HFD) were treated with the gut microbiota-modifying antioxidant tempol suggested that inhibition of intestinal FXR signaling could be advantageous in amelioration of obesity, insulin resistance, and nonalcoholic fatty liver disease [184,185]. This led to the search and the finding of Gly-MCA compound, a natural conjugated bile salt with prevalence in humans. Mechanistic studies revealed that FXR regulates genes

involved in ceramide synthesis, and that ceramides mediate the metabolic effects of Gly-MCA [186]. Specific inhibition of intestinal FXR may be a reasonable therapeutic strategy for the treatment of human metabolic disorders and the prevention of chronic inflammatory events leading to cancer development in the gut.

Epidemiological studies have shown that oxidants and free radicals-induced DNA damage are one of the predominant causative factors for cancer pathogenesis. The capabilities of arsenic in inducing generation of ROS, expression of miRNAs, and the generation of cancer stem cells have been recently reviewed [187]. Hence, oxidants are attractive targets for chemoprevention and therapy. Both avoidance of specific risks that are associated with genetic susceptibility and decreasing oxidative stress in general should delay carcinogenesis [188]. The work by John *et al.* [189] reported a list of dietary agents that can potentially target oxidative stress, in turn delaying, preventing, or treating cancer development. Some of these agents are currently in use in basic research, while some have been launched successfully into clinical trials.

Dietary agents are known to exert an antioxidant property that is one of the most efficient preventive strategies in cancer progression. These include vitamins A, C, E, epigallocatechin gallate (EGCG), and flavonoids [183]. In addition to targeting redox-sensitive signaling proteins and transcription factors, dietary alpha-keto acid metabolites of methylselenocysteine (MSC) and selenomethionine can alter HDAC activity and histone acetylation status in prostate and colon cancer cells [190,191].

Ulcerative colitis (UC) is a chronic inflammatory condition and it is associated with increased risk of colorectal cancer [192,193]. Synthetic anti-inflammatory drugs are used as preventive and therapeutic agents of UC, but some of them revealed severe side effects in the clinics [194]. Dietary antioxidant polyphenolics derived from fruits and vegetables have demonstrated anti-inflammatory effects via the inhibition of NF-κB and induction of antioxidant defense systems [195]. Tannin-rich fruits have been evaluated as alternative prevention strategies for colorectal cancer based on their anti-inflammatory properties. The recent study by Kim *et al.* [196] compared tannin-rich preparations from mango (rich in gallotannins) and pomegranate (rich in ellagitannins) in the dextran sodium sulfate-induced colitis rat model.

In rats, mango and pomegranate beverages decreased intestinal inflammation and the levels of proinflammatory cytokines in mucosa and serum. Mango polyphenols inhibited the IGF-1R-AKT/mTOR axis, and pomegranate polyphenols downregulate the mTOR downstream pathway through reductions in ERK1/2. *In silico* modeling indicated a high binding-docked of gallic acid to the catalytic domain of IGF-1R, which may suppress the activity of the enzyme. Ellagic acid docked effectively into the catalytic domains of both IGF-1R and EGFR. Experimental assays using polyphenolic extracts from each beverage, as well as pure compounds, confirmed the computational predictions (Figure 14.1). Overall,

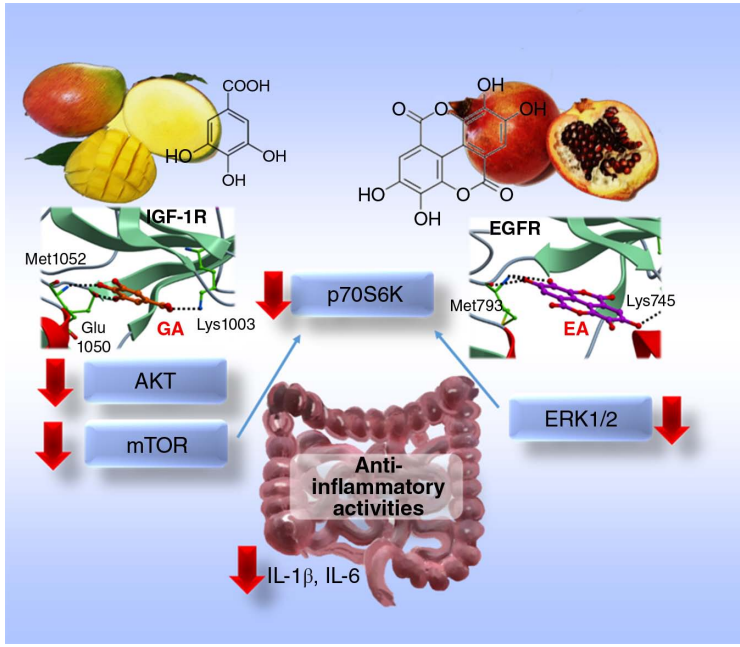

Figure 14.1 Mango beverage (rich in gallotannins) inhibited the IGF-1R-AKT/mTOR axis, and pomegranate beverage (rich in ellagitannins) downregulated the mTOR downstream pathway through reduction of ERK1/2 in DSS-treated rats. Gallic and ellagic acid were predicted *in silico* to bind to specific targets, such as IGF-1R and EGFR. *Source:* Reproduced from Ref. [196] with permission of John Wiley & Sons.

these results suggest that different molecular targets, triggered by dietary antioxidants polyphenols, can be involved in the protection against chronic inflammatory ulcerative colitis [196].

14.6 Tumor Control of the Microenvironment

In this chapter, we have reviewed how inflammation and the microenvironment contribute to carcinogenesis and how specific carcinogens seem to mediate their cancer properties through their affects on these biological programs. The cellular programs are complex, and individual host immune and inflammatory cells can have opposing affects on both contributing to and suppressing cancer formation. Recently, drugs that restore an immune response have shown dramatic affects in eliciting tumor regression even to otherwise therapeutically refractory human cancers.

In general, this begs two questions: How do cancers causally initiate changes in the tumor microenvironment and immune system that results in

tumorigenesis? How and when can one reverse cancer by blocking protumorigenic properties of the tumor microenvironment and/or by restoring an immune response? In general, the answer seems to be that specific oncogenes can causally dysregulate the tumor microenvironment and block the host immune response and that inhibition of these oncogenes may lead to a reversal of the properties that can result in tumor regression [197].

Over a decade ago, multiple laboratories simultaneously described that oncogenes that initiate cancer in an animal model, when inactivated, result in a complete and sustained reversal of tumorigenesis. Tumors were described as "oncogene-addicted" [198]. Subsequently, it was found that upon oncogene inactivation, there were two sets of changes that occurred: Tumor cells became autonomously growth arrested, differentiated, and/or died; and the host tumor microenvironment underwent dramatic normalization associated with the suppression of angiogenesis and restoration of an immune response against the tumor. Importantly, many of the changes that occurred in the tumor causally required host immune effectors [199].

Then, most recently, it has been shown that oncogenes may directly regulate the expression of host immune checkpoints, such as PD-L1, and that this is causally required for oncogene-induced tumor initiation as well as oncogene inactivation-induced tumor regression [200,201]. Hence, there is a potential unifying explanation for how normal cells become cancer cells and fuel carcinogenesis and how inactivation of a oncogene in cancer cells reverses this neoplastic process. Thus, specific oncogenes directly couple hallmark changes that occur in the cancer cell as well as in the tumor microenvironment. Many oncogenes are likely to contribute, and the mechanisms are likely to be related to the regulation of not just immune checkpoints and cellular cytokines, but also many other host and immune regulatory molecules [199].

From this model, several predictions could be made with experimental, investigational, and clinical implications: First, that it is really critical in therapeutic development to utilize model systems that have an intact host and host immune response. Second, that genes implicated in the pathogenesis of cancer are likely to contribute to tumorigenesis through both tumor-intrinsic and host-dependent effects. Third, therapies that target specific oncogenic signaling pathways may contribute to tumor regression by restoring a host immune response. Fourth, specific genetic features of human tumors may predict particular sensitivity to specific types of immune therapy. Fifth, carcinogens may work in concert to perturb inflammation and/or immune response and thus may contribute to cancer formation.

Regardless of the precise molecular mechanisms, these observations also suggest that measurements in the tumor microenvironment, host immune system, and inflammation would be highly useful in interrogating the mechanisms by which carcinogens contribute to tumorigenesis, and the mechanisms

by which novel therapies can be used to prevent, block, or reverse these processes [4,139–141].

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15

Metabolic Dysregulation in Environmental Carcinogenesis and Toxicology

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15.1 Introduction

Intermediary metabolism enjoys a dichotomous relationship with many toxic responses, and the ability of metabolism to both promote and oppose individual responses to toxic exposures poses a number of special challenges to the understanding and study of metabolic contributions to toxicological susceptibility and environmental carcinogenesis [1]. These promotional and oppositional metabolic responses can also take a number of distinct forms. For example, changes in metabolism can serve to either mediate or mitigate toxic responses involving oxidant stress [1]. Toxicants can also directly or indirectly target metabolic processes to alter overall metabolic fitness in the promotion of toxicity, suggesting the potential for nonmutually exclusive reciprocal interactions between toxicity and metabolism. The dysregulated metabolism that characterizes cancer exhibits similar dichotomous roles [1]. Chronic toxicity and carcinogenic latency enjoy even more complex relationships with metabolism, in large part, due to the longer time frames over which chronic processes evolve and the associated exponential potential for complex indirect metabolic contributions to temporally distant endpoints. The timing, duration, and pattern of both toxic and carcinogenic exposures play important roles in determining ultimate associated biological outcomes, and there is increasing awareness of critical exposure windows as major determinants of both immediate toxic responses and long-term outcomes such as cancer development. Metabolism plays a major role in normal developmental plasticity and strongly influences fetal reprogramming in response to altered embryonic environmental conditions [2]. As such, metabolism seems well suited to play similar roles in the development of cancer.

Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer, First Edition. Edited by Michael D. Waters and Claude L. Hughes.

Environmental contributions to cancer development are widely recognized and involve a diverse array of factors, including lifestyle, occupational exposures, environmental pollutants, medical therapies, geophysical factors, and infectious agents [3,4]. Corresponding effects on intermediary metabolism and their ultimate causal contributions to the development of cancer and its associated phenotypic characteristics, however, have been incompletely explored [1]. In addition, little is known about the temporal and mechanistic relationships between specific exposures, metabolic dysregulation, carcinogenesis, and the development of other phenotypic cancer hallmarks, including dedifferentiation, loss of normal growth constraints, and invasive/metastatic potential [1,5–9]. Identification and characterization of specific causal relationships between environmental exposures, cancer development, and associated metabolic changes is particularly challenging, in part, because the causative exposures and their critical exposure windows are frequently undefined and may occur over prolonged – and potentially discontinuous – periods of time in the context of complex environmental mixtures and at concentrations not commonly examined in standard toxicity or carcinogenicity testing. Where examined, the biological effects of individual low-dose exposures have frequently revealed biphasic dose–response relationships – or hormesis – sometimes involving directionally opposite biological responses unanticipated on the basis of prior traditional testing [10,11]. In addition, complex environmental exposures hold the potential for both exaggerated and novel combinatorial actions. Moreover, the timing, duration, and order of key individual exposures may be as important to ultimate carcinogenic outcomes as their fundamental natures and specific identities.

The development, selection, and progression of cancer represent a pathologic continuum extending over highly variable periods of time [1]. In fact, latency in cancer development following known carcinogenic exposures is very common and can span years or even decades. Hence, much of our knowledge of the processes contributing to environmental carcinogenesis is largely inferred from historical exposures and the ability of short-term toxic exposures to experimentally promote the development of cancer or individual cancer-like phenotypes. Recent attempts to identify low-dose exposures capable of promoting the development of cancer-associated phenotypic hallmarks, both individually and in combination, provides an excellent conceptual framework for the characterization of environmental carcinogenesis [9,12], but the ability to establish unambiguous causal relationships between initial exposures, distant outcomes, and requisite intermediate stages of cancer development involving these phenotypes remains elusive [1]. A recent workshop convened by NIEHS confirmed the need for a better understanding of the mechanistic underpinnings and interrelationships between exposures, carcinogenesis, and associated phenotypic hallmark development as a major unmet need in the field [9], underscoring the fundamental importance of such causal triangulation. As such, iterative interrogation of causative interactions between exposures and

associated phenotypes is needed from both ends of the cancer continuum extending from initial exposures to cancer initiation and progression.

From a mechanistic perspective, metabolic changes may be either a cause or a consequence of cancer development. Alternatively, metabolic changes may represent epiphenomena of cancer development with causal associations at the level of cancer selection or progression, rather than initiation. None of these possibilities are mutually exclusive. With these caveats in mind, this chapter largely focuses on the inherent complexity of metabolic dysregulation in established cancers, as well as the potential contributory roles played by intermediary metabolism along the pathologic continuum of cancer initiation, selection, and progression. Much of the pertinent literature on cancer metabolism fails to unambiguously distinguish between early cancer-specific metabolic changes and qualitatively similar noncarcinogenic toxic effects or acute adaptive cellular responses. Many HTS platforms employed for the study of carcinogenesis are also adapted from toxicity screening platforms and do not directly address metabolism. Although primarily focused on cancer, many of the considerations herein are thus similarly applicable to the study of toxicology. An attempt is also made to highlight areas where translational toxicology can be extended to address mechanistic contributions of carcinogenic exposures to the development and progression of cancer and its associated phenotypic hallmarks, including dysregulated metabolism. Lastly, this chapter addresses both fundamental knowledge gaps and key unmet needs from the perspective of the characteristic metabolic features of cancer and addresses some fundamental limitations of current experimental approaches to the problem of cancer metabolism.

15.2 Metabolic Reprogramming and Dysregulation in Cancer

Dysregulated metabolism is one of the most recognizable and characteristic features of cancer biology [13–15]. Increased glycolytic lactate generation by cancer was first described by Warburg nearly a century ago [16], and this constitutes one of the earliest recognized phenotypic hallmarks of cancer [1]. Warburg and his contemporaries went on to establish many of the fundamental metabolic characteristics of cancer [16–19] before interest in cancer metabolism declined and shifted instead to the molecular biology of mutagenesis, oncogenes, and regulated gene expression [1]. Interest in metabolism has subsequently reemerged, in part, due to increased recognition of the central importance of metabolism to cancer biology, and also to address the mechanistic underpinnings and functional importance of altered gene expression profiles and epigenetic changes accompanying dysregulated cancer metabolism. The associated metabolic phenotypes are not necessarily fixed [7] and can change in response to both substrate availability and the metabolic demands of proliferation, growth, and cell

survival [1]. Cancer cells alter their ability to metabolize carbohydrates, lipids, and peptides to meet increased energy demands and to provide anabolic precursors needed to support proliferative cell growth [13,14,20]. Nonidentical anabolic and catabolic pathways can share both common substrates and required cofactors; so these processes are tightly integrated and, in general, serve to expand the potential metabolic repertoire available to cancer cells, thereby augmenting their flexibility to adjust to increased cellular demands, changing environmental conditions, and fluctuating substrate availability [1].

Warburg originally hypothesized that fixed mitochondrial defects were primarily responsible for both cancer development and its associated glycolytic phenotype, despite the fact that his own data and that of his contemporaries clearly demonstrated preserved oxidative metabolism in cancer [1,16–18,21–23]. This hypothesis is now widely discounted, but these early pioneering attempts at the biochemical characterization of cancer set the tone and paved the way for much of the subsequent work informing our present understanding of cancer metabolism [1]. Although not widely appreciated, Warburg demonstrated persistent oxidative metabolism following withdrawal of all exogenous substrates [16], suggesting the crucial capacity to oxidatively utilize endogenous substrates when exogenous substrates are unavailable [1,17,24]. Corresponding rates of carbon dioxide formation and oxygen utilization (i.e., the "respiratory quotient") in these experiments were consistent with either lipid or amino acid oxidation, ostensibly derived from the metabolism of structural or functional pools of intracellular lipids and proteins [16,17,22]. Endogenous protein utilization is also probably reflected in increased ammoniagenesis observed under both aerobic and anaerobic conditions [1,16,22,25]. The ability of glucose (Glc) to attenuate ammoniagenesis suggests protein-sparing effects that are in addition to its known lipid-sparing effects [1]. Parallel increases in cancer-associated ammoniagenesis and glycolysis [16] are also consistent with coordinate dysregulation and functional coupling between these processes.

The mechanistic underpinnings of common cancer-associated changes in metabolism remain incompletely defined. In general terms, metabolic changes can reflect alterations in either capacity or control – or both. With this in mind, metabolic capacity greatly exceeds cellular demands in both cancer cells and normal cells [1,26], so the specific advantages conferred by increased capacity are somewhat obscure. It is likely that control is of equal or greater importance than capacity [26]. For a given metabolic pathway, however, flux control does not reside at any single point, but rather is variably distributed across all steps of that pathway [26–28]. In addition, controlling factors may differ between intact cells and *in vitro* assay conditions [29], so observed changes in individual pathway components may not always translate into corresponding alterations in metabolic flux and vice versa [1,14,17,19,26,30].

Cancer cells exhibit both nutritional preferences and metabolic dependencies in meeting their fundamental catabolic and anabolic needs, just like normal

cells [1]. These preferences and dependencies can fundamentally shape cellular responses to nutrient availability and stress, but there are no requirements that nutritional preferences must always reflect metabolic dependencies. Many metabolic intermediates also exhibit signaling functions that serve to trigger, guide, or coordinate cellular responses. As a metabolite class, this principle is probably best exemplified by lipids such as diacylglycerol or phosphatidylinositol phosphates. Metabolic signaling functions may have evolutionary origins with primitive tropisms guiding cellular movement either toward nutrient sources or away from disadvantageous environments serving as their original *raison d'être*. The evolutionary conservation of AMP kinase, the prototypic energy sensor of all eukaryotic cells [31], is compatible with such speculation. Interestingly, Akt signaling – which couples trophic factor signaling to both cell survival and metabolism and has been implicated in cancer development – is similarly conserved across species, although its role in metabolic regulation predates its well-characterized role in antagonizing apoptosis [13,32].

The phenotypic characteristics of cancer cells can vary considerably and are typically neither fixed nor specific for cancer [1,7,17,25,33]. Distinct cell types and tissues can also exhibit fundamentally different responses to common extrinsic stimuli, including hormones, mechanical stimuli, environmental stress, and chemical exposures [11,30]. Cancer has been associated with a number of metabolic alterations considered both fundamental to its nature and essential for its continued growth and survival. The pertinent literature is largely descriptive or associative in nature, however, and there are limited data to suggest primary causal metabolic links between environmental exposures and cancer development [1]. The continually "evolving, dynamic, and heterogeneous" nature of cancer [7,33] also poses fundamental problems for both cancer study and treatment, so a better understanding of the specific determinants and functional consequences of cancer heterogeneity is also needed [30].

Mesotrophic or oligotrophic conditions within tumors can differ markedly from those encountered in more structured surrounding normal tissues and can challenge the ability of cancer cells to meet their anabolic and catabolic demands. Metastatic cells adapted to previously constrained intratumoral conditions can be further challenged by broad variations in trophic conditions encountered as they migrate into extended host environments and invade normal tissues. Oncogenic changes that reprogram cells to exhibit enhanced metabolic plasticity are thus more likely to possess adaptive fitness under rapidly and widely changing environmental conditions, and adaptive flexibility itself may be favored by selection over any given fixed metabolic characteristic.

15.2.1 Carbohydrate Metabolism in Cancer

All mammalian cells metabolize Glc to help meet catabolic demands and support anabolic carbon needs [13]. This is of particular importance to rapidly

proliferating cancer cells where tumor growth rates correlate with glycolytic activity [34]. Although cancer cells utilize exogenous substrates other than carbohydrates, they generally show a preference for Glc when multiple substrates are available [16,17,22,35]. The central importance of glycolysis – and, by extension, the downstream interacting citric acid cycle – can largely be ascribed to their combined amphibolic contributions to meeting cellular catabolic and anabolic demands. Cancer cells characteristically exhibit increased Glc utilization and can serve as an effective systemic Glc trap [13,17,34]. In fact, the ability of cancer cells to increase glycolytic lactate production in both the presence and absence of oxygen – traditionally referred to as the Warburg effect – was the earliest signature metabolic feature described for cancer [1,13,16,17,19,23]. Glycolytic capacity and Glc flux rates, however, greatly exceed both the anabolic and catabolic needs of normal cells [36], as well as cancer cells [17,26,34]. The ability of oxygen to partly suppress lactate production in normal cells – a response commonly referred to as the Pasteur effect – is preserved in cancer [18]. Nonetheless, cancer cells still exhibit increased lactate generation in both the presence and absence of oxygen [16,17]. This so-called aerobic glycolysis is observed in conjunction with preserved mitochondrial oxidative metabolism [1] and likely reflects the ability of GAPDH in cancer cells to engage in NAD^+/NADH redox coupling with both LDH and mitochondria in a nonexclusive manner. This situation contrasts with most normal cells, where GAPDH redox coupling tends to be binary with coupling to either the mitochondrial malate-aspartate shuttle system in the presence of oxygen or to LDH in its absence – but typically not to both [1,24,37]. Although the precise underlying mechanisms are not known, cancer-associated mitochondrial uncoupling may promote cytosolic NADH recycling to NAD^+ to support glycolytic flux into both oxidative Glc metabolism and lactate production [38,39]. The corresponding ability of glycolysis to inhibit respiration – the so-called Crabtree or Reverse Pasteur effect – plays a reciprocal role with the Pasteur effect in the bidirectional coordination of glycolysis and oxidative metabolism in both normal cells and cancer cells [1,17,40,41]. Like the Pasteur effect, the Crabtree effect has been attributed, in part, to competition between glycolysis and mitochondria for available ADP and inorganic phosphate [17,19,41]. Feedback inhibition of HK activity [19,41] and HK–mitochondria interaction [13,32,42] may also contribute to these effects. The specific underlying mechanisms, however, remain incompletely defined, and it is likely that neither the Pasteur effect nor the Crabtree effect has a single mechanistic explanation [19].

Hexokinases (HK) catalyze the phosphorylation of Glc, which is the first committed step of its metabolism. This reaction maintains the favorable downhill concentration gradient that permits facilitated Glc entry into cells and represents the initial branch point for all major physiological pathways of Glc utilization [13]. The high affinity HK1 and HK2 isoforms also physically and functionally interact with mitochondria [32,43] to coordinate intra- and

extramitochondrial metabolism, promote cell survival, and directly antagonize apoptogenic signals converging on mitochondria [13,32]. HK1 is constitutively expressed in most cells, whereas the inducible HK2 isoform is commonly overexpressed in cancers [13,44]. Both HK1 and HK2 compete with each other for interaction with mitochondria [43], but the specific molecular determinants, relative isoform contributions, and functional implications of this competition are still unknown. HK1 and HK2 are kinetically suited for distinct functional roles and are well positioned to direct both location-specific and isoform-specific metabolic channeling [32]. For example, HK1 is well suited to directing Glc metabolism in a catabolic direction, whereas HK2 is better suited to channeling Glc flux into anabolic pathways [43,45–47]. Most cancer-associated changes in cellular Glc phosphorylating capacity have been attributed to the high-affinity HK2 isoform. Increased HK2 expression thus likely affords increased metabolic flexibility for cancer cells to respond to the increased catabolic and anabolic demands of rapid proliferative growth [45].

As the principal end product of glycolysis, pyruvate has three major metabolic fates in mammalian cells – namely, gluconeogenesis, mitochondrial oxidative metabolism, and nonoxidative cytosolic conversion to lactate. Of these fates, only lactate generation is freely reversible, and gluconeogenesis is not ubiquitously observed in all cells and tissues like the alternatives. Pyruvate conversion to lactate by LDH is generally favored in the absence of oxygen, whereas the pyruvate dehydrogenase (PDH) complex oxidatively decarboxylates pyruvate and irreversibly commits it to citric acid cycle metabolism when oxygen is available. PDH also serves as a major metabolic control point that integrates important regulatory feedback by the principal PDH reaction products, acetyl-CoA and NADH. As such, PDH plays key roles in coordinating intra- and extramitochondrial metabolism that are dependent on a variety of factors, including thiamine availability [48].

Glc-6-P flux through the pentose phosphate pathway (PPP) also directly supports cancer proliferation via a number of mechanisms. First, the PPP generates both ribose and NADPH for nucleotide and nucleic acid biosynthesis [49]. PPP flux via both G6PDH and 6-phophogluconate dehydrogenase (6PGDH) is also redox-coupled to reduced glutathione (GSH) generation required for glutathione peroxidase-mediated detoxification of both organic and inorganic peroxides [13,50]. Catalase can also detoxify inorganic peroxides, including those generated by superoxide dismutase (SOD), but not organic peroxides. As such, GSH and glutathione peroxidase activity both assume major roles in cellular responses to chronic oxidant stress involving lipid peroxidation. PPP flux also importantly provides cellular NADPH required for both fatty acid and cholesterol biosynthesis [51]. In addition, PPP flux can antagonize apoptogenic signaling via direct coupling with NADPH-dependent caspase inhibition [13,52,53]. Although other non-PPP oxidoreductases, such as malic

enzyme and extramitochondrial IDH, can potentially substitute for G6PDH and 6PGDH in redox coupling to these important cellular functions [1,54], they play much less important roles in most normal cells [5]. Nonetheless, the potential for alternative redox coupling mechanisms in cancer cannot be presently discounted and must be evaluated individually for a given cell type and set of conditions [55]. The recent identification of mammalian NAD kinase (NADK), which is overexpressed in cancer and catalyzes the direct phosphorylation of NAD^+ to form $NADP^+$ [56], suggests additional mechanisms whereby cancer cells can adaptively remodel intrinsic redox coupling mechanisms. In support of this contention, overexpression of either wild-type or gain-of-function mutant NADK increases both $NADP^+$ and NADPH abundance, enhances PPP-coupled antioxidant coping mechanisms, and promotes both cancer development and progression [57,58].

Cancer is also characterized by increased hexosamine biosynthesis from Glc, which is required for the production of glycoproteins, glycosaminoglycans, and glycosphingolipids [59,60]. Posttranslational *O*-linked protein glycosylation associated with increased hexosamine biosynthesis pathway (HBP) flux has been associated with several key nonmetabolic features of cancer, including enhanced proliferation, apoptotic resistance, and increased invasive potential [61,62]. The ability of *N*-acetylglucosamine (GlcNAc) to rescue Glc-deprived KRas-transformed cells from cell death [63] is consistent with these observations, although the underlying mechanisms remain obscure. GlcNAc competitively inhibits HK activity [64], but its ability to substitute for Glc as a mtHK substrate and thereby antagonize apoptogenic stimuli to promote cell survival has not been directly addressed [65]. Hexosamine flux also activates trophic factor signals that control glutamine (Gln) uptake by cancer cells, thereby coupling Glc and Gln metabolism [59]. Another potential coupling mechanism involves the ability of *O*-linked G6PDH glycosylation to increase PPP flux and enhance tumor growth [66], suggesting specific mechanisms for crosstalk between the HBP and the PPP that can contribute metabolic reprogramming in cancers characterized by increased flux through both pathways [60].

Few normal tissues, and probably even fewer cancers, can be characterized as functionally gluconeogenic [1]. In fact, cancers arising from the principal gluconeogenic tissue, the liver, have been characterized by a marked transition from a gluconeogenic phenotype to a robust glycolytic phenotype that appears essential for hepatic cancer development [18,47,67]. It is not unusual, however, to see reports of altered "gluconeogenic" gene expression in cancer [1]. In the absence of accompanying functional validation, it is unlikely that these cancers are truly gluconeogenic as suggested. It is more likely that these observations can be attributed to the fact that gluconeogenesis shares common reaction steps with both glycolysis and glyceroneogenesis, which both play established functional roles in cancer metabolism [1,68,69]. The key gluconeogenic steps shared with glycolysis are sequentially reversed and directionally opposite to their glycolytic counterparts, requiring catalysis by separate enzymes to bypass the

irreversible rate-controlling glycolytic reactions catalyzed by HK, phosphofructokinase (PFK), and pyruvate kinase (PK). Not surprisingly, glycolysis and gluconeogenesis are reciprocally regulated and spatiotemporally segregated in distinct cell types and intracellular compartments. Glycolysis is the principal source of 3-phosphoglycerate (3-PG) for glycerol and triacylglycerol (TAG) synthesis in most normal tissues, although glyceroneogenesis from mitochondria-derived malate can also produce 3-PG to support lipogenesis, serine (Ser) biogenesis, and one-carbon metabolism essential for cancer progression and growth [68,69].

15.2.2 Lipid Metabolism in Cancer

The observations of Warburg and his contemporaries focused most early attention on dysregulated glycolysis in cancer metabolism, but it has been estimated that cancers typically derive less than half of their energy from glycolysis [17]. Moreover, cancer cells are able to increase their oxidative metabolism of non-glycolytic substrates (Crabtree effect) and to survive for prolonged periods of time when completely deprived of Glc if oxygen is available [17]. Not surprisingly, cancer-associated metabolic alterations involving alternative substrates, including lipids, are now widely recognized [1,14,17,70–72]. In fact, increased lipogenesis is considered a hallmark of many aggressive cancers [73,74], where endogenous *de novo* fatty acid (FA) synthesis supports both membrane biogenesis and the energetic demands of proliferation even if extracellular lipid is available [14,73–75].

Phospholipids, including phosphoacylglycerols and sphingomyelins, represent the principal constituents of cell membranes. In addition to serving as important structural components of polar lipid bilayers, phosphatidylinositols also play important roles in cellular signal transduction, providing a tightly regulated source of the intracellular second messengers diacyl glycerol and inositol phosphates. Membrane lipids derived from endogenous lipogenesis exhibit increased acyl saturation [74], which influences both membrane structure and fluidity, and are associated with reduced intrinsic susceptibility to peroxidation. In contrast, acyl groups derived from exogenous lipids tend to be more polyunsaturated and exhibit greater susceptibility to peroxidation [74]. Cellular susceptibility to oxidant injury can therefore be closely coupled to cellular choices in lipid metabolism, and lipid signal transduction can be similarly affected [74,76,77].

De novo FA synthesis is dependent on cytosolic acetyl-CoA [78], which is largely derived from pyruvate. Although pyruvate is generally derived from Glc, it can also be generated from alternative sources such as alanine (Ala) and glutamine (Gln) [79]. The intramitochondrial PDH complex irreversibly directs pyruvate from all of these sources into the citric acid cycle [68], thereby promoting citrate formation for export into the cytosol for

conversion to acetyl-CoA and malonyl-CoA through the sequential actions of ATP-citrate lyase (ACLY) and acetyl-CoA carboxylase (ACC) [80]. Direct extramitochondrial acetyl-CoA generation from acetate has also been reported and is catalyzed by acetyl-CoA synthetase 2 (ACSS2), which is overexpressed in cancer [81,82]. Fatty acid synthase (FASN) then catalyzes the condensation of malonyl-CoA and acetyl-CoA to form long-chain FA. Both ACC and FASN, which are rate-controlling for *de novo* FA synthesis, are also overexpressed in cancer [73]. Interestingly, ACC is also coupled to epigenetic regulation through direct competition with histone acetylation for available acetyl-CoA [83].

Increased Glc flux supports lipogenesis at several levels [73,80]. Glycolysis provides pyruvate for acetyl-CoA production and *de novo* FA generation, as well as 3-phosphoglycerate (3-PG) for glyceroneogenesis. In parallel, branched pathway flux through the PPP supplies NADPH to provide reducing power for lipid biosynthesis as well as ribose moieties for nucleotide biosynthesis. The concept of neutral carbon balance is crucial to understanding the operation of the citric acid cycle and its anabolic contributions to cancer metabolism, insofar as this cycle cannot effectively proceed at a carbon deficit. Cataplerotic carbon losses must always be matched by anaplerotic mitochondrial carbon uptake [1,68]. As a consequence, citrate carbon exported to support acetyl-CoA formation and *de novo* lipid biosynthesis in the cytosol must be continually replaced by anaplerotic sources such as Glc-derived pyruvate or Gln-derived α-ketoglutarate (αKG). Of these, pyruvate is typically most important. However, reductive acetyl-CoA synthesis from Gln via αKG can also occur under hypoxic conditions favoring the conversion of pyruvate to lactate [78,84,85]. This pathway may also operate when HK2 is unable to properly direct Glc flux into anabolic fates [47].

Cancers are also capable of lipolytic metabolism of both endogenous and exogenous lipids [1,17,35,71]. FA retrieval from neutral intracellular lipids is mediated by intracellular monoacylglycerol lipase (MAGL), which is overexpressed in cancer [86]. Transformed cells can also retrieve FA from exogenously scavenged lysophospholipids [84]. Cancer cells coexpress extracellular lipoprotein lipase (LPL) and the long-chain fatty acid translocase, CD36, which together permit the uptake and utilization of FA derived from extracellular TAG de-esterification [71,87,88]. Although incompletely characterized, cancer stem cells share metabolic features with established cancers [89]. The fact that they exhibit increased CD36 expression [88] could suggest possible roles for exogenous lipids in both stem cell-derived cancer and stem cell-based therapeutic resistance.

Lipogenic and lipolytic phenotypes are not mutually exclusive in cancer, and both phenotypes can be observed in the same cells [17,35,71]. Resulting FA are channeled into biosynthesis of both structural and signaling lipids [77]. Endogenous lipid recycling via lipophagy can also provide FA for cellular use in

cancer [90–92]. The availability of multiple FA-generating mechanisms to meet cellular needs [71,93] is compatible with the notion that cancer cells possess an expanded metabolic repertoire well suited for adaptative flexibility to changing substrate availability that could convey important selection advantages for cancer.

15.2.3 Protein Metabolism in Cancer

Cancer cells conserve endogenous proteins and amino acids more avidly than normal cells [94]. They also scavenge systemic nitrogen and act as "nitrogen sinks" that contribute to both positive tumor nitrogen balance and associated host cachexia [17,34,94,95]. It is therefore of considerable interest that both Warburg and his contemporaries observed ammoniagenesis in cancer that is augmented in the absence of exogenous substrate and reduced in the presence of Glc [16,22,25]. Taken together, these observations suggest the ability to utilize endogenous proteins under oligotrophic conditions. They also suggest protein-sparing effects for exogenous Glc. Since surplus intracellular proteins and amino acids are typically metabolized not stored, it is likely that cancer cells recycle endogenous functional and structural proteins, although selectivity in targeting specific proteins for proteolysis remains to be directly addressed. Proteasomal degradation of endogenous regulatory proteins has been implicated in cancer development [96,97], but broader metabolic roles for the ubiquitin–proteasome system have not been rigorously interrogated. The anabolic or catabolic benefits of protein recycling have historically been viewed as by-products of other primary cellular processes, rather than their *raison d'etre*. Autophagy, which plays important roles in recycling excess or damaged intracellular components in normal cells, may also contribute to nutrient recycling in cancers [92,98–100].

Gln is the most abundant circulating amino acid and plays a number of important roles in cancer metabolism [95,101,102]. Cancer cells avidly abstract Gln from their environment and are also capable of endogenous Gln biosynthesis to help support solid tumor adaptations to both nutrient deprivation and hypoxia [103]. Glutaminolysis is a substantial contributor to cancer energy metabolism and represents an important nonglycolytic source of lactate generation [79,104]. In addition to supporting transamination reactions required for both purine and pyrimidine biosynthesis, Gln-derived αKG supports reductive acetyl-CoA biosynthesis needed for lipogenesis in cancer cells under hypoxic conditions [78,85], suggesting novel metabolic flexibility to adapt to variations in both substrate availability and environmental conditions. Like Glc and FA, Gln is a major oxidative substrate for cancer cells, even under hypoxic conditions [84,105]. However, only a small fraction of all available Gln is ultimately oxidized or otherwise diverted for anabolic purposes [106]. It is widely accepted that high rates of metabolic flux are needed to support

sustained proliferation [106], but the rate of glutaminolysis – like that of glycolysis – greatly exceeds the fundamental catabolic and anabolic needs of cancer cells [19,26,107,108]. Such seemingly excessive rates of major pathway flux have important metabolic control implications for anabolic branched pathway flux and should probably be considered as associated metabolic costs, rather than waste [20,26].

Amino acid biosynthesis is required to support cellular needs that cannot be met by substrate abstraction from the environment. For example, cancer cells exhibit increased Ser biosynthesis [47,69,109–111], which accounts for as much as half of all anapleurotic Gln flux [109]. These processes are intimately intertwined with Glc metabolism and require anabolic input from glycolysis and/or the citric acid cycle. Ser, in turn, importantly supplies methylene groups for one-carbon metabolism in both the folate and methionine (Met) cycles [69,110,111]. Both Ser and homocysteine (Hcy) then play important roles in the biosynthesis of other amino acids [69,111], including cysteine (Cys) and glycine (Gly), which are crucial substrates for glutathione generation needed to maintain intracellular redox homeostasis. The Met cycle also supports methyltransferase reactions responsible for histone modifications important for epigenetic regulation [110,112]. Ser biosynthesis is initiated by PGDH, which is strongly induced by protein restriction and employs glyceroneogenic 3-PG as a substrate [69]. In principle, PGDH can compete with glycolytic GAPDH for available NAD^+, which could favor the use of glyceroneogenic 3-PG derived from malate and the citric acid cycle [69].

Aspartate (Asp) is generated by the transamination of oxaloacetate and actively participates in the mitochondrial transmembrane exchange of reducing equivalents via the malate-aspartate shuttle [113]. Mitochondrial ETC activity involving complex I is coupled to intramitochondrial Asp biosynthesis via regeneration of NAD^+, which is a required cofactor for the upstream conversion of malate to oxaloacetate [113–116]. As such, mitochondria play a central role in Asp biosynthesis, although the LDH-catalyzed conversion of pyruvate to lactate in the cytosol can substitute for mitochondrial ETC activity in providing reducing equivalents for the extramitochondrial generation of oxaloacetate from malate under conditions where ETC activity is either inhibited or defective [113–117]. This explains the ability of pyruvate to mitigate the dependence of Asp biosynthesis on mitochondrial ETC activity, as well as the development of pyruvate dependence in respiration-deficient cells [117]. The presence of redundant redox coupling mechanisms for oxaloacetate generation – vis-á-vis Asp biosynthesis – is roughly analogous to the redundant NAD^+/NADH coupling mechanisms with GAPDH that help maintain glycolytic flux under both aerobic and anaerobic conditions. Both of these examples illustrate the importance of both metabolic redundancy and environmental context when considering cancer biology.

15.3 Moonlighting Functions

In its simplest form, the central paradigm of molecular biology holds that each individual gene yields a single corresponding protein encoded by its cognate transcript and ostensibly possessing a singular cellular function [118]. Although generally valid, exceptions to this rule are commonplace, and both retrograde and alternative pathways of biological information transfer are now well described [1,118]. In addition, the notion that individual proteins possess singular functions is not absolute, and numerous examples of so-called moonlighting proteins capable of performing multiple independent functions have been reported [119]. Despite coordinated regulation and catalytic activities emphasizing metabolism as their principal functions, the existence of secondary – or so-called moonlighting – functions for multiple glycolytic enzymes such as HK, phosphoglucose isomerase, GAPDH, enolase, and LDH [32,119,120] are compatible with the notion that their regulated expression is not solely for metabolic purposes. These potential exaptations have obvious implications for both intermediary metabolism and cancer biology.

PKM2, an allosterically regulated low-affinity embryonic PK isoform, is overexpressed in cancer where it diverts Glc flux into anabolic branched pathways including the PPP and the Ser biosynthesis pathway [55,104,121]. In addition to its classical enzymatic role in promoting anabolic Glc flux, PKM2 also interacts with multiple cellular regulatory factors [122], resulting in additional pleiotropic actions through novel moonlighting functions as both a transcriptional coactivator and a protein tyrosine kinase [30,120]. Major moonlighting functions described for other glycolytic enzymes and a number of citric acid cycle enzymes suggest the very real, albeit underexplored, possibility that these and other metabolic enzymes may contribute to carcinogenesis via mechanisms other than their canonical enzymatic functions [32,119,120,123]. It has been suggested that the development of moonlighting functions could represent an intermediate evolutionary stage; so, in the absence of adaptive conflicts, the acquisition of novel moonlighting functions could confer adaptive fitness and thereby provide a basis for selection completely unrelated to canonical functions [119]. The enzymatic promiscuity of aldose reductase, which can potently detoxify reactive aldehydes and is overexpressed in cancers [124], is compatible with such a contention.

15.4 Cancer Metabolism in Context

15.4.1 The Gestalt of Intermediary Metabolism

> ". . . the whole is greater than the sum of its parts . . . by limiting our analysis to the behavior of single enzymes and simple pathways, we miss

> the point that in real life, thousands of enzyme reactions are occurring, and tens of metabolic pathways interact." –
>
> Paul Srere [28]

Intermediary metabolism is not a singular entity. Rather, it is a highly integrated and tightly regulated network of individual biochemical reactions organized into myriad intersecting metabolic pathways that are ultimately responsible for the synthesis, transformation, and transport of every molecular constituent of living cells. In aggregate, this organizational arrangement affords intrinsic metabolic flexibility – and, in many cases, important functional redundancy – to allow cells to respond to changing extrinsic trophic conditions or altered amphibolic demands without compromising cellular viability or sacrificing overall organizational economy. At the most fundamental level, individual metabolites also represent important signaling effectors capable of conveying critical information about both the state of the cell and its local environment to the cell's innate adaptive machinery. This ability to transduce signals cannot be fully separated from specific biochemical roles in metabolism. These generalizations are true of cancer cell metabolism, just as they are of normal cell metabolism [17–19,125], although they can assume different quantitative or qualitative importance in cancer. As such, metabolism for a given cell or tissue cannot be properly considered outside the context of the cellular gestalt [1,24,116]. In other words, a holistic understanding of cancer-associated changes and their myriad interactions with one another is essential to properly understand cancer metabolism.

The complex interconnected series of biochemical processes that together comprise intermediary metabolism can individually drive, augment, or counterbalance one another in promoting associated functional responses. One cannot simply switch one part of intermediary metabolism off or on and expect other connected pieces to work completely and independently without fundamental consequences. As such, secondary, compensatory, or coupled responses can potentially achieve pathophysiological importance equal to or greater than their corresponding proximate carcinogenic changes. In other words, if metabolic flux through a given pathway promotes pathology development, flux via alternative reciprocal paths may have opposite effects. As such, the relative contributions of individual pathways in counterbalancing or augmenting the development of pathology may be more important than the absolute magnitude of individual contributing processes. As an example, oxidative metabolism represents a major source of reactive oxygen species (ROS) [126], whereas PPP flux is a major driver of counteracting antioxidant quenching mechanisms [49,50]. The balance of these opposing contributions helps determine net oxidative stress (Figure 15.1). The abilities of both α-ketoacids (e.g., pyruvate and αKG) and α-hydroxyacids (e.g., lactate) to directly detoxify ROS [127–132] also suggests additional mechanisms whereby metabolic intermediates derived from different pathways can directly oppose oxidant stress [1].

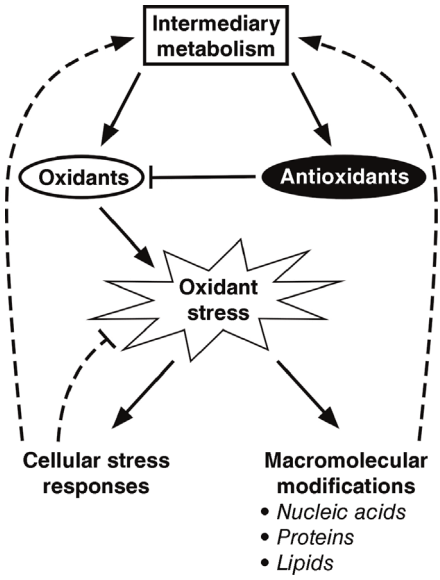

Figure 15.1 The dichotomous relationship between cellular metabolism and oxidant stress. Intermediary metabolism contributes to both oxidant species generation and opposing antioxidant coping mechanisms. Local imbalances between cellular oxidants and antioxidants can lead to net oxidant stress and functionally contribute to cancer development by triggering or amplifying intrinsic cellular stress responses and by promoting oxidative modification of cellular constituents. Feedback from these changes can also directly or indirectly modulate metabolic flux and thereby attenuate or intensify cellular oxidant stress.

Examination of individual enzymes or pathways in isolation always risks overlooking important organizational and control principles operating in intact cells [26,28,29,133]. Experimental consideration of cancer metabolism as a system thus requires multiple complementary approaches that include both classical biochemistry and molecular biology. Metabolic flux and control analysis, in particular, is crucial to understanding the functional relevance of individual changes in gene expression or biochemical activity, insofar as alterations in substrate or product abundances alone give limited information regarding metabolic flux [26]. Similarly, if metabolic capacity exceeds cellular demands and is therefore not limiting, then individual enzyme or transporter abundances may not quantitatively reflect either cellular needs or metabolic flux. Even where increased metabolic capacity or functional reserves can be demonstrated, it is likely that cancer cells seldom, if ever, operate at maximum capacity [1,17–19,26,34,36,107].

Highly coordinated changes in cellular metabolism are required to support the increased anabolic and catabolic demands of rapidly proliferating cancer cells [1,14]. These demands can vary broadly in both magnitude and direction and will obligatorily reflect a variety of tumor-specific factors, including anatomic location, local environment, and specific cell phenotype [7,24,33]. Endergonic – or free energy-requiring – biochemical processes thus require coupling to more thermodynamically favorable exergonic – or free energy-liberating – processes in order to proceed. Neither endergonic nor exergonic biochemical processes can operate independently and must be coupled to one

another. As such, energy metabolism is closely coupled to anabolic activity and other energy-requiring processes like active transport [17,19]. The fundamental importance of matched ATP generation and its hydrolysis has been recognized for decades [19,107,134,135], but its central role in metabolic control is still widely underappreciated. Cells can never operate at an energy deficit, and the capacity for cellular energy generation uniformly exceeds its utilization in intact cells. At the most fundamental level, ATP allows coupling of thermodynamically unfavored biochemical reactions to more thermodynamically favored counterparts [19,34,36,107,108]. ATP conservation occupies a central position in metabolic regulation. Energy demand drives ATP generation, not vice versa [19,24,136]. In fact, it was the recognition of these fundamental relationships that originally led to the concept of cellular adenylate energy charge (AEC) as a major controlling factor in metabolic regulation [134,137,138]. Catabolic processes are favored by low AEC values and elevated AMP levels, whereas high AEC values and increased ATP abundance favor anabolic processes. These counterbalancing effects serve to ensure that dynamic cellular demands can be met by appropriate diversion of available cellular resources. For example, ATP hydrolysis is a crucial driver of glycolysis, and the activities of key glycolytic enzymes such as PFK and PK vary inversely with AEC as befits their established roles in catabolism [44]. In contrast, HK activity exhibits a novel relationship with AEC values with activity observed at low AEC values that increases with higher AEC values, consistent with its postulated roles in both catabolism and anabolism [44]. These complex relationships, however, make cause-and-effect inferences regarding glycolytic flux and ATP abundance difficult without direct examination.

Metabolic flux is never fixed and can change over time in response to a variety of intrinsic and extrinsic parameters that include substrate availability, allosteric control, and varying cellular anabolic and catabolic demands. All metabolic flux occurs under nonequilibrium conditions, and both the magnitude and direction of flux are ultimately determined by the degree to which each individual reaction in a given pathway is displaced from its equilibrium [139]. As such, metabolic flux control is variably distributed across all component pathway steps [27,106,139]. One corollary of this arrangement involves the fact that, under steady-state conditions, those reactions most removed from equilibrium are best positioned to restrict metabolic flux and exert control [139].

Lastly, the central importance of the major amphibolic pathways of glycolysis and the citric acid cycle in supporting both the anabolic and catabolic needs of all cells cannot be overemphasized. Both pathways are fundamentally essential to the characteristic metabolic and proliferative phenotypes of cancer cells, and a functioning citric acid cycle relies on tight coupling with glycolysis and other catapleurotic substrate sources to balance anapleurotic substrate losses and remain carbon neutral. This interconnectedness and the associated requirement for overall balance are illustrative of the gestalt of intermediary metabolism and

are fundamental to the nature of both normal metabolism and dysregulated cancer metabolism.

15.4.2 Cancer Tissues, Cells, and Organelles as Open Systems

Since cancer cells and tissues are not closed systems, the availability of external substrates and essential cofactors, as well as exogenous trophic signaling effectors, can represent critical determinants of metabolic flux control [139]. Altered clearance of downstream reaction products via either terminal metabolism or extrusion into the extracellular environment can similarly influence flux via modulation of normal metabolic feedback. These factors are of particular importance to metabolic phenotype development in cancer cells that depend upon either *de novo* synthesis or macromolecular recycling to supply critical substrates that are variably and unreliably available in the extracellular environment. One cellular strategy to mitigate the impact of preferred exogenous substrate variability and associated mesotrophic or oligotrophic extracellular conditions involves adaptation to utilize more diverse metabolic substrates of both internal and external origins. Cancer cells appear to have broadly adopted this strategy, albeit with a general preference for extracellular Glc even when alternative substrates are available [17]. Such hierarchical preferences probably serve to protect endogenous lipids and proteins from unnecessary recycling under eutrophic conditions. It is important to distinguish metabolic preferences from true dependence or auxotrophy, however, insofar as preferences do not obligatorily connote specific substrate dependencies if alternative nonpreferred substrates are available and can be effectively utilized instead. Since environmental substrate availability can vary widely, it is also easy to see how metabolic dependency can be contextually defined by the spatiotemporal availability of suitable alternative substrates [1,116].

15.4.3 The Endosymbiotic Nature of Cancer

An important corollary of the fact that cancer cells are not closed systems involves highly dynamic interactions between cancers and their hosts. Cancer cells arise from normal cells, but, once established, they interact closely with their external environment and serve as “metabolic traps” that effectively compete with their normal counterparts for limited local and systemic resources [34]. Such competition can be qualitative as well as quantitative [140], and the ability to competitively scavenge exogenous nutrients helps promote cancer cachexia to the ultimate detriment of the host [17,34,79,140,141]. Cancers represent obligate endosymbionts because they cannot exist independently outside their hosts. Because this relationship favors malignant tumor growth at the expense of the host, cancers have frequently been referred to as “parasitic” in nature, with obvious parallels to lessons drawn from true host–parasite relationships [79,142,143]. The preferential

utilization of host resources for growth and metastasis thereby enhances the inherent fitness of cancer cells while reducing the corresponding fitness of their host [95].

Cancer cells produce large quantities of lactate [17], and intratumoral lactate accumulation represents a poor prognostic factor associated with both increased metastasis and mortality [144]. Lactate is an important gluconeogenic substrate, although cancer cells typically exhibit little, if any, intrinsic gluconeogenic activity [1]. In contrast, lactate exported to the extracellular environment is readily transported to the host liver where increased hepatic gluconeogenesis plays a crucial role in maintaining increased Cori cycle activity between cancer and host [79,145]. The nonessential amino acid Ala, which is formed by reductive transamination of pyruvate and similarly exported by cancer cells, can also serve as a major gluconeogenic substrate in the host liver and maintain parallel Ala–Glc cycle activity between cancer and host [101]. Together, these cycles contribute to negative energy balance in the host benefitting the cancer and helping promote host cachexia. Similar local tissue interactions have been reported involving reciprocal changes in lipid metabolism between cancers and adjacent normal adipose tissue [72], suggesting coordinated metabolic reprogramming between cancer and adjacent normal tissues that also favors cancer growth at host expense. As such, cancer metabolism must always be considered in the context of host metabolism [79,140], particularly when considering therapeutic approaches to cancer involving metabolism [79,146]. Perhaps not surprisingly, intratumoral symbiotic interactions involving metabolic substrate exchange between cancer cells and stromal cells have also been reported [82].

15.4.4 Catabolic and Anabolic Support of Cell Proliferation

Glycolysis and the citric acid cycle represent sequential interactive amphibolic pathways that support both the anabolic and catabolic requirements of rapidly proliferating cancer cells [1,13,14,68,69]. Catabolic support roles for metabolism have historically received the greatest attention, but the importance of anabolic support of the proliferative cancer phenotype is now also widely recognized [13,14]. Cellular proliferation requires the ready availability of macromolecular building blocks to support associated increases in nucleic acid biosynthesis, membrane biogenesis, protein synthesis, and overall biomass [20]. Newly synthesized proteins also frequently require posttranslational modifications for proper targeting and function [69,147–149]. These biosynthetic processes and the asymmetric transmembrane movement of exogenous substrates and ions are both supported, in turn, by cellular energy that is largely derived from glycolysis and terminal oxidative metabolism. Specific requirements for citric acid cycle carbon balance [68] and specific cofactor coupling arrangements serve to help coordinate these catabolic and anabolic

contributions with other metabolic functions [1]. In general, catabolic processes converge and involve oxidative reactions employing NAD^+ as a major cofactor, whereas anabolic processes diverge and tend to employ NADPH to power reductive biosynthesis [5].

The metabolic phenotypes of cancer cells can reflect primary changes in either metabolic control or capacity – or both [1,15,24,106,134,135]. Substrate availability, intracellular compartmentalization of metabolism, and both catabolic and anabolic demands can also serve as major phenotypic determinants [5,13,28]. A direct relationship exists between cellular ATPase activity and ATP generation [19,107,135], and in the absence of limiting substrate availability, cellular energy production largely changes in response to demand, not vice versa. This well-described, albeit widely underappreciated, relationship is an important driver of metabolism in both normal cells and cancer cells alike.

15.4.5 Cancer Heterogeneity

The broad morphological, functional, and behavioral heterogeneity of cancer is multifactorial in origin and has both intrinsic and extrinsic determinants [7,150–152]. Intrinsic determinants include genetic mutations, epigenetic modifications, and autocrine factor elaboration. Extrinsic determinants include local environmental conditions, host immunosurveillance, paracrine and endocrine factors, and functional interactions with host cells or intratumoral stromal cells. The phenotypic diversity observed both within and across individual solid tumors reflects both cellular pedigree [15,95] and evolutionary selection [153,154]. From an evolutionary perspective, ongoing mutagenesis promotes both genetic and phenotypic diversity, and bidirectional interactions between associated changes in intrinsic cancer biology and extrinsic factors promote the selection of advantageous phenotypes from the resulting pool. Since microenvironmental selection pressures vary widely both within and across tumors, there are ample opportunities for heterogeneous selection pressures due to diverse favorable and unfavorable niche-specific mismatches across the tumor mass. At the most fundamental level, this heterogeneity affords tumors the flexibility to both shape and adapt to local evolutionary selection pressures and thereby promote cancer progression in the context of rapidly changing microenvironmental conditions [155,156].

Both stem cells and somatic cells can independently give rise to cancer [157–159], and these distinct cellular origins – which are characterized by unique phenotypic characteristics and associated intrinsic potential for plasticity – are not mutually exclusive and can both contribute to heterogeneity [89,152]. Importantly, an innate capacity for plasticity is not restricted to stem cells, and fundamental changes in cellular identity due to differentiation, dedifferentiation, and transdifferentiation appear to be major drivers of heterogeneity [160]. In principle, these lineage-specific changes can be either

stochastic or hierarchical in origin. They are also associated with metabolic correlates. For example, dedifferentiation associated with cancer development can be accompanied by reactivation of embryonic or developmental gene expression patterns and the reacquisition of stem cell-like features [161–163]. Cancer development has also been associated with shifts in enzyme isoform expression with markedly different kinetic and/or regulatory characteristics [13,18,34,81,82,161,162]. The well-described switch from low-affinity hepatic glucokinase expression to high-affinity HK expression in hepatomas [161,162] and the corresponding switch from the normal brain HK1 isoform expression to HK2 expression in brain tumors [1] are prototypic examples of such changes. As such, both dedifferentiation and isoenzyme shifts may have specific implications for metabolic phenotype development and heterogeneity in cancer.

Unfortunately, both current and proposed systems biology approaches to the problem of cancer heterogeneity do not directly address functional heterogeneity [1,156]. This is particularly problematic for the study of associated dysregulated cancer metabolism, where metabolic changes can reflect not only intrinsic cancer biology but also extrinsic host and/or tumor environmental factors. Moreover, changes in the metabolome do not linearly reflect – and can even qualitatively differ from – specific corresponding changes in the transcriptome or the proteome for a given cell, tissue, or microenvironmental niche (Figure 15.2) [1]. Metabolic feedback can influence both transcriptional and posttranscriptional metabolic gene regulation, as well as downstream encoded protein functions. This complex interplay of factors establishes mechanisms for dynamic bidirectional interactions between the metabolome, the genome, the transcriptome, and the proteome in individual cells and has profound implications for both heterogeneity and systems biology approaches to the interrogation of cancer metabolism and its dysregulation [1].

No single metabolic feature accounts for the changes in intermediary metabolism associated with cancer development [30]. These changes are also not fixed, as intratumoral cells are exposed to a broad array of extracellular nutrient conditions during tumor growth that can vary along a continuum from eutrophic to oligotrophic. Other associated microenvironmental variations, including changes in both pH and oxygen availability, can influence metabolism and vice versa. As such, there is potential for bidirectional interactions between cancer cells and their local environment that lends itself to “active Darwinism” where cells can interact with their environment to influence their own selection and growth [152,155]. Accompanying differences in the intracellular compartmentalization of metabolism within individual cells can further contribute to metabolic complexity and heterogeneity [28,32,115,164]. Together, these metabolic changes and associated tumor-specific intercellular crosstalk can contribute to both overall tumor heterogeneity and malignant potential [7].

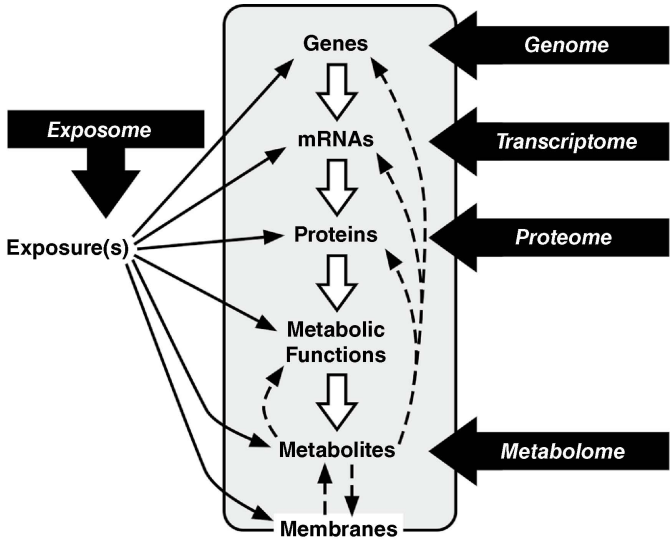

Figure 15.2 Exposome interactions with metabolism and genome-encoded metabolic information. Exposome-mediated effects on metabolic gene expression and function can occur at a number of nonexclusive levels along the entire path of genetic information flow specified by the central paradigm of molecular biology. Exposures are capable of both direct and indirect interactions with the genome, as well as with its associated transcriptome, proteome, and metabolome. The depicted relationships are neither linear nor fixed, and they reflect complex, dynamic, multilevel interactions between exposures, metabolic gene expression, and metabolism (solid arrows). Intermediary metabolism can also contribute to the bidirectional flow and control of genomic information via metabolic feedback and coupled epigenetic changes (dashed arrows), which has profound implications for the use of integrated systems biology approaches to address cancer metabolism.

At the genomic level, tumor heterogeneity clearly reflects branched evolutionary growth, and up to two-thirds of all detectable somatic mutations exhibit geographically restricted distributions within a given tumor [153]. This has obvious practical implications for tissue sampling in the assessment of cancer genomes to guide precision medicine or in the characterization of specific genotype–phenotype relationships *in vivo* [153,156].

To summarize, cancers are highly dynamic and complex heterogeneous systems, but whether the dynamic functional and structural heterogeneity that characterizes them reflects organized or disorganized complexity is debatable [7,161,165,166]. What is clear is that solid tumors are organized very differently than normal tissues, and these differences pose specific problems for both the study and treatment of cancer [1,7,33]. As such, a better understanding of both the determinants and functional consequences of genomic, phenotypic, and organizational variation in cancer is needed [30].

15.4.6 Phenotypic Relationships between Cancer Cells and Their Parental Cell Origins

The importance of the specific cellular origins of cancer has historically been underconsidered as a determinant of cancer-associated metabolic phenotypes [1]. For example, all mammalian cells utilize Glc, but different normal tissues and the cancers that arise from them vary widely in both their relative and absolute dependence upon Glc as a primary energy substrate [1]. Altered Glc utilization can therefore have very different functional implications for cancers derived from highly glycolytic normal tissues than for cancers arising from parental tissues that exhibit preferences for – or a greater dependence upon – nonglycolytic energy substrates. Some structurally and functionally heterogeneous normal tissues, such as pancreatic islets and kidneys, are also comprised of multiple distinct cell types with a broad range of dependencies for Glc as a primary energy substrate. As such, any shift toward Glc dependence in cancers derived from different cell types within these tissues may have fundamentally different meanings. In addition, the availability or unavailability of alternative substrates can strongly influence substrate utilization patterns in both quantitative and qualitative terms for cancer cells with metabolic repertoires that differ from their tissue of origin. At least in principle, cancer cells with an expanded metabolic repertoire or altered control characteristics could be metabolically indistinguishable from their parental cell types under one set of conditions and markedly different under another.

Cancers arising from phenotypically distinct parental tissues retain many of the gene expression patterns and metabolic features of their tissues of origin [15,95]. Observed differences can be both quantitative and qualitative in nature, so a nonarbitrary frame of reference is needed to identify changes specific for both carcinogen exposure and subsequent multistage cancer development. Ideally, direct comparisons between normal and neoplastic cells with common identifiable parental origins would obviate the potential for cell- or tissue-specific confounding effects when comparing cell types or tissues of widely divergent origin. Normal tissues of origin can therefore provide suitable frames of reference with the caveat that some normal tissues are highly heterogeneous, necessitating more targeted comparisons with specific cell types of origin. As such, the use of metabolic gene expression profiles without normalization for corresponding parental cell type or tissue expression patterns could be misleading when assigning importance or specificity to changes associated with cancer development. Corresponding functional assessments may require similar comparisons.

Malignant transformation has pathological implications for all living cells, but specificity derived from parental origins and attributable to specific cancer types needs more attention. For example, although implicit, it is unlikely that a hepatocyte can serve as a suitable model for the transformation of myocytes or neurons and vice versa. Applicability to individual cancer types and assumed

parental cell types will likely require more targeted models and cell-based screening platforms.

15.4.7 Evolutionary Perspectives of Metabolic Fitness and Selection in Cancer Development

It is widely accepted that the accrual of genomic mutations, regardless of their origin, is conducive to cancer genesis. As a consequence, cancer research has been heavily focused on those factors capable of directly promoting oncogenic changes via mutagenesis [167]. In contrast, selection has largely been viewed as the backdrop against which intrinsic cancer biology plays itself out. At the most fundamental level, however, stochastic mutagenesis provides the genetic diversity that affords selectable changes in adaptive fitness [165]. Despite widespread acknowledgement of the crucial importance of selection in the successful establishment of cancer, its relative contributions and underlying mechanistic determinants have been both understudied and incompletely defined. By virtue of the increased metabolic support requirements for many, if not most, of the cardinal features of cancer [1], the dysregulated intermediary metabolism that characterizes neoplastic transformation is uniquely suited to serve as a major basis for its selection, regardless of whether the associated changes are a cause or a consequence of transformation. Evolutionary medicine, which provides a unique heuristic platform for understanding both disease susceptibility and development [168], may therefore have special utility in the study of metabolism in cancer development and progression. Selectable adaptations to physiological challenges are known to drive organismal evolution, which suggests evolutionary correlates for cancer selection at the cellular level within the host. In addition to providing a framework for the identification and characterization of both selection pressures and selectable phenotypes crucial for cancer establishment, an evolutionary perspective also affords a conceptual basis for explaining both cancer heterogeneity and well-described deviations from modeled multistage cancer behavior, including Peto's paradox [154].

Metabolic fitness can generally be defined as the propensity of a given metabolic phenotype to be selected, recognizing that fitness can differ under varying conditions. Since fitness, like intermediary metabolism, is neither a fixed property nor a unitary entity, it is reasonable to speculate that selection reflects the ability to adaptively alter metabolic traits and available resource utilization to match cellular demands under varying environmental conditions. In fact, a fixed metabolic phenotype could prove disadvantageous to a nascent cancer cell under changing heterotrophic conditions, whereas the contrasting ability to adaptively alter the metabolic gestalt to mitigate selective disadvantages represents a potentially selectable form of adaptive metabolic fitness. Both adaptive and maladaptive metabolic responses can occur across the entire range of physiological and pathophysiological environmental conditions. Cancer cells with the

intrinsic phenotypic plasticity to adapt to changing environmental conditions may thus be better suited for survival than cells with any particular fixed characteristic. Given broad variability in both the type and amounts of nutrients available to cells along the carcinogenic continuum, the ability to adapt to mesotrophic or oligotrophic conditions and to bypass associated metabolic bottlenecks may provide competitive growth advantages that translate into selection. A corollary of such a relationship would assert similar selective advantages for cells during rapid clonal expansion, tissue invasion, and metastasis. Although adaptive metabolic fitness represents one plausible requirement for both selection and clonal expansion, it is unlikely to be the only one.

The juxtaposition between fixed or restricted intrinsic cell biology and the changing tumor microenvironment poses numerous opportunities for functional mismatches relevant to selection. The extratumoral migration of cells previously adapted to intratumoral microenvironments during either metastasis or local tissue invasion can also pose additional opportunities for selectable mismatches to arise. Evolutionary mismatch theory is thus readily applicable to discussions of transformed cell selection, recognizing that functional mismatches can contribute to cancer initiation as well as its selection. For example, oxidative stress, which results from imbalances between oxidant stressors and cellular antioxidant-coping mechanisms, can influence the entire carcinogenic spectrum. Mismatches between intrinsic trophic demands and external trophic conditions can also arise and potentially influence selection, particularly in physically and nutritionally constrained environments and during periods of high anabolic and catabolic demands, such as during rapid proliferation, clonal expansion, or tissue invasion. Mismatches can also arise when fixed or restricted oncogenic changes (e.g., auxotrophy) confront limiting host factors or environmental conditions. These issues are discussed further in Section 15.5.

> "A modification of one cell or of any small proportion of the order characteristic of mutation – that is, $1{:}10^5$ per cell life – will have no detectable effect on the function of the tissue unless one condition is fulfilled. This is that the mutation results in a differential survival advantage for descendants of the mutant cell as compared with the descendants of normal cells" –
>
> Macfarlane Burnet [169].

The role of selection, while implicit, has been incompletely integrated into both existing carcinogenic models and high-throughput screening (HTS) assays. These may not be trivial oversights insofar as selection can be reasonably expected to play a major primary role in the ability of nascent transformed cells to both survive and propagate [154]. In principle, carcinogenic mutations and other procarcinogenic changes are unlikely to result in cancer development if affected cells are not selected (Figure 15.3). In other words, oncogenic

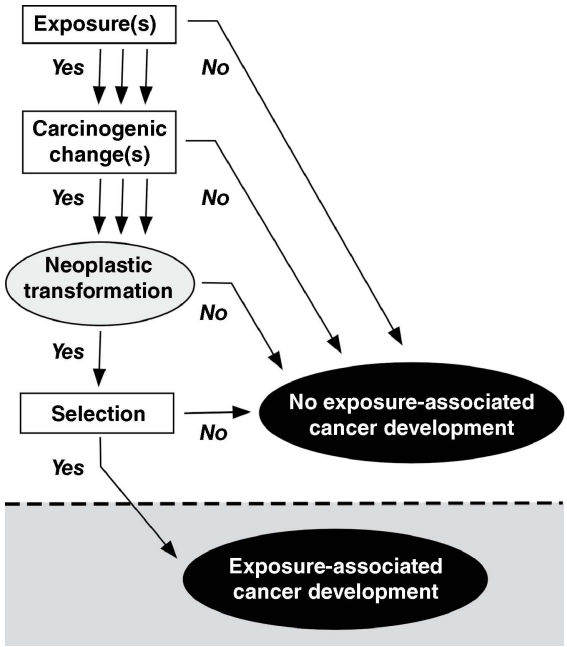

Figure 15.3 Selection is fundamentally required for successful exposure-associated multistage cancer development. Not every cell transformation event results in cancer development. Transformed cells with selectable fitness advantages over both normal cells and other competing transformed cell lineages are more likely to successfully establish cancer. A simplified model depicting a single selection cycle is shown, although selection can theoretically occur at multiple transition stages during multistage cancer development and involve both general and stage-specific selection pressures. As such, there are myriad opportunities for multiple sequential and/or simultaneous determinative selection events involving either positive or negative selection pressures during the course of cancer development.

transformation is a necessary but insufficient condition for the successful establishment of cancer. Although speculative, a corollary of this assertion should probably hold that while selection may not guarantee the successful establishment of cancer, its absence has fitness implications that may ensure its failure or help determine the clinical aggressiveness of any rare established tumors unfavored by selection. Selectable phenotypes also need not be carcinogenic *per se*. They require only more proximate association with oncogenic changes capable of establishing a transformed phenotype. The accepted requirement for multiple genomic "hits" in cancer initiation may have similar underexplored phenotypic requirements in downstream selection. As such, selection can be considered separately, but not independently, from oncogenic transformation. Cancer models must take into account not only those

carcinogenic factors that directly or indirectly promote oncogenic transformation, but also associated selectable phenotypes that allow their ultimate selection, propagation, and successful establishment as progressive tumors. Dysregulated metabolism is ideally suited to serve as one basis for such selection. If inherent fitness advantages depend upon the availability of extrinsic metabolic substrates or cofactors that can become limiting in the environment, then such fitness is, by definition, contextual in nature.

> ". . . biological systems and the molecules which comprise them can only be understood in terms of their history. They are what they are now because of what they were previously and how at that time they interacted with a changing environment – itself partially the product of other biological processes." –
>
> Steven Rose [155]

Sir Karl Popper eschewed fundamentally teleological views of a passive role for selection in evolution, advocating instead for a form of active Darwinism "in which the organism itself is not passive and neutral, waiting to be selected, but instead actively participates in its own selection, by choosing appropriate environments and modifying inappropriate ones; organism and environment interpenetrate and modify one another in ways which are determined in part by their own mutual history" [155]. Both views can easily be extended to cancer cell selection in tumor microenvironments, as well as during metastasis and host tissue invasion. By virtue of the fact that altered intermediary metabolism can potentially represent both a cause and a consequence of carcinogenesis – and given the well-described reciprocal relationships between cancer cell metabolism and the local tumor microenvironment – there is probably no characteristic feature of cancer better equipped to enable active Darwinism than dysregulated metabolism, particularly within the setting of rapidly growing tumors [170]. Of course, active Darwinism is not mutually exclusive of more unidirectional, less interactive modes of selection at fundamentally different stages of multistage cancer development – or during the development of therapeutic resistance. Cancer resistance to antimetabolite therapy has been widely attributed to selection of advantageous preexisting mutants, rather than induction of *de novo* mutagenesis [168,169,171], and is fully consistent with the latter view.

15.5 Dual Roles for Metabolism in Both the Generation and Mitigation of Cellular Stress

Cellular stress plays a central role in cancer development. Basal somatic cell mutation rates have been estimated to be approximately 10^{-7}–10^{-6} per gene for each mitotic event [168,172]. Since mutagenesis rates increase in cells under

stress [173] and following oncogenic transformation [174], cells acquiring transforming oncogenic mutations have the potential to establish a vicious cycle of stress-induced mutation and mutation-induced stress. All cellular stress is a net function of the balance between the magnitude and nature of the incident stresses and the corresponding adequacy of intrinsic cellular coping strategies (Figure 15.1). There is considerable heterogeneity in both cellular stress responses and stress-associated outcomes in different cell types or tissues, even under indistinguishable conditions. In principle, metabolic reprogramming can contribute to both the propensity for cancer development and transformed cell selection via either the metabolic promotion or alleviation of stress. For example, an expanded metabolic repertoire may enhance the inherent adaptive flexibility of cancer cells [1,24,109,175,176], thereby enabling them to thrive under highly variable conditions and to favorably adapt to the myriad conditions and stresses invariably encountered during rapid proliferation. On one hand, metabolic stress, including oxidant stress, is associated with carcinogenesis [168]. On the other hand, metabolism is able to antagonize, as well as promote, such stress, suggesting multiple direct and indirect mechanisms whereby metabolism can positively and negatively contribute to cancer genesis, initiation, progression, and control. Some illustrative examples of the broad stress categories relevant to cancer biology are briefly considered in the following sections.

15.5.1 Metabolism and Oxidative Stress

Oxidative stress plays major established roles in carcinogenesis [126,167]. ROS are capable of modifying and modulating all major classes of biomolecules. For example, lipids, especially polyunsaturated FA, are highly susceptible to oxidation, which promotes formation of reactive aldehyde lipoperoxidation products. ROS are also capable of damaging nucleic acids and functioning as either mutagens or clastogens if not sufficiently opposed by intrinsic cellular antioxidant coping mechanisms [177]. They can also indirectly promote both the carbonylation and nitrosylation of proteins.

Cellular oxidative stress arises when oxidant species generation exceeds or overwhelms intrinsic antioxidant coping capacity (Figure 15.1) [1,178]. As such, oxidative stress can arise from primary increases in oxidant species, reduced antioxidant coping capacity, or both [1]. Since metabolism represents a source of both oxidant species and antioxidant factors, oxidant stress can represent either a cause or a consequence of metabolic alterations [1,179]. The catalases, peroxidases, and SOD that serve as major intrinsic antioxidant defenses are also highly conserved across both normal cells and cancer cells. The metabolic processes that support the reactions catalyzed by these enzymes are similarly conserved, establishing a mechanistic basis for both amplification and attenuation cycles between intermediary metabolism and oxidant stress.

Historically, the contributions of nonenzymatic detoxification of oxidant species have received much less attention than redox-coupled enzymatic mechanisms. Several amphibolic intermediates, however, possess direct antioxidant properties in addition to their classical metabolic roles. For example, α-ketoacids such as pyruvate and αKG are potent antioxidants and are sufficient to alter cellular oxidant responses [127,128,130,180]. α-Hydroxyacids such as lactate exert similar protective effects [129,131]. Taken together, these observations suggest intrinsic buffering of the pro-oxidant effects of metabolism and the possibility of specific antioxidant roles for both glycolytic and citric acid cycle metabolites that are in addition to those traditionally ascribed to coupled PPP flux and GSH generation.

Both inorganic and organic peroxides represent major endogenous oxidant species. Of these, organic peroxides, particularly lipid peroxides, are probably of greatest biological importance. Glutathione peroxidase detoxifies both inorganic and organic peroxides, whereas catalase is capable of detoxifying only inorganic peroxides, including those generated by SOD. Glc flux via the PPP plays a major role in redox homeostasis through $NADP^+$/NADPH coupling with glutathione reductase, and primary increases in Glc phosphorylation – which gates entry into this pathway – increases PPP flux and protects against oxidative stress [13]. Increased NADK expression, which is overexpressed in cancer and catalyzes the direct conversion of NAD^+ to $NADP^+$, has been reported to have similar effects [56,58]. In addition to their pathogenic roles in oxidant stress, ROS can also transduce mitogenic signals at concentrations not traditionally associated with oxidant stress or macromolecular damage [56,181,182]. Taken together, these observations suggest multiple links between – and tight control of – normal metabolism, oxidant species accumulation, and cellular signal transduction that may be of particular relevance to oxidant stress and environmental carcinogenesis.

Nitrosative stress can accompany oxidative stress and reflects cellular imbalances in the production and neutralization of nitric oxide and related reactive nitrogen species (RNS). RNS, in turn, can result in direct protein modifications via either Cys nitrosylation or tyrosine (Tyr) nitration [183]. *S*-nitrosation of redox-sensitive protein thiol groups has established functional consequences in a number of metabolic enzymes such as GAPDH and HK [184]. Similarly, *S*-nitrosylation of the von Hippel–Lindau protein (pVHL) has been associated with increased HIFα accumulation and enhanced expression of hypoxia-regulated genes, including genes important for metabolism [185,186]. Intriguingly, the tissue specificity of these changes has been invoked to account for observed variations in the hypoxic responsiveness of normal tissues, suggesting potential mechanisms that could similarly contribute to cancer heterogeneity, as well as more proximate carcinogenic stage transitions. The classification of nitrates and nitrites as Group 2A carcinogens by the WHO International Agency for Research on Cancer under conditions that promote endogenous nitrosation is certainly compatible with this notion.

Increased metabolic flux has also been associated with covalent protein modifications involving carbonyl groups derived from the autooxidation of carbohydrates, lipids, and amino acids. As such, carbonylation can serve as a biochemical marker of oxidative stress, sometimes referred to as carbonyl stress. These modifications also hold the potential to alter cellular functions and contribute to carcinogenesis [187–189].

15.5.2 Metabolism and Hypoxic Stress

Cells located within rapidly growing tumors experience widely varying microenvironmental oxygen tensions [85,190], and associated changes in hypoxic signal transduction play established roles in regulating gene expression programs associated with both cancer development and metabolism [85]. Warburg postulated that repeated sublethal exposures to respiratory toxins (or so-called chemical hypoxia) were sufficient to induce cancer formation due to associated primary structural and functional changes in mitochondria [23]. Although a primary role for mitochondrial damage in cancer genesis is now widely discounted [1,13,17,18,24,161,191], the reported ability of chronic intermittent hypoxia to promote transformation of cultured myocardial fibroblasts [192] is consistent with the notion that hypoxia can represent a carcinogenic stress. However, these findings have not been independently validated, and hypoxia *per se* has not been shown to unambiguously increase either spontaneous or inducible cancer development *in vivo* [17]. Nonetheless, the ability to tolerate broad variations in oxygen tension has profound implications for cancer cell survival and selection during tumor growth, tissue invasion, and metastasis. Established roles for reduced oxygen tension and hypoxia-regulated gene expression in both stem cell and cancer cell tropisms [193,194] also suggest specific mechanisms for metastatic homing and coordination between metabolic reprogramming and tissue targeting.

15.5.3 Nutritional Stress and Metabolism

Cancer cells within heterogeneous tumor microenvironments are exposed to highly variable external nutrient concentrations [1,17]. Given the increased anabolic and catabolic demands placed on rapidly proliferating cells, these variations in nutrient availability can pose major challenges for clonal cancer development and progression. Cells exiting these heterogeneous microenvironments are exposed to additional variations in trophic conditions during metastasis and host tissue invasion that may neutralize – or render moot – fixed adaptive changes previously beneficial within specific tumor microenvironments. Competition for limited available resources can also serve as a basis for selection, particularly under mesotrophic or oligotrophic conditions, and this may ultimately prove to be of equal or greater importance than more

proximate carcinogenic changes in successful cancer establishment. Extrinsic selection pressures can also indirectly help shape the metabolic reprogramming characteristics of cancer. Consistent with this notion, nutrient deprivation responses, including autophagy and induction of metabolic gene programs, have been associated with the development of resistance to therapies targeting trophic signaling pathways [195].

As a corollary of the postulated teleological relationships above, nutritional excesses, like nutritional restrictions, may also pose determinative stresses and play either causal or permissive roles in cancer development through augmented ROS generation, nutrient-dependent signal transduction, and/or metabolite-sensitive modifications of cellular macromolecules. These considerations may be particularly important in cancers associated with dietary composition, obesity, or metabolic syndromes [3,71]. Compatible with this notion, local nutrient excess associated with inappropriate trophic signals, such as those observed following oncogene activation, have been associated with increased ROS accumulation [59].

15.5.4 Metabolism and Physical Stress

Cancer cells are exposed to a highly variable and diverse array of physical forces during local tumor expansion, tissue invasion, and metastasis. Cells within rapidly growing tumors are subject to both intrinsic and extrinsic compressive forces, as well as to tensile stresses. These forces reflect a variety of factors, including tumor biomass expansion, anatomic spatial constraints, extracellular matrix (ECM) remodeling, and heterogeneous tissue densities [196,197]. Both hydrostatic and oncotic pressure changes can also contribute to increased interstitial fluid pressure within solid tumors [198,199], and these changes can be amplified by alterations in tissue rigidity [200].

Metastatic cell migration through both interstitial and vascular compartments is associated with shear stresses, as well as with broad variations in ambient hydrostatic and oncotic pressure conditions. Deforming rheological stresses play important roles in metastatic selection [201], and malignant cells exhibit both altered tensional homeostasis and increased resistance to shear stress [197,202]. Perhaps not surprisingly, aberrant mechanosensitive signaling has been implicated in both cancer development and metastasis [166,200]. Intermediary metabolism strongly influences both the composition and physical characteristics of cell membranes and directly supports membrane repair [203,204], so it is not unreasonable to speculate that at least some of these differences have underlying metabolic determinants.

Rapid tumor growth can also result in the compression or distortion of adjacent normal tissues, including blood vessels and lymphatics. These changes can potentially alter both local tissue communication and metabolic exchange between tumor and host, thereby contributing to the development of vicious cycles that can

enhance intratumoral microenvironmental change, accelerate competition for limited nutrients and/or oxygen, and increase associated selection pressures.

15.5.5 Metabolism and Other Forms of Cellular Stress

Homeostatic mechanisms serve to ensure the constancy of the *milieu intérieur* in normal tissues and thereby prevent most normal cells from experiencing overt physicochemical stress due to widely varying environmental conditions [205]. The organizational and functional changes associated with rapidly growing tumors, however, can expose cancer cells to stresses that differ both qualitatively and quantitatively from cells in normal tissues. As a consequence, other forms of stress with the potential to influence, select, or interact with cellular metabolism also warrant brief consideration. Such conditions can have a primary metabolic basis or promote metabolic adaptive responses – or both. For example, recently postulated roles for caspase I in inflammation-associated metabolic regulation [206] suggest novel roles and potential reciprocal functional interactions between apoptogenic signaling effectors and metabolism that may have pathophysiological relevance in the setting of inflammatory stress. In addition, the intratumoral microenvironment is typically more acidic than normal tissues [17,190]. The ability of glycolysis to influence microenvironmental pH is well described, but the common attribution of these changes to Glc-derived lactic acid accumulation [34] represents a significant causal oversimplification [1]. The pK_a of lactate is 3.87, so very little, if any, lactic acid exists within the physiological pH range [207]. Intratumoral pH alterations better reflect metabolic carbon dioxide generation and accumulation [208], as well as the variable contributions of uncoupled metabolic generation of H^+ and its extracellular extrusion via both secondary active Na^+/H^+ exchange and cotransport with monocarboxylates such as lactate [85,209]. The coupling of H^+ export with lactate export probably helps explain the established utility of lactate as a marker of metabolic extracellular acidification [1]. It does not, however, explain the discordant spatial heterogeneity of intratumoral pH changes and oxygen tensions [210] or the corresponding spatiotemporal divergence of intratumoral pH and lactate accumulation [211,212]. The ability of glycolysis-deficient transformed cells to acidify their extracellular environment like glycolytic cells [208] suggests major nonglycolytic determinants of intratumoral pH and reinforces the notion that microenvironmental pH changes provide an imperfect proxy for glycolysis.

15.6 Models of Carcinogenesis

Cancer development occurs at the nexus of host genetics, aging, and the environment. Mutagenesis, regardless of origin, is deemed central to this

process and is strongly reflected in all major models of carcinogenesis. It is, however, also widely recognized that processes other than mutagenesis are integral to cancer development [1,154,213], and the importance of mutagenesis may be greatest for those exposures sufficient to produce cancer in isolation [213]. While space does not permit an exhaustive accounting of their historical development or experimental basis, a brief overview of the prevailing multistage models of carcinogenesis is given below. Despite the established central role of mutagenesis in neoplastic cellular transformation, an argument is advanced that evolutionary heuristics and a greater emphasis on the selection of phenotypes associated with oncogenic transformation (e.g., dysregulated metabolism) are needed insofar as unselected transformed phenotypes are unlikely to ultimately result in successful cancer establishment within the host.

15.6.1 Traditional Multistage Models of Cancer Development

First proposed in the 1950s [214,215], multistage cancer development is now widely accepted and supported by a wealth of experimental and epidemiological evidence [213,216]. In its simplest form, the two-hit model was originally developed to help explain differences in malignant transformation between heritable and nonheritable forms of certain cancers [216]. In this model, heritable germline tumor suppressor gene mutations predispose to cancer development following a second critical acquired mutagenic event, whereas sequential acquired mutations are required for nonheritable forms of these cancers. In both heritable and nonheritable cancers, nascent or transitional forms of cancer arise as a consequence of multiple sequential carcinogenic changes [172,214–216]. The specific number of steps required for cell transformation in different somatic tissues can vary widely, but once established, it can be argued that the final selection rate of otherwise fully transformed cells can ultimately become rate-limiting in carcinogenesis. Cancer progression, in turn, ostensibly reflects changes associated with antecedent initiation and selection, as well as acquired resistance to both intrinsic and extrinsic checks on cell growth and survival.

Ongoing DNA damage and changes in the corresponding integrity and fidelity of intrinsic repair mechanisms are both integral to all multistage models of environmental carcinogenesis [216], although the relative importance of mutagenesis to cancer development may be greatest at the initiation stage [213]. Background mutagenesis rates and the basal propensity for a given cell type or tissue to undergo malignant transformation without specific associated environmental exposures, however, are difficult to quantify and can vary widely across both cell types and species [217]. Basal somatic mutation rates have been estimated to be as high as 10^{-6} per genetic locus for each cell division, and these ongoing stochastic changes help generate the genetic diversity necessary for both physiological and pathophysiological adaptive evolution to occur [158,172].

Carcinogenic exposures ostensibly accelerate these rates either directly or indirectly, and carcinogen-induced mutagenesis is always operating in addition to these basal propensities for stochastic mutagenesis and associated transformation. The associated rates of transition for sequential stages in multistage cancer development can vary broadly and are ostensibly nonlinear [217]. Multistage cancer models should ideally accommodate these variations and reflect the sum of all the sequential stage transitions that culminate in carcinogenic initiation, as well as prior basal mutagenic propensities and subsequent natural selection [217], recognizing that unselected transformed cells are unlikely to successfully establish cancer (Figure 15.3) [165,218].

Both mutagenic and nonmutagenic carcinogens can influence different stage transitions during multistage cancer development, either individually or as cocarcinogens [213]. In addition, individual carcinogens can also potentially act at multiple stages, thereby increasing the potential interactive complexity of multiple agents acting in combination [219]. This has obvious implications for complex combinatorial environmental exposures.

15.6.2 Role of Replicative Mutagenesis in Cancer Development

Tomasetti and Vogelstein recently attempted to explain variations in cancer development by noting a correlation between the number of normal intrinsic stem cell divisions and lifetime risk of cancer development, suggesting that stochastic replicative mutations may play a much larger role in cancer development than previously thought [220]. These authors concluded that only a third of all variation in cancer risk could be attributed to either environmental or genetic factors, a contention that has subsequently been vigorously challenged [159,221]. In fact, increased stem cell abundance in tissues with higher rates of cancer formation is also consistent with the notion that parental stem cell populations give rise to cancers independent of total replicative events [159]. Although replicative mutagenesis undoubtedly contributes to cancer development, its relative contribution to the total presently remains unknown and is likely much lower than this estimate [159,213,222].

15.6.3 Acquired Mismatch Model of Carcinogenesis

Evolutionary medicine provides a number of useful heuristics for the consideration and modeling of multistage cancer development, including an emphasis on selection based upon both favorable and unfavorable mismatches arising between the intrinsic biology of cancer cells and their local external microenvironments. It also promotes the view that a sufficient rate of mutagenesis provides a mechanistic basis for the genetic and phenotypic diversity necessary to produce selectable fitness differences within a given cell population. Mutagenesis is generally random, whereas selection is not. Selection also requires

fitness advantages that are generally environment specific. As noted previously, primary oncogenic changes that do not directly result in – or are not independently accompanied by – selectable alterations in cellular fitness are unlikely to ultimately result in the successful establishment of cancer (Figure 15.3). The inefficiency of the evolutionary process by which successful cancer clones emerge and are selected is widely accepted, and not every oncogenic change ultimately culminates in cancer development [143,165,168,173]. Fundamental imbalances arising between multistage oncogenic changes that favor cellular transformation and corresponding extrinsic selection pressures – both within tumors and across the extended host environments – establish favorable and unfavorable mismatches that are capable of both driving selection and ultimately determining the extent and nature of successful cancer establishment and spread. Fitness is environment specific, so selection pressures are also environment specific and can spatiotemporally vary within solid tumors and across different normal host compartments encountered by metastatic or invading cancer cells. These selection pressures can contribute not only to niche-specific cell phenotypes in different microenvironments but also to the development of resistance to therapeutic agents capable of imposing external selection constraints that promote phenotype establishment that might not otherwise be observed, as in the example of antimetabolite therapy promoting its own resistance through artificial selection of preexisting mutants [169,171]. A given set of selection pressures can also differentially affect independent stages, either individually or in combination, during multistage cancer development [218]. Ultimately, only a very small fraction of all procarcinogenic changes will result in cancer development, and a strong argument can be advanced to experimentally address early carcinogenic changes as potentially separate from – albeit intimately linked to – selection.

Dysregulated metabolism is unique among the characteristic phenotypic hallmarks of cancer insofar as intermediary metabolism fundamentally supports or enables virtually every other cancer hallmark either via direct catabolic and anabolic support or through associated trophic signals [1]. As such, cancer-associated changes in metabolism can represent enabling mechanisms for both the selection of oncogenic changes and the release of normal cellular growth constraints that characterizes cancer [8]. Imbalances between intrinsic trophic demands and external trophic conditions have obvious implications for the growth, function, and survival of cancer cells. Trophic constraints associated with competition for limited resources can also take a number of different forms involving limiting quantities of major energy substrates, oxygen, or essential cofactors. For example, the essential trace element copper, which is central to the catalytic function of the mitochondrial metalloenzyme cytochrome *c* oxidase, can serve as both a tumor promoter and a limiting factor for tumor growth [223]. As noted previously, metabolism also enjoys dichotomous roles in both the generation and alleviation of cellular stresses relevant to carcinogenesis, so similar

dichotomies may be observed in cancer development. Longevity is a major risk factor for cancer development [126,154,172,214,215], but age-associated increases in cancer likely reflect more than the simple accumulation of critical genetic mutations over time. They likely also reflect age- or environmental exposure-associated changes in metabolism, either declines in metabolic protective functions or the accrued effects of contributory pathogenic metabolic changes [143]. Given the prolonged time frames involved, particularly where latency is observed, accumulated primary direct carcinogenic changes may be functionally indistinguishable from indirect secondary changes.

In the simplified acquired mismatch model depicted in Figure 15.4, procarcinogenic mutations and associated stresses accrue over time and approach a theoretical threshold for overwhelming intrinsic cellular coping mechanisms. At the same time, changes in cellular processes that result in incremental declines in counteracting strategies for coping with procarcinogenic changes can lower the threshold at which these protective mechanisms are overwhelmed, thereby contributing to accelerated development and selection of cancer-promoting changes over time. Obviously, both the time course and

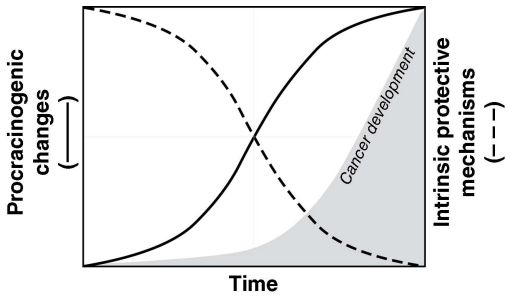

Figure 15.4 Simplified acquired mismatch model of multistage cancer development. Multistage cancer development (shaded area) reflects both the accrual of procarcinogenic cellular changes (solid line) and the reciprocal decline in competing intrinsic cellular protective or reparative functions over time (dashed line). Cancer initiation occurs when acquired imbalances, or mismatches, between these net competing pro- and anti-carcinogenic functions achieve some critical threshold for oncogenic transformation. Individual procarcinogenic changes can be either mutagenic or nonmutagenic and involve one or more stage in multistage cancer development. Successful cancer establishment from an individual transformed cell also requires that transformation is accompanied by at least one selectable fitness advantage favored by prevailing extrinsic selection pressures over more mismatched competitors. As such, functional mismatches can play important, sometimes opposing, roles at all stages of cancer development, including initiation and selection. In principle, favorable and unfavorable selection pressures can ultimately promote or inhibit the successful establishment of cancer, respectively, and help determine the phenotypes of successful clones. The depicted net effects therefore reflect the complex contributions of a number of augmenting or competing factors, which can be spread over multiple nonexclusive stages. Latency may simply reflect temporal delays in the development or selection of sufficient changes in either or both functional arms.

nature of these interactions will vary widely for individual cell types and tissues. The specific number, identity, and sequence of requisite stages necessary for multistage cancer development can also vary [172,213,216].

At the organismal level, cancer suppression mechanisms can be viewed as selectable traits that oppose early cancer establishment (i.e., before reproductive age) by promoting the loss of general tumorigenic fitness within populations [154]. At the cellular level, however, an obvious corollary of this evolutionary perspective suggests that the characteristic hallmarks of cancer, including metabolic dysregulation, are selectable variations capable of bypassing these important cellular or host defenses against the establishment of cancer. As a consequence, specific selections favorable at the organismal level may be deleterious to the host or the cancer cell at the cellular level [158].

15.7 Potential Metabolic Targets for Environmental Exposures

15.7.1 Conceptual Overview of Potential Metabolic Targets

Toxicological data for many suspected or known environmental carcinogens are plentiful, but they frequently lack sufficient mechanistic or functional information to address specific roles for exposures as determinants of metabolic hallmark development. The fundamental contributions of – and requirements for – metabolic reprogramming in environmental carcinogenesis are also still incompletely delineated and, in many cases, have not been directly examined. Procarcinogenic exposures generally fall into one of three categories: (i) directly genotoxic, (ii) indirectly genotoxic, or (iii) nongenotoxic [1,143,224,225]. Exposures that are not directly genotoxic can promote indirect genotoxicity via mechanisms involving cellular metabolism, particularly exposures with primary effects on oxidant stress or its amelioration. There is no single common pathway to indirect genotoxicity, however. DNA mutagenesis can be determined by a variety of independent cellular factors, including ROS generation, antioxidant responses, the intrinsic nature of the primary exposure, and prevailing environmental conditions. For example, agents that impair intrinsic stress response or repair mechanisms via metabolism can indirectly contribute to mutagenesis and enhance the genotoxicity of other concomitant exposures. As such, alterations in metabolism can represent both a cause and a consequence of genotoxicity (Figure 15.1). Direct and indirect genotoxic or mutagenic stresses can also affect both nuclear and mitochondrial genomes, but the contributions of toxicant-induced mitochondrial dysregulation to cancer development have been poorly studied, and not every toxic response that mimics a phenotypic hallmark of cancer is necessarily carcinogenic.

Chronic oxidative stress is strongly associated with cancer development [226] and correlates with DNA structural changes that predate other characteristic histopathological and clinical features of cancer [227,228]. Chronic oxidative stress indirectly contributes to nuclear genomic instability via secondary genotoxicity, although the extent to which these effects require accompanying DNA repair mechanism defects is not known. Mutagenesis of the mitochondrial genome is less well understood but is also frequently attributed to primary metabolic alterations capable of promoting oxidant stress and associated mitochondrial genomic instability. This has not been directly demonstrated, however, and it has recently been suggested that cancer is associated with decreased, rather than increased, mitochondrial genome instability [229]. The accompanying reduction in mitochondrial genomic diversity is also apparently not associated with reduced cancer progression. This seemingly paradoxical stabilization of the mitochondrial genome in cancer could reflect metabolic alterations that reduce mitochondrial ROS and the associated accumulation of mitochondrial mutations that contribute to normal senescence [229,230]. Although speculative, this could also reflect selectable fitness advantages for cancer cells with functionally intact mitochondria or, less likely, mitophagic recycling of defective organelles. If validated, future studies will need to address this apparent discrepancy in mitochondrial and nuclear genomic instability and its relevance to cancer and dysregulated metabolism.

Genotoxicity can mechanistically influence metabolic gene expression via mutagenesis of either coding or regulatory sequences of genes of interest [1]. Traditional frameworks for addressing genotoxicity have focused on the direct consequences of mutagenic changes [167], but it is important to remember that indirect effects can also be mediated through mutagenic changes that directly affect upstream regulatory factors or disrupt epistatic regulatory interactions [1]. Only a very small fraction of human genetic variation occurs within exon boundaries, and the overall importance of variation within *cis*-acting regulatory elements and distant epistatic loci to the development of diseases, including cancer, has been increasingly recognized [231]. As such, mutagenesis, like both metabolism and cancer, is not a singular entity. In principle, multiple distinct types of functional mutagenic events can therefore yield similar metabolic gene expression phenotypes. Viewed from such a mechanistic perspective, mutagenesis can influence metabolic target gene expression and corresponding downstream metabolic functions via a number of nonexclusive direct and indirect mechanisms depicted in Figure 15.5. Mutations directly involving either the coding region or associated *cis*-acting regulatory sequences of genes encoding metabolic enzymes or transporters can directly alter the expression and/or function of their cognate proteins and thereby impact metabolic phenotype development. Similar types of mutations involving genes encoding upstream *trans*-activating regulators of metabolic target genes can also indirectly influence target gene expression. Alternatively, mutations

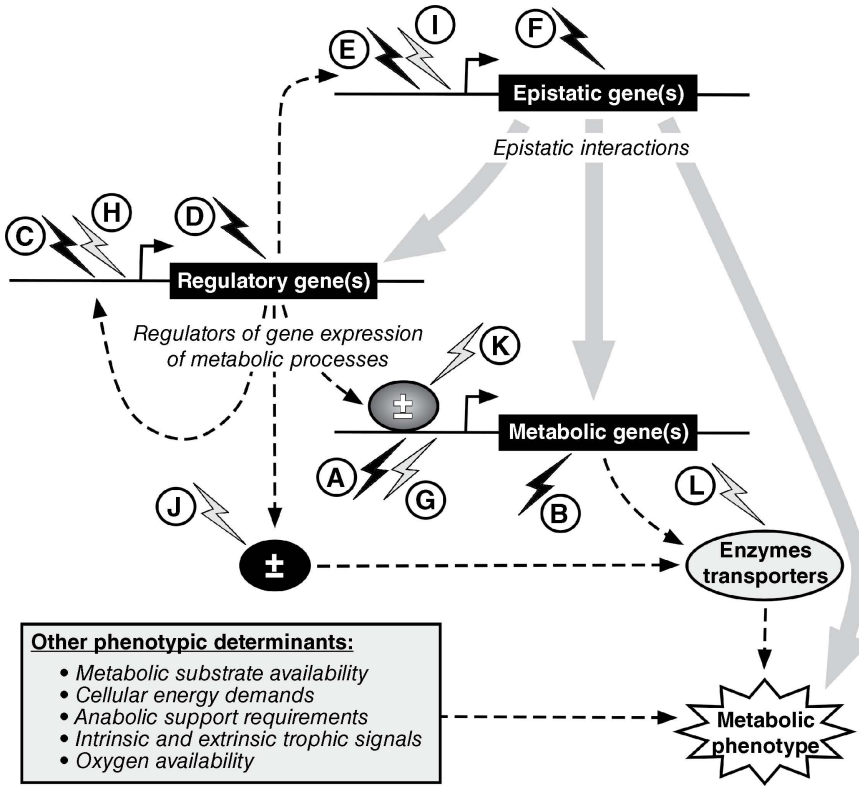

Figure 15.5 Complex direct and indirect genotoxic and nongenotoxic contributions to metabolic dysregulation. Genotoxicity can directly influence metabolism via mutations (*black lightning bolts*) involving the regulatory (A) or coding (B) regions of metabolic genes that result in altered expression (A and/or B) and/or function (B) of their cognate gene products. By extension, genotoxicity involving disruption of upstream regulatory gene product expression (C and/or D) and/or function (D) can indirectly influence the same processes. Alternatively, genotoxic effects (E and/or F) can disrupt epistatic interactions between distant genetic loci that are phenotypically indistinguishable from the effects of direct mutagenesis of known metabolic or regulatory gene loci (A–D). Nongenotoxic effects, including epigenetic modifications (*gray lightning bolts*) of metabolic genes (G), upstream regulatory genes (H), epistatic genomic loci (I), posttranscriptional regulatory effectors (J and/or K) or metabolic gene products (e.g., enzymes and transporters; L), can also strongly influence metabolic phenotype development. These complex interactions are not mutually exclusive, and, by definition, both direct and indirect genotoxic effects – as well as nongenotoxic effects – will interact with a number of dynamic drivers of metabolism (e.g., substrate availability and both anabolic and catabolic demands) to determine the cell's metabolic phenotype, which is typically not fixed.

occurring at distant epistatic gene loci can indirectly result in gene expression phenotypes indistinguishable from those generated by direct mutagenesis of either metabolic target genes or associated regulatory genes. Epigenetic changes can mimic mutagenic changes in each of these different scenarios, and non-genotoxic agents can influence metabolism via direct or indirect disruption of metabolic protein functions. These complex alternative possibilities are not mutually exclusive, and, in principle, can be phenotypically indistinguishable.

15.7.2 Identification of Key Targetable Contributors to Metabolic Dysregulation and Selection

By definition, all selection reflects fundamental differences in fitness between phenotypically distinct cells. Fitness, particularly metabolic fitness, can be highly contextual and can be considered in both relative and absolute terms. In principle, however, metabolic adaptations can represent either a cause or a consequence of selection. Selectable changes can also be either universally or contextually adaptive – or even nonadaptive [232]. As such, adaptive traits that enhance cellular fitness in response to environmental challenges or extrinsic signals may be indistinguishable from qualitatively similar cellular phenotypes arising through independent selection of other unrelated traits – or so-called exaptations. Selection pressures can also act directly at the level of specific selectable traits, such as altered metabolism, or at the level of programmatic flexibility permitting adaptations to rapidly changing environmental conditions such as those encountered during rapid proliferative tumor expansion or metastasis. Invariant selection pressures should act similarly on a given phenotype, whether fixed or adaptively flexible. In contrast, variable selection pressures in different or rapidly changing microenvironments could have divergent effects on both similar and different metabolic phenotypes. Since environmental selection pressures and individual selectable phenotypes can vary over both time and space within and across these niches, associated selection can be highly context dependent and could favor cells with either flexible or broader fixed metabolic repertoires. These possibilities have specific implications for both cancer biology and its study.

Procarcinogenic exposures can target cellular metabolism at a number of different levels via both direct and indirect mechanisms. In principle, multiple independent contributing mechanisms can also combine to yield a common phenotype, and changes in a given metabolic pathway can engender reciprocal or complementary changes in other competing or coupled pathways. Distinguishing between primary and secondary metabolic derangements is thus crucial to understanding the causal relationships between specific exposures and associated procarcinogenic and metabolic changes, particularly following prolonged latent periods in the setting of exposure-associated cancer

development. Persistent cancer-specific changes also need to be distinguished from phenotypically similar short-term toxic responses, which may or may not ultimately translate into cancer. In general, exposures can directly target discrete gene products responsible for (i) key metabolic reactions, (ii) cellular transport, or (iii) regulatory factors responsible for the coordination, control, or integration of sequential metabolic steps. The possibility must also be entertained that procarcinogenic effects may be indirectly mediated by changes in substrate or cofactor availability, allosteric feedback, or environmental alterations that physicochemically favor or disfavor unrelated procarcinogenic events. Exposures may also target metabolism indirectly through changes in cellular organization. This can involve targeted disruption or inhibited formation of supramolecular complexes crucial for cellular structure and function or disruption of metabolic compartmentalization important for metabolic channeling or its control.

Potential targets for metabolic dysregulation generally fall into one of several broad functional categories listed in Table 15.1. Although not intended to be either exclusive or comprehensive, this list of prototypic targets emphasizes selected metabolic processes with established functional importance or demonstrated regulatory differences in cancer. Given their established importance, any of these factors could potentially serve as direct or indirect targets for metabolic dysregulation, although exclusion from this list should not be construed to suggest that unlisted factors are either uninvolved or unimportant. For potential targets with multiple isoforms, targeting may be restricted to specific isoforms or isoform subsets. Critically important amphibolic pathways such as glycolysis and the citric acid cycle represent particularly attractive targets for primary or secondary carcinogenic dysregulation and reprogramming. Glycolysis has historically garnered the greatest attention due to its prominence in cancer metabolism, its central position in intermediary metabolism, and its role as a major determinant of flux through both anabolic branched pathways and the citric acid cycle. Other metabolic pathways can also constitute primary targets, but, of necessity, accompanying changes in amphibolic flux via glycolysis and the citric acid cycle are also required to fully support the anabolic and catabolic needs of rapidly proliferating cancer cells. The selected targets represent biologically plausible and coherent examples of primary metabolic or regulatory targets suitable for additional study that are derived from our knowledge of the types of metabolic changes associated with cancer, our understanding of their underlying biochemical mechanisms, and their known regulatory characteristics.

Major rate-controlling steps in essential metabolic pathways represent obvious potential targets for metabolic reprogramming, insofar as they represent important nodes for both integration and flux control through major and branched pathways alike. In principle, however, any essential step in a series of nonredundant reactions can be targeted to alter metabolism and/or its control.

Table 15.1 Selected metabolic pathway targets implicated in dysregulated cancer metabolism.

Individual pathway targets	Metabolic importance
Glycolysis (amphibolic)	
Hexokinase (HK)	• Catalyzes the first committed step of Glc metabolism, which represents the entry point to all major physiological pathways of Glc utilization [13] • High-affinity HK1 and HK2 isoforms physically and functionally interact with mitochondria and directly couple intra- and extramitochondrial metabolism. They are major mediators of the antiapoptotic functions of trophic factors [13,32] • The inducible HK2 isoform is overexpressed in cancer and favors anabolic metabolism, whereas the constitutive HK1 isoform favors catabolic Glc flux [43,46,47]
Phosphofructokinase (PFK)	• Major irreversible rate-controlling step of glycolysis [233,234] • PFK1 regulated by adenylate energy charge and PFK2 • PFK2 activated by AMPK
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	• Mediates binary glycolytic NAD^+/NADH coupling with either mitochondria or LDH to maintain glycolytic flux in the presence or absence of oxygen, respectively
Pyruvate kinase (PK)	• Major irreversible rate-controlling step of glycolysis • The low-affinity PKM2 isoform is strongly expressed in cancers and may serve to redirect glycolytic flux into anabolic pathways that support lipid, nucleotide, and Ser biosynthesis [30,55,121,235,236]
Lactate dehydrogenase (LDH)	• Important source for NAD^+ required for glycolytic flux via GAPDH in the absence of oxygen [237,238]
Pyruvate dehydrogenase (PDH) complex	• Mediates the critical step committing the products of glycolysis to an oxidative fate via the citric acid cycle, namely, the irreversible pyruvate decarboxylation to yield intramitochondrial acetyl-CoA
Pentose phosphate pathway	
Glucose-6-phosphate dehydrogenase (G6PDH)	• Rate-controlling PPP enzyme and the principal source of NADPH for both reductive lipid biosynthesis and the antioxidant activity of GSH-Px [5,239]
6-Phosphogluconate dehydrogenase (6PGDH)	• Lys acetylation upregulates 6PGDH activity and promotes tumor growth [240]

(*continued*)

Table 15.1 (*Continued*)

Individual pathway targets	Metabolic importance
Citric acid cycle (amphibolic)	
Isocitrate dehydrogenase (IDH)	• Cancer-associated mutations in both IDH1 and IDH2 promote oncometabolite formation [78,175,241–244] • Contributes to reductive synthesis of acetyl-CoA from Gln-derived αKG under hypoxic conditions [78]
Fumarate hydratase (FH)	• FH mutations associated with cancer [241] • Reduced FH activity promotes fumarate accumulation and disruptive nonenzymatic succination of Cys residues in cellular proteins [241]
Succinate dehydrogenase (SDH)	• Shared component of both the citric acid cycle and the ETC (complex II) [245] • Oxidizes succinate to form fumarate and $FADH_2$, thereby mediating e^- transfer to ubiquinone in the ETC • SDH mutations associated with cancer [241]
De novo lipogenesis	
ATP:citrate lyase (ACLY)	• Generates acetyl-CoA for lipogenesis and regulatory protein acetylation from cataplerotic citrate • Upregulated in cancers [15]
Acetyl-CoA carboxylase (ACC)	• Catalyzes the first rate-controlling step in *de novo* lipogenesis • Demonstrated roles in epigenetic regulation [83]
Acetyl-CoA synthetase ACSS)	• ACSS2 overexpressed in cancer and directly catalyzes the formation of acetyl-CoA from acetate in the cytosol [81]
Fatty acid synthetase (FASN)	• Important rate-controlling step in lipogenesis • Upregulated in cancers [246,247]
Lipolysis	
Lipoprotein lipase (LPL)	• Mediates extracellular FA retrieval from triacylglycerols for uptake and utilization [71,246–248]
Monoacylglycerol lipase (MAGL)	• Mediates intracellular FA retrieval from triacylglycerol stores [86]
Amino acid biosynthesis	
Phosphoglycerate dehydrogenase (PGDH)	• Major role in Ser biosynthesis [55,69,109,249] • Commonly amplified in cancer [245]

Mitochondrial electron transport chain assembly and function	
Complex I (NADH-ubiquinone oxidoreductase)	• Catalyzes electron transfer from NADH to ubiquinone with associated membrane proton translocation [116,250,251] • Plays a central role in Asp biosynthesis from oxaloacetate by directly supporting the conversion of malate to oxaloacetate [116]
Complex II (SDH)	• Only membrane-bound member of the citric acid cycle • See also SDH above
Complex III (ubiquinol-cytochrome *c* oxidoreductase)	• Catalyzes electron transfer from ubiquinol to cytochrome *c* with associated membrane proton translocation • The Q_o site serves as a cellular oxygen sensor and serves to transduce a hypoxic signal and stabilize HIFα stabilization via ROS release [252]
Complex IV (cytochrome *c* oxidase)	• Sole irreversible respiratory chain component • Catalyzes oxidation of cytochrome *c* • Subject to inhibitory binding by CO, NO, cyanide, and azide; physiological levels of NO reduce oxygen affinity [253]
Hexosamine biosynthesis	
Glutamine:fructose-6-phosphate amidotransferase (GFAT)	• First committed step of hexosamine biosynthesis that controls *O*-GlcNAc modification of proteins • Hexosamine biosynthetic pathway flux is required for trophic signaling support to maintain Gln uptake needed for both growth and survival [59]
Cellular transport mechanisms	
Facilitated hexose transporters (GLUT)	• Facilitate cellular Glc uptake via functional coupling with intracellular phosphorylation by HK [1,13]
Long-chain fatty acid translocase (CD36)	• Mediate cellular lipid uptake [71,248]
Monocarboxylate transporters (MCT)	• Mediate the bidirectional transport of monocarboxylates such as lactate and pyruvate with protons [85,254]
Voltage-dependent anion channel (VDAC)	• Outer mitochondrial membrane channel that partners with ANT in the inner mitochondrial membrane to form anionic metabolite exchange conduits at mitochondrial contact sites • Implicated in mitochondrial permeability transition pore formation and apoptogenic cytochrome *c* release in response to proapoptotic Bcl-2 protein binding • Molecular target of GSK3β signaling and mitochondrial HK binding responsible for regulating anion exchange and antagonizing apoptogenic signals at the level of the mitochondria

(*continued*)

Table 15.1 (*Continued*)

Individual pathway targets	Metabolic importance
Adenine nucleotide translocator (ANT)	• Inner mitochondrial membrane channel that partners with VDAC in the outer mitochondrial membrane to form anionic metabolite exchange conduits at mitochondrial contact sites
Other	
Tp53-induced glycolysis and apoptosis regulator (TIGAR)	• Promotes Glc entry into the PPP in cancer cells to enhance nucleotide biosynthesis and antioxidant activity [255] • Originally classified as a low-affinity FBPase, this biochemical identity has recently been called into question [256,257] • Relationship to p53 incompletely delineated [255] • Interacts directly with mitochondrial HK [255]
AMP kinase (AMPK)	• Prototypic energy-sensing enzyme in eukaryotic cells • Contributes to Pasteur effect via direct phosphorylation and activation of PFK2 • Inactivates key biosynthetic enzymes [137,258]
NAD kinase (NADK)	• Catalyzes the direct phosphorylation of NAD^+ to form $NADP^+$ [56,57] • Gain-of-function mutations associated with cancer development and progression [58]
Sirtuins	• NAD^+-dependent deacylases that regulate posttranslational acylation (i.e., acetylation, succinylation, and malonylation) of diverse target proteins, including histones [259,260]
Lysine acetyltranferases (KAT)	• Catalyze acetylation of metabolic proteins [261]
Aldose reductase	• Catalyzes major rate-controlling step for polyol pathway flux in cells exposed to supraphysiological Glc concentrations • Capable of detoxifying endogenous and exogenous reactive aldehydes, including HNE, MDA, and acrolein

The overall metabolic impact of any given change will be dictated by a number of considerations, including the presence or absence of functional redundancy, the existence of alternative paths for metabolic flux, relative cellular dependence on affected pathways, and, where indicated, the availability of alternative substrates.

15.7.2.1 Glycolysis

The glycolytic enzymes HK, PFK, and PK represent obvious targets for metabolic dysregulation due to their major established roles in glycolytic flux control. GAPDH warrants similar consideration due to the crucial dependence of this key glycolytic enzyme on NAD^+ availability in order for glycolysis to proceed. This critical cofactor is classically derived from NAD^+/NADH coupling between GAPDH and the mitochondrial shuttle systems in the presence of oxygen or between GAPDH and LDH in its absence [13]. In normal cells, these alternative redox coupling mechanisms are largely exclusive of one another [13,212,237,238]. In contrast, simultaneous coupling between GAPDH and both mitochondria and LDH appears permissible in cancer [1]. As such, exposures leading to alterations in either the magnitude or control of these coupling mechanisms or that involve substitution of alternative modes of redox coupling would be well equipped to promote both cancer development and selection. Certain isoforms of HK and PK may have specific relevance to cancer. For example, HK2 is overexpressed in cancer and promotes both anabolic metabolism and cell survival [13,47]. PKM2 similarly diverts Glc flux into branched anabolic pathways, including the PPP and the Ser biosynthetic pathway, in cancer [55,104,121].

15.7.2.2 Lipogenesis, Lipolysis, and the PPP

A number of key enzymatic targets in both *de novo* lipogenesis (e.g., ACLY, ACC, ACSS, and FASN) and lipolytic metabolism (e.g., LPL, MAGL, and SCD) have been implicated in cancer development [70,71,73]. As such, these processes and their control represent excellent targets for metabolic dysregulation, both individually and in combination. Given the essential support roles played by PPP flux in lipogenesis, nucleic acid biosynthesis, and resistance to oxidative stress, both G6PDH and 6PGDH and their associated upstream regulators also represent major candidate targets meriting additional study [49,262]. Two potential novel regulators warrant specific mention here. The first involves the recently demonstrated ability of lysine (Lys) acetylation to specifically upregulate 6PGDH activity and promote tumor growth [240], which could suggest novel upstream regulatory roles for lysine acetyltransferase activity in these responses [261]. The second involves NADK, which is independently capable of influencing both cellular redox status and associated redox-coupled functions by catalyzing the phosphorylation of NAD^+ to form $NADP^+$ [56,57].

15.7.2.3 Citric Acid Cycle

Cancer-associated mutations in citric acid cycle enzymes – including isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH; ETC complex II), and fumarate hydratase (FH) – are well described [54,126,241]. Hereditary cancers associated with both SDH and FH mutations have been attributed to ROS generation and associated oxidant stress-induced mitochondrial mutagenesis [126]. Normal ETC-linked isocitrate oxidation requires irreversible mitochondrial NAD^+-dependent isocitrate dehydrogenase (IDH3) activity, whereas mitochondrial (IDH2) and cytosolic (IDH1) $NADP^+$-dependent isoforms catalyze bidirectional isocitrate-αKG interconversion [242]. The latter reaction can directly couple with lipogenesis and epigenetic acetylation via reductive acetyl-CoA formation by ACC [78,83]. Cancer-associated mutations in both IDH1 and IDH2 promote NADPH-dependent generation of 2-hydroxyglutarate, a novel oncometabolite capable of inhibiting αKG-dependent enzymes important for hypoxic gene regulation [175]. Associated competition for available NADPH also interferes with a variety of redox-coupled cellular functions, including antioxidant protection, biosynthetic processes (e.g., lipogenesis), signal transduction, and epigenetic regulation [54,78,241–243,263,264].

15.7.2.4 Organizational or Compartmental Targets

The specific intracellular location where individual metabolic events occur can influence both the ultimate metabolic fate and functional importance of individual reaction products. Both widespread metabolic compartmentalization [29,46,133,265] and the archetypal example of mitochondria–HK coupling [13,43,46] are compatible with this notion. As such, some abnormalities observed in cancer could involve altered compartmentalization and redirection of flux to fates that enhance metabolic fitness or reciprocal metabolism-directed compartmentalization of other factors or cellular functions capable of promoting cancer cell growth and survival (e.g., mitochondrial-HK interaction) [13,32,266].

In principle, procarcinogenic exposures can also affect intermolecular interactions required for the formation and function of complex organizational structures, including cell membranes, organelles, chromatin, and metabolons [164,267] or ETC supercomplexes [268]. Such targeting can be considered in both structural and functional terms and can involve both individual component functions and higher order integrated complex functions. For example, fundamental contributions by mitochondrial ETC activity to carcinogenesis are widely accepted and can reflect both functional and structural mitochondrial changes [16]. mtDNA-deficient cells are pyrimidine auxotrophs due to obligatory requirements for intact ETC function and dihydroorotate dehydrogenase activity in pyrimidine biosynthesis [117]. With the exception of complex II (SDH), all respiratory complexes physically and functionally participate in dynamic supercomplexes such as the respirasome [268,269]. Formation of these complexes influences both overall ETC function and individual respiratory complex

turnover [268], suggesting mechanisms whereby ETC function may be targeted at the level of supercomplex assembly rather than at the level of individual respiratory complex components. As such, both individual ETC complex activities and supercomplex assembly are potentially attractive targets for carcinogenic disruption [250,268,270]. Mitochondrial targeting could also involve altered ETC functional coupling with transmembrane metabolite exchange and/or redox-driven extramitochondrial processes. In addition to their fundamental catabolic and anabolic roles, mitochondria also serve as major ROS generators [178,271]. If unopposed by intrinsic antioxidant coping mechanisms [178], ROS accumulation can promote oxidant stress, oncogenic signaling, and genomic instability. Mitochondria also importantly buffer cytosolic calcium concentrations [271] and initiate and control apoptosis via permeability transition pore formation and apoptogenic cytochrome *c* release [32,271].

Other potential organellar targets include the endoplasmic reticulum and the plasma membrane, the latter containing both cell surface receptors important for trophic factor signaling and specific transport mechanisms for cellular metabolite uptake. Membrane functions involving transport or signal transduction are dependent upon membrane organization, so alterations in either membrane composition or structure can indirectly influence these cellular functions just like direct targeting of transport or signal transduction. Changes that alter membrane integrity or generate cell surface clearance signals can also influence cellular lifespan. Importantly, not all intracellular compartmentalization involves bounding by cellular membranes [28], so exposures that alter the normal establishment of nonorganellar compartments or intracellular chemical gradients (e.g. H^+, Ca^{++}, adenine nucleotides, and nicotinamide adenine nucleotides) could also contribute to metabolic dysregulation.

15.7.2.5 Metabolite Transport Mechanisms

Cellular transporters responsible for the transmembrane movement of essential metabolic substrates, including hexoses, monocarboxylates, lipids, and amino acids, represent obvious potential carcinogenic targets [1]. Given their central role in cellular energy metabolism, ATPase activities coupled to transmembrane ion movements responsible for generating and maintaining electrochemical gradients and asymmetric transmembrane metabolite partitioning also represent viable potential targets [19].

Mitochondrial HK also promote cell survival, in part, via direct coupling with mitochondrial metabolite exchange [32]. The voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane participate in the formation of an anion exchange conduit that gates the ingress and egress of anionic metabolites such as adenine nucleotides, P_i, pyruvate, and succinate between the cytosol and mitochondria. ATP–ADP exchange via this conduit directly couples intramitochondrial ATP generation with extramitochondrial

ATP hydrolysis and is controlled by HK binding to mitochondrial contact sites [1,13,32,272]. VDAC and ANT have also been implicated in mitochondrial permeability transition pore formation, and competition between HK and apoptotgenic effectors for binding to VDAC at mitochondrial contact sites is thought to directly couple metabolism to the antagonism of apoptogenic stimuli [13]. By directly contributing to the coordination between intra- and extramitochondrial metabolism, these coupling mechanisms may also directly contribute to both the Crabtree and Pasteur effects [1,13].

15.7.2.6 Signal Transduction Effectors

Signaling effectors capable of transducing cellular trophic, stress, and energy status signals also frequently modulate metabolism and/or couple metabolism with essential proliferative and cell survival functions. These pathways frequently overlap or intersect with oncogenic signals and can assume particular importance in cancer. Trophic signal transduction pathways constitute particularly attractive targets for metabolic reprogramming and dysregulated metabolism [14,42,273].

Hypoxic regulation of metabolism is also highly integrated with cellular signaling cascades involved in proliferation and stress responsiveness. As such, metabolism can be indirectly targeted via a variety of factors capable of modulating signal transduction pathways or associated coupling mechanisms that are capable of exerting metabolic control.

AMPK is a major sensor and regulator of cellular energy balance that mediates the effects of the tumor suppressor LKB1 [274]. LKB1 activates AMPK under appropriate conditions, and its loss is common in cancer [274]. AMPK activation promotes a shift from anabolic to catabolic processes [275]. Direct metabolic effects attributed to AMPK include increased Glc utilization and FA oxidation with corresponding reductions in lipogenesis and protein synthesis, which can be partly attributed to the direct inactivation of key biosynthetic enzymes [258]. These changes partly underlie the rationale for using pharmacologic activators of AMPK (e.g., metformin and salicylates) to treat selected cancers [274,276]. The relationships between metabolism and energy signals are not fixed, and both metabolism and its regulation by LKB1/AMPK/mTOR signaling are highly contextual in nature [277]. Similar relationships exist between metabolism and trophic factor signaling.

Sirtuins are NAD^+-dependent deacylases with established roles in intermediary metabolism, cellular stress responsiveness, and DNA maintenance and repair [259,260]. They influence genomic stability via primary effects on Glc and lipid metabolism and secondary effects on both oxidant stress resistance and epigenetic histone acylation [259,278]. In addition to effects in cancer cells, sirtuins can indirectly influence cancer cell survival and growth via immunomodulatory effects in activated host immune cells [279,280].

The reversible acetylation of histone Lys residues by lysine acetyltransferases (KAT) plays important roles in epigenetic regulation of gene expression. This

activity is highly regulated and is closely coordinated with corresponding deacetylation by histone deacetylases to influence overall chromatin structure and gene expression [281]. Interestingly, KAT are similarly capable of regulating metabolic activity via direct Lys acetylation of metabolic enzymes [240,261,281]. Taken together, these observations suggest major direct and indirect roles for KAT activity in both metabolic reprogramming and cellular plasticity involving stem cells and somatic cells alike.

Metabolic pathways importantly transduce cellular signals in addition to their conventional enzymatic and metabolic functions [1,89,282]. As such, metabolic disruption can have profound extrametabolic consequences that are not reflected in conventional metabolic profiles or assays. The effects of altered flux through a given metabolic pathway may also be strongly influenced by exhaustion of – or competition for – limited quantities of shared cofactors that alter normal metabolic coupling mechanisms (e.g., disruption of oxidoreductase coupling via redox sink generation). Metabolic niche signals and signal transduction pathways controlling cancer dormancy or reactivation also represent attractive candidates for study [283].

Adipose tissue can potentially serve as a depot for lipophilic toxins and carcinogens, so metabolic changes that culminate in either the expansion or reduction of adipose in the host can indirectly influence both the storage and systemic release of such agents. This provides a potential mechanistic basis for both sustained carcinogen release and temporally delayed systemic exposures that could contribute to cancer latency. An obvious corollary of these possibilities involves the potential to confound interrogation of the relationships between environmental exposures and carcinogenic or toxic outcomes.

15.8 Metabolic Changes Associated with Exposures to Selected Agents

15.8.1 Selected Agents Classified by the World Health Organization's International Agency for Research on Cancer (IARC)

The WHO IARC Monographs program (*IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*) was initiated in 1971 to formally evaluate carcinogenic risks associated with human chemical exposures. Since that time, the program scope has expanded to include chemical classes, complex mixtures, occupational exposures, physical and biological agents, and lifestyle factors [284], and nearly a thousand agents have been formally evaluated [285]. Priority for evaluation has generally been given to agents with both widespread exposure and perceived carcinogenic potential. The specific criteria employed to assess carcinogenic risks were initially focused on short-term mutagenicity testing "based on the observation that most carcinogens are also mutagens,

although not all mutagens are carcinogens" [213,286]. Subsequent iterations of these criteria have been updated and expanded to accommodate other types of information, including evidence of carcinogenic mechanisms of action, but mutagenic capacity remains a centerpiece of much of this cumulative body of analysis [284,287].

As of January 26, 2017, a total of 119 agents have been classified by the WHO IARC as "carcinogenic to humans" (Group 1), and an additional 81 agents have been classified as "probably carcinogenic to humans" (Group 2A). Another 292 agents are classified as "possibly carcinogenic to humans" (Group 2B). Although a comprehensive assessment of all 492 Group 1 and 2 agents is not feasible here, a number of these agents have been independently associated with metabolic changes characteristic of many cancers. Unfortunately, there are many more classified agents for which there are little or no relevant information about either direct metabolic effects or metabolic roles in associated cancer development. Agents not presently classified as either Group 1 or 2 are not generally considered herein, but the IARC has appropriately warned that "no determination of non-carcinogenicity or overall safety should be inferred" for unclassified agents [284]. In fact, a number of agents originally classified as having limited evidence of carcinogenicity in humans have subsequently been reclassified as carcinogenic with the accrual of additional evidence [287]. By definition, the capture of potentially carcinogenic agents by this classification framework is limited to those agents that have been selected for analysis and for which relevant testing data exist. It provides no information about untested or unselected exposures. As a consequence, this list likely represents only a fraction of all potential environmental exposures and should therefore not be viewed restrictively [287]. By virtue of the fact that IARC classification reflects limited carcinogenic interrogation of selected agents under conditions not designed to be either physiologically or environmentally relevant, both incomplete capture of environmentally relevant carcinogens and underestimation of their associated true risks are highly likely. This classification framework also does not directly address metabolic contributions to the key characteristics of carcinogens [167] or specific metabolic requirements for cancer development. Unfortunately, the available supporting literature does little to address these deficiencies. With these caveats in mind, several representative examples of IARC-classified exposures with associated metabolic alterations are provided in the following sections.

15.8.1.1 IARC Group 1 (Carcinogenic to Humans)

Agents classified as carcinogenic in humans are expected to alter factors or processes essential for cancer development; so observed metabolic changes in response to exposure are compatible with the notion that they contribute to associated cancer development. The absence of such demonstrable changes,

however, does not exclude metabolic contributions, as negative results can reflect the confounding influences of a number of factors, including sampling bias and incompletely defined lower threshold exposures – in terms of both dose and duration – for such contributions. Several selected Group 1 agents representative of their chemical classes are also addressed below.

Polycyclic Aromatic Hydrocarbons (PAH)

The prototypic PAH, benzo[a]pyrene (BaP), is an established carcinogen with a diverse array of exposure sources, including coal tar and food pyrolysis [3]. Although acute BaP exposure can disrupt mitochondria–HK interaction [288], chronic exposure is associated with increased Glc metabolism *in vivo* with a reduced threshold for induction of metabolic alterations when coexposed with phenol [289]. The additivity, timing, exposure threshold, and mechanistic underpinnings of these effects have been incompletely interrogated, however, and neither their persistence nor long-term consequences have been well delineated. Similar combinatorial effects with sulfur dioxide coexposure have also been observed [289]. Interestingly, a single episode of systemic BaP exposure has been reported to increase glycolytic HK, PFK, PK, and LDH activities in murine lung for as long as 28 days [290]. This importantly represents one of the few reports of sustained metabolic alterations following a single exposure to an individual agent. Similar changes are observed following exposure to other PAHs like methylcholanthrene [290,291], suggesting possible class effects. Interestingly, both PK and LDH are also induced in fetal lung following transplacental exposure to these agents [291]. Characterization at environmentally relevant levels in conjunction with other exposome constituents has not yet been directly addressed.

Dioxins and Dioxin-Like Compounds

Halogenated PAH derivatives, including dioxins, polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans, commonly exhibit endocrine-disrupting effects [292]. Like BaP, dioxins influence both systemic Glc and lipid metabolism [293–295] and are capable of modulating normal hepatic glucokinase and lipogenic enzyme expression via aryl hydrocarbon receptor (AhR)-dependent mechanisms [296] Coplanar PCBs and their congeners also activate AhR and are similarly capable of modulating both endocrine function and metabolism, particularly lipogenesis and systemic Glc homeostasis [297]. It is therefore of considerable interest that AhR agonism by chemically distinct dioxins and PAH have been implicated in glycolytic enzyme induction [290,291,298], suggesting at least some common potential mechanisms of action involving AhR that can be either genomic or nongenomic in nature [11]. Dioxin toxicity is highly variable across species, however, with humans exhibiting greater tolerance than other species [11]. Given both its environmental relevance and its prolonged biological half-life in humans (~10 years) [11], dioxin warrants closer mechanistic scrutiny,

particularly in the context of complex low-dose exposures with other agents. An examination of the specific roles played by metabolism in dioxin-associated cancer development should be an obligatory part of that scrutiny.

Sulfur Mustard

Sulfur mustard is a highly reactive Group 1 carcinogen [286,299]. Its toxicity and carcinogenicity have been broadly attributed to direct alkylation of a variety of macromolecules, oxidant stress, and enhanced mutagenesis. Interestingly, chemical warfare studies conducted by the British Defense Ministry during the early 1940s suggested specific pathogenic importance for selective HK inactivation by sulfur mustard, ostensibly due to direct sulfhydryl group alkylation [300]. Although Cys residues do not directly participate in HK-catalyzed Glc phosphorylation, they do form internal disulfide bridges of potential conformational importance [43]. The ability of arsenite – another Group 1 carcinogen similarly capable of sulfhydryl group modification – to inactivate HK2 [43] is compatible with common mechanisms of inactivation. Interestingly, HK1 can also be readily and potently *S*-nitrosylated *in vitro* [184], and limited stable *S*-nitrosylation of this isoform may occur endogenously [301]. These latter observations raise the mechanistic possibility of functionally similar changes under conditions of nitrosative stress, although there is limited available evidence to address the potential physiological or pathophysiological relevance of such changes [184,301].

Metals

Metals are ubiquitous throughout all biological systems, as well as the environment, and have been broadly implicated in cancer development [302–305]. Many, albeit not all, metals have been classified as Group 1 agents based on this ubiquity and their demonstrated ability to promote cancer in humans. Many metals are classified into different IARC Group based on chemical forms. Interestingly, metalloproteins comprise approximately half of all enzymes, which underscores the biocatalytic importance of metals in normal biology and helps explain the profound pathophysiological consequences of disrupting metal homeostasis [303,306]. Both organic and inorganic forms of heavy metals play well-established roles as carcinogens [302,305]. In addition to disruption of normal biocatalytic functions, many unliganded metal ions such as iron, cadmium, copper, cobalt, chromium, and vanadium are capable of generating ROS via either Haber–Weiss or Fenton-type reactions [304]. Chromium is also capable of generating thiol radicals via direct interactions with cysteine, and arsenic can activate NADPH oxidase to increase superoxide formation [304]. As a class, metal ions thus represent important exogenous sources of ROS, and metal-induced oxidant stress and lipid peroxidation have been implicated in both disruption of normal cell signaling [307] and carcinogenesis [304,308]. Metalloestrogenic contributions to hormone-responsive cancers have also been reported [309].

As a general class of agents, metals are thus attractive candidate effectors in both carcinogenesis and cancer hallmark development. Nickel, in particular, has been implicated as a promoter of cancer hallmark development [12]. Exposure to nickel compounds is also associated with cancer development in humans [287], although these carcinogenic effects may be more commonly ascribed to protein modifications and secondary epigenetic changes, rather than via primary DNA mutagenesis [310,311]. Nickel has been shown to specifically interact with heat shock proteins and to promote general intrinsic protein disorder that can broadly impact cellular functions, not only in affected mammalian cells but also in the gut where nickel may alter microbiome functions in ways that could be relevant to cancer development [311]. The ability of nickel to mimic hypoxic responses [310–312] suggests additional mechanisms whereby this metal can directly contribute to metabolic alterations that mimic cancer. Other metals, including arsenic and lead, also have established roles in carcinogenesis [3]. Arsenic is both naturally occurring and widely distributed in the environment [302,305] and selectively inactivates many enzymes, including HK, via covalent thiol modifications. HK induction in normal mesangial cells by low-dose arsenic mimics changes observed in cancer, although this may simply represent an acute compensatory adaptive response to impaired Glc metabolism [313]. Interestingly, adaptation to chronic low-level arsenite exposure has been associated with oncogenic transformation accompanied by both DNA hypomethylation and adaptive increases in reduced glutathione generation [314]. The latter changes are associated with increased glutathione reductase expression and, although incompletely characterized, are consistent with changes that would typically accompany increased PPP flux.

Oncogenic Viruses

A number of oncogenic viruses, including human papillomaviruses, hepatitis B and C viruses, herpes viruses, human T-cell lymphotropic viruses, human immunodeficiency viruses, and Epstein–Barr virus, are also associated with cancer and have been classified as Group 1 agents [221,315,316]. Early studies on viral transformation by many of these agents led to the identification of viral oncogenes and clearly demonstrated associated changes in metabolism [17,315]. Increased Glc utilization is observed very early during viral transformation, and major causal roles for both increased glycolytic enzyme activities and mitochondrial HK association have been suggested [5]. More recently, specific roles for HIF and hypoxia-regulated metabolic gene programs in viral carcinogenesis have been implicated in these changes [316].

Plant Toxins

The Group 1 plant toxins aristolochic acid and aflatoxins also warrant brief mention here. Both of these agents represent serious natural food contaminants that promote mutagenic DNA adduct formation and have been associated with

both oxidant stress and nonspecific acute alterations in both lipid and amino acid metabolism [219,317–319]. Interestingly, aflatoxin B1 exposure has been assigned a carcinogenic mode of action associated with chronic metabolic or physiological alterations [219], despite the fact that very little is known about its primary or secondary effects on metabolism. With this in mind, glycolytic enzyme induction (HK, PFK, PK, and LDH) has been reported in murine lung nearly a month following systemic exposure to aflatoxin B1 [290], and transplacental induction of PK and LDH in fetal lung has also been reported in aflatoxin B1-treated pregnant mice [291].

15.8.1.2 IARC Group 2A (Probably Carcinogenic to Humans)

Ethyl Carbamate (Urethane)

Urethane is a nonspecific respiratory poison associated with lung cancer formation [23]. The metabolic consequences of urethane exposure are incompletely characterized, but sustained glycolytic enzyme induction (HK, PFK, PK, and LDH) has been reported in murine lung nearly a month following systemic exposure [290]. Transplacental exposure also increases fetal lung PK and LDH activities and alters LDH isoform expression patterns in a manner that mimics cancer, effects not associated with noncarcinogenic pulmonary toxicant exposures [291,320].

Organophosphates

Prototypic exposures to Group 2A organophosphate insecticides such as diazinon and malathion are common and are associated with increased cancer risk [1]. This chemically diverse class of agents is characterized by the shared ability to irreversibly inactivate cholinesterases and other Ser hydrolases via covalent modification of catalytically active Ser residues [308]. Organophosphates exhibit endocrine-disrupting properties [11,321] at very low doses, reflecting the sensitivity of the endocrine system to disruptive exposures [11]. Endocrine actions have established relevance to many cancer hallmarks, including dysregulated metabolism, altered apoptotic susceptibility, and proliferation [11,292]. Although direct cholinergic contributions to cancer development have been suggested, secondary organophosphate-induced oxidant stress and associated genotoxic effects are thought to have greater causal importance [308].

Low-level organophosphate exposures during development have been associated with persistent postnatal abnormalities in both Glc and lipid homeostasis in rodents [322]. The ability of organophosphates to covalently modify and inhibit cellular lipases, which are also Ser hydrolases [323], suggests at least one mechanism whereby these agents may directly influence intermediary metabolism and promote compensatory reprogramming. Other direct effects relevant to metabolism are not well delineated.

The herbicide glyphosphate has also been classified as a Group 2A agent [324]. Like other organophosphates, it exhibits endocrine-disrupting properties [325]. Interestingly, both glyphosate and its principal degradation product, aminomethylphosphonic acid, are also Gly analogs, which may be capable of disrupting amino acid metabolism by interfering with endogenous hydroxymethyltransferase activity in Ser biosynthesis from Gly [326]. Glyphosate also directly chelates metal cations [325,327,328] and exhibits ionophore-like properties capable of promoting mitochondrial uncoupling [328]. Specific metabolic effects in the context of environmental carcinogenesis have been underexplored.

15.8.1.3 IARC Group 2B (Possibly Carcinogenic to Humans)

Many agents classified as Group 2B belong to classes of agents represented in Groups 1 and 2A (e.g., metals and plant toxins). Recognizing that different groupings can represent both differing levels of evidence and fundamentally different chemical or functional characteristics, two selected overlapping broad classes of agents are briefly addressed herein.

Trace Metals

Cobalt is a ubiquitous natural trace element that is essential to humans and an integral component of vitamin B_{12}. It has been classified as a Group 2B carcinogen and can also function as a metalloestrogen [287,309,310]. Like its fellow transition metal nickel – which is also classified as a Group 2B agent – cobalt can serve as a hypoxia-mimetic agent [310,312]. By this shared mechanism, these metals are capable of similarly recapitulating hypoxic modulation of a number of gene expression programs affecting metabolic pathways such as glycolysis [85].

Mycotoxins

Exposures to Group 2B plant mycotoxins, such as ochratoxin A and trichothecenes, have demonstrated effects on lipid metabolism and have been associated with lipid peroxidation [317,319,329]. Like their Group 1 plant toxin counterparts, however, very little is known about their primary effects on metabolism.

15.8.1.4 Other Agents

Bisphenol A

Environmental exposure to bisphenol A (BPA), presently classified as an IARC Group 3 agent, is both widespread and increasing [305,330]. This ubiquitous chemical is xenoestrogenic and exhibits endocrine-disrupting effects at low doses [292,331]. It is also capable of independently promoting the development of numerous phenotypes that mimic cancer hallmarks in a diverse array of models [12]. In addition, BPA exposure has been associated with increased

breast density, an established risk factor for breast cancer [197,332], although its carcinogenicity remains a topic of debate [10,333–335]. Brief systemic exposure to BPA also induces prolonged increases in glycolytic HK, PFK, PK, and LDH activities in murine lung [290]. Other metabolic consequences of BPA exposure have been incompletely characterized. Like other potential carcinogens reviewed herein, the persistence of associated metabolic effects, their causal relationship to cancer development and progression, their relevance to environmentally encountered BPA levels, and corresponding roles in metabolic dysregulation remain incompletely defined [1].

Reactive Aldehydes

Reactive aldehydes, such as acrolein (IARC Group 3), are ubiquitous in the environment and exhibit carcinogenic potential in animals [336]. Acrolein forms DNA adducts and inhibits nucleic acid repair mechanisms, thereby amplifying both its own toxicity and that of other mutagenic agents. Unlike nuclear DNA, mitochondrial DNA lacks robust nucleotide excision repair mechanisms, which amplifies its intrinsic susceptibility to mutagenic damage by these agents [337]. Acrolein and other reactive aldehyde lipoperoxidation products like 4-hydroxynonenal (4-HNE), oxynonenal (ONE), and malondialdehyde (MDA; IARC Group 3) are also produced endogenously via unsaturated FA peroxidation by ROS [338], suggesting both endogenous and exogenous sources of exposure and a specific basis for mechanistic interactions with other exposures or conditions capable of promoting oxidant stress. The fact that 4-HNE can be clastogenic at concentrations achievable *in vivo* under conditions of severe oxidative stress [339] suggests major potential roles for endogenous reactive aldehydes in stress-associated cancer development. As a class, reactive aldehydes are also capable of adduct formation with a variety of cellular proteins, including numerous metabolic enzymes [336,338]. For example, 4-HNE forms adducts with histone *N*-methyltransferases that contribute to the epigenetic regulation and altered expression of both metabolic enzymes (e.g., PK and LDH) and metabolite transporters (e.g., VDAC and GLUT) relevant to intermediary metabolism [338,340].

Formaldehyde is a known environmental carcinogen (Group 1) that can also be produced endogenously [189]. Like other endogenously produced reactive aldehydes, formaldehyde is capable of adduct formation with both DNA and proteins and can be both genotoxic and cytotoxic. Low-level exposures result in genotoxicity, cytotoxicity, increased lipid peroxidation, and downregulation of a number of important metabolic enzymes, including HK1, glutathione reductase, and carbonic anhydrase 2 [341]. Acetaldehyde (Group 2B), the first metabolite of ethanol oxidation, can also form DNA adducts and may have similar effects [287].

In addition to detoxification via glutathione interaction [189], reactive aldehydes can be detoxified by aldose reductase (AR), a metabolic enzyme

overexpressed in cancers [342] but best characterized as the rate-limiting enzyme for polyol pathway flux in end-organ targets of diabetes exposed to supraphysiological concentrations of Glc. The adaptive advantage of AR overexpression in cancers [342] is difficult to explain in simple metabolic terms given its low affinity for Glc and the pathophysiological irrelevance of supraphysiological Glc exposures for most cancers. Interestingly, however, AR is a member of the aldehyde dehydrogenase superfamily and its affinity for reactive aldehydes and their glutathione conjugates is several orders of magnitude greater than for Glc [124], so AR overexpression in cancer could suggest an alternative role in the detoxification of reactive aldehydes (e.g., 4-HNE and MDA) that is independent of its canonical role in gating flux through the nonphysiological polyol pathway in normal cells (i.e. a novel moonlighting function). The use of increased aldehyde dehydrogenase activity as a biochemical marker of cancer stem cells is compatible with such speculation [343].

A number of other unrelated agents with the potential to modulate metabolism and promote carcinogenesis were recently identified by the Halifax Project (see Section 15.8.2.3) and are detailed elsewhere [1].

15.8.2 Environmentally Relevant Combinatorial Exposures

Given strong epidemiological evidence for environmental carcinogenesis, both the paucity of corresponding validated experimental data reflecting environmentally relevant complex exposures and strong historical biases for carcinogenic testing at maximal tolerated doses together provide a compelling rationale for greater carcinogenic testing under environmentally relevant conditions.

15.8.2.1 Occupational and Common Environmental Exposures

Both direct tobacco use and indirect exposure via second-hand smoke have been classified with Group 1 agents by the IARC. Tobacco smoke – containing numerous individual Group 1 carcinogens, including BaP, benzene, formaldehyde, arsenic, and cadmium, as well as a number of other suspected carcinogens such as acetaldehyde (Group 2B) and acrolein (Group 3) – promotes glycolysis and ketogenesis in fibroblasts and has been implicated in metabolic remodeling favoring microenvironmental tumor growth [344]. The active constituents responsible for these changes and their corresponding targets have not yet been identified. Evidence for specific metabolic responses to other complex environmental exposures is similarly scant. For example, soot was first identified as an environmental contributor to scrotal cancer in chimney sweeps in the late eighteenth century and constitutes one of the earliest reported occupational carcinogenic exposures [3]. Presently classified as a Group 1 exposure, soot is highly variable in composition, containing both inorganic and organic constituents that include known Group 1 carcinogens like PAH (e.g., BaP) and arsenic.

Interestingly, chronic exposure to BaP, which a major constituent of soot, is associated with increased Glc metabolism that is enhanced by coexposure to sulfur dioxide, also a major soot constituent [289]. Direct effects of soot on metabolism, however, have not been reported.

No discussion of environmental exposures would be complete without consideration of natural dietary carcinogens – some already mentioned previously. The importance of dietary exposures is widely recognized, but much, if not most, of the focus on diet has been directed toward synthetic agents despite the fact that dietary carcinogens can come from a variety of different sources and can be either natural or synthetic in origin [3,345]. In general, dietary carcinogens include both natural constituents and natural or synthetic contaminants of ingested foods, whereas others can be pyrolytic products generated during cooking. Alternative sources involve microbial processing of procarcinogens within foodstuffs either during storage [3] or within the enteric microbiome [346,347]. This latter possibility represents an important understudied contributor to environmental carcinogenesis. In fact, Ames noted just over a quarter century ago that "very low exposures to pesticide residues or other synthetic chemicals should be compared to the enormous background of natural substances" [345]. In other words, natural toxins and carcinogens may be of equal or greater concern than synthetic toxins and carcinogens in environmental carcinogenesis [345,348].

Gut microbiota play important roles in processing dietary nutrients, toxins, and carcinogens [346,347,349]. Metabolism by enteric microflora can redefine not only the chemical forms of intestinal contents but also the bioavailability and ultimate biological consequences of ingested carcinogens, procarcinogens, and/or carcinogenic antagonists. As intermediary processors of enteric contents and potential targets for xenobiotics [347,349], intestinal microflora can exert both primary and secondary influences on human environmental exposures. In essence, they serve as a filter or lens through which the gut "sees" much of the external environment. Recently postulated roles for microfloral metabolites such as deoxycholic acid in linking obesity to cancer [350] are fully consistent with this notion.

15.8.2.2 Environmentally Relevant Low-Dose Combinatorial Exposures

Environmental carcinogenesis specifically relates to carcinogenic interactions between the host and its environment, broadly defined as the sum of all exposures to external agents and conditions that could directly or indirectly contribute to cancer development in the host. The IARC has estimated that environmental toxic exposures are responsible for as many as one in five human cancers, although the true burden of environmentally induced cancer is likely much higher [12,351]. Despite the inherent difficulty of quantifying the relative contributions of environmental exposures to cancer development, the ability of

individual exposures to promote or enable carcinogenesis-associated processes has been scientifically validated [12,287].

> ". . . without studies of the mechanism of carcinogenesis, the fact that a chemical is a carcinogen at the [maximum tolerated dose] in rodents provides no information about low-dose risk to humans." –
>
> Bruce Ames [345]

In vivo exposure testing in rodents has traditionally been regarded as the gold standard for assessing the ability of a given agent to induce cancer, typically at doses sufficient to elicit rapid and robust toxic or carcinogenic responses. Once evidence of carcinogenesis is obtained for a given exposure, serial testing is typically performed to define lower exposure limits for overt carcinogenic responses and thereby establish apparent "safe" exposure thresholds. Unfortunately, this approach disregards both the inherent complexity of cancer development and the likelihood of multiple underlying contributing mechanisms in environmental carcinogenesis. The effects of single agents examined in isolation also cannot be simply extrapolated to complex mixtures, particularly at low concentrations [3,10,11,352].

Searches for carcinogenic agents have historically focused on agents with the potential for widespread exposures that are independently capable of cancer induction, frequently at acutely toxic concentrations over time frames that are far shorter than those typically associated with environmental carcinogenesis where latent periods between initial exposures and cancer establishment can last years or decades [9,158]. Unfortunately, traditional approaches do not directly address the nontrivial possibility that a single agent, independently incapable of cancer promotion, could potentially combine with other exposures to collectively promote carcinogenesis [9,12]. Cancer is a complex disease characterized by multiple phenotypic changes involving myriad cellular structures, functions, and signaling pathways. As such, it is possible – even likely – that cumulative environmental exposures may act differently in combination than in isolation [12]. Consistent with this notion, many ubiquitous environmental exposures are capable of enabling cancer hallmark development [1,9,12]. Even if unable to individually serve as "complete carcinogens," exposures to these agents could potentially act either additively or synergistically in combination to promote cancer development in a manner not predictable via conventional toxicological and carcinogenic testing [9,12]. Combinatorial exposures to multiple agents can also be variably distributed in both place and time and, in addition to the broader theoretical potential to influence more than one critical transition stage in multistage cancer development, they may affect both cells destined to become cancer and host cells critical for determining environmental characteristics or systemic surveillance.

15.8.2.3 The Halifax Project

In 2011, the Canadian nonprofit organization Getting to Know Cancer (http://gettingtoknowcancer.org/) launched an international initiative entitled the Halifax Project with the explicit purpose of generating comprehensive literature reviews to assess the known contributions of environmentally relevant exposures to the development of both cancer and its characteristic phenotypic hallmarks [1,9,12]. Using the phenotypic hallmarks of cancer described by Hanahan and Weinberg [6,353] as a conceptual framework for analysis, individual multidisciplinary teams were assembled to broadly interrogate the published literature relevant to each characteristic hallmark and its interactions with other hallmarks [12]. To address the hallmark of dysregulated metabolism and metabolic reprogramming, authors were specifically charged with identifying key metabolic targets for disruption or dysregulation, as well as prototypic environmental exposures with the potential to modulate these targets and influence cross-hallmark interactions [1]. Primary consideration of known carcinogens was specifically discouraged to focus efforts on the identification of novel potential environmental contributors to the development of both cancer and its associated hallmarks. The overarching goal of this initiative was to explore the possibility that low-dose chemicals incapable of promoting cancer development alone might somehow combine to promote cancer phenotype development and potentially drive environmental carcinogenesis. These efforts revealed both a limited amount of relevant functionally validated metabolic data in the pertinent literature and strong publication biases for both nonmetabolic effects and large monotonic responses in the relevant cancer and toxicology literature [1]. Demonstrations of sustained metabolic changes unambiguously linked to both specific environmentally relevant exposures and cancer initiation were particularly sparse.

A number of specific metabolic targets implicated in dysregulated cancer metabolism were identified, and an attempt was made to single out potentially disruptive exposures worthy of further examination [1]. To focus the search for metabolic targets, a restricted set of prototypic targets amenable to modulation by environmentally relevant exposures were selected, and iterative cross-hallmark comparisons were made to identify possible interactions between specific dysregulated metabolic features and other cancer hallmarks [1,12]. These efforts were primarily limited by the paucity of unambiguous published evidence for direct causal relationships between specific exposures, dysregulated metabolism, and carcinogenesis [1]. In general, the published literature was highly biased by associative and descriptive studies that were neither designed nor intended to directly address specific metabolic contributions to carcinogenesis. In addition, only previously studied exposures found in the published literature were included in the list selected for cross-hallmark comparison [1]. By definition, this list was incomplete, as important unstudied or understudied exposures and known carcinogens were not represented in

these efforts. These limitations notwithstanding, a number of exposures capable of modulating selected prototypic metabolic targets were identified with the corresponding potential to either promote or antagonize the development of other nonmetabolic hallmarks based upon directional responses to common exposures. Evidence for directionally opposite cross-hallmark promotion and antagonism for the same hallmark was identified for many prototypic agents, likely due to differences in exposure conditions, model systems, and experimental endpoints [1]. That said, dysregulated metabolism is not a singular entity, so multiple directionally divergent relationships between "metabolism" (broadly defined) and other individual hallmarks are not only possible, but expected.

The Halifax Project importantly established the theoretical potential for individual environmental exposures to specifically enable or activate multiple key driver mechanisms associated with cancer development and identified numerous gaps in our present understanding of the carcinogenic potential of low-dose environmental exposures [9,12]. Although not all exposures capable of promoting cancer hallmark development are necessarily carcinogenic, this work provides a compelling rationale for further interrogation of the carcinogenic potential of low-dose combinatorial exposures, especially since the present absence of direct evidence for carcinogenicity following low-dose combinatorial exposures does not constitute evidence of an absence of carcinogenicity. Only rigorous direct interrogation of the long-term outcomes of environmentally relevant low-dose exposures will address these deficiencies.

15.9 A Conceptual Overview of Traditional and Emerging Toxicological Approaches to the Problem of Cancer Metabolism: Implications for Future Research

15.9.1 General Experimental Considerations in the Study of Metabolism *In Vitro*

Mammalian cell culture has enabled studies that have contributed greatly to much of our present mechanistic understanding of cancer biology and is integral to many current cell-based HTS approaches to both toxic and cancer risk assessment. As such, some discussion of the suitability of these *in vitro* HTS assays and associated standard culture conditions to the study of metabolism is warranted.

Standard conditions for mammalian cell culture were originally established to maximize cell viability in continuous culture under defined conditions [354,355]. They were never designed nor intended to facilitate evaluation of physiological relevance. As a consequence, an enormous body of work has examined biological processes *in vitro* under profoundly nonphysiological conditions and under the

unvalidated assumption that supraphysiological nutrient availability will not quantitatively or qualitatively influence the associated experimental results. Close interactions between metabolism and both cell function and survival, however, are now widely recognized [1,17], and the cell's trophic environment has been shown to contextually influence both cellular stress responsiveness and metabolic substrate dependence independent of intrinsic cellular factors [116,127,128,130–132,180].

Experiments conducted in the presence of supraphysiological concentrations of Glc (>1 g/l) are commonplace, which can profoundly influence both the magnitude and direction of metabolic flux within the cells. In fact, standard culture medium preparations frequently contain Glc concentrations as high as 25 mM (4.5 g/l), which is nearly fivefold greater than physiological and seems more relevant to the study of uncontrolled diabetes than cancer. Moreover, some subsets of intratumoral cancer cells are more likely to experience mesotrophic or oligotrophic conditions than their normal counterparts, so it can be argued that testing under such potentially limiting conditions would be more appropriate. Unfortunately, verification of results at physiological substrate concentrations is not routinely pursued, but this crucial methodological requirement should be an obligatory part of all toxic and carcinogenic testing. Testing under potentially limiting heterotrophic conditions, ranging from eutrophic to oligotrophic, would also further inform our understanding of the roles played by dysregulated metabolism in cancer biology. By extension, supraphysiologic Gln, pyruvate, and amino acids require similar experimental attention, and serum supplementation serves as an incompletely considered source of large quantities of lipids, lactate, and other common circulating metabolic substrates. Supraphysiological concentrations of pyruvate and lactate can also potentially influence results via their intrinsic antioxidant quenching properties as outlined in Section 15.5.1. One important caveat of using more physiological culture medium, however, involves an accompanying requirement for more frequent medium exchanges, as many standard cell lines can completely exhaust their Glc supply at physiological levels in less than 24 h if not replenished.

Direct comparisons between cells cultured under markedly differing conditions are also of particular concern when addressing the literature, as most studies pay limited attention to the specific roles of growth factor and energy substrate availability in determining experimental outcomes. A major problem relates to the widespread use of diverse nonphysiological culture media differing in both the types and amounts of energy substrates available to cells. In addition, both serum and exogenous trophic factor supplementation, which are neither standardized nor optimized for the study of metabolism, strongly influence both the types and magnitude of intermediary metabolism observed *in vitro*. As such, rigorous controls are required to obviate unwanted experimental bias. Accompanying time course data are also needed to control for

different rates of substrate utilization, to guide optimal sampling, and to ensure that exhaustion of key metabolic substrates or cofactors do not become limiting and influence metabolic results in experiments of longer duration (e.g., >12–24 h). For example, extracellular lactate accumulation reflects the net balance of intracellular generation, extracellular extrusion, and counterbalancing uptake and reutilization as a cellular energy substrate. Lactate can also be elaborated from sources other than glycolysis [79,104], which is important to consider given the fact that changes in lactate accumulation are not uniformly accompanied by comparable changes in Glc disappearance.

15.9.2 Systems Biology and Current Approaches to *In Vitro* Toxicology Screening

Experimental approaches to carcinogenic risk assessment have traditionally emphasized robust short-term exposures capable of promoting rapid *in vivo* cancer development. However, given the practical limitations, inefficiency, and expense of *in vivo* testing for carcinogenic potential [352], increased recent emphasis has been placed on probabilistic cell-based HTS assays using surrogate *in vitro* endpoints [225]. More emphasis has also been placed on establishing "safe" exposure thresholds for individual agents [352]. Unfortunately, conventional toxicological assays and current HTS methods alone are poorly suited to identify or define specific roles for dysregulated metabolism in carcinogenesis. Toxicity signatures generated by HTS platforms provide important correlative information, albeit with limited specificity for – and limited direct mechanistic insights into – cancer metabolism. Given the highly contextual nature of metabolism, assay conditions and the biochemical appropriateness of specific metabolic changes may be as important as their fundamental nature or direction. Alterations in metabolic control, which can be as important as alterations in capacity [1,26], may also not be reflected in conventional gene expression profiles. Additional functional testing, including specific metabolic flux analysis, is thus required to validate metabolic relevance, provide mechanistic insights, and establish causal relationships. Specificity for cancer is also key, as promiscuous or nonspecific assays are likely to identify promiscuous agents or nonspecific effects. Newer systems biology approaches to toxicological screening and evidence-based toxicology bring numerous strengths to the table and, in theory, have the power to markedly expand chemical testing capabilities. Unfortunately, they are also uniquely limited in their ability to address dysregulated metabolism. For example, the NIEHS ToxCast and Tox21 screening platforms address toxicity and toxic response pathway activation, but the results are not cancer specific and do not directly address metabolism [1]. HTS screening systems, as presently employed, also do not directly address either intermediary metabolism or metabolic control. These represent major shortcomings as tools for identifying and understanding crucial differences in

cancer metabolism. As such, individualized conventional metabolic analysis under biologically relevant conditions is still needed to fully interpret the metabolic significance of data obtained by using these screening platforms. In addition, sampling at single fixed time points risks overlooking crucial sequential effector involvement or transient intermediate states that do not temporally coincide with sampling. As such, positive results obtained using these platforms may be informative, whereas negative results may be completely uninformative.

The EPA Toxicology Forecaster (ToxCast) platform is a diverse collection of *in vitro* HTS assays used to identify agents capable of promoting gene expression changes that directionally mimic toxicity or disease development *in vivo*. Unfortunately, the individual component assays do not directly assess intermediary metabolism, and their monotonic single-endpoint nature limits their ability to provide important spatiotemporal and functional information needed to delineate specific metabolic contributions, address the reversibility of observed changes, or distinguish between acute toxicity and more sustained carcinogenic effects involving common effectors. Another potential downside to these cell-based assays involves their inability to detect test agent effects requiring specialized metabolism to generate active metabolites – effects that often require intact *in vivo* systems for detection. They also do not adequately recapitulate the complexity or heterogeneity of *in vivo* biological responses to the exposome. For example, Myc oncogene *trans*-activation is associated with altered expression of target genes regulating both Glc and Gln metabolism and plays established roles in cancer energy metabolism [225,356]. These facts notwithstanding, ToxCast screening failed to experimentally validate these known associations using a standard Myc reporter gene assay designed for this purpose [1,225]. This negative result does not exclude Myc involvement in dysregulated cancer metabolism, however, and may have technical explanations [1]. First, these assays employ a single hepatocarcinoma cell line (HepG2) stably transfected with chimeric Myc reporter gene constructs containing *cis*-acting Myc binding motifs fused to a minimal promoter–reporter gene construct [357,358]. Because these assays measure the ability of simple *cis–trans* interactions to drive the expression of heterologous promoter constructs that do not retain the fundamental spatial or cooperative *cis*-acting sequence relationships of endogenous target genes, the potential importance of additional flanking sequences, spatial relations with the transcription start site(s), and requirements for other associated enhancer or repressor motifs are completely discounted. These assays also presume that (i) regulation in this cell culture model is representative of that expected in diverse *in vivo* target tissues and (ii) the requisite upstream signaling pathways responsible for Myc activation are not only present in these cells but are also activated in a manner identical to that expected in cancer [1]. A unitary mode of *trans*-activation is also assumed. Despite the fact that reporter gene expression is consistent with the ability of a

specified exposure to activate endogenous target genes, these assays do not establish gene-specific *trans*-activation [1]. As such, positive results require validation of individual endogenous transcriptome targets, and negative results, as in the case of Myc, may be completely uninformative. These discrepant results are illustrative of the basic limitations of ToxCast screening assays and the need for independent biochemical validation and characterization [359]. The use of *in vitro* p53 activation for genotoxicity screening is similarly problematic [360,361], as p53 is activated by a diverse array of stress signals [362,363] and is not specific for DNA damage [321,364].

The Toxicology in the 21st Century program (Tox21) seeks to extend the reach of existing *in vitro* toxicology screening platforms by integrating HTS resources at multiple US federal agencies, including the Environmental Protection Agency, the National Institute for Environmental Health Sciences, the Food and Drug Administration, and the National Center for Advancing Translational Sciences. Through platform integration, Tox21 hopes to identify compounds with the potential to disrupt human processes with long-term adverse health consequences such as cancer. Notably, all of these HTS platforms emphasize monotonic *in vitro* assays that are neither designed nor well suited to directly address metabolism. As such, their specific utility in both the detection and characterization of dysregulated metabolism is limited [1].

No universal metabolic gene expression signatures have yet been identified in cancer, and the confounding influences of both parental tissue origin [15] and heterogeneous tumor sampling biases [153,156] on gene expression profiles are increasingly recognized. Experimental approaches designed to detect large changes in gene expression also frequently assume that changes in capacity are sufficient to account for metabolic phenotype development while ignoring the dynamic controlling influences of substrate availability, allosteric feedback, and cellular energy demands in intact cells (Figure 15.3). As such, they may fail to detect crucial determinants of dysregulated metabolism. With this in mind, the routine use of fixed nonphysiological culture conditions for screening poses additional methodological causes for concern. The nutrient largesse associated with standard culture conditions fail to recapitulate *in vivo* growth and selection conditions and may also strongly influence results.

Following the successful completion of the Human Genome Project and publication of the first complete representative human genome sequence in 2003, numerous ongoing targeted sequencing initiatives have been launched to identify specific somatic mutations associated with cancer development [365–367]. These efforts have been driven, in part, by the recognition of numerous cancer-associated somatic mutations, including coding mutations in genes encoding known oncoproteins, tumor suppressors, trophic signaling pathway components, DNA repair and maintenance factors, and metabolic genes implicated in dysregulated metabolism [241,366,368]. Disease-associated mutations are not restricted to coding regions, however, but rather are

disproportionately localized to noncoding genomic regions, where the importance of nonlinear epistatic gene interactions and both mutagenic and epigenomic *cis*-acting regulatory element modifications in disease development have been increasingly recognized (Figure 15.5) [231]. These observations have expanded interest to the whole cancer genome [231,366] and have prompted calls for more integrated systems-based approaches incorporating biological understanding into genotype–phenotype analysis and interpretation [1,231]. The ultimate metabolic consequences of any given mutation cannot be simply extrapolated from observed metabolic target gene alterations and must be empirically determined via conventional experimental methods.

The WHO International Programme on Chemical Safety (IPCS) utilizes a structured mode of action (MOA) framework to estimate the carcinogenic relevance of individual chemical exposures [369–371]. In this framework, cancer-associated risk assessment is largely restricted to the analysis of chemicals acting via common postulated MOA for a given tissue and a single specified biological endpoint [372]. Alternative endpoints, tissues, or MOA for a given chemical are considered separately [370,372], and conceptual attention to chemical interactions, including chemical synergism and joint mechanistically independent effects, is limited [373,374]. Moreover, the notion that agents incapable of individually promoting cancer development might jointly combine to cause or contribute to cancer is generally overlooked [9]. This framework also ignores the fact that many, if not most, chemical carcinogens exhibit more than one MOA [219] and are capable of interactions with each other in complex mixtures [12,167,219]. As a consequence, the restrictive scope of the IPCS MOA framework likely encourages underestimates of overall carcinogenic risk [9]. Unfortunately, these are not trivial oversights, and the direct support and advancement of this framework by the International Life Sciences Institute (ILSI), a nonprofit member organization comprised largely of representatives of the food and beverage, agricultural, chemical, and pharmaceutical industries, could raise conflict-of-interest concerns in the promotion of a framework with such a restricted experimental focus. The limitations of the IPCS MOA framework have been detailed elsewhere, including the limitations of attempting to establish causal relationships from epidemiological data [1,219,375]. Despite their conceptual attractiveness, unitary toxicological MOA are frequently unpredictable [303]. When inferred or assumed, they require independent empiric validation, especially for nonfixed, dynamic, and interactive processes such as intermediary metabolism. For complex, multistage processes such as carcinogenesis, where no universal mechanistic set of requirements has yet been identified, these considerations assume additional importance.

To address the limitations of the frameworks and platforms above, novel complementary approaches are ultimately needed to address the metabolic consequences of environmental exposures and their specific contributions to

cancer development, selection, and progression. Systems biology approaches – genomic, transcriptomic, proteomic, and metabolomic (Figure 15.3) – provide powerful means to identify gene expression signatures and/or patterns of metabolite accumulation that distinguish cancer cells from their normal counterparts and help focus additional targeted study. In its simplest form, however, metabolomic data provide static snapshots of highly dynamic biochemical processes [125,376]. Individual metabolic intermediates can also be shared by multiple different pathways [139], and metabolomic analysis can be complicated by both intratumoral heterogeneity and intracellular compartmentalization of metabolism [377]. As such, supplementary metabolic flux data are typically required to fully interpret such information. By definition, the experimental relationships between the exposome and the metabolome will not be fixed (Figure 15.3), so these types of studies also need to be carefully designed and standardized, as the type and magnitude of metabolic flux within cells dynamically reflect a variety of intrinsic and extrinsic experimental variables, including substrate availability, cell growth state, environmental conditions, and extant energy demands. As such, perturbational profiling strategies [238,378] may be needed to enhance or complement conventional transcriptomic, proteomic, metabolomic, and functional screening approaches to the identification of mechanistic determinants of metabolic change.

Probably the greatest weakness of current methods of risk assessment is not technical in nature, but rather lies in assumptions of unitary characteristics or principles. Even in cells that are highly predisposed to cancer development, both modeling and overall risk assessment will be complicated by the nontrivial roles of chance and a "multiplicity of unquantifiable modifiers" as determinants of which cells will ultimately become successful cancers [232] and what they will look like. In fact, it can be argued that heterogeneity in cancer is both a predictable and expected outcome on this basis alone. In this sense, the parallels between cancer development and evolution are striking. Cancer is not a single disease, nor does it have a single cause [232]. As such, no single model is sufficient to address the complex and heterogeneous metabolic changes that support cancer development and progression, and many common associated cellular phenotypes like proliferation and loss of normal growth constraints may exhibit diverse underlying mechanistic bases and metabolic dependencies [30].

15.10 The Nosology of Cancer and Cancer Development

Finally, the nosology of cancer and its development also warrants brief mention. Medical nomenclature and associated disease classification schemes typically reflect our fundamental understanding of underlying pathogenesis and strongly influence both experimental and therapeutic approaches to disease [379,380]. The etymology of cancer has a deep pathological and historical basis dating

back to Hippocrates [380]. More recently, however, changes in cancer nosology have been proposed as a behavioral tool to reduce unnecessary cancer screening [380,381]. By restricting the term cancer to lesions with the highest malignant potential, proponents of these proposed changes hope to minimize screening behaviors for lesions with a greater statistical likelihood of indolence [381,382]. Although well-intentioned, these proposed taxonomic reforms promote the binary reclassification of cancers as either lethal or indolent based on statistical analysis of population outcomes, thereby ignoring individual variations in disease progression and making no attempt to incorporate current understanding of the underlying determinants of both pathologic variability and progression [380]. In fact, both indolent and malignant behaviors can strongly reflect both host and environmental factors in addition to intrinsic cancer biology [1,24,380]. Even where the risk of malignant progression is small – as in the case of ductal carcinoma *in situ* [381] – low risk is not synonymous with no risk [382], and binary classification schemes that fail to make this distinction could establish a false dichotomy and thereby represent a disservice to inappropriately stratified patients with a finite risk of clinical progression, however low. Improved understanding of the fundamental determinants of cancer behaviors and their mechanistic underpinnings should naturally lead to the identification of better progression markers and could ultimately provide a basis for meaningful nosological reform. Understanding should always precede such reform, however, and not vice versa.

Like intermediary metabolism, cancer is not a singular fixed entity. It is also not binary in nature [380]. Rather, cancers are highly heterogeneous and dynamic open systems that actively interact with their host and reflect a continuum of characteristic biological features that are capable of both qualitative and quantitative changes over time [1,7,33]. Cancer nomenclature should therefore reflect the full spectrum of intrinsic cancer biology and attempt to emphasize specific mechanistic determinants of different clinical outcomes, including malignant transformation and variations in therapeutic responsiveness. This integrated focus on both clinical and molecular pathological features to improve disease taxonomy is a fundamental goal of precision medicine [383].

These nosological considerations are not restricted to cancers *per se*, but also apply to carcinogens and all processes associated with cancer development. For example, the terms carcinogenesis and mutagenesis are frequently used interchangeably, reflecting, in part, the central importance accorded to mutagenesis in cancer development. These terms are not synonymous, however, and unnecessarily restricting consideration of carcinogenic factors to mutagenesis alone risks overlooking important nonmutagenic contributions to cancer development. In fact, Peto recognized four decades ago that both mutagenic and nonmutagenic processes can contribute to neoplastic transformation and proposed an expanded definition of carcinogens to include "any agent which makes it more probable that a fully transformed cell will proliferate successfully

rather than be eliminated or held in check" [213]. As illustrated by the foregoing extensive discussion of potential metabolic contributions to the initiation, selection, and progression of cancer, exposures that directly or indirectly influence metabolic fitness are well suited to contribute to the entire spectrum of cancer development.

Lastly, the pertinent literature on cancer metabolism spans many scientific disciplines, so common nomenclature is of particular importance [156]. For example, the term "low dose" can easily – and inappropriately – be misconstrued to suggest an absence of associated biological effects. Conventional toxicological dogma notwithstanding, there is probably no exposure threshold that is completely without biological effects [1,10,11,352]. Uniform definitions of cancer, carcinogenesis, and carcinogen are also important, but they should be firmly based on current biological knowledge. Where different associated clinical outcomes are observed, they should help provide experimental focus for attempts to classify and characterize the underlying mechanistic basis of such differences. In general, nosological refinements should follow biological understanding and should represent all events, stages, and processes that lie on an associated biological continuum. Ultimately, "any taxonomy that does not provide a framework for scientific discovery and mechanistic understanding is, for that reason, undesirable" [380].

15.11 Discussion

Cancer metabolism is intrinsically complex, and its individual characteristic features are typically neither fixed nor specific for cancer. In fact, many of the metabolic features of cancer are shared with normal developing embryonic tissues [320], stem cells [89,384], and some highly proliferative or specialized tissues such as the retina [16,17]. The metabolic features that distinguish cancer from these other tissues and cell types are incompletely defined and can be highly contextual in nature [1,17,116].

This chapter attempts to clarify in very broad terms what is – and is not – known about dysregulated cancer metabolism and its contributions to both environmental carcinogenesis and the successful multistage establishment of cancer. There are large amounts of published associative data describing metabolic changes in cancer, but very limited published data that unambiguously establish specific requirements or clearly defined mechanistic roles for metabolism in exposure-associated cancer development. The paucity of unambiguous functionally validated data addressing causal roles for metabolism in environmental carcinogenesis was previously identified as a key deficiency in the pertinent literature by the Halifax Project [1], a finding confirmed during the preparation of this chapter. No single alteration accounts for cancer, and specificity for cancer may not ultimately reside in any single individual

change, but rather in how multiple individual changes are coordinated and packaged together in the context of the overall cellular and environmental gestalt. The intrinsic complexity of cancer metabolism is emphasized throughout to both stimulate interest in the field and to focus more basic research on the fundamental mechanisms underlying dysregulated metabolism and their specific causal relationships with environmental carcinogenesis.

Although the framework of Hanahan and Weinberg [6,353] provides a useful structured platform for conceptually and experimentally addressing the characteristic features of cancer [12], it is not without limitations [1,7,33]. For example, the original omission of dysregulated metabolism as a cancer hallmark [353], followed by its subsequent inclusion as an "emerging hallmark" over a decade later [6], is curious given the fact that metabolic alterations represent the earliest described and most recognizable characteristic features of cancer [1,12,13]. Omission of the "missing hallmark" of dedifferentiation [7] is also notable, suggesting a possible need to extend this framework and to incorporate additional flexibility to fit our evolving understanding of cancer biology and not vice versa [1]. The unique abilities of cancer cells to invade normal tissues and to metastasize constitute what are arguably their most cancer-specific phenotypes [1,33,165]. The loss of normal growth constraints, including both contact inhibition and anoikis, is also highly characteristic of cancers and such changes have been linked with metabolism [5,8]. Other classical hallmarks of cancer can be individually shared with both normal tissues and benign tumors [33]. As such, an attempt has been made herein to extend this framework by providing a more nuanced and detailed view of the salient features and inherent complexity of cancer metabolism. In particular, the discussion of intermediary metabolism is extended to include not only energy metabolism but also anabolism and metabolic coupling with non-metabolic cellular functions. Because energy metabolism represents only a fraction of the intermediary metabolism relevant to cancer, a much broader view of the gestalt of cancer metabolism is advocated.

Using evolutionary heuristics, the potential importance of complex multistage selection in cancer development is repeatedly emphasized (Figure 15.3), as unselected transformed cells are unlikely to go on to successfully establish cancer [165,218]. An evolutionary framework emphasizing selection also helps explain both cancer heterogeneity and the differing characteristics and requirements for cancer establishment in different parental tissues [154]. Metastasis, which is both a highly selective and inefficient process [201,385], similarly lends itself to evolutionary analysis. Observed variations in the metastatic potential of cells with common parental origins strongly suggests a crucial role for selection in both invasive and metastatic success [218,386]. These differences are ostensibly a function of both intrinsic cancer cell biology and environmental selection pressures [218]. Both local tissue invasion and metastasis entail migration through heterogeneous trophic and physical environments [218],

so changes in adaptative fitness that equip cells to tolerate and survive associated environmental transitions are thus excellent candidates for selection. Given the broad variability in environmental conditions and substrate availability encountered by cancer cells, metabolic fitness seems ideally suited to serve as a basis for selection [386]. Bidirectional metabolic interactions between cancer cells and their local tumor microenvironments also represent significant opportunities for cells to participate in their own selection via active Darwinism [386,387]. Neither cellular metabolism nor external environmental conditions are fixed for cells within rapidly growing tumors or during local tissue invasion or metastasis, which results in heterogeneous environment-specific selection characteristics [7,210]. In contrast, a fixed environmentally restrictive metabolic phenotype would be potentially maladaptive under such conditions [1,24,232]. The ability of cancer cells to help shape their microenvironment can also directly enhance both their tissue invasiveness and metastatic potential via redox-sensitive ECM remodeling [271].

Epigenetic regulation has been broadly characterized as adaptive in nature [143] and is intimately tied to metabolism. It can also occur at multiple levels via the modification of nucleic acids, histones, and signaling proteins. Although not traditionally considered heritable, there is accumulating evidence that some epigenetic changes can be transgenerationally retained [1,388,389]. Given the known reciprocal relationships between metabolism and epigenetic modifications such as methylation, acetylation, phosphorylation, and glycosylation [389], this suggests specific nonmutagenic mechanisms whereby metabolism may couple to durable – and possibly even heritable – changes relevant to the development of cancer and its phenotypic hallmarks. Although specificity for cancer cannot presently be asserted for many epigenetic endpoints or interactions, such specificity may not represent an absolute requirement for carcinogenic relevance [1]. Epigenetic and nongenotoxic effects of environmental carcinogens remain an understudied area representing a recently identified unmet need in both toxicology and cancer biology [9].

Classical signal transduction pathways typically involve the highly regulated phosphorylation and dephosphorylation of specific amino acid residues in signaling effector proteins. These phosphorylation–dephosphorylation events are organized in signaling amplification cascades and carry specific associated energy requirements of potential relevance to the overall metabolic gestalt. That said, not all signal transduction is mediated by protein phosphorylation and dephosphorylation. Metabolite profiles closely reflect both cell state and corresponding environmental conditions and are capable of transducing cellular signals that can be translated into specific functional responses and gene expression programs that ensure that cellular needs are met. The overlap between classical signal transduction and metabolite-mediated information transfer in cancer represents another area warranting increased conceptual and experimental attention.

Host–tumor metabolic interactions represent an additional underexplored frontier in cancer biology. Because cancers are open systems that are obligatorily dependent upon their hosts for growth and survival, both the host and the cancer itself can represent viable targets for carcinogenic environmental exposures and therapeutic intervention [1,146]. For example, the supportive roles played by host gluconeogenesis in highly glycolytic tumor survival and growth [79] represent one obvious area for more focused inquiry in host–cancer interaction. Other metabolic contributors to both host cachexia and immune surveillance warrant similar attention [390,391].

The past half dozen years have seen the launch of several crosscutting scientific initiatives of relevance to cancer research, including the National Cancer Moonshot Initiative, the Precision Medicine Initiative (PMI), and the VA Million Veteran Program (MVP). The National Cancer Moonshot Initiative was launched in 2016 with the specific aim of providing new resources and incentives to accelerate the overall pace of cancer research. This nascent initiative – when coupled with large ongoing correlational genomic sequencing initiatives like the PMI and the closely related VA MVP – launched in 2015 and 2011, respectively – holds specific promise for the identification of novel genomic markers of both susceptibility and therapeutic responsiveness in complex multigenic diseases such as cancer. The VA MVP is already well over halfway to its goal of enrolling 1,000,000 representative veterans for such efforts. Although these genomic sequencing initiatives are not cancer specific, a major early focus of the PMI will be on cancer. Its initial focus on precision oncology will be overseen by the National Cancer Institute (NCI) and will directly address the genomic basis of a number of cancer-associated problems, including cancer susceptibility, tumor heterogeneity, metastatic potential, therapeutic responsiveness, and resistant phenotype development. The NCI is also working to build a National Cancer Knowledge System where cancer-specific genomic information can be stored and correlated with associated clinical and therapeutic outcomes data. Ideally, these initiatives will ultimately stimulate and help direct – but not supplant – further investigator-initiated basic "reverse translational" research into the underlying cancer biology associated with specific genetic markers.

There is no single cause for cancer [232]. The continuum of cancer initiation, selection, progression, and metastasis is a multistage multifactorial process that can vary widely both within and between individual tumors – and across cancer types [222]. The defining metabolic characteristics of individual stages along this continuum remain incompletely delineated, and corresponding selection pressures can vary widely. Once established, oncogenic mutations will influence the behavior and carcinogenic susceptibility of all cells descendant from the mutant parental lineage, whether stochastic or hierarchical in origin [169]. Selection, however, becomes the essential limiting factor in successful cancer establishment once transformation is achieved. Metabolism, broadly defined, is ideally suited to serve as a basis for such selection. Both the complexity and the

dynamic nature of intermediary metabolism require holistic consideration of the metabolic gestalt in its entirety within specific cellular and environmental contexts, rather than piecemeal as individual metabolic reactions or pathways under standardized conditions with no requirements to mimic *in vivo* conditions [1,115,116]. For these reasons, no single model is sufficient for the study of cancer – much less cancer metabolism. There is thus an overarching need for multiple complementary cancer models, including physiological models whose metabolism can be directly modulated and monitored to provide specific mechanistic insights into the myriad roles and causal relationships played by metabolism in cancer development, as well as its selection and progression.

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Part Five

Biomarkers for Detecting Premalignant Effects and Responses to Protective Therapies during Critical Windows of Development

16

Circulating Molecular and Cellular Biomarkers in Cancer

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16.1 Introduction

Circulating cancer biomarkers are molecules found in blood or other biological fluids that derive from cancer cells or are produced by normal cells in response to the development of a cancer. The availability of highly selective biomarkers can have a great impact on the early detection of cancer, the follow-up of the disease, and the monitoring of the response to therapy. Cancer biomarkers detectable in body fluids, so-called liquid biopsies, have the great advantage of being identified by minimally invasive methods compared with markers found in cancer tissues, which require invasive practices, such as biopsies, surgical excisions, or needle aspirates, to obtain a tumor sample. The latter procedures, in addition, are not always practicable, for example, if the tumor is not easily accessed or has to be frequently monitored to follow the response to therapy.

In the middle of the nineteenth century, Jones [1] described the first cancer biomarker, showing that high levels of immunoglobulin light chains were present in the urine of 75% of multiple myeloma patients. Since then, several cancer biomarkers in different body fluids, mainly proteins, enzymes, and hormones, have been described for different tumors and some of them are used in clinical practice (a list can be found at the National Cancer Institute website link: http://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-fact-sheet). However, so far, neither biomarkers valid for every type of cancer have been described nor every cancer has been associated with a specific marker. Moreover, even cancer-specific biomarkers may fail to be present in all the patients suffering from that cancer, and in some cases their expression can be induced by noncancerous pathologies.

For these reasons, the search for highly specific and selective biomarkers is a field in constant development, including the improvement of minimally

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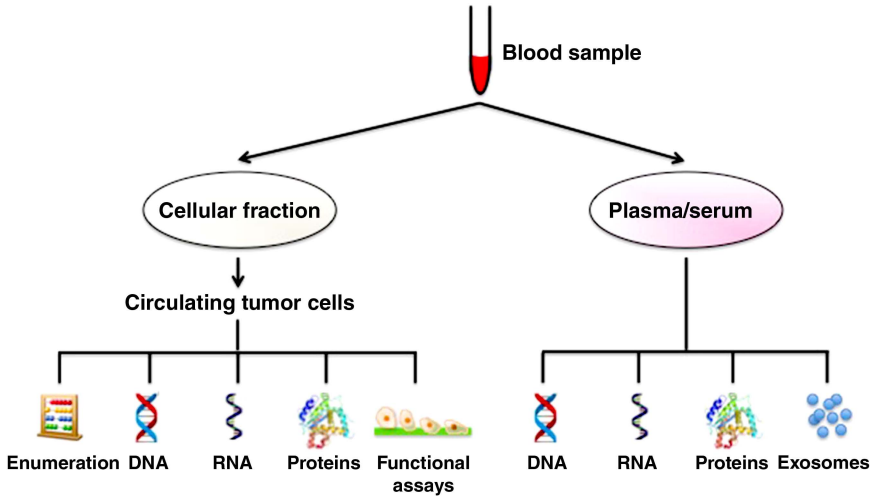

Figure 16.1 Sources of cancer biomarkers in blood. Blood plasma and serum are sources of nucleic acids and proteins, which can be directly extracted and analyzed. Exosomes can also be purified from the blood, counted, and then analyzed for their content. From the blood cellular fraction, cancer tumor cells can be isolated. Enumeration of these cells can give information on patients' disease status, their molecular characterization can highlight the genomic alterations occurring in the tumor of origin, as well as its expression profiles both at the proteomic and transcriptional level. Moreover, functional assays (e.g., invasion capacity) can clarify their biological role.

invasive and highly efficient techniques for their detection. To this point, not only new plasma proteins have been identified as cancer markers but also proteomic approaches have been developed for the generation of circulating plasma signatures possibly predicting tumor outcome [2]. Moreover, the identification of genetic and epigenetic changes specifically occurring in cancer cells has widened the area of cancer biomarkers, and the possibility to detect these changes not only in tumor tissues but also in circulating cell-free nucleic acids (cf-NAs), exosomes, or circulating tumor cells (CTCs) has opened new paths for cancer diagnosis and follow-up. The aim of this review is to give an overview on cancer proteomic, genetic, epigenetic, and cellular markers detectable in body fluids, in particular in blood, and on their possible clinical relevance (Figure 16.1).

16.2 Proteins in Body Fluids: Potential Biomarkers

Proteins present in body fluids have been extensively investigated since the pioneering work of Henry Bence Jones [1], who found a myeloma marker in urine and validated it as a diagnostic marker of multiple myeloma (known a

century later as the "Bence Jones protein") [3]. To date, few blood protein cancer biomarkers are widely used in clinical practice, such as PSA (prostate specific antigen) for prostate carcinoma, α-fetoprotein for hepatocellular carcinoma, and CEA (carcinoembryonic antigen) for colorectal cancer; thus, there is an extreme need for specific cancer biomarkers. The clinical use of circulating protein biomarkers also includes human epididymis protein 4 (HE4) that is now considered (when expressed at high level) a strong and independent indicator of poor prognosis in epithelial ovarian cancer patients. HE4 may be compared with the classical marker CA125 [4,5] and progastrin-related peptide (ProGRP) that assumes a clinical diagnostic value when evaluated by a standard detection kit to measure its overexpression in small cell lung cancer patients with poor prognosis, who are usually diagnosed only at an advanced stage [6,7].

For a long time, the standard assays to evaluate the presence and nature of proteins in body fluids have been two-dimensional gel electrophoresis and ELISA. The further development of advanced techniques such as liquid chromatography–mass spectrometry (LC–MS) allowed the rapid quantitative evaluation of single protein levels in body fluids, thus making possible the identification of robust cancer biomarkers [8–10]. Technical advances in MS, such as MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and SELDI (surface-enhanced laser desorption/ionisation)-TOF MS, have enabled high-throughput proteome analysis [3,11].

As reviewed by Wu and Qu [10], cancer serological protein markers (often low-molecular-weight proteins secreted into the bloodstream) are present in low amounts in blood (estimated concentration: pg to ng/ml), so their analysis suffers from several difficulties. Indeed, the use of blood is biased by the fact that 99% of total serum proteins is represented by 22 abundant, constitutive proteins [12], which mask less abundant proteins that can be isolated only by a labor-intensive procedure. Innovative strategies have been developed to bypass this problem, focusing on proteins such as albumin and transferrin that are not only very abundant in serum but are also able to bind other proteins.

Albumin can be isolated from blood samples by immunoaffinity devices, allowing the characterization of the so-called albuminome [13]. As an example of albuminome exploitation, MALDI-TOF MS profiling of peptides bound to albumin for early detection and staging of hepatocarcinoma provided useful information about the stage of liver tumor development [14]. However, due to the various protocols of albuminome purification, consisting in sequential steps followed by the analysis of the single components, there is still an active search for an unequivocal and reproducible procedure [15]. Transferrin has also been exploited for this purpose; the use of a resin-capturing transferrin in blood samples allowed the isolation of transferrin-bound proteins to be further characterized in a proteomic workflow [16]. For example, this approach has been used to analyze transferrin and associated proteins isolated from serum samples of normal controls and breast cancer patients by label-free mass

spectrometry; few candidate markers were identified, among which the most promising fibronectin and fibrinogen were differentially expressed in serum from advanced (but not early) breast cancer patients and healthy donors [16].

According to the "Updated Guidelines From the European Group on Tumor Markers" [5], different categories of protein cancer biomarkers are of interest: (i) screening/diagnostic biomarkers, for early detection of cancer in asymptomatic people; (ii) prognostic biomarkers, for classifying patients on the basis of selected therapy and estimation of disease outcome; and (iii) surveillance/predictive biomarkers, for disease monitoring and treatment response. Representative examples of each category within the context of circulating biomarkers will be described.

16.2.1 Diagnostic Protein Biomarkers

An ideal biomarker should detect the disease at a very early stage, based on the assumption that the earlier the neoplastic lesion is detected, the better the clinical outcome is; thus, the identification of biomarkers with a predictive value seems to be instrumental for diagnostic surveys, especially for cancers causing a high mortality rate, such as colorectal cancer. This issue has been addressed, for example, by a prospective proteome analysis performed on plasma samples collected before the diagnosis from individuals at risk of colorectal cancer. In this population, compared with matched controls, eight circulating proteins, namely, apolipoprotein C-II, complement components C4-B and C9, clusterin, α-2-HS-glycoprotein, mannan-binding lectin serine-protease, mannose-binding protein C, and *N*-acetylmuramoyl-L-alanine amidase, were identified as early diagnosis candidate biomarkers [17]. Further analysis of the data revealed that the most promising biomarker was the secreted form of the glycosylated protein clusterin (also known as TRPM-2, testosterone-repressed prostate message), being its elevated blood expression associated with a high risk to develop colorectal cancer. However, this association was found only for men, and cannot be generalized, thus indicating that sex-related/hormone-dependent factors could influence the final outcome, adding a further level of complexity to the identification of circulating protein cancer biomarkers [17]. A screening for colorectal cancer biomarker candidates both in tumors and blood focused on glycoproteins identified a multiprotein "signature," represented by ceruloplasmin, serum paraoxonase/arylesterase 1, serpin peptidase inhibitor, clade A, leucine-rich alpha-2-glycoprotein, and tissue inhibitor of metalloproteinases 1 [18], having a diagnostic [18] and prognostic [2] value for colorectal cancer.

A cohort of patients monitored for atrial fibrillation and free from major neurological symptoms were screened for central nervous system (CNS) cancer prognostic biomarkers. Intriguingly, 3 out of 191 patients exhibited high serological levels of neuropeptide Y and S100 B calcium-binding proteins

and secretagogin; 2 of them developed malignant gliomas 1 year later, thus suggesting that elevated levels of these biomarkers could predict CNS tumor development [19].

16.2.2 Prognostic Protein Biomarkers

Serum protein profiling aims to detect circulating biomarkers not only to improve diagnosis but also to predict outcome for cancer patients. For colorectal cancer, the routinely used blood marker to monitor tumor development and treatment response is the CEA. Because of the fact that CEA levels are also influenced by multiple factors besides tumor development, an active search for additional reliable colorectal cancer-specific circulating markers is done. Protein profiling of sera from colorectal cancer patients compared with healthy individuals identified five proteins that were distinctive of colorectal cancer patients, including apolipoproteins A-1 and C-I, even more sensitive than CEA in detecting early phases of colorectal cancer [20]. However, when colorectal cancer protein profiles were compared with those of other cancer types (breast, ovarian, and prostate carcinoma), no net specificity for colorectal cancer was found, thus lowering the impact of these data on the search for specific colorectal cancer markers [20].

The protease inhibitor SPINK1 (serine peptidase inhibitors Kazal type), also named TATI (tumor-associated trypsin inhibitor), was found to be overexpressed in body fluids from cancer patients; its increased serum concentration compared with normal samples was recorded in a number of tumors, including bladder, breast, colorectal, hepatocellular, ovarian, pancreatic, and prostate cancer (reviewed in Ref. [21]). The enhanced expression is often associated with poor prognosis [21]; for example, the analysis of SPINK1/TATI in serum from colorectal cancer patients before and after surgery revealed a correlation between its elevated levels and a poor outcome [22]. For these reasons, SPINK1 can be considered as a valuable cancer biomarker possibly with a prognostic value. A study aiming at identifying specific serum biomarkers in patients with and without colorectal cancer recurrence [23] reported the presence of nine protein species (not yet identified) differentially expressed in patients with colorectal cancer recurrence; of course, this set of preliminary data requires further confirmation.

The search for circulating biomarkers for esophageal squamous cell carcinoma revealed an optimal diagnostic potential for secreted clusterin (sCLU), above described as a colorectal cancer diagnostic marker. In fact, comparing its concentration in serum from normal subjects and cancer patients, it was evident that cancer patients overexpressed serum sCLU compared with healthy controls; of note, the analysis of patients before and after surgery established a correlation between elevated sCLU and better prognostic value [24]. This evidence is in disagreement with a previously published paper showing a

decreased concentration of CLU in serum from esophageal squamous cell carcinoma patients [25], pointing to a need for comparable and reproducible analytical procedures.

Elevated levels of the glycoprotein angiopoietin-like 2 (ANGPTL2) were measured in serum from patients affected by gastric cancer compared with healthy subjects [26]. Promising data came from the analysis of ANGPTL2 in samples representative of progressive disease stages, suggesting a significant correlation between a high biomarker concentration and advanced tumor progression [26]; however, it should be noted that high circulating ANGPTL2 levels have also been described in noncancer diseases [27].

In breast cancer, a panel of five serum protein biomarkers (apolipoproteins A-I, C-I, and H, complement protein C3a-desArg, and transthyretin) was identified, which can discriminate between serum from women with breast cancer and healthy controls. Of note, the prognostic value of the five protein markers seemed to be restricted to women with estrogen receptor (ER)-negative tumors, which are characterized by their low expression level and a more favorable prognosis [28].

HMGB1 protein (high-mobility group box 1), a well-known mediator of inflammation overexpressed in tumor tissues, has been proposed as a potential circulating biomarker for ovarian cancer by comparing its levels in serum from patients with epithelial ovarian cancer, patients with benign ovarian tumor, and healthy individuals [29,30]. In fact, increased serum HMGB1 levels were found in patients with ovarian cancer and also in patients with ovarian cancer recurrence, thus identifying HMGB1 as a biomarker of tumor invasiveness and poor prognosis [29–31]. These data are in line with the various results on a panel of cancer types, supporting the general positive correlation of HMGB1 with tumor development and poor outcome (reviewed in Ref. [32]).

Blood-based proteomic profiles were used to detect lung cancer biomarkers in clinically relevant cohorts [33]; the study identified a panel of proteins, including the matrix metalloproteinases MMP7 and MMP12, carnosine dipeptidase CNDP1, carbonic anhydrase CA6, C-reactive protein, complement component 9, and SERPINA3, all implicated in tumor proliferation, stress response, and inflammation. In this analysis, the authors also focused on the reliability of the well-established lung cancer marker HSP90, detected at high levels in the serum of lung cancer patients. However, the distribution of HSP90 in the healthy control group revealed a high preanalytic variability due to sample handling (hemolysis, bacterial protein contamination, and degradation) that could impair the significance of HSP90 as biomarker [33].

Circulating biomarker panels have been researched in patients affected by CNS tumors; few reliable biomarkers have been so far identified in blood from glioblastoma multiforme patients, including matrix metalloproteases, coagulation, and angiogenic factors (reviewed in Ref. [34]).

16.2.3 Protein Biomarkers of Drug Response

To monitor the efficacy of cancer treatments, patients' blood samples are usually collected before, during, and after the standard therapy, in order to search for circulating protein biomarkers modulated by cancer treatment and possibly acquiring a predictive value of clinical outcome.

Serum from non-small cell lung cancer patients was collected before and after the treatment with the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitors erlotinib and gefitinib. A significant correlation was found between the expression of EGFR ligands TGF-α and amphiregulin and disease progression/overall survival after drug administration, facilitating the classification of patients into subgroups on the basis of the differential outcome after drug treatment [35]. A similar study revealed that high baseline plasma levels of amphiregulin (but not of TGF-α) are associated with adverse prognosis in patients with advanced non-small cell lung cancer [36].

The serial analysis of colorectal cancer patients treated with the first-line drugs oxaliplatin and capecitabine identified apolipoprotein A-I as a potential predictive biomarker for chemotherapy response, being its serum levels lower in responding than in nonresponding patients. This may indicate that the change in serum levels of apolipoprotein A-I could be useful to monitor the efficacy of chemotherapy [37]. The analysis of biomarker changes in response to the TRC105 antiangiogenic monoclonal antibody (raised against endoglin) was recently carried out in plasma from patients with different advanced solid tumors [8]. As expected, some circulating angiogenic factors (Ang-2, PDGF, AA, PDGF-BB, and VEGF-D) were significantly decreased by the treatment, thus suggesting that their monitoring could be used to modulate therapy.

Most studies on circulating cancer biomarkers are performed in serum or plasma; however, for cancers involving kidney and urinary systems, urine is the best fluid to analyze using a modified proteomic tool, high-throughput peptidomics. This technology examines endogenous protein fragments instead of intact proteins, given that mainly in urine peptides are created by the action of metabolic enzymes, including proteases [38,39].

In conclusion, many hurdles still exist in this field, mainly due to a low specificity and sensitivity of circulating protein markers; so, integrated omics studies on proteome and peptidome have to be applied, sustained by associated bioinformatics tools.

16.3 Circulating Cell-Free Nucleic Acids

Cf-NAs were detected for the first time in human blood by Mandel and Metais in 1948 [40]. However, the link between this discovery and cancer became clear many years later when, in 1994, two independent groups detected circulating DNA fragments bearing cancer-associated mutations in cancer patients [41,42].

Cf-NAs, which include DNA, mRNA, miRNA, and ncRNA, are generally extracted from serum or plasma to reduce contamination with blood cell nucleic acids. They are mostly released into the circulation by cells that undergo cell death, mainly by apoptosis or necrosis. The level of circulating cf-NAs is commonly higher in cancer patients than in cancer-free individuals, thus making this same parameter a possible tumor marker [43]. Nevertheless, other pathological conditions can increase cf-NA blood levels, indicating that further analyses of this marker are important to disclose its relationship with cancer [44].

16.3.1 Circulating Cell-Free Tumor DNA

Circulating tumor cf-DNA (hereafter abbreviated as ct-DNA) is released not only by primary tumor cells but also by CTCs or micrometastasis [45]. Cell-free DNA fragments have a length ranging between 150 and 200 bp and up to more than 20 kb [46]. The length of the short DNA fragments is compatible with the internucleosomal cleavages occurring during apoptosis; in fact, circulating nucleosomes can also be detected in the blood [47]. In a recent paper, Snyder *et al.* [48] have shown that deep sequencing of circulating cf-DNA gives a genome-wide map of *in vivo* nucleosome occupancy, which can inform on the tissue of the origin of the DNA and, in the case of ct-DNA, of the tumor.

Ct-DNA can be a very early tumor marker considering that an amount of DNA sufficient for its isolation and characterization can be released into the blood by tumors below the detection limit of radiological examinations, that is around 50×10^6 cells [49]. Cf-DNA generally remains in the circulation for about 15 min, but can last up to a few hours, and is then cleared by the kidney and the liver [50]. The rapid turnover of the ct-DNA allows monitoring tumor changes over days rather than long time intervals, as is typical with biopsies [51].

The analysis of the cf-DNA as a tumor biomarker can have different endpoints, as the search for cancer-specific mutations, including copy number variations and chromosomal aberrations, microsatellite instability and loss of heterozygosity (LOH), DNA integrity, and epigenetic changes. In all cases, ct-DNA studies require highly sensitive techniques, given that ct-DNA is diluted in the circulating cf-DNA by the DNA released by normal cells (for a review, see Ref. [52]). PCR-based techniques, and more recently digital PCR, are mainly used for the analysis of the ct-DNA, together with next-generation sequencing (NGS) technologies, which provide information on wide genomic regions without *a priori* knowledge of the genomic alterations to look for. From the clinical point of view, ct-DNA can be used for the detection of the disease, to monitor tumor burden, cancer heterogeneity, the response to therapy, and the minimal residual disease, as well as the possible development of therapy resistance. The possibility of multiple and serial samplings of the ct-DNA, simply using blood samples, is clearly an added value for the determination of these parameters.

16.3.1.1 Cf-DNA Integrity, Microsatellite Instability, and LOH

Ct-DNA features not related to a specific type of tumor could be used as a general marker for cancer detection. The size of cf-DNA fragments could be one of such parameters, linked to the fact that cancer cells probably undergo different types of cell death besides apoptosis, which is the main type of death in normal cells [53]. Nevertheless, controversial results have been reported regarding its possible relationship with cancer. A large body of evidence suggests that in breast cancer the ratio between longer and shorter DNA fragments is higher in cancer patients than in normal individuals [54–57]. In these studies, the length of blood ALU and/or LINE (transposable) DNA repetitive elements was determined by quantitative PCR, and the ratio between longer and shorter fragments was found not only to be higher in patients than in normal individuals but also as a possible marker of breast cancer progression toward the metastatic stage. Moreover, Iqbal *et al.* [57] found a decrease in DNA integrity after surgery in breast cancer patients. Similar results have also been described in other types of tumors as, for example, colorectal cancer and nasopharyngeal tumors [58,59].

However, discrepant results have also been reported showing a higher proportion of small DNA fragments from malignant cells than from normal ones (60,61). Recently, Jiang *et al.* [62] analyzed the size of circulating DNA in hepatocellular carcinoma patients by massively parallel sequencing. To distinguish between DNA derived from cancer cells and normal cells, the authors identified chromosome arms either amplified or deleted in cancer cells, postulating that tumor DNA fragments should have been more represented among the DNA fragments derived from the tumor-amplified regions, while the fragments originated from normal cells should have been more represented among the fragments derived from the tumor-deleted regions. Using this approach, Jiang *et al.* [62] found that the shortest fragments preferentially derived from tumor DNA. The different techniques used in the different studies could be at the basis of contrasting results obtained; the application of multiple approaches at the same circulating DNA samples could help resolve this controversy.

LOH and microsatellite instability have been successfully detected through PCR-based methods in cf-DNA from different tumor patients [63]. However, the presence of these markers can be masked by wild-type cf-DNA, making difficult to obtain clear-cut results [45].

16.3.1.2 Tumor-Specific Genetic Alterations

It is well known that tumors bear a high load of gene mutations. For some tumors, driver mutations in specific genes have been identified, which are used in the clinic to aid in diagnosis, determining therapy and prognosis. *BRAF* mutations in melanoma patients [64], *EGFR* mutations, *BRAF* mutations and *ALK* gene rearrangements in non-small cell lung cancer [65], *KRAS* mutations

in colorectal cancer [66] and Her2/neu overexpression and *PIK3CA* mutations in breast cancer [67] are examples of such genetic lesions. The presence of these genetic alterations is generally determined in primary tumor tissues or metastasis; however, a large body of studies has shown that these mutations can also be detected in ct-DNA.

In a prospective study, Beaver *et al.* [68], screening for the presence of common *PIK3CA* mutations in DNA extracted from early breast cancer specimens and matched plasma samples, showed that 93.3% of the mutations found in tumors were also detected in plasma DNA by droplet digital PCR, indicating a high sensitivity of this approach. The approach seemed also very specific, given that no *PIK3CA* mutations were found in 10 plasma DNA samples from healthy individuals. Ct-DNA can also be a highly sensitive and specific biomarker for metastatic breast cancer [69] and to follow tumor response to therapy and relapse [70,71].

In a retrospective study, Olsson *et al.* [72] evaluated the possible use of serial analysis of cf-DNA for an early detection of metastasis, studying 20 women with primary breast cancer and a long follow-up. After a low-coverage whole genome sequencing of primary tumors, they highlighted the presence of ct-DNA in patients' plasma samples by quantifying tumor-specific rearrangements using droplet digital PCR. After surgery, the reappearance of ct-DNA could accurately distinguish between patients with or without clinical recurrence. In patients with a long-term free survival, ct-DNA was not detected, while in 86% of patients with recurrence, ct-DNA detection anticipated the clinical diagnosis of metastasis by months to years. Moreover, ct-DNA levels were prognostic for a low survival. Despite the limited size of the patient sample analyzed, this study clearly shows that ct-DNA is a feasible marker for cancer patient management.

Patients' specific mutations in ct-DNA have also been proved to be good markers for surveillance and prognosis in different types of tumors, among which are melanoma [73,74], pancreatic cancer [75–78], gastrointestinal tumors [60,79–82], non-small cell lung cancer [83,84], and gynecological tumors [85].

Detection of a spectrum of tumor rearrangements in serial samples of plasma DNA can give a picture of tumor genetic heterogeneity that cannot be represented in a single biopsy [86]. The feasibility of this approach was demonstrated in a proof of principle study on a breast cancer patient with bone and liver metastasis at diagnosis [86]. DNA samples from primary archival tumor and metastasis specimens, and several plasma samples collected before and during therapy with an AKT inhibitor, were analyzed by massively parallel sequencing using a platform of 300 cancer genes. Not all the mutations identified in the metastasis were found in the primary tumor, while all the mutations detected in the primary tumor and/or in the metastasis were found in the ct-DNA, indicating that a deep-coverage mutational analysis of ct-DNA can give a faithful representation of the mutational landscape of tumors and

metastasis. Moreover, after therapy, an increase in the fraction of mutant alleles was observed earlier than radiological assessments showed disease progression, giving further support to the possible use of ct-DNA to monitor tumor burden.

In a large study on 640 patients affected by different tumors at different stages, Bettegowda *et al.* [87] confirmed the usefulness of ct-DNA as tumor marker. They found circulating DNA bearing tumor-associated mutations, either point mutations or rearrangements, in more than 75% of the patients with advanced tumors and about 50% of those with primary tumors. Gliomas were characterized by the lowest levels of ct-DNA, with only 10% of the patients positive for this marker, possibly because the blood–brain barrier hampers ct-DNA entry into the circulation. Looking for rearrangements in CTCs, the authors analyzed blood cells and the plasma from the same patient. They found that in all the cases in which rearrangements were detected in the cell compartment, they were also detected in the plasma, but the reverse was not true. This suggests that ct-DNA could be a better marker than CTCs; however, it must be noted that in this study CTCs were highly diluted by patients' normal blood cells, since no CTC enrichment was performed.

16.3.1.3 Tumor Genetic Alterations and Therapy Resistance

Ct-DNA genetic profiling can be highly useful for the identification of mutational mechanisms associated with the acquisition of therapy resistance. Serial sampling of patients' blood after therapy offers the opportunity to monitor tumor evolution while avoiding repeated biopsies. Murtaza *et al.* [88] followed six patients with advanced breast, ovarian, or lung cancers over 1–2 years. Performing exome sequencing on two to five plasma samples for each patient, the authors were able to identify an increased fraction of mutant alleles that are known to be associated with resistance to drugs as cisplatin, tamoxifen, and trastuzumab.

In a subset of tumors, the identification of mutations causing the constitutive activation of proto-oncogenes that drive tumor growth has led to the development of therapies that specifically target the activated oncogene. However, the appearance of tumor clones resistant to the drug frequently occurs either because of counteracting mutations in the target gene or in genes that reactivate the targeted pathways. The analysis of ct-DNA has been proved to be effective for the elucidation of resistance mechanisms, which can give indications for further therapeutic approaches. For example, in melanoma patients carrying the $BRAF^{600}$ mutation and not responding to BRAF inhibitors, plasma ct-DNA was detected bearing mutations in *NRAS* (Q61R mutation), which could confer therapy resistance [89,90]. Moreover, in some patients detection of circulating *NRAS* mutations preceded the radiological detection of disease progression [90].

EGFR receptor is activated in a subset of different tumors, and ct-DNA analysis has been able to highlight distinctive mechanisms of resistance to anti-EGFR therapies. In lung cancer patients carrying druggable activating *EGFR* mutations,

Oxnard *et al.* [91] detected plasma circulating *EGFR* DNA fragments bearing the T790M mutation, which confers resistance to the drug. The finding of the circulating T790M mutation anticipated the radiological detection of the disease up to 16 weeks. Mutations in *KRAS* codon 12 or 13 were also found in circulating DNA from lung cancer patients not responding to anti-EGFR therapy [92], as well as in colorectal cancer patients who were resistant to anti-EGFR therapy [49]. Using plasma DNA next-generation sequencing, Mohan *et al.* [93] found different mechanisms of resistance to anti-EGFR therapy, including an increase in *KRAS* copy number or focal amplifications of *MET* or *HER2*. *HER2* amplification was also found in circulating DNA from colorectal cancer patients treated with an anti-EGFR antibody-based therapy [94].

16.3.1.4 Tumor Epigenetic Alterations: DNA Methylation

A change in the DNA methylation pattern, with a general demethylation of the genome and the hypermethylation of CpG islands, is a peculiar feature of almost all cancers [95]. Methylation of tumor suppressor gene promoters, and the consequent transcriptional silencing, frequently occurs early in cancer development, thus being a possible early cancer marker. Methylation is a stable DNA modification that can be detected in cf-DNA. Cancer-associated methylated DNA circulating in blood is actually a promising biomarker for cancer initiation, progression, and response to therapy [96,97].

Methylation sensitive restriction enzymes can be used to analyze DNA methylation, although sodium bisulfite DNA treatment is the method of choice. Sodium bisulfite converts cytosine, but not methylcytosine, to uracil, leading to a modified DNA that can be analyzed by PCR with primers specific for the methylated and the unmethylated DNA, or by DNA sequencing, including next-generation sequencing technologies [98,99].

Despite the fact that methylation of several gene promoters is shared by different types of tumors, there are some tumor suppressor genes whose methylation preferentially occurs in specific tumors. Among these, *SEPT9* and *SHOX2* are relevant markers for colorectal cancer and lung cancer, respectively. *SEPT9* was found to be hypermethylated in colorectal cancer tissues compared with normal adjacent tissues and blood lymphocytes and, when analyzed in plasma samples from colorectal cancer patients and healthy individuals, showed a 69% sensitivity and a 86% specificity [100]. Several retrospective studies have shown high sensitivity and specificity of *SEPT 9* methylation when plasma samples of colon cancer patients and healthy individuals were analyzed [96]. However, in a large prospective study performed on more than 7900 individuals programmed for colonoscopy, the sensitivity of the test dropped to 48%, while specificity remained very high (91%) [101], indicating that further technical improvements have to be introduced to make this test more sensitive for screening of asymptomatic individuals. Other possible methylated biomarkers for colorectal cancers are the *ALX4* and *APC* genes [96,102].

In lung cancer, the level of *SHOX2* methylation has been found to correlate with the disease [103]. Analysis of *SHOX2* methylation in body fluids is used to confirm the cancer diagnosis when cytological and histological tests are inconclusive [104]. Despite the fact that its sensitivity is not high enough to be used for cancer screening, optimization of the test, together with the discovery of other markers to be analyzed in cf-DNA in combination with *SHOX2* methylation, can improve the utility of this marker. To this point, *SEPT9* and *DCLK1* promoter methylation in cell-free circulating DNA have been described as potential noninvasive markers for lung cancer [105,106].

Recent studies on breast cancer have found panels of genes whose methylation could be tested in blood samples for breast cancer diagnosis [107,108] or the identification of metastatic breast cancers [109,110]. In a pilot study, Fackler *et al.* [109] showed that the analysis of the metastatic signature in ct-DNA from breast cancer patients faithfully reflected patients' response to therapy.

Promoter methylation of the *O*-6-methylguanine-DNA methyltransferase (*MGMT*) DNA repair gene has been found as a strong marker for tumor response to alkylating agents [111]. Barault *et al.* [112] have recently developed a digital PCR quantification of *MGMT* methylation in plasma cf-DNA, which improves the sensitivity of this biomarker for the prediction of the response to alkylating agents in glioblastoma and metastatic colorectal cancer.

Methylated promoters detectable in circulating cf-DNA have been proposed as biomarkers for different types of cancers (e.g., as gastric cancer reviewed in Ref. [113]; hepatocellular carcinoma [114]), but much work has still to be done to define markers that could be of possible clinical relevance.

16.3.2 Circulating Cell-Free RNA

Besides DNA, also circulating cell-free gene transcripts and noncoding RNAs are found in blood and other body fluids and have a potential role as cancer biomarkers. Although RNA is, generally, easily degraded by nucleases, circulating RNA is stable, being enclosed in membrane vesicles, bound to proteins, or packaged into apoptotic bodies [45,115,116]. Circulating RNAs are mainly analyzed by microarray technologies and reverse-transcription quantitative PCR (RT-qPCR) or, more recently, by next-generation sequencing technologies, such as RNA-seq [116,117]. Among RNAs, miRNAs have drawn particular attention as possible circulating cancer biomarkers.

16.3.2.1 Circulating Cell-Free microRNA

MiRNAs are short single-stranded RNA molecules (17–25 nt long), which regulate the expression of a large number of genes by binding to complementary regions of the target RNAs. MiRNAs repress gene expression either leading to RNA degradation or inhibiting translation [118], but some studies have shown that they can also upregulate RNA translation [119,120]. The expression of

several miRNAs is deregulated during tumorigenesis, and miRNAs can function as oncogenes or tumor suppressor genes, thus being useful for cancer diagnosis and treatment [121]. MiRNAs are released into the blood circulation mainly by cells dying by apoptosis or necrosis, although they can also be actively secreted [117]. Both normal and cancer cells can release miRNAs, as well as cells of the tumor microenvironment. In contrast to ct-DNA, which can be distinguished from normal DNA (thanks to the presence of specific mutations or epigenetic modifications (see above)), miRNAs are identical in normal and cancer cells and what makes them possible circulating biomarkers is their differential level in the blood of cancer patients relatively to healthy individuals. Thus, miRNA amount in plasma or serum must be accurately determined for their possible use in cancer diagnosis and prognosis. The lack of standardization of the multiple steps required to measure circulating miRNA levels, from blood collection and processing to miRNA extraction and analysis, and difficulties in finding proper reference standards are probably the cause of the lack of complete concordance between the results of different studies performed in the same type of cancer [116,122–125]. Optimization and standardization of the analytical techniques are thus required to make miRNAs clinically useful circulating cancer biomarkers, to get the most from their potentially relevant features, among which are their high stability in blood and ease of analysis [126].

MiRNA signatures, that is miRNAs differentially expressed in cancer patients versus healthy individuals, potentially useful for cancer diagnosis and prognosis have recently been reviewed for several types of tumors, as, for example, gastric cancer [127], breast cancer [128–130], melanoma [131], hepatocellular carcinoma [132], pancreatic cancer [133], gliomas [134], and lung cancer [135]. Here, we will cite some of the most recent papers regarding the possible use of miRNAs as cancer biomarkers.

As far as lung cancer is concerned, Sozzi *et al.* [136] and Montani *et al.* [137] proposed a plasma 24-miRNA signature (named MSC: microRNA signature classifier) and a serum 13-miRNA signature (named miR-Test), respectively, for the early detection of this tumor. The two signatures shared five miRNAs. Both groups validated the signatures in large cohorts of about 1000 subjects enrolled in two independent lung cancer screening programs involving heavy smokers and persons older than 50 years, and found that these signatures could distinguish cancer patients from healthy subjects. The sensitivity and specificity of MSC were 87 and 81%, respectively, while for the miR-Test they were 74.9 and 74.8%, respectively. The analysis of these miRNA signatures could parallel, or even precede, patient screening with low-dose computed tomography, the current screening test for lung cancer, which entails high cost and a high rate of false positive results. Further analysis of the two signatures in the same cohorts could possibly help in defining a signature with an increased diagnostic potential and clinical utility.

In breast cancer, several studies have independently identified circulating miRNAs possibly usable as cancer biomarkers (reviewed in Ref. [128]). Recently, Madhavan *et al.* [138], performing a circulating miRNA global profiling approach in metastatic breast cancer patients, followed by validation in two independent cohorts (~400 individuals in total), identified a plasma 16-miRNA signature (miR-141, miR-144, miR-193b, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-215, miR-365, miR-375, miR-429, miR-486-5p, miR-801, miR-1260, miR-1274a) that was associated with overall survival. Moreover, six miRNAs (miR-200a, miR-200b, miR-200c, miR-210, miR-215, and miR-486-5p) could predict the onset of metastasis up to 2 years before clinical detection. Thus, these miRNAs are potential early biomarkers of metastasis in breast cancer patients.

Different miRNAs were found to be deregulated in plasma of triple-negative breast cancer patients relatively to healthy subjects [139]. In particular, miR-16, miR-21, and miR-199a-5p expressions were lower in cancer patients than in healthy controls and increased again after surgery, suggesting that the level of these miRNAs could be used to predict tumor relapse.

Circulating plasma miRNAs associated with colorectal cancer and possibly predicting the cancer stage have been described by Sun *et al.* [140]. The authors, performing a two-stage approach, with the screening of 754 miRNAs in a small number of healthy subjects and colorectal cancer patients from stages I to IV (10 subject for each class) and the subsequent validation in a total of 187 cancer cases followed up for an average of 28 months, and 47 healthy controls, identified four miRNAs possibly useful for colorectal cancer identification and prognosis. In particular, the authors found that miR-96 distinguished stage I–IV colorectal cancer from healthy controls; miR-203 separated stage III–IV colorectal cancer patients from stage I–II, and miR-141 differentiated stage IV colorectal cancer from stage I–III patients. In addition, plasma miR-96 and miR-200b were independent prognostic factors for overall survival. It is worth noticing that miRNA-200b and miR-141 were also found as possible biomarkers for metastatic breast cancer in the study by Madhavan *et al.* [138].

High levels of plasma miR-25 have been found as a potential biomarker for esophageal squamous cell carcinomas in two independent screenings [141,142]. High miR-25 levels were associated with poor survival [142]; moreover, Komatsu *et al.* [141] showed that miR-25 expression decreased in postoperative plasma samples and increased again during recurrence, suggesting that it could be used for patient follow-up after surgery.

As mentioned at the beginning of this section, circulating miRNAs can be found in exosomes and can be extracted from these vesicles after their isolation from the blood. A description of extracellular vesicles and examples of exosomal miRNAs as possible biomarkers of different types of cancer will be reported in Section 16.4.2.

16.4 Extracellular Vesicles: General Features

Cancer cell biomarkers can be present in body fluids not only as free entities but also encapsulated in extracellular vesicles (EVs). As recently reviewed [143,144], cell-derived extravesicles were first isolated in 1946 as coagulation components by high speed centrifugation of platelet-free plasma [145] and further characterized by electron microscopy in 1967 by Wolf [146] as microparticles produced by plateletes ("platelet dust"). Then, two independent groups provided the evidence that EVs were detectable during the maturation of reticulocytes into erythrocytes [147,148]. Nowadays, it is known that EVs are composed of organelle-free cytosol surrounded by a lipid bilayer membrane, generally characterized by the externalization of phosphatidylserine (PS); their diameter ranges from 50 to 150 nm. EVs are released by the cells in the extracellular milieu and have been detected in all biofluids, including urine, blood, saliva, ascites, bile, semen, breast milk, amniotic, and cerebrospinal fluid [149,150]. The active involvement of EVs in many pathological conditions, including cancer, is now widely accepted.

EVs contain (and can transfer to host cells) cell components such as receptors, lipids, proteins, and nucleic acids (non coding RNAs, mRNAs, microRNAs, and DNA) [151], so that they can be defined as "bioactive organelles carrying a wide range of protein and nucleic acid cargoes" [152]. The energy-dependent uptake of EVs by the host cells is extremely rapid and occurs through various endocytic pathways, such as macropinocytosis, phagocytosis, and lipid raft-mediated internalization, depending on the proteins/glycoproteins present on the surface of EVs and target cells. As recently reviewed [150,153], heparan sulfate proteoglycans, lectins and tetraspanins, integrins, and immunoglobulins could regulate the interaction between vesicles and cells.

To organize researchers working in this rapidly expanding field, the *International Society for Extracellular Vesicles* (*ISEV*) was established in 2011 and the *Journal of Extracellular Vesicles* was launched in the same year by Co-Action Publishing. A database named *Exocarta* [154] has been built, within a more comprehensive database named *Vesiclepedia* (http://microvesicles.org) [155]; also *EVpedia* (http://evpedia.info) includes EV-related publications and high-throughput data [156,157].

16.4.1 Classification of EVs

According to the recommendation of the ISEV [158], the heterogeneous EVs can be categorized into three main classes, that is, exosomes, microvesicles (MVs) (also referred to as ectosomes or microparticles), and apoptotic bodies. They differ in their origin, being exosomes derived from multivesicular endosomes, microvesicles from plasma membranes, and apoptotic bodies from the fragmentation of apoptotic cells [149,159].

In 1987, the term exosome was first used by Johnstone *et al.* [160] to describe "small membrane vesicles formed by vesiculation of intracellular endosomes and released by exocytosis", with a cup-shaped morphology and a diameter ranging from 50 to 150 nm. The notion of exosomes as mediators of intercellular communication was acquired later on [161,162].

MVs are larger than exosomes (50–2000 nm) and are released from the plasma membrane. Like exosomes, MVs are now widely accepted as important mediators of cellular cross talk and actors in cancer-related processes including chemotherapy resistance, inflammation, angiogenesis, and metastasis [163].

Apoptotic bodies are formed during the late apoptotic phase, which is characterized by an extreme plasticity of the cytoskeleton, thus favoring membrane blebbing, coupled to nuclear fragmentation into round-shaped bodies, concomitant with protein/DNA degradation [164]. Apoptotic bodies are irregularly shaped, made by a double-layer membrane surrounding a portion of cytoplasm, with a size ranging from 50 and 5000 nm; their destiny is to be rapidly engulfed by phagocytic cells [165]. Of note, given that many nuclear proteins are released into the cytoplasm during apoptosis, they can contain different molecular species of nuclear origin as well as fragments of cytoplasmic organelles [166,167], thus giving rise to potentially dangerous autoimmune reactions [168–170].

The isolation of EVs can be accomplished by differential centrifugation at increasing speed according to the EV size, eventually coupled to filtration or size-exclusion chromatography or sucrose gradient sedimentation; more recently, commercially kits have been developed, based on immunoprecipitation procedures (reviewed in Ref. [150]). The need to develop and apply standardized protocols for sample collection/handling as well as EV isolation/analysis has strongly been recommended [171].

16.4.2 EVs and Cancer

EVs are secreted both by healthy and pathological cells and have been implicated in many disorders, including cancer, based on their general increased amount in the fluids of cancer patients [172]. Cancer cell-derived EVs (often called TDMV, tumor-derived microvesicles, oncosomes or TEX, tumor-derived exosomes) are considered as clinically relevant and noninvasive cancer biomarkers [173] and can be easily isolated (even from a limited volume of fluids) by sequential centrifugations coupled to filtering using commercially available kits, and characterized by microscopic observation. Proteomic analysis of proteins and characterization of the RNAs (especially miRNAs) contained in EVs are powerful approaches to define the content of vesicles in different cancer types and investigate whether their amount/composition could assume a diagnostic/prognostic value.

Serum is typically the most used body fluid to isolate EVs; when collected from patients affected by breast [174,175], ovarian [176] and pancreatic [177]

cancer, and melanoma [178], it was found to contain an exosome amount significantly higher than serum from healthy donors. Many groups reported quantitative alterations of exosomes in other body fluids, such as urine of patients with prostate [179–181] and ascites for colorectal cancer patients [182].

Extensive proteomic characterization allowed the demonstration that exosomal features can discriminate between normal and cancer samples; for example, the comparison of exosomes isolated from the oral fluid of healthy donors and from patients affected by oral cancers revealed a significant difference not only in their number but also in size, being larger in pathological samples [183]. Moreover, specific biochemical markers have been detected in cancer samples, possibly exploitable for early diagnosis. Using sophisticated proteomics techniques, it has been possible to discover that exosomes derived from ovarian cancer cell lines contain proteins and miRNAs different from those isolated from normal cells, providing a "barcode" useful as a diagnostic tool for early detection of ovarian cancer [184]. Another example is represented by the analysis (through Agilent Human miRNA microarrays and qRT-PCR) of exosome-associated miRNA in malignant ascites and intraoperative peritoneal lavage fluids in gastric cancer patients after surgery [185]. Specific exosomal miRNAs were found in fluids from patients with serosa invasion and peritoneal metastasis, thus suggesting that miRNA expression profiles could be used as biomarkers to predict the peritoneal premetastatic phenotype of gastric cancer, a sign of poor prognosis [185]. Analogously, a signature represented by a significantly increased amount of exosomal miRNA-21 and miRNA-146a was identified in the cervicovaginal lavage from patients affected by cervical cancer; this association was especially strong in HPV^+ patients [186].

Exosome-associated proteins (http://www.exocarta.org/) are mainly members of the HSP or tetraspannin family, or belong to the groups of multivesicle related or membrane proteins; in few cases, a possible identification of genuine cancer protein biomarkers has been reported (reviewed in Ref. [187]). For example, a meta-analysis approach revealed that high serum levels of exosomal caveolin-1 are correlated to poor prognosis of patients affected by genitourinary (bladder, prostate, and renal) cancers, thus suggesting that the level of exosomal caveolin-1, if confirmed, could have a predictive value for these diseases [188]. Intriguingly, a proteomic analysis carried out by applying the ExoQuick TC™ procedure revealed the presence of high levels of IAP (inhibitors of apoptotic proteins) (both proteins and mRNA), including survivin, in several solid tumor cell line exosomes, thus suggesting that the evasion of apoptosis typical of cancer cells could also be modulated by EVs [189] and confirming previous data on the relevance of survivin [190]. A survey performed on prostate cancer patients matched with normal donors revealed the presence of survivin within plasma exosomes isolated from both normal subjects and cancer patients, but at a much higher amount in prostate cancer samples [191]. Analogously, the

fraction of exosome-packaged survivin was measured in blood samples from breast cancer patients and found to be higher than that in healthy subjects [192]. These observations suggest that the analysis of exosomal survivin could be a helpful plasma-based diagnostic biomarker for cancer, potentially useful also to monitor treatment efficacy [193]. However, the validation of this marker is far from being firmly established.

16.4.3 EVs as Mediators of Cell-To-Cell Communication

Macromolecules contained in cancer cell-derived EVs not only can be exploited as cancer biomarkers, but can also be transferred by EV themselves into recipients cells and play a role in cancer development and progression. EVs can thus act as mediators of cell-to-cell communication, affecting the phenotype of recipient cells [194,195]. By consequence, the release of EV content, that is, oncoproteins, oncogenes, chemokine receptors, soluble factors, and proangiogenesis proteins, could have an impact on cancer development. For example, exosomes isolated from cancer cell lines are able to promote epithelial to mesenchymal transition (EMT) in recipient cells through the elevated content of factors involved in transdifferentiation, thus conferring more migratory and metastatic power to cancer cells [196,197].

Many investigators focused on EV role in the cross talk between the primary tumor and distal organs through the modulation of the multiple steps leading to metastasis, including prevention of host immune response, stimulation of angiogenesis, intravasation, and pre-/prometastatic niche formation [163,195]. Also, the molecular chaperone HSP90, which is involved in cancer cell invasion, could be secreted via EVs stimulating cancer cell motility and invasiveness through the interaction with tissue plasminogen activator [198]. EVs can be involved in multidrug resistance mechanisms; as reviewed by Gong *et al.* [163], through the horizontal transfer of RNAs and proteins to neighboring or distant cells, EVs can promote tumor development and spread by releasing drug-resistant proteins such as P-glycoprotein (P-gp) and Multidrug Resistance Protein 1 (MRP-1).

The capacity of EVs to include and transfer bioactive molecules during intercellular communication stimulated the idea that EVs can be used as potent gene delivery systems, being natural products, so small and flexible that can cross biological membranes, and able to protect their cargo from degradation [177,199]. Remarkably, stem cells, such as iPSCs, ESCs, hemopoietic, mesenchymal, and neural stem cells, are capable of secreting exosomes that keep the parental phenotype, thus being attractive for developing strategies against various diseases. Given that tumor exosomes sustain cancer development and spread by modulating the host microenvironment, they can be considered as a suitable tool to reduce the advantage of tumor cells once armed with appropriate factors [187,200–202].

16.5 Circulating Tumor Cells

Solid tumors shed part of themselves into the bloodstream in the form of CTCs, which can be used as cancer biomarkers to assess early diagnosis, prognosis, and response to therapy in cancer patients. CTCs originate from different types of solid tumors, both of epithelial origin (such as breast, colon, lung, and prostate cancer) [203] and mesenchymal origin, mainly sarcomas; sarcoma CTCs are still poorly studied [204]. In addition to CTCs, circulating endothelial cells derived from the tumor region are also present in the bloodstream and their role as biomarkers of angiogenesis in different cancer types is under investigation [205].

16.5.1 Two-Step Processing of Blood Samples: Enrichment and Identification of Circulating Tumor Cells

The first and major challenge in the utilization of CTCs is their number; in fact, there are 1–10 CTCs/mL of blood compared with millions of white blood cells and billions of red blood cells, with an overall estimated ratio of $1:10^9$ [206], thus enrichment protocols must be applied to isolate and characterize CTCs.

The enrichment step is replete with pitfalls because it should be performed quickly, in appropriate culture conditions and without any fixative to keep cells alive and suitable for further analysis; at the same time, it should be efficient and accurate, selecting virtually all the CTCs, minimizing the background of blood cells. So far, dozens of enrichment methods have been described, based on biological (commonly defined label-dependent) or physical properties or a combination of them [203,207].

Protein expression-based technologies for CTC enrichment exploit the different origin of carcinomas and leukocytes, epithelial and mesenchymal, respectively; the most common epithelial marker for CTC positive selection is the cell adhesion protein EpCAM, while for the subtraction of white blood cells the CD45 antigen is used (reviewed in Ref. [208]). The "gold standard" method based on the EpCAM marker is the CellSearch® system, to date the only one cleared by the Food and Drug Administration (FDA). CellSearch and other analogous systems, such as MagSweeper™ and AdnaTest®, work with ferrofluid or magnetic beads coated with EpCAM. With a similar technology, MACS® and EasySep® allow the depletion of red and white blood cells from the blood sample [206]. A device for the *in vivo* isolation of CTCs has also been developed, the CellCollector™, an EpCAM-coated wire that is applied for 30 min in the peripheral arm vein of the patient [209]. This method actually improves the CTC rescue, picking out cells from about 1–1.5 l versus few milliliters of standard blood samples; however, since it works with an undefined blood volume, it cannot be used for counting CTCs during the disease follow-up [210].

Many CTC enrichment techniques exploit the difference in specific physical properties between CTCs and normal blood cells [203]. The most common are

based on cell size or density (ISET® filtration system and Ficoll gradient, respectively) and stiffness [211], given that CTCs are larger and stiffer than blood cells. A new device, the Jetta™ microfluidic chip, consists of a single spiral microchannel that allows a size and deformability segregation of single CTCs in chambers [212]. Moreover, all the label-dependent techniques can be coupled to physical methods in order to maximize both the number and purity of CTCs. A good example is the device developed by Brinkmann *et al.* [213]: after removing red blood cells by centrifugation (cell-density depletion), cells targeted with biotin-conjugated EpCAM antibodies are captured on a streptavidin-coated platform encapsulated in a microfluidic chip for maximizing the contacts between target and bait. Similarly, CTC-iChip applies first a size-based enrichment and then a positive or negative label-based cells' selection [214].

All the methods described above show peculiar caveats. For instance, in size- or deformability-based enrichment protocols, a number of CTCs might escape selection from the general population because they are smaller or less stiff. Moreover, when an anti-epithelial marker antibody is used, tumor cells that have undergone EMT can evade selection; on the other hand, being blood cells of mesenchymal origin, the use of these markers increases not only CTCs' rescue but also the background. It is likely that physical-based purification cannot overcome this problem because, after EMT, cells probably have the same stiffness as leukocytes [203]. One way to bypass this issue is to include markers overexpressed in the tumor, but not regulated during EMT, for CTC isolation. Markers fulfilling this feature that have facilitated very good results are as follows: the plastin-3 antigene in colorectal cancer [215], HER2 in breast cancer [216–218], and melanoma-associated antigene (MAGE) family in testis cancer [219]. Moreover, a large body of evidence demonstrates that CTCs showing an intermediate phenotype, displaying partial downregulation of epthelial markers (low EpCAM), and a partial upregulation of mesenchymal markers are endowed with high plasticity and are able to metastasize when injected into nude mice [220].

After the enrichment step, the proportion between CTCs and normal blood cells rises up to $1{:}10^2$–10^3 [206]; thus, for a precise enumeration, the identification of CTCs at the single cell level is required. With label-dependent technologies, the enrichment and identification steps are coupled; otherwise, cells should be collected onto a support suitable for further analysis by immunocytochemistry (ICC) [209,221,222]. The most common antigens used for CTC identification are cytokeratines as epithelial markers, while to recognize leukocytes CD45 is used; other relevant markers can be used in a tumor-specific manner, such as EGFR, PSA, the epidermal growth factor receptor 2, the androgen receptor, the prostate-specific antigen, and N-cadherin or vimentin as EMT markers [223–225]. Due to the high background, a conventional "by-hand" microscopic analysis is unsuitable; automated cell image capturing and identification are routinely performed on devices equipped

with four different fluorescence channels in order to analyze two specific tumor markers, CD45, to exclude blood cells, and nuclei counterstained with DAPI or Hoechst 33342 [226].

16.5.1.1 CTC Number as a Cancer Biomarker

In cancer patients, the mere enumeration of CTCs provides useful information on the disease stage and response to therapy. Patients' CTC status is defined as negative for <5 CTCs/7.5 ml blood or positive when CTCs are ≥5/7.5 ml blood [203,206].

During the first "Advances in Circulating Tumor Cells (ACTC): from Basic Research to Clinical Practice" meeting in 2012, several scientists confirmed the independent prognostic relevance of CTCs, inferred from clinical trials with breast cancer patients [227]. Specifically, a high number of CTCs before chemotherapy predict poor disease-free survival (DFS) and overall survival (OS); in addition, the presence of tumor cells in the blood of patients undergoing chemotherapy denotes an elevated risk of relapse and short overall survival, irrespectively of their amount before treatment [203,227–229]. Therefore, sequential CTCs counting can provide useful information on the treatment efficacy, being an early indicator of response to therapies [228]. In recent years, several reports of prospective studies about the prognostic relevance of CTCs in very large cohorts of breast cancer patients have been published [228–230]. In particular, Bidard *et al*. [229] have collected data on about 2000 patients with metastatic breast cancer from 20 studies performed in 17 European centers and their results confirmed the independent prognostic value of CTC enumeration on DFS and OS; interestingly, serum levels of standard tumor markers, such as carcinoembryonic antigen and cancer antigen 15-3, were not as informative. Currently, many hundreds of clinical trials based exclusively on CTC enumeration are being implemented (registered at https://clinicaltrials.gov/ct2/results?term=CTC&Search=Search).

16.5.2 Characterization of CTCs

16.5.2.1 Molecular Characterization of CTCs

CTC molecular characterization can give a large body of information on the tumor of origin, disease stage, response to therapy, and metastatic potential. Genomic analysis of cancer-specific point mutations or amplifications in single CTCs can provide insights into tumor heterogeneity. However, for genomic analysis of single cells, DNA must be amplified before being sequenced with conventional or next-generation sequencing technologies, but, although substantial improvements have been made, this technique might introduce a bias or false findings [231]. To overcome the amplification issue, populations of CTCs can be used, although this approach goes to the detriment of key information on intratumor heterogeneity [203]. Alternatively, single-cell DNA can be analyzed,

without amplification, with extremely sensitive mutation analysis technologies such as digital PCR to find out tumor specific mutations/amplifications [224,232]. Coupled to CTC immunocytochemistry identification, also fluorescence *in situ* hybridization is sensitive enough to point out DNA mutations in single cells [218,233].

Mutational analysis of CTCs can increase the window on disease status obtained through primary tumor biopsies. For example, only in 20% of primary breast tumors, the *HER2* oncogene is amplified ($HER2^+$) and therefore targeted for treatments, but several groups have reported that approximately a further 30% of patients show *HER2* amplification in distant metastasis and, interestingly, also their CTCs are $HER2^+$ [234,235]. Furthermore, in patients with breast cancer expressing the *ER*, *ER*-CTCs were found; these cells likely escape ER-targeting therapies and, if an alternative therapy is not applied, they might be responsible for the development of ER^- metastasis after several years [222]. Moreover, in metastatic non-small cell lung cancer patients treated with tyrosine kinase inhibitors, a new mutation in *EGFR* gene, which confers drug resistance to tumor cells, has been identified in CTCs [236]. Thus, CTC analyses can detect primary tumor heterogeneity and changes acquired by tumor cells during disease progression, driving toward the best therapeutical choices with the right timing.

Also, the transcriptomic profile of CTCs can be exploited both to monitor disease progression and to evaluate tumor heterogeneity. In a pioneering study, Stathopoulou *et al.* [237] quantified CK19 mRNA in breast cancer patients' CTCs before and after adjuvant chemotherapy: in early-stage cancer patients, the high CK19 RNA levels were significantly reduced after treatment, indicating that CK19 RNA can be a valid marker for monitoring therapy efficacy; on the contrary, in patients with overt metastasis CK19 RNA expression was not informative, being maintained at high levels. It is worth underlining that CK19 can be used as marker both at mRNA and protein level, as mentioned in Section 16.5.2.2. With a technical improvement, for the first time in 2012, Powell *et al.* [238] demonstrated that a single-CTC gene expression analysis is possible, bypassing the issue of leukocytes contamination that can limit the expression profile utilization. The authors performed the analysis on 87 cancer-related genes and demonstrated that 31 of them were highly expressed only in a subset of CTCs, confirming a CTC phenotype heterogeneity, which reflects the intratumor heterogeneity. Markou *et al.* [239] developed a multiplexed PCR array to simultaneously evaluate the expression of six genes, including *CK19* and *HER2*, on pulled CTCs; with this approach some information is lost, for example, sample heterogeneity, but a markers' panel, allows a broad and useful characterization of CTC expression profile.

Recently, the RNA-seq approach has been exploited by Miyamoto *et al.* [240] to determine the expression profiles of single CTCs isolated from prostate cancer patients. CTCs from each patient showed a high grade of heterogeneity

in the expression of several genes. Retrospective analysis of CTCs from patients not responding to the treatment with an androgen receptor (AR) inhibitor revealed that a subset of their CTCs had mutations in the *AR* gene or activation of a noncanonical Wnt signaling pathway, which could account for therapy failure. This paper shows that performing a genome-wide analysis, such as RNA-seq, on single CTCs actually widens the amount of information on the disease progression and response to therapies.

16.5.2.2 Functional Characterization of CTCs

For an exhaustive characterization, CTCs can also be subjected to functional assays both *in vitro* and *in vivo*. The EPISPOT assay has already been used in several clinical studies; it detects tumor-specific proteins secreted by CTCs during *in vitro* culture on a membrane coated with the antibodies of interest [241,242]. CK19, HER2, cathepsin D, and Muc-1 are good examples of biomarkers for breast cancer, and moreover, the releasing of CK19 correlates with an unfavorable outcome [203,242]. CK19 is also a marker for colon cancer, being secreted by colon cancer CTCs [241], whereas PSA and fibroblast growth factor 2 have been used as markers for prostate cancer [243]. Interestingly, the efficacy of drugs on CTCs can be tested in the EPISPOT assay monitoring the number and intensity of the immunospots [244]. The result of this drug screening has been defined as an "oncogram," a very useful tool to set up a personalized treatment [245]. Functional tests to evaluate the invasion capacity of CTCs are especially relevant to assign metastatic potential to these cells. One of these tests evaluates invasion by measuring CTCs' ability to metabolize a fluorescently labeled adhesion matrix [246].

Significant information on CTC features can be achieved from xenotransplantation of patient-derived CTCs in immunodeficient mice. Cell inoculation can be performed immediately after collection or following a short-period of culture, which can increase the cell number but might affect the relative abundance of some subtypes [245]. If inoculated CTCs give rise to metastasis, it means that cells were endowed with high invasive potential; this result has, *per se*, a prognostic value for the assessment of the disease-free survival [203]. Moreover, to find out which organs are targeted by invasive cells and characterize the explanted metastasis are also of utmost importance in characterizing CTCs and their tumor of origin [220]. Recently, it has been demonstrated that CTCs collected from patients with chemosensitive or chemorefractory tumors give rise to metastasis with the same characteristics when injected into mice [247]. Similarly, Baccelli *et al.* [220] showed that breast cancer CTCs generated $EpCAM^{low}MET^{high}CD44^{high}CD47^{high}$ metastasis, implying the presence of circulating cells with an EMT-MET (mesenchymal to epithelial transition) intermediate phenotype. In addition, $EpCAM^{high}$ CTCs obtained from prostate cancer patients were found in mouse bone marrow and spleen, demonstrating that epithelial cells also have invasive ability [248].

Animals xenografted with human-derived CTCs are, *bona fide*, a good replica of the disease of a single patient and could be a model for tumor and metastasis progression. In this view, xenografts might be very appropriate models to apply in personalized medicine, for example, in testing drug efficacy. However, at present, xenograft experiments require a high number of CTCs, thus limiting the application to few patients in advanced stages of cancer [203].

The importance of CTCs as cancer biomarkers is not at issue, but their low concentration in peripheral blood hampers a deep functional characterization of these cells; this drawback might be overcome by the use of stable cell lines derived from blood-collected CTCs. However, since cell culture techniques have been developed to obtain primary cultures and cell lines from tumor/metastasis biopsies, which contain millions of cells, the low number of CTCs limits this approach and, only recently, reports of successfully *in vitro* cultured CTCs have been published [245]. In chronological order, the first CTC primary culture has been established by Zhang *et al.* [233] from breast cancer patients. Although these cells could not be propagated indefinitely, pivotal results were obtained; first, a potential signature of brain metastasis was identified in cultured EpCAM-negative CTCs and, then, brain and lung metastasis were actually detected in mice xenografted with these cell populations. In another study on breast cancer-derived CTCs, oligoclonal cultures were obtained and three out of five were tumorigenic in nude mice. CTC lines were used both for genomic sequencing, which revealed new mutations other than the primary tumor, and for testing drugs against new targets [249]. For *ex vivo* expansion of lung cancer CTCs, Zhang *et al.* [250] applied an innovative and peculiar technique, simulating the tumor environment with a 3D coculture. This has been the first example of *in vitro* culture of CTCs isolated from an early-stage cancer patient, and this detail can probably explain why sequencing of their genome did not reveal additional mutations compared with the primary tumor.

With the aim of establishing a colon cancer CTC line, Cayrefourcq *et al.* [251] performed only a negative selection to deplete blood cells and maintained the collected $CD45^{-}$ cells in nonadherent culture conditions. The resulting cell line, named CTC-MCC-41, was propagated for more than 2 years and is the first CTC-derived cell line described for colon cancer. CTC-MCC-41 cells resemble characteristics of the primary colon cancer of the patient, and show epithelial features with stem cell characteristics, such as an $EpCAM^{low}$ MET^{high} phenotype, tumorigenic potential when xenografted, capacity of inducing angiogenesis, and an osteomimetic signature, suggesting that, *in vivo*, they had been located in the bone marrow before entering again into the bloodstream (see Section 16.5.4). The positive results presented above open the way to new attempts in culturing CTCs of different origin. However, a careful examination of the features of longtime propagated cell lines derived from CTCs is required, given that some morphological and molecular parameters can be altered by a prolonged *in vitro* culture, thus becoming no longer representative of the original cell source.

16.5.3 Single CTCs *versus* CTC Clusters

CTCs are present in the bloodstream as single entities or as clusters, formed by 3–50 cells [206]. Far from being artifacts, CTC clusters, although very rare, represent a resource to implement the utilization of CTCs as biomarkers [252,253]. In the literature, CTC clusters are also called circulating tumor microemboli (CTM); in lung cancer patients, a correlation between the presence of CTM and high incidence of venous thromboembolism (VTE) and pulmonary embolism (PE) has been reported [254]. Among the several methods for CTC enrichment described above, a number of them are able to efficiently catch CTM, such as CellSearch and CellCollector®, based on $EpCAM^+$ fishing, and the filtration-based technology ISET [210,252,255]. The immunocytochemical analysis of breast cancer-derived clusters revealed a high expression of mesenchymal markers and low levels of the epithelial ones [218] and, in xenografted mice, clusters showed a metastatic potential from 23- to 50-fold greater than single CTCs [253]. It has been speculated that, as it occurs in collective migration in normal tissue, one or a few leader cells with mesenchymal properties might drive all the cluster into the journey from the primary tumor to distant organs, where the epithelial cells play a role in metastasis formation [206,256]. In breast cancer, single-cell RNA-seq of single CTCs versus CTC clusters highlighted the overexpression of plakoglobin in CTC clusters, a marker of desmosomes, and adherent junctions [253]. Moreover, both high levels of plakoglobin in the primary tumor and the abundance of CTC clusters predicted a poor overall survival. In agreement with these observations, Aceto *et al.* [253] showed that a breast cancer mouse model knockout for plakoglobin lacked CTC clusters and did not develop metastasis. Taken together, these results suggest that groups of cells sharing high expression of plakoglobin (intratumor foci) might leave the primary tumor as oligoclonal entities and move through the blood in a cooperative manner.

Besides regrouping with each other, CTCs can also cluster with platelets and stromal components. It has been demonstrated that platelets can induce a TGF-β-mediated EMT in breast and colon cancer cells both *in vitro* and *in vivo* [257]. According to this, Yu *et al.* [218] have been shown a positive staining with CD61 platelet-specific marker of $EpCAM^{low}$ MET^{high} CTC cluster. Another possible partner for CTCs is their own soil, that is, the stromal components of the primary tumors; in this case, the function of the cotraveling is to protect tumor cells into the bloodstream, but, especially, to facilitate the colonization of distant organs [258]. At the moment, counting CTC clusters in cancer patients can give information on the disease-free survival after cancer treatment and response to therapies; in perspective, plakoglobin and other adhesion molecules implicated in cluster cohesion might be exploited as therapeutic targets. Obviously, further studies on CTC cluster biology are needed to define their role in different type of carcinomas, for example, in determining the organotropism of metastatic tumors.

16.5.4 In Hiding Before Getting Home, the Long Journey of CTCs

In 2004, Jonathan Uhr's group determined that the half-life of CTCs was 1-2.4 h, counting them just before and at different time points after removal of the primary tumor [259]. It is worth specifying that this measure is referred to CTCs released from primary tumors, whereas CTCs from metastasis might have a different turnover [259]. In the same report, the authors looked for the presence of CTCs in 36 breast cancer patients many years after mastectomy and without tumor relapse or overt metastasis; surprisingly, 13 out of 36 patients had CTCs. The discrepancy between the very short CTC half-life and their presence into the bloodstream decades after the tumor surgical removal might be explained assuming the existence of micrometastasis or niches that continually refill the circulating population of tumor cells [203]. Notably, in patients affected by different types of cancer, tumor cells have been found in the bone marrow, and have been named disseminated tumor cells (DTCs). Cells can be present in this district with or without the development of overt metastasis, therefore, it has been suggested that bone marrow can be, among other organs, a "dormancy-inducing organ" for cancer cells. The presence of prostate-derived tumor cells in bone marrow niches has been demonstrated in a mouse model by Shiozawa *et al.* [260]. Two main conclusions were drawn in this report: first, CTCs directly compete for the niche occupancy with hematopoietic stem cells and drive their terminal differentiation, and second, cancer cells can be mobilized from the bone marrow when standard clinical protocols for hematopoietic stem cell activation are applied.

In mouse models of breast cancer, CTCs stored in the bone marrow have been characterized and transcriptome analysis revealed an osteoblast-like phenotype; it is conceivable that this adaptation does not occur exclusively in the bone marrow, but CTCs can probably gain the phenotype of the organ in which they hide [261]. It is also possible that cells from primary tumors already have specific protein expression signatures that allow them to target specific organs [262]. The latter case is supported by the seminal study on brain metastatic breast cancer (BMBC) by Zhang *et al.* [233], in which the authors identified a potential brain signature $HER2^{+}EGFR^{+}HPSE^{+}Notch1^{+}$ in CTCs of BMBC patients. Subsets of CTCs with signatures that determine an organ-specific homing can actually be the seeds of cancer metastasis; knowing if a patient has or not these particular subsets of CTCs is fundamental in choosing a proper and prompt therapy [233,238].

16.6 Conclusions

The search for tumor-circulating biomarkers is an expanding field. In recent years, more and more evidence has shown that tumorigenesis can be monitored

looking for cancer-associated changes in blood samples, thus avoiding, or reducing, invasive interventions [263]. However, a series of problems are encountered for circulating marker analysis: (i) Studies performed on serum/plasma suffer not only from perturbations in blood collection and processing that may affect reproducibility of the analysis but also from the complex blood dynamic physiological/pathological behaviors. (ii) The evaluation of circulating cancer proteins is limited by their extreme dilution within blood proteins, which implies a not easy and reproducible isolation and identification. (iii) For nucleic acids markers, the nonstandardized steps of extraction, quantification, and normalization are often at the basis of inconsistencies found among different papers, in particular regarding the identification of possible miRNA markers in different tumors. (iv) CTCs are in a very low concentration in peripheral blood, therefore an enrichment of several log units is needed before further analysis; moreover, because of their heterogeneity, tumor-specific multimarkers techniques have to be developed to maximize their recovery.

The growing interest toward the identification of circulating cancer markers stimulated the setting up of more and more advanced procedures to bypass the above problems. Illumina has recently launched a new company whose mission is to look for tumor-specific DNA or RNA in the blood that could reveal the disease before clinical symptoms. Given the high sensitivity of the mutational analysis of ct-DNA, specific cancer mutations could be found prior to the detection of tumor; this could however make difficult an opportune immediate intervention, but could also be of extreme value in patient follow-up and intervention as early as possible. The improvement of MS by MALDI-TOF and SELDI-TOF allowed the development of high-throughput proteomic/peptidomic platforms especially suited for the identification of protein signatures by the analysis of circulating proteins as well as of proteins encapsulated within EVs. Moreover, a specific analysis of glycosylated species, which are significantly modulated in cancer cells, can be made possible through new-generation glycoproteomics platforms [264]. Also, for the enrichment and identification of CTCs, a number of advanced procedures have been developed, starting from the FDA-cleared CellSearch system and the CellCollector medical wire that allows a sensitive positive capture of $EpCAM^+$ CTC cells, up to innovative functional assays for their characterization.

How informative circulating cancer markers are depends on cancer types and stages of the disease [265]. For example, the possibility to look for specific genetic/epigenetic alterations, miRNA expression, protein profiling, and CTC features after surgery or therapy of cancer patients could help monitoring eventual tumor burden and therapy resistance without repeated biopsies or instrumental analysis, and in some cases, even earlier than these clinical approaches. This can be particularly useful when alternative therapeutic strategies can be applied to defeat the tumor. Nevertheless, it has to be

mentioned that, so far, the information retrieved with blood samples are used in a manner complementary to routine clinical and imaging methods.

Based on the results obtained to date "Many single biomarkers may be associated with malignancy. However, no single biomarker has shown sufficient sensitivity/specificity to be considered as a diagnostic tool for the disease." [266]. Ideal biomarkers have to be highly specific to avoid false positives and be able to discriminate among cancers to direct the analysis for diagnosis confirmation and follow-up. Much cooperative work is still required that focuses on the preanalytical, analytical, and postanalytical quality requirements to identify and validate reliable cancer markers. The realization of multicenter studies performed on biological material collected in a standard manner and analyzed with a common procedure will then pave the way to the identification of ideal circulating biomarkers, making "liquid biopsies" a fundamental clinical tool.

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17

Global Profiling Platforms and Data Integration to Inform Systems Biology and Translational Toxicology

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17.1 Introduction

For centuries, biochemistry has provided the underpinnings that aid in understanding biological events and clinical outcomes. From the earliest days of medicine, metabolites – low molecular weight biochemicals circulating in blood or other biofluids – have been an important interpretive tool for clinicians. Centuries ago, the chemical properties of urine aided physicians in the diagnosis of medical conditions. But it was at the beginning of the twentieth century that the English physician, Archibald Garrod, first postulated that human disease was caused by missing or altered steps in the body's chemical pathways, establishing the concept of inborn errors in metabolism that has persisted to date [1]. His work and discoveries ultimately established a fundamental linkage between genetics, metabolic composition, and phenotype.

The connection of metabolism to phenotype was further developed over the next 50 years during the golden age of biochemistry. Leading scientists, including Otto Heinrich Warburg, Gertrude Cori, and Hans Adolf Krebs, sought to understand how biochemistry impacts complex biological processes; for example, muscle metabolism, diabetes, and cancer. Through their research efforts, these scientists were able to create and populate the first metabolic pathway maps. Today, we use this biochemical framework to derive mechanistic understanding from the measurement of metabolites.

The 1944 discovery that genetic information was conferred by deoxyribonucleic acid (DNA), the 1953 discovery of DNA's structure, and cracking of the genetic code in 1966 – showing how the sequence of chemical bases in DNA codes for the synthesis of proteins – opened the door to an explosion of knowledge in molecular biology. In the nucleus of the cell, a gene's DNA serves

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as a template for synthesis of messenger ribonucleic acid (mRNA); mRNA then travels from the nucleus to the cytoplasm and serves, in turn, as a template for the synthesis of a protein molecule. Proteins are involved in virtually every biological function, including cell growth, differentiation, regulation and coordination of physiological processes, metabolism, immune function, disease processes, and death. Gene expression or transcription refers to the construction of mRNA molecules (transcripts) from the template provided by a specific gene.

Knowledge of how gene expression is controlled and coordinated, as well as the roles of specific proteins in cellular functions and in disease processes, has informed our understanding of the modes and mechanisms of action by which chemicals produce toxic effects in cells and whole organisms. The introduction of higher throughput dideoxynucleotide DNA sequencing in 1977 [2] made it feasible to determine the sequences of entire genomes, ushering in the era of genomics. This technology was used in the Human Genome Project, which was proposed in the late 1980s and begun in 1990. The reference human genome sequence – consisting of about 23,000 genes that code for proteins, made up of 3 billion chemical base pairs – was made available in 2000 [3].

Since then, high-throughput genome sequencing has become faster and less expensive. To capitalize on the enormous potential of genome-wide DNA sequence information, new molecular technologies and bioinformatics tools have been developed that make it possible to generate and analyze biological data sets of unprecedented magnitude and complexity [4]. Beyond DNA sequence analysis, postgenomic technologies enable analysis of the transmissible epigenetic modifications of chromatin and DNA (epigenomics); gene expression or the production of mRNA transcripts (transcriptomics); the production and modification of the proteins themselves (proteomics); and the production of metabolites (metabolomics).

The technologies of transcriptomics, proteomics, metabolomics, and epigenomics reflect expression of genes, proteins, metabolites, and epigenetic modifications, respectively. Although genomes are relatively static, transcriptomes, proteomes, metabolomes, and epigenomes are dynamic – displaying time-dependent changes in response to diet, stress, disease processes, and exposure to toxicants and stressors. Consequently, their analysis must be linked to the state and condition of the biologic system under investigation. Organisms and individual cells continuously adapt to perturbations in their environment. Such changes are particularly sensitive to fluctuations in the availability of energy substrates. The cellular transcriptional machinery and its chromatin-associated proteins integrate environmental inputs to mediate homeostatic responses through gene regulation. Numerous connections between products of intermediary metabolism and chromatin proteins have recently been identified. Chromatin modifications that occur in response to metabolic signals are both dynamic and stable and could be inherited transgenerationally [5]. These emerging

concepts have biological relevance across the spectrum of biochemical responses: influencing an organism's ability to maintain tissue homeostasis, to adapt while aging, and to respond to exogenous exposures or toxicity [6].

Exposure to exogenous agents, whether drugs or environmental pollutants, may elicit modifications in specific mRNAs, proteins, and metabolites as well as changes at the chromosome level (i.e., epigenetic modifications). These changes observed under defined conditions of cellular location, dose level, time, and biologic context could provide meaningful information about biologic responses following these exposures. Typically, effects can be detected at subcellular and molecular levels at time points before they are manifested at the level of tissues, organs, or the whole organism. Toxicant-specific alterations in gene expression, protein synthesis, and metabolite production may correspond with observable or phenotypic responses of cells, tissues, and organisms. The process of relating molecular expression data to toxicity and pathology observed in conventional toxicology tests and using histopathological evaluation has been described as "phenotypic anchoring" [7,8].

Advances made since the completion of the Human Genome map and in assessments made using postgenomic technologies have stimulated the emergence of interdisciplinary fields of study known as systems biology and high-dimensional biology (HDB). Systems biology is in essence a discipline that brings together varied data streams holistically to interpret and understand biological events, behaviors, responses, or outcomes [9,10]. High-dimensional biology is a parallel approach, during which multiple omics tools are used simultaneously to study a research question to promote a more comprehensive understanding of a biological system. Advances made in computational and mathematical modeling are used to integrate data streams to develop or understand biological networks; whether these be metabolic networks, cell signaling networks, or adverse outcome pathways [11–13].

This chapter will provide background information on the current state of omics technologies that provide global profiling data across the biological space. Advances made in these areas along with efforts to integrate data streams for clinical translation will provide key perspectives on how these tools and the subsequent findings and analyses can be applied to inform research needs in translational toxicology and therapeutics related to reproduction and cancer.

17.2 Global Omics Profiling Platforms

17.2.1 Genomics

Genomics is the study of any organism's genetic composition, which is comprised of both coding regions, which can be transcribed into RNA, and noncoding regions, which are important in structure and function. The Human

Genome Project and the mapping of the reference human genome is obviously one of the key global omics profiling efforts that has laid the groundwork for those genomics and postgenomics efforts that have continued since its completion in 2000. For the purposes of this chapter, discussion will look beyond this pivotal effort that has been covered in detail elsewhere [14,15] and focus on recent efforts.

Despite being relatively static in nature, particularly when compared to the transcriptome, a human's genetic background can still be an important determinant in health and disease. Single nucleotide variations, (i.e., single nucleotide polymorphisms (SNPs), small insertions or deletions), or structural variations (i.e., large insertions/deletions, copy number variants, inversions) are all known to contribute to genetic mutations, deletions, and duplications as well as aberrant or variable gene transcription – all of which can modulate one's response to drugs or disease [16].

Several technologies, ranging from the original Sanger technique to next-generation (Next-Gen) sequencing, are being actively used in current genomics assessments, including assessments of single nucleotide variants and structural variants in DNA and genome-wide association studies (GWAS). Briefly, Sanger sequencing is a method that uses *in vitro* DNA replication and DNA polymerase to facilitate the selective incorporation of one of four of the standard dideoxynucleotides into a DNA sample, the specific sequence and progress of which is then tracked using fluorescent or radioactive labeling [2,17]. DNA microarrays are also commonly used and involve the hybridization of the DNA sample with a set of predefined oligonucleotide probes distributed across the entire genome or enriched around regions of interest [18]. Next-Gen sequencing methods fragment genomic DNA that is subsequently sequenced and aligned to a reference sequence. Although more expensive than DNA microarrays, which are based on *a priori* knowledge of DNA sequence and variants, Next-Gen sequencing strategies allow the discovery of novel changes as well as a targeted in-depth sequencing at coding and noncoding regions of interest [19].

GWAS are typically undertaken to genotype a cohort of interest and to identify variants associating with a particular trait in a discovery-driven approach. Typically performed using DNA microarrays, an explosion in GWAS in recent years has led to the publication of over two thousand association studies in which over 15,000 SNPs have been linked with various diseases and traits [20]. Unfortunately, they typically do not explain the entire genetic contribution to a particular trait [21]. However, GWAS outputs include a list of SNPs evaluated for their frequency in relation to the trait under study. It bears noting that most reported associations in GWAS are intronic or intergenic, affecting DNA structure and gene expression rather than protein sequence [22].

Although most of the SNPs associating with a certain trait have a small effect size, they provide important clues on disease biology and may even indicate

therapeutic responsiveness or suggest new treatment approaches. A recent effort integrating GWAS findings with clinical outcome data successfully linked lung cancer patient overall survival rates with SNPs associated with platinum-based chemotherapy responsiveness [23]. Effectiveness of platinum-based therapies, widely used across multiple cancer types, has long been plagued with reduced therapeutic effectiveness and resistance, prompting several efforts to utilize systems biology approaches on this issue [24]. Another study identified BCL11A as a gene controlling fetal hemoglobin level. This finding has spurred follow-up studies to characterize the molecular mechanisms behind fetal globin regulation that could in turn lead to identification of therapeutic approaches against beta-thalassemia and sickle cell anemia [25–27]. These GWAS efforts indicate successful approaches that link clinical outcomes and biological processes with traits along with strategies for follow-up functional investigations [28].

17.2.2 Epigenomics

Gene function is not only determined by DNA sequence information, but also by regulatory changes that can occur through alterations at the chromosome level. Epigenetics is the study of these heritable modifications that can lead to changes in phenotype that occur without any change in nuclear DNA sequence. These modifications can lead to significant effects on individual variation in susceptibility and disease, particularly during the vulnerable stages of developing embryo, fetus, and newborn [29,30]. Temporally regulated, epigenetic changes modulate cellular differentiation and responses to environmental stimuli through dynamic and reversible changes in chromatic structure and gene expression. The study of epigenomics to elucidate gene–environment interactions will provide key opportunities to develop gene-specific diagnostic tests and epigenetic biomarkers for a broad range of pediatric disorders, including developmental delay and intellectual disability [31].

The two primary and best documented epigenetic mechanisms are histone modifications (e.g., phosphorylation, acetylation, methylation, etc.) and DNA methylation. Modifications to histones, the proteins around which DNA is stored, can affect access to and transcription of DNA by cellular machinery, resulting in genetically identical cells achieving diverse phenotypes that may be differentially responsive to environmental stimuli [30]. DNA methylation is the most common covalent modification of genomic DNA and also the most amenable to measurement using genomic strategies. This methylation occurs primarily at cytosines within CpG dinucleotide islands, which are disproportionately located at the 5′ regulatory regions of genes [32]. Typically, methylation of these islands leads to transcriptional repression, providing an added layer of regulatory control that cells utilize during developmental stages, in tissue differentiation and to maintain the stability of the genome [31].

Nutritional status can induce epigenetic changes, as folate and vitamins B-6 and B-12 are required cofactors in the DNA methylation reaction. Work in the 1970s first revealed the functional relevance of DNA methylation in the context of cancer [33].

Technologies to measure methylation can be categorized as either gene specific or genome wide. Discovery of methylation hot spots within a larger genomic context can be done using restriction landmark genomic scanning (RLGS). In RLGS, large numbers of methylated residues can be detected using direct end labeling of genomic DNA digested with a restriction enzyme and separated by high-resolution two-dimensional electrophoresis [34]. In methylated DNA immunoprecipitation (MeDIP), purified DNA is immunoprecipitated with an antibody against methylated cytosines, giving rise to genomic maps of DNA methylation that can be screened on oligonucleotide microarrays [35]. The discovery that sodium bisulfite could be used to specifically convert nonmethylated residues to uracil in the 1990s led to the development of several methylation detection techniques. These include whole genome bisulfite sequencing [36], reduced representation bisulfite sequencing (which combines restriction enzymes with bisulfite sequencing for CpG island enrichment) [37], and high-density methylation arrays [38]. In the latter, the methylation state of over 500,000 CpG sites is measured in a single reaction using multiplexed genotyping of bisulfite-treated genomic DNA. Gene-specific methylation techniques use methylation sensitive restriction enzymes to digest DNA before analysis by Southern blot or PCR amplification; sites that were methylated were identified by their resistance to the enzymes. These techniques offer high-resolution analysis that can be complementary to the other techniques.

A recent upsurge in genomic DNA methylation studies has revealed direct links between aberrant DNA methylation and disease, indicating a promising future as a diagnostic tool. In particular, alterations in global DNA methylation profiles have been noted in autoimmune, neurodevelopmental, and metabolic conditions [39,40]. DNA methylation pattern changes in response to drug treatment also indicate the use of DNA methylation as a biomarker for monitoring treatment efficacy, treatment response, and prognostic outcome [41]. Cell-free circulating DNA in blood, demonstrated to retain methylation markers for various diseases, provides a noninvasive way to utilize this epigenetic marker to assess longitudinally treatment response and efficacy. Targeted developments of epigenetic biomarkers for clinical use are now being actively pursued.

17.2.3 Transcriptomics

The transcriptome represents the set of all mRNA molecules present in a biological system at a given time. As such, it represents the genes that are being actively expressed at that point in time, providing a snapshot in a dynamic system that is constantly changing in response to both endogenous and

exogenous factors. Transcriptomics, the field that investigates global measurements of mRNA transcripts, and the explosion in both discovery-based and targeted technologies along with the computational tools to facilitate data interpretation has ushered in an era that relies on genotype and gene expression data to reveal the underpinnings of xenobiotic effect and disease manifestation [42]. The continual refinement and maturation of these technologies has and will continue to shape our thinking and advances for years to come.

High-density microarrays, a dominant transcriptomic technology for years, consists of a solid matrix support (e.g., glass, quartz, silicon) to which complementary DNA (cDNA) are immobilized [43–45]. RNA isolated from a biological sample and amplified using polymerase chain reaction (PCR) technologies is labeled with a detection tag (e.g., biotin, fluorescent) prior to incubation with the support or array. After washing and processing, sample RNAs that hybridize to cDNAs on the arrays are detected and quantitated using a laser-based scanner. Relative gene expression levels are determined after a series of normalization and background adjustment steps, the nature and extent to which will vary depending on the specific platform. The results of microarray assays have been shown to correlate well with measurements of the expression of single genes by methods such as quantitative real-time polymerase chain reaction (qRT-PCR); a low-throughput technology believed to represent a gold standard approach in mRNA quantitation [46]. Microarrays corresponding to essentially all known human genes and representing the genomes of animal models used in conventional toxicology are widely available commercially.

The refinement and maturation of microarray technology has facilitated dramatic advances in its capabilities and confidence in the approach as a transcriptomic tool over the years. Implementation of shorter oligonucleotide probes has increased the microarray capabilities, allowing over a million probes to be spotted per array. Standardization of sample preparation, establishment of reproducible protocols, and methods for data normalization and analysis were instrumental in its adoption. However, these efforts have also identified technological limitations. The dynamic range in the technology is finite, limited by the detection strategies utilized as well as issues with probe set binding. Also, ultimately, the gene expression measures are relative rather than absolute values, and at low expression levels, microarrays often have poor resolution of transcript binding due to issues of background and nonspecific binding. Further, despite development of higher throughput instrumentation and robotics, the high costs and limited throughput to screen thousands of biological samples limit the long-term viability of this technology.

Whole transcriptome Next-Gen sequencing (RNA-Seq) offers an alternative method for estimating transcript abundance and has the potential to overcome many of the limitations associated with microarrays. It does not rely on predetermined probe sequences for expression measurements and is based on simple counting of reads that can be reliably aligned to a reference sequence.

As count data, RNA-Seq has effectively no limit to the dynamic range of signal detection, and, in theory, can provide a higher degree of accuracy and precision in estimating relative expression levels [47]. However, RNA-Seq data also has potential challenges which remain less well explored. Reliable quantification of expression levels appears highly dependent on read depth, and low transcript abundances are characterized by high variance, providing uncertainty regarding the true sensitivity of this technology. Additionally, methods for normalization and statistical analysis of RNA-Seq data are less mature, and no established best practices exist for RNA-Seq data analyses [47]. However, continual advances in the technology and informatics approaches have greatly lowered the cost of this technology, positioning it to overtake microarrays as the transcriptomic technology of choice.

Several other approaches that have gained attention as providing high-throughput quantitative gene expression profiling make use of oligonucleotide ligation-mediated amplification [48–51]. All of these strategies use oligonucleotide ligation to mRNAs in a sample to elute desired targets prior to PCR amplification, which can be conducted on standard PCR instrumentation. This approach eliminates the need for DNA or RNA extraction from samples, which in turn obviates long-standing issues of RNA recovery and yields with the other technologies. As a result, sample amplification is no longer required, and in some instances single-cell assessments can be conducted. Although providing a more targeted screening approach (i.e., not whole transcriptome coverage), advances in the technology, depending on the platform, are enabling the multiplexing of hundreds to thousands of detector oligonucleotides within a sample. Also, as the technologies are amenable to automation, thousands of samples can be screened, allowing for large-scale interrogation across a range of biological pathways or regulatory gene networks. Two platforms of particular note are (RASL-Seq) RNA-mediated oligonucleotide Annealing, Selection, and Ligation with Next-Gen SEQuencing [50] and the L1000 technology, which performs detection of resulting PCR amplicons of 978 landmark gene targets using a multiplexed, luminex bead-based methodology and flow cytometry [51]. The L1000 is the high-throughput transcriptomic tool used as a part of the National Institute of Health's Library of Network-based Cellular Signatures (LINCS) project. LINCS aims to create a network-based understanding of biology by cataloging changes in gene expression and other cellular processes that occur when cells are exposed to a variety of perturbing agents (http://www.lincsproject.org/). LINCS and the Connectivity Map concept will be discussed in more detail in Section 17.7.

Fueled by the completion of the genome and the increasing standardization and accessibility of microarray technology, gene expression data generation blossomed and, along with it, attempts to discern patterns of expression or informative networks that could aid in predicting disease incidence or therapeutic efficacy. One of the earliest successful efforts to explore existence of a cancer

classification signature focused on differentiation between acute myeloid leukemia and acute lymphoblastic leukemia using gene expression data from bone marrow samples [52]. Using the top 50 genes most strongly statistically correlated with the two cancers as a signature, an additional set of leukemia samples was successfully classified. On the basis of these findings, another group hypothesized that such a disease phenotype signature could be used to identify potential bioactive drugs that could modulate that phenotype, even in the absence of a known drug target [53]. Screening a bioactive compound library for molecules that induced expression of the signature genes [52] in a leukemia cell line indeed resulted in identifying the drug gefitinib as a promising leukemia therapy drug candidate worthy of a clinical trial [54]. Tools to mine gene expression data for signatures and connections across the disease–phenotype–drug continuum have flourished, leading to numerous other success stories in cancer and therapeutic drug discovery [49,55,56].

17.2.4 Proteomics

Proteomics is the study of the complete set of proteins found in a particular cell type or biological system at a given time. Ultimately, the myriad of functions and responses of any living system are controlled by proteins that are themselves subject to a range of modifications and interactions that dictate both the type and extent of these outcomes. Existence of these multiple, modified forms, all of which differentially control protein function, activity, specificity, and stability, make it impossible to adopt a single approach that can comprehensively monitor proteomic changes. In addition, protein adduct formation following either exogenous chemical exposure and reactive intermediate formation or endogenous oxidative stress can also perturb endogenous regulatory protein modifications [57]. Consequently, multiple varied proteomic approaches are needed to adequately explore the relevant functional underpinnings underlying drug action and disease modality.

Exposure to xenobiotics or perturbagens can result in two main types of proteome changes: (1) changes in protein levels due to changes in gene expression, mRNA stability, protein stability, or some combination of these three and (2) changes in the relative levels and interactions of two or more modified forms of a protein, which may be more critical to function than the absolute protein levels [58]. Proteomic assessments can be divided broadly into mass spectrometry (MS) or non-MS strategies. MS approaches are discovery driven, theoretically allowing survey of all proteins present in a sample or complex mixture. Non-MS approaches focus on a targeted subset of proteins known to be critical in a signaling pathway and focus on evaluating protein presence and protein–protein interactions. MS approaches may also incorporate gel-based separation prior to chromatographic separation and use of protein labeling to enable quantitative assessments.

Of the label-free MS approaches, gel-based proteomics provides a strategy that allows visualization of separated proteins that can then be quantitated following image analysis. In this approach, proteins are separated by electrophoresis across the two dimensions of mass and pH (i.e., two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis, or 2D-PAGE), visualized using fluorescent or colorimetric protein dyes prior to selection for subsequent mass spectrometry (MS) analysis. Selected proteins undergo proteolytic digestion to form peptides prior to MS analysis. The resulting tandem mass spectra of the peptides are evaluated against large-scale peptide libraries using one of the variety of available database search engines and data analytics to link peptide fingerprints to protein identifications [59,60]. Comparative 2D-PAGE with differential fluorescent labeling, also known as two-dimensional differential gel electrophoresis (2D-DIGE), allows quantitative comparisons of proteomes [61,62]. With this technique, resolution is often sufficient to allow separation of posttranslationally modified from unmodified proteins, allowing separate characterization and quantitative analysis. Although 2D-PAGE has been used mostly for global analyses of complex proteomes, the method is also useful for comparative analyses of smaller subproteomes.

Advances in instrumentation and automation over the past 15 years has significantly increased the use of shotgun proteomics as a label-free technology that can detect up to tens of thousands of proteins rapidly, depending on the biological matrix being investigated. In shotgun proteomics, protein mixtures are digested to complex mixtures of peptides, which are then separated using high-performance liquid chromatography combined with MS [63,64]. As with 2D-PAGE peptide fingerprinting, databases are then searched to match the resulting peptide tandem MS spectra with corresponding peptide sequences, and software is used to reassemble the collection of peptide sequences into proteins. Shotgun proteomics is the most effective technology for automated analysis of complex peptide mixtures [65,66].

In addition to the 2D-DIGE, label-based MS methodologies have provided the proteomics field strategies to globally quantitate proteins, a key limitation of MS-based platforms. Current methodologies use stable isotope tagging of samples using protein derivatization or stable isotope labeling by amino acids in cell culture (SILAC) [67,68]. Briefly, these methodologies tag proteins in samples by exploiting protein structural groups (e.g., thiol-reactive groups on proteins or amino- or carboxy-termini on proteins) or exploit the cellular metabolic machinery for isotope incorporation and make use of paired experimental designs to measure relative amounts of proteins in a sample. Shortcomings of these approaches include the need for derivatization of the samples and the need to perform quantitative assessments by pair-wise comparison. An alternate method that provides absolute protein quantitation spikes in known quantities of stable isotope-labeled standard peptides into proteolytic digests from complex mixtures. Levels of the protein of interest in the sample can then be quantitated

relative to the levels of the spiked-in standard [69]. Although these approaches have demonstrated promise for quantitative candidate biomarker analysis in biofluids [70,71], researchers need to consider the clinical validation process more fully to facilitate widespread adoption and regulatory acceptance [72].

Non-MS-based proteomic approaches are available and are used to provide targeted and applied rather than discovery-based assessments. In 2000, two teams described platforms that exploited the printing technology developed for gene expression microarrays to spot proteins and antibodies onto similar arrays [73,74]. Although challenges in antibody affinities and reactivities hinder the widespread acceptance of antibody arrays, reverse phase protein arrays (RPPAs) have emerged as an alternative that are amenable to analysis of large numbers of samples and have demonstrated promise across a range of applications, including pharmacodynamic assessments, tumor classification, therapeutic target assessment, and biomarker validation [75–77]. Briefly, tissue or cell lysates or biofluids (e.g., serum, cerebrospinal fluid) are spotted onto a matrix support and incubated with an antibody or panel of antibodies through the use of multiplexing [78]. Antibody optimization is still needed, but this approach allows the interrogation of a full network or signaling pathway for a targeted, yet comprehensive understanding of the mode of action.

Another targeted non-MS-based strategy with great potential is an array-based technology that is analogous to the nucleic acid hybridization microarray – array making use of aptamers as targets. Aptamers are short single-stranded oligonucleotides that fold into diverse and intricate molecular structures that bind with high affinity and specificity to proteins, peptides, and small molecules [79–81]. This aptamer-based multiplexed proteomic technology has been used to make quantitative measurements of approximately 1000 proteins from biofluids, exhibiting a dynamic range over seven orders of magnitude and identified potential biomarkers of chronic kidney disease [82]. Another study reported capture and analysis of just over 800 proteins from patient sera that ultimately led to identification of a 12-protein panel with high sensitivity and specificity to be a diagnostic biomarker candidate for non-small cell lung cancer [83].

There are several challenges that affect the utility of proteomics assessments. Given that the abundance of proteins represented in a proteome can range over six orders of magnitude, higher abundance proteins tend to preclude assessments of lower abundance proteins, particularly in complex mixtures. While this is readily apparent in gel-based approaches, where the dynamic range of fluorescent dyes typically spans only 200–500-fold, it should be noted that this challenge is not unique to the gel-based approaches [84]. Also, despite efforts to incorporate quantitation to shotgun proteomics and other nongel-based methods, no reliable method exists as of yet, which severely hampers application of these assessments in xenobiotic efficacy and mode of action studies. Database utilization for peptide or protein identification is confounded by multiple issues. Biologically, the existence of degenerate peptides (i.e., peptides shared by two or more proteins)

can hinder identifications, as can the existence of many identical protein subsequences due to alternative splicing in higher eukaryotes [85]. Informatically, different search algorithms used to interrogate the same data will yield different identifications. These issues along with the high cost, the relatively large sample requirement for discovery approaches, the varied nature of the proteomic instrumentation and technologies, and the requirement for extensive analysis and support have hindered it from entering into more widespread use.

17.2.5 Metabolomics

Metabolomics is the study of collections of molecules (intermediates and products) generated through metabolic processes in a given biological system, fluid, cell, or tissue at a given point in time. Biochemical characterizations of these molecules and their fluctuations related to internal (i.e., genetic) and external (i.e., environmental) factors can thus provide a direct assessment of health and all of its influences [86,87]. The potential value of this technology is now being realized, with hundreds of studies conducted over the past decade demonstrating the insight metabolomics can provide to nearly all areas of biology, serving as a key integrator for genomic, transcriptomic, and proteomic data. We now understand that an individual's metabolic profile can reflect alterations in homeostasis that underlie health, disease, and response. These profiles are key to understanding health influences that operate by changing metabolism, including genetics [88], microbiota [89], environment, diet, epigenetics [90,91], and combined effects.

The primary technologies used in metabolomic analyses are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS, with both gas or liquid chromatographic separation). NMR-based technology has been shown to be robust and reproducible in laboratories that follow similar analytical protocols [92], and consensus standards for analytical standardization and data representation in metabolomic analyses have been implemented [93]. NMR is valuable for identifying patterns of spectra reflecting global metabolic changes, while MS-based analyses offer the advantage of greater sensitivity – in some instances by as much as 10,000-fold. Both technologies can detect differences in metabolic profiles that correspond to various modes of toxicity, but integration of the technologies will allow for a more comprehensive approach.

NMR-based metabolomics studies of urine have been demonstrated to provide a global snapshot of metabolic changes that can occur throughout the organism and have led to landmark findings in the field. Principal component analysis (PCA) of urinary NMR data have shown that the development and resolution of chemically induced tissue injury can be followed by plotting trajectories of PCA-derived parameters [94]. Follow-up identification of specific metabolites within these spectra and mapping onto known metabolic pathway maps facilitated linking biochemical and cellular consequences and mechanisms of injury [95].

These efforts led to the identification of endogenous bacterial metabolites as key elements of diagnostic metabolomic profiles [96–98]. Although the interplay of gut bacteria with drug and chemical metabolism had been known previously, recent NMR metabolomic studies indicate that interactions between host tissues and gut microbes have a much more pronounced effect on susceptibility to injury than had been appreciated previously [99].

Recent translational efforts have shown great promise in harnessing the potential of the metabolome to provide information related to specific exposures in concert with an indication of disease risk. This approach, known as "meeting-in-the-middle," has been conceived as a research strategy to identify biomarkers that are specific of certain exposures and, at the same time, biomarkers of a particular risk or disease outcome [100]. Early work exploring this concept identified a list of putative biomarkers confirming exposure to dietary compounds and then successfully identified linkages between these exposures to breast and colon cancer outcomes [101]. More recent efforts revealed linkages between lifestyle variables (incorporating information on diet, anthropometry, lifestyle choices) identified using metabolomic profiling of risk of hepatocellular carcinoma incidence [102]. These and future efforts hold great promise to disclose useful information on the exposure-to-disease pathway, possibly identifying a risk exposure measure that could in turn aid in identifying a targeted prevention scheme.

A critical issue in the application of metabolomics is the standardization of methods, data analysis, and cross-laboratory reporting. Indeed, a consequence of the chemical diversity of metabolome components is the difficulty of comprehensive analysis with any single analytical technology. As mentioned earlier, recent cooperative study by the Consortium for Metabonomic Toxicology indicated that NMR-based technology is robust and reproducible in laboratories that follow similar analytical protocols [92]. Investigators in the field recently have agreed on consensus standards for analytical standardization and data representation in metabolomic analyses [103]. Even with these concerns, metabolomics is closer than proteomics to being introduced into clinical practice and translational efforts. Metabolite analyses using MS are already routinely adapted in clinical laboratories for drug monitoring screening and in disease diagnostics [11] for inborn errors in metabolism. Given metabolites' proximity to phenotype, it is not surprising that many scientists increasingly view metabolomics as an important tool for unlocking the full potential of disease research, genomics, and precision medicine.

17.3 High-Throughput Bioactivity Profiling

17.3.1 High-Throughput Bioactivity and Toxicity Screening

Although microarray or RNA-Seq-based methods provide useful information about transcriptional responses in biological systems to chemical compounds,

the current technologies are still too costly to serve as a screening tool in xenobiotic mode of action studies, where assessments across a range of concentrations, cell types, and time points are needed to generate sufficiently robust data. In contrast, a battery of reliable, reproducible high-throughput screening (HTS) assays currently exist to interrogate transcriptional, protein, or endpoint responses individually and efficiently across thousands of chemicals, across robust concentration ranges. Compared with more extensive conventional toxicology and toxicogenomics studies, *in vitro* screening tests are designed to be efficient and cost-effective, used in the triage of small molecule libraries for a specific molecular or biological activity. Primarily established for use in pharmaceutical drug discovery, initial screens typically employ higher and fewer chemical concentrations, limited replicates, and time points for a broad survey of possible bioactivities or liabilities. HTS thus provides a practical method to investigate more than 100,000 compounds per day in miniaturized *in vitro* assays in order to identify those with the potential to cause adverse effects [104]. For safety evaluation and toxicity testing, significant activities or "hits" in the screening assays correspond to biological pathways that are known to lead to adverse outcomes. With sufficient accumulated data, it may be possible to use structure–activity analysis to predict HTS hits, so that potential targets can be predicted prior to screening. The application of robotic HTS as a useful complement to conventional toxicology has been expanding over the past 10 years [105–108].

HTS assays fall into two broad categories: cell-free and cell-based assays. Cell-free assays generally measure direct effects on specific molecular targets of interest. These assays have been used to measure enzymatic activity [109–111], binding of substances to receptors [112], ion channel activity [113], nuclear receptor activity [114], and protein–protein interactions [115]. Because they involve homogenous reactions, biochemical assays are readily miniaturized. However, not all targets can be prepared satisfactorily for biochemical testing. Furthermore, a chemical's activity measured in cell-free assays does not necessarily correspond to its activity in the intact cell, which may be affected by the presence of intracellular cofactors, issues of membrane permeability, cytotoxicity, and other influences on the target molecule. In contrast, cell-based assays measure the effects of chemicals on pathways of interest in the physiological environment of a cell, without the need to specify a molecular target. Examples include functional assays [116,117], reporter gene assays (which use "marker" genes to signal activation of target genes) [118–120], and phenotypic assays for processes such as inflammation [121], cell migration [122], or cell division [123]. Because cell-based assays measure effects on entire pathways, perturbations can be assessed at more than one step in a pathway. Cell-based HTS in 1536- or even 3456-well plate formats is not uncommon [124–126].

17.3.2 *In Vitro–In Vivo* Extrapolation

HTS bioactivity and toxicity screening assays are actively being used by scientists with the US Environmental Protection Agency to fill significant data gaps in toxicity information across the tens of thousands of chemicals in commercial use in the United States [105,127]. One early limitation to the adoption of these *in vitro* data into chemical safety assessment and testing prioritization efforts was the inability to relate the *in vitro* nominal concentrations at which bioactivity was observed out to relevant external exposures. Activities observed in these *in vitro* assays lack consideration of *in vivo* toxicokinetic processes – which include absorption, distribution, metabolism, and excretion (ADME) – that will ultimately dictate the extent of toxicity and/or potency of these chemicals *in vivo*. No matter how active or potent a chemical may be in certain *in vitro* assays, if it is not absorbed into the human body upon exposure it will not be bioavailable to elicit any effect. Similarly, a chemical that is rapidly cleared may not achieve a sufficiently high concentration to elicit an effect.

In vitro–in vivo extrapolation (IVIVE) is a process that utilizes *in vitro* experimental data to predict phenomena *in vivo*. IVIVE to predict chemical pharmacokinetics has gained broad acceptance in the pharmaceutical industry, where experimental data from *in vitro* pharmacokinetic assays that monitor chemical pharmacokinetic properties can be employed along with scaling factors to predict *in vivo* chemical clearance or pharmacokinetics. In toxicology, IVIVE has been used in conjunction with various pharmacokinetic modeling strategies, including physiologically based pharmacokinetic (PBPK) modeling, to relate a known external exposure to an internal blood or target tissue dose [128]. This process is known as forward dosimetry. Alternately, reverse dosimetry is often used to relate a known internal dose (i.e., either from blood biomonitoring data or an *in vitro* assay bioactivity concentration) to an external chemical dose required to achieve blood concentrations equal to those internal doses observed [129].

Recently, an IVIVE approach amenable to incorporation with HT screening data was presented that predicted external doses required to achieve internal steady-state blood concentrations similar to those eliciting activity in *in vitro* HT screening assays [130,131]. This approach incorporated key determinants of chemical steady-state pharmacokinetics to estimate chemical steady-state behavior. A similar strategy can be utilized to estimate maximum concentration (i.e., C_{max}) values after chemical administration. This particular approach was designed with throughput in mind: only processes deemed critical to drive pharmacokinetics were considered; and anything not measured experimentally was set to a conservative or human health protective assumption. Comparison of the IVIVE predictions demonstrated good reproducibility compared to those chemicals with existing *in vivo* data [132]. Where the predictivity was not ideal,

the error tended to be a conservative error and protective of human health – an important aspect in the world of toxicology and chemical safety.

17.4 Biomarkers

Biomarkers provide a critical bridge between the discovery-based and hypothesis-driven laboratory research arena for clinical translation and ultimate use at the bedside. Given the stakes, it is important to understand relevant working definitions by key stakeholders. In 2001, the Biomarkers Definition Working Group defined a biomarker as a trait that can be objectively measured and evaluated; therefore, it can be used as an indicator of biological processes (i.e., normal versus disease) or of pharmacologic response upon therapeutic intervention [133]. The FDA has defined a biomarker as a measurable endpoint that may be used as an indicator of a disease or a physiological state of an organism. According to these definitions, such indicators may include physiological measurements, blood tests and other chemical analyses of tissue or bodily fluids, genetic or metabolic data, and imaging-based or laboratory-measured biomarkers. Their roles are wide ranging in areas including discovery research, clinical practice, and public health practice [134].

Clinical application of biomarkers applies across several areas, including risk assessment, screening, diagnosis, prognosis, prediction of therapeutic response (effect modifiers), prediction of clinical outcome (surrogate endpoints), and patient monitoring during and after treatment. More information is provided in Table 17.1. Regardless of the intended application, a three-step biomarker evaluation framework consists of analytical validation (focusing on accuracy, reliability, reproducibility); qualification (or clinical validation; association with clinical outcome); and clinical utility (i.e., benefit/risk ratio) [134,135]. In addition, it is important to recognize that the nature of the evaluation of a biomarker will be wholly dependent on the intended use; if a test's validation does not reach the level needed for its intended use, the test could be sent back for further development [134,136].

Three main aspects entail omics-based test development: analytical development, computational modeling of the predictor, and its clinical utility validation. Given the multidimensional and rich information generated by omics data, mathematical modeling, including machine learning and chemometric methods, will be critical to building classifiers for effective medical decision-making [11]. McShane *et al.* [137] provides an in-depth discussion of the main issues to take into account during omics-based biomarker development, which include consideration of samples, analytical development of assays, computational model development, clinical utility assessment, and regulatory issues. Criteria that should be assessed for effective biomarker validation are also discussed [137].

Table 17.1 Use of biomarkers in the clinic.

Biomarker use	Objective
Disease risk stratification	Assess the likelihood a disease will develop or recur
Screening	Detect and treat early-stage disease in the asymptomatic population
Diagnosis/differential diagnosis	Definitively establish the presence and precise description of disease
Classification	Classify patients by disease subset
Prognosis	Estimate the risk of or the time to clinical outcomes
Prediction/treatment stratification	Predict response to particular therapies and choose the drug most likely to yield a favorable response in a given patient
Therapy-related risk management	Identify patients with a high probability of adverse effects of a treatment
Therapy monitoring	Determine whether a therapy is having the intended effect on a disease and whether adverse effects arise
Posttreatment monitoring	Early detection and treatment of advancing disease or complications

Source: Adapted from Ref. [136].

HT and omics technologies may theoretically lead to data-driven assessments that may ultimately be more clinically predictive, but a key intermediate step in the translation is the identification, development, curation, and widespread utilization of appropriate metadata, reference data sets, and analytics. Moreover, multiomics approaches require integration of heterogeneous data sets across different platforms that add to the already confounding effects of complexity, bias, and noise. Translational success will depend greatly on combining expertise across several wide ranging disciplines, including clinicians, medical laboratory professionals, data scientists computational biologists, biostatisticians, clinical bioinformaticists, and lawyers [11,138].

17.5 Exposomics

The exposome was first coined by Dr. Christopher Wild in 2005 to provide a complementary concept to the genome for use in characterizing gene–environment interactions in epidemiological studies [139]. Moreover, it emphasized what had emerged as a critical need to refine and expand exposure assessments to comprehensively characterize all exposures across a life span, including the *in utero* period [140]. It is important to emphasize that the exposome goes beyond cataloging only chemical exposures and is meant to also capture diet, behavior,

and lifestyle factors: all of which will have an effect on the internal environment, which in turn, in concert with the genetic background, will dictate the biological responses and, potentially, disease outcomes. Recently, an effort was launched to redefine the original definition to provide greater emphasis to the need for a more holistic exposure assessment, and suggested rephrasing the exposome definition to: "the cumulative measure of environmental influences and biological exposures throughout a lifespan and how these exposures relate to or influence health and biological responses" [141].

Technologies used to provide exposomic characterization overlap with those used for metabolomic studies and include primarily MS instrumentation, coupled with a range of separation and detection methods. Of particular note is high-resolution MS, which measures large numbers of chemicals based on mass resolution and mass accuracy. These characteristics allow prediction of elemental composition of a chemical using the accurate mass/charge (m/z) values; these values can be queried against human metabolite databases (Kyoto Encyclopedia of Genes and Genomes (KEGG); the Madison Metabolomics Consortium Database (MMCD)) for metabolite mapping and identification. Expanded coverage is possible with different technologies (e.g., dual chromatography Fourier transform-MS) with potential resolution of up to 10,000 chemicals, which could include representative chemicals of each of the components of the pan-metabolome [142]. Such coverage paves the way for metabolome-wide association studies, which would facilitate greater linkages between diet and disease. As with proteomics and metabolomics, limitations in the ability to quantitatively measure molecules is an issue, and gaps in metabolite databases hinder identification. For instance, the Human Metabolome Database, which contains data and analytics on approximately 2500 metabolic intermediates, provides coverage across only 10% of the metabolites of intermediary metabolism in nutrition studies [142].

17.6 Bioinformatics to Support and Data Integration and Multiomics Efforts

The magnitude and complexity of the data generated by any one of the omics technologies in conjunction with the need to integrate data across disciplines requires the use of advanced computational techniques. "Bioinformatics" is the branch of computational biology focused on the collection, management, analysis, and integration of numerical biologic data. Bioinformatics encompasses the integration of data across the omics technologies as well as observations and measurements from other data sources (e.g., HTS data), and the integration of all these data in databases and related information resources. At a basic level, bioinformatics is represented by information resources such as GenBank, a repository of gene sequence data and associated information

structured for easy retrieval. At an intermediate level are tools, such as BLAST or SAGEmap, that perform insightful sequence alignment and function and structure analysis. Finally, sophisticated information systems (e.g., expert systems) integrate data from numerous sources to solve multifaceted problems.

Table 17.2 provides a listing of selected open-source web-based platforms that provide a whole suite of databases and tools for use by experimental

Table 17.2 Selected open-source bioinformatic tools for data analysis and integration.

Tool	Platform	Description	Reference
Bioconductor	Genomics, molecular biology	Platform with software tools (>934 interoperable statistical and bioinformatic packages (in R)); experimental data	[143]
EMBL-EBI (European Molecular Biology Lab-European Bioinformatics Institute)	Cross-platform[a)]	Databases with associated analytical tools and query functions for data mining; web-based interface	[144]
ExPA Sy (Expert Protein Analysis System)	Cross-platform	Strong in proteomics (home of Uni-Prot), now expanded across other platforms. Databases, web-based, and downloadable software tools	[145]
The Gene Index	Genomics	Genome sequence and expressed sequence tags (ESTs) from >100 species; annotation, functional assignments, etc.	[146]
The Genotype Tissue Expression Project (GTEx)	Genomics–transcriptomics	Resource database and associated tissue bank to explore links between gene expression and genetic variation	[147]
MassTRIX	Transcriptome–metabolome	Uses existing KEGG[b)] annotations to visualize transcriptome and metabolome data	[148]
OMICtools	Cross-platform	Metadatabase of >4400 web-accessible tools, cross all omics platforms, designed for big data processing and integration	[149]

a) Cross-platform: coverage across more than two omics platforms.
b) KEGG: Kyoto Encyclopedia of Genes and Genomes.

scientists, clinicians, and bioinformaticians alike. In recent years, an explosion in online open-source analytic tools and software for use in omics and multiomics assessments is widely apparent. However, caution is recommended as online availability does not necessarily indicate adequate peer review, reliability, or quality, and no one tool is appropriate for all tasks. An understanding of the principles behind the bioinformatic tools in conjunction with an understanding of one's research needs is tantamount to selecting the appropriate tool for your research.

17.7 Data Integration: Multiomics and High-Dimensional Biology Efforts

Integration is defined as the process through which the different streams of data (e.g., omics data) can be brought together to provide a higher level assessment, with greater informative power, than any one technology or field on its own. Ultimately, this can feed into a higher dimensional, systems biology approach that can leverage the experimental power of genomics and transcriptomics technologies along with the more translationally relevant fields of metabolomics and proteomics that provide the functional rationale for a particular disease, clinical effect, or prognosis/diagnostic outcomes. Figure 17.1 provides a high-level view of how the various omics data platforms fit within a continuum to answer research questions in translational therapeutics and toxicology. The research problem of interest, along with the decision context, need to be

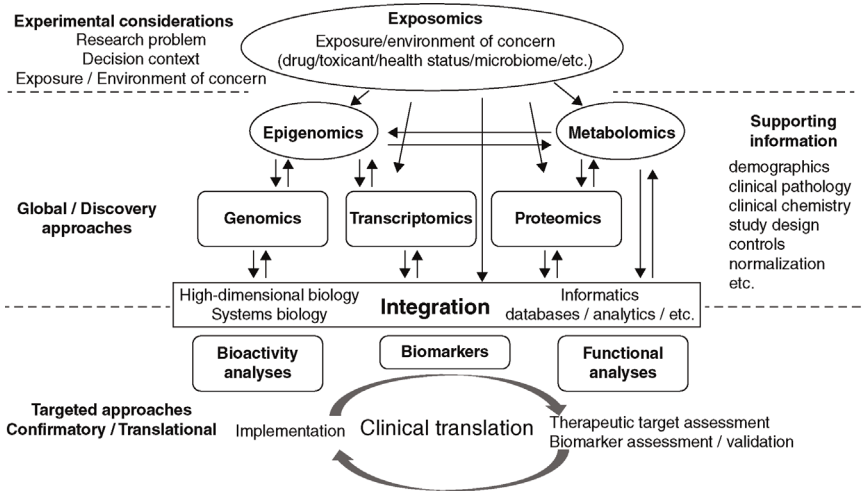

Figure 17.1 Schematic of design, workflow, and execution considerations in the conduct of omics studies and data integration to inform translational research.

considered during problem formulation and experimental design. The first phase represents the global or discovery phase, where data within the omic platforms required to address the research problem are generated and analyzed, but also to facilitate long-term usefulness within a data repository or reference database. Next comes the data integration step, which will make use of appropriate analytics and tools based on the needs of the project. The findings following integration can then feed into designing the required targeted studies to confirm hypothesized mode of action or bioactivities; alternately, the required clinical utility and validation studies can commence for biomarker validation. A few examples are provided that further demonstrate successful integration across two or more of the omics platforms, many focused in the zone where data integration interacts directly with clinical engagement. These examples provide a few snapshots of visionary thinking that has shaped progress to date and may serve to guide future efforts in this translational space.

Knowledge that close to 90% of trait-associated variants detected across GWAS fall within noncoding regions suggests that these variants have an important regulatory role in DNA metabolism. With this in mind, several key efforts have been underway to integrate genomic regulatory and binding information and epigenomic data with transcriptomic data to enhance phenotype-related interpretation. The Encyclopedia of DNA elements (ENCODE; [150,151]) represents an international effort that has collated binding information of 119 transcription-related factors in over 450 distinct experiments to create a dense metanetwork, comprised of transcription factor binding data (including combinatorial coassociations of transcription factors), and other genomic information (e.g., microRNA regulation). This data set can be accessed directly through ENCODE and is poised to increase general knowledge about the physiology and metabolism of DNA. This regulatory information has laid the groundwork for enhanced interpretation of personal genome sequences and understanding basic principles of human biology and disease.

Defining connections between diseases, a disease-modifying gene product and a chemical modulator of that protein is ultimately what many strive to do in biomedicine. Unfortunately, defining these connections can be very challenging given the great disparity between the disciplines of clinical medicine, molecular genetics, and chemistry. Landmark work conducted at the Massachusetts Institute of Technology set out in essence to short-circuit some of these challenges by translating the disparate areas into the common language of gene expression. These scientists devised a strategy to identify functional connections between drugs, genes, and diseases and, through the use of mapping similarities in the profiles, could identify complementarity in function or behavior. To achieve this, a large reference cataloge of gene expression data from cultured human cells perturbed with many chemicals or agents (i.e., perturbagens) was created using Affymetrix gene expression data. A

connectivity map was then assembled for use to rank gene expression profiles of a perturbagen of interest against those within a reference database to identify connections between drugs, diseases, and therapeutic targets [55]. This approach has been used to successfully discover unknown therapeutic activities of a range of natural products and drugs against cancer, androgen signaling, and several other targets [49,56]. The Connectivity Map database is available as an open-source tool, with funding as a part of NIH's LINCS program.

An emerging paradigm in the study of cellular function and biological response is the reciprocal regulation that occurs between cellular signaling pathways and metabolism. Signaling-dependent regulation of metabolism is known to be exploited by cancer cells, with nutrient uptake and metabolism induced by oncogene activation used to support continuous macromolecular biosynthesis and cellular proliferation. Recent efforts to integrate metabolomic and transcriptomic data sets in cancer research have borne these findings out, providing very promising leads in the pancreatic and breast cancer areas.

In the first, a global metabolite profiling analysis identified a fatty acid network that was highly coregulated and decreased in pancreatic cancer biopsy samples [152]. Gene expression profiles were then mined for transcripts that were coregulated with key metabolites, successfully identifying a target that associated with poorer survival in two independent cohorts. This effort identified a lipolytic pathway that may play an important role in the development and progression of pancreatic cancer and may provide potential targets for therapeutic intervention.

Based on the knowledge that dysregulated miRNA could mediate malignant phenotypes, potentially through metabolic reprogramming, Koufaris *et al*. [153] interrogated a panel of tumor cell lines for associations between miRNAs and metabolomic data. A miRNA cluster was identified that strongly associated with both c-Myc expression and global metabolic variation. Follow-up analysis revealed that miR-22 repressed a subset of metabolic enzymes involved in fatty acid metabolism; expression of these genes were shown to be associated with poor outcomes in breast cancer patients. This integrated and systematic analysis established the role of this miRNA as a novel regulator of tumor cell metabolism also identifying miRNA-directed gene targeting as an effect modifier in breast cancer outcomes. Such a finding could be exploited to identify treatment options and to improve patient prognosis.

High-dimensional biology (HDB) refers to the simultaneous study of the genetic variants (DNA variation), transcription (mRNA), peptides and proteins, and metabolites of an organ, tissue, or an organism in health and disease [154]. The fundamental premise is that the evolutionary complexity of biological systems renders them difficult to comprehensively understand using only a reductionist approach. Such complexity can become tractable with the use of "omics" research. Romero *et al.* [155,156] provide a HDB assessment of data generated to understand preterm parturition syndrome, reviewing data derived

from (1) genomics to examine predisposing factors for preterm birth; (2) transcriptomics to determine changes in mRNA in reproductive tissues associated with preterm labor and preterm prelabor rupture of membranes; (3) proteomics to identify differentially expressed proteins in amniotic fluid of women with preterm labor; and (4) metabolomics to identify the metabolic footprints of women with preterm labor likely to deliver preterm and those who will deliver at term. This assessment suggested links between exposure to environmental factors and epigenetic reprogramming, leading to an alteration in both the fetal and maternal immune systems, and was critical in identifying future research needs in this research area [155,156].

17.8 Conclusion

The completion of the sequencing of the human genome at the turn of the century set off a string of major advancements in technologies that support global profiling platforms. Advances in sequencing technologies, mass spectrometry and related proteomic, metabolomic, and exposomic platforms, high-throughput robotics, informatics, and the computational tools to support predictive assessments quickly followed allowing expansion into fields that assess profiling strategies from genetic out to functional and phenotypic changes. Meanwhile, recognition for the value in releasing data in the public domain and the need for standardization and quality control has provided statically powerful data sets that can be effectively mined for discovery-based assessments of gene expression networks, signaling pathways, and interaction networks.

It is important to recognize, however, that the ultimate success of this effort depends on the ability to bridge some of the technological advancements of the past 20 years into strategies that more directly impact clinical needs and patient outcomes. Bridging strategies that better relate transcriptomic findings to phenotypic outcomes which then in turn can identify biomarkers and sufficiently validate them for prognostic or diagnostic utility will require forging of multidisciplinary efforts that effectively bring in stakeholder input throughout the process. Thankfully, advances discussed in this chapter highlight successes that have paved the way for progress in ongoing and future translational toxicology efforts.

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18

Developing a Translational Toxicology Therapeutic Portfolio for Cancer Risk Reduction

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18.1 Introduction

Toxgnostics is an emerging term within the field of oncology. The term refers to the systematic and prospective identification of genetic predictors of potential adverse effects of anticancer therapy [1]. The ultimate aim of toxgnostics is for clinicians to be able to accurately prescribe the most effective anticancer treatment regimen with the lowest possible toxic side effect profile, personalized to individual patients.

Currently, the systemic treatment of solid tumors often produces only a modest increase in survival rates, while concomitantly exposing the patient to a very real prospect of significant and potentially life-threatening toxicity. One example can be found in the management of stage 2 colorectal cancer. Chemotherapy can provide a survival benefit to a modest 4% of the patient population; however, significant side effects occur in an order of magnitude of more patients (Common Toxicity Criteria (CTC) Grading level 3; Table 18.1) [2].

Personalized medicine can be defined as the practice of utilizing an individual's genetic profile to tailor treatment decisions regarding the prevention and management of disease. With the completion of the Human Genome Project at the turn of the millennium, the hope was that personalized medicine could be realized. Increasingly, both genome-wide studies and target gene approaches are being utilized to select the most effective anticancer therapy for a patient, with increasing success and efficacy. However, this individualized approach has not yet extended to minimizing any treatment-associated adverse effects.

Currently, the decision process, a clinician undertakes when selecting a treatment regimen for a patient, involves risk stratification to minimize

Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer, First Edition. Edited by Michael D. Waters and Claude L. Hughes.

Table 18.1 Common Terminology Criteria for Adverse Events (CTCAE) [3].

Common Terminology Criteria for Adverse Events	Common symptoms
Grade 1	Mild abdominal pain Increase of <4 stools per day from baseline Fatigue relieved by rest Mildly hypocellular bone marrow
Grade 2	Moderate abdominal pain; limiting activities of daily living (ADLs) Increase 4–6 stools per day from baseline Fatigue not relieved by rest; limiting instrumental ADLs Moderately hypocellular bone marrow
Grade 3	Severe abdominal pain; limiting self-care Increase of ≥7 stools per day from baseline Fatigue not relieved by rest; limiting self-care Severely hypocellular bone marrow
Grade 4	Life-threatening consequences from diarrhea, interventions needed Aplastic persistent for >2 weeks
Grade 5	Death related to adverse event

potential toxicity. This relies upon long-established predictors of associated toxicity such as patient performance status and gross organ function such as hepatic and renal function. Performance status is a scale developed by the Eastern Cooperative Oncology Group (ECOG), and is used by researchers and clinicians to assess how patients' disease state affects their daily living abilities (Table 18.2) [4].

Table 18.2 ECOG performance status.

Grade	ECOG performance status
0	Fully active, able to carry out all predisease activities, without restriction
1	Restricted by strenuous physical activity, but able to carry out light work
2	Ambulatory but unable to carry out any work. Up and about for >50% of waking hours
3	Capable of limited self-care. Up and about less than 50% of waking hours
4	Unable to carry out any self-care. Totally confined to bed or chair
5	Dead

Toxgnostics is a more agnostic approach to identify germ line variants that influence toxicity. The field is still in its infancy, but we anticipate that the most practical methodology to use would be next-generation sequencing (NGS) to facilitate high-throughput analysis, with the aim of identifying more frequent variants with perhaps more subtle effects upon toxicity profiles. Such information could be used to optimize risk–benefit analysis of treatment – The maximum treatment benefit with the lowest associated toxicity, which could be selected *a priori*.

Alterations in DNA can affect all facets of cancer, including its origin – They are the initiating lesion that allows the cell to escape the normal cellular regulations and to become cancerous. The DNA mutations may also determine the diseases response to treatment; it is now increasingly common for a tumor to be sequenced to allow targeted therapy, especially with the increasing use of drugs targeted to specific somatic tumoral mutations, for example, Crizotininb selectively suppresses growth of non-small cell lung cancers, which carry the ALK gene rearrangement. It is becoming ever more apparent that specific germ line polymorphisms are responsible to the presence and grade of any treatment adverse events.

18.2 The Identification of Novel Predictors of Adverse Events

18.2.1 Candidate Gene Studies

Traditionally, enzyme kinetics, and pharmacodynamics, have been the studies used to correlate toxicity and pharmacological parameters. The founding belief for such relationship is that plasma–drug associations, which affect drug absorption, are primarily affected by genetic, environmental, and physiological factors [5]. This assumption may be an oversimplification due to significant intra- and interindividual variations in pharmacokinetics that are at play for most cytotoxic drugs.

One classic example, which has found success in the field of oncology, is the routine clinical testing of thiopurine methyltransferase activity levels prior to the administration of 6-mercaptopurine and 6-thioguanine. Thiopurine methyltransferase (TPMT) methylates mercaptopurine and thioguanine, necessary for their metabolism and excretion. Low levels of TMPT activity result in high levels of active metabolite. TPMT activity is inherited as a monogenic codominant trait. Patients who are homozygous (1 in 178 to 1 in 3736) for two inactive TMPT alleles experience profound myelosuppression; indeed, heterozygous (3–14% of the population) patients may also experience significant myelosuppression. Homozygous wild-type patients (86–97% of the population) are at a much lower risk of such adverse events. Interestingly, and

pertinent for the field of toxgnostics, there are significant differences in frequencies of low activity alleles within different ethnic populations [6–10].

Interestingly, three TMPT SNPs account for the majority (>90%) of dramatic variations in drug response, making routine testing a feasible prospect.

Thiopurines are used as treatment options for both malignant and non-malignant diseases. For nonmalignant conditions, the treatment range tends to start in the lower spectrum to minimize risk of significant treatment-associated adverse events. However, in doing so, the clinicians risk the transformation to a malignant process [11]. Conversely, with malignancies, dosing regimens tend to start at the upper end of the spectrum, as doses have been calculated from clinical trials, remembering that up to 97% of the population are homozygous wild type [12,13]. This obvious risk from such high dosing is significant myelosuppression in homozygous-deficient patients. If homozygous-deficient patients could be prospectively identified, their treatment should undergo a 10-fold reduction. Assays for enzyme activity have recently gained widespread acceptance within the field and are increasingly used prior to the initiation of treatment.

To date, candidate gene studies have been utilized to target pathways known to be involved in the distribution, metabolism, and excretion of drugs. Unfortunately, despite the identified several hundred polymorphisms, which have been attributed to toxicity, only a minimal subset have so far been validated in clinical trials. A further issue that has arisen is that the majority of candidate gene studies that have been examined so far have been done so in small, single-center retrospective trials, therefore have resulted in insufficient statistical power. Another limitation is that such small studies increase the chances of including false positives within the data.

One obvious constraint of the candidate gene approach is that all discoveries are confined to targets within known biochemical pathways involved in the metabolism of the drug. The rate-limiting step is therefore the further characterization of existing pathways and identification of novel ones, and is therefore dependent upon other research areas. To advance this field, new methodologies of gene discovery need to be considered.

18.2.2 Genome-wide Associations

One validated alternative to the single candidate gene methodology is the agnostic approach permitted by genome-wide association studies (GWAS). GWAS refer to the practice of comparing the genotype of interest at various polymorphic loci (single-nucleotide polymorphisms, SNPs), with that of the designated wild type, or control. One major advantage of this approach is that millions of loci can be studied. If this practice is carried out across large patient populations, we can be afforded sufficient statistical power to identify true associations, which can then be validated in further clinical studies.

GWAS have been used successfully in the field of oncology, from studies identifying susceptibility to certain cancers, such as a recent study identifying 15 new susceptibility loci to breast cancer (and in doing so explaining 14% of familial cases) [14], to abnormal processing of anticancer treatments such as methotrexate. Methotrexate is a chemotherapy agent that has been used for more than 50 years. The drug is an antifolate agent, which inhibits folate synthesis and hence DNA synthesis, resulting in cell apoptosis. One issue regarding the prescription of methotrexate is the wide interpatient variation of methotrexate clearance. This variation affects both its clinical effectiveness and its toxicity to the patient [15,16]; in extreme examples, this can result in irreversible kidney damage and ultimately death. Ramsey *et al.* [17] used GWAS in a cohort of just under 700 children with acute lymphoblastic leukaemia (ALL) to identify polymorphisms associated with abnormal methotrexate clearance [17]. Through genome-wide analysis, the group identified a single locus, *SLCO1B1*, organic anion transporter that mediates disposition of certain drugs, which was significantly associated with methotrexate clearance. The authors identified both common and rare variants at the loci. The conclusion of the paper was a successful proof of principle of GWAS in pharmacogenomics, which we would extend to the emerging field of toxgnostics.

To date, with the exception of the above example and small number of other studies, GWAS have been primarily used for the identification of proposed treatment advantages and of cancer susceptibility, as opposed to the toxgnostics approach of identifying patients who would be at an increased risk from certain treatments. We anticipate that the field of toxgnostics is a natural next step for GWAS.

In order for genome-wide association studies to gain the necessary statistical significance, the studies need to include large patient numbers. To make such studies feasible, high-throughput DNA sequencing and analysis is needed. One methodology that could facilitate such analysis is next-generation sequencing.

18.2.3 Next-Generation Sequencing

NGS is one of the most advanced technologies scientists have in their arsenal in the battle against cancer. This technique facilitates the generation of high-throughput, quality data, and is an alternative to the traditional Sanger Method. The term is a 'covers all' for all automated, high-throughput, non-Sanger DNA sequencing. Since the turn of the century, the demand for high quality, quick, and affordable DNA sequencing has driven the advancement of NGS. In a nutshell, NGS involves the parallel sequencing of millions of DNA fragments from the same sample, enabling entire genomes to be fully sequenced in less than a day. Compare this with the sheer scale and time frame necessary for the first complete sequencing of the human genome. Currently, two main platforms

have been used – LifeTechnologies Ion Torrens Personal Genome Machine (PGM) and Illumina MiSeq [18]. Both systems generally use similar steps.

First, the DNA template is prepared, and a library is produced, which is then clonally amplified. The libraries are created by fragmenting the DNA samples and ligating synthetic oligonucleotides. The fragments then act as a template for *de novo* DNA synthesis. This occurs by cycling a flooding and washing of nucleotides with a known and predetermined sequence. As the nucleotides are incorporated onto the DNA ends, the order is digitally recorded. This is a differentiating step between MiSeq, which detects fluorescence, and PGM, which records pH changes. The raw data are then analyzed and converted into a useable format for researchers.

In recent years, NGS has allowed personalized medicine to advance and start to fulfill its anticipated potential and rewards are now being reaped in the oncology field as a result.

In 2011, the first pilot study was published, which explored the practicalities of high-throughput screening in oncology. This study, known as MI-ONCO-SEQ (Michigan Oncology Sequencing Project), enrolled patients who had been diagnosed with advanced cancer, and who had been previously deemed eligible for clinical trials. The aim of this pilot was to identify tumor-specific mutations in individual patients that could ultimately be used to tailor treatment. This was achieved through whole genome sequencing, targeted exome sequencing of tumor and somatic DNA, along with tumor transcriptome sequencing. Furthermore, the goal was to obtain this personalized analysis within a time frame acceptable for clinical decision-making (4 weeks, the standard washout period that patients must wait between clinical trials).

The study identified a variety of proposed cancer-forming mutations, ranging from point mutations and copy number changes to structural rearrangements and gene expression alterations. One example was the identification of a Ras mutation in a patient with malignant melanoma, which then provided the clinicians with subsequent treatment rationale [19].

This is a prime example of how high-throughput DNA sequencing can be utilized to influence personalized anticancer treatments. In the field of toxgnostics, we anticipate that this technology can be extended to identify any specific mutations that are markers of potential treatment-associated adverse effects, and thus avoiding the need to expose patients to such risks.

18.3 Proof of Principle Toxgnostics

As with all novel approaches, the argument for use of toxgnostics is strengthened with proof of principle examples. Even though there has been an abundance of potential toxgnostic markers published to date, only a small subset is currently utilized in clinical practice.

In potentially curative cases of colorectal cancer, the definitive treatment is surgical excision. Adjuvant chemotherapy is given to increase the odds of survival; however, this increase is modest in comparison. 5-Flurouracil (5-FU) continues to be the main player in anticancer treatment of colorectal cancer; its cytotoxic action is in the inhibition of the production of thymidine and through the incorporation of its metabolites into DNA and RNA [20]. The survival advantage of 5-FU is a 5% increased long-term survival.

5-FU is partnered with other agents to form common chemotherapy regimens, for example, 5-FU with leucovorin and irinotecan forms FOLFIRI regimen [21]. Capecitabine is a prodrug, which undergoes conversion to 5-FU in malignant tissue. Capecitabine is partnered with oxaliplatin to form XELOX [22].

Exposure to 5-FU can result in significant toxicity to the patient; depending upon the regimen and dosage, up to 30% of patients experience significant toxicity (Common Toxicity Criteria Grading level 3), ranging from diarrhea and nausea to myelosuppression and ultimately death in approximately 1% of cases [23,24]. Due to the modest advantage of 5-FU therapy and the relatively common adverse effects, a delicate assessment of risk–benefit analysis needs to be considered. One approach could be to use biochemical and kinetic assays to measure enzyme activity, which could, in turn, be used to predict patient response to the treatment. However, in reality, such experiments are generally too expensive and labor intensive to be routinely used in large scale. Genetic polymorphisms within the population have been predicted to influence the metabolism and distribution of 5-FU and its active metabolites. Potentially, such polymorphisms could ultimately be identified and prospective dose modifications made.

The biochemical pathway of capecitabine and 5-FU action has been well established, and provides 25 candidate genes that have been proposed to affect 5-FU toxicity [25,26] Despite significant published work investigating inherited genetic variants and associated toxicity, only a few have been identified with any significant confidence [26].

Rosmarin *et al.* [26] examined associations between capecitabine toxicity and candidate polymorphisms in patients from the QUASAR2 trial (Quick and Simple and Reliable trial 2). The authors also carried out meta-analysis of data from their evaluation and previously published studies, amounting to analysis of 36 polymorphisms. Perhaps surprisingly, only four polymorphisms (*TYMS 5'VNTR 2R/3R*, *TYMS 3'UTR 6bpins-del*, *DPYD 2846T>A*, and *DPYD* **2A*) were shown to be associated with grade 3+ adverse events, and then only in 5-FU monotherapy [26] DPYD is a protein necessary for the first step of the 5-FU catabolism to the inactive metabolite dihydroflurouracil. The meta-analysis by Rosmarin *et al.* [26] identified two variants of DPYD (*DPYD 2846TA* and *DPYD* $*2A$), which were found be associated with a relatively high risk of capecitabine-associated toxicity (odds ratio, 5.51).

A candidate gene study of capecitabine-related toxicity has been performed as an extension of this meta-analysis [27]. In the published study, 25 genes in the capecitabine and 5-FU pathway were investigated for novel variants that are associated with capecitabine toxicity. In all, 1046 capecitabine-treated patients from the QUASAR2 trial were recruited, and grade 3+ toxicity was recorded for just over one-third of patients. Genomic data were available for approximately 90% of the patients (genome-wide tagSNPs arrays and exome arrays), thanks to blood banking at the start of the trial. This study identified four new genetic variants associated with capecitabine toxicity, two of which map to DPYD locus, but are independent of previously identified associations. One example of a toxicity-associated allele is rs12022243. This allele was found to be relatively common, with a study population frequency of 0.22, and was found to induce a moderate effect upon toxicity (OR global binary = 1.8). Despite such potentially catastrophic outcomes, prescreening is not currently a routine practice. The authors of this study propose that in future patients are subject to a highly sensitive test used to detect patients at risk of severe, life-threatening toxicity, the result of rare *DPYD* alleles [27]. By doing so, the authors are suggesting the integration of toxgnostics in everyday anticancer treatment.

18.4 Proposed Protocol

18.4.1 Integration within Randomized Control Trials

For all new toxgnostics studies, no matter what methodology chosen, we propose that the following steps should be adhered to in order to identify rigorous associations, resistant to the robust testing.

First, the gold standard of toxgnostics should be that all studies are embedded within large, well-designed, and prospective randomized control trials (RCTs) of novel anticancer agents, or dramatic changes to exiting regimens. RCTs are the most rigorous way of determining whether a cause-and-effect relationship exists between treatment and patient outcome [28]. We would hope to extend this to correlate genetic markers with treatment adverse effects.

The advantages of this are manifold. As they are within RCTs, the recruitment of the patients will ensure that the control and the treatment arm have stratified patients for age, disease status, and comorbidities, as well as lifestyle choices such as smoking status. The more we start to understand regarding the complex interplay between a patient's genetic profile and their environment, the more this will provide invaluable information. This approach will also reduce the likelihood of including any confounding bias. Second, the large sample size of RCTs will minimize the chance of including false positives with in the data. Third, the statistical power of the association will also be increased. This can be illustrated with the following example: If 270 patients are recruited, studying an event with a rate of 30%, and 761 genes are analyzed with Cox

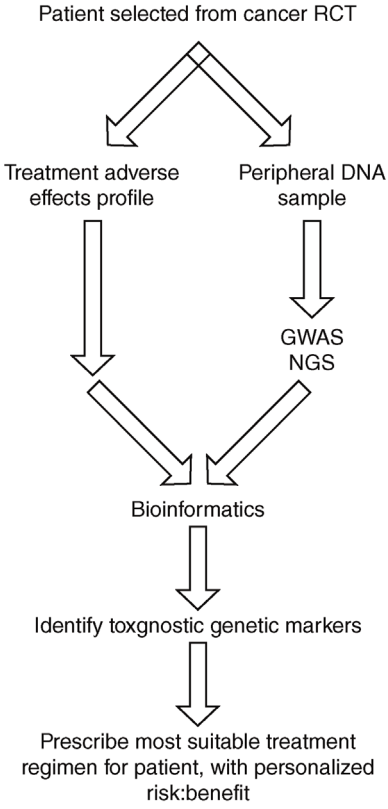

Figure 18.1 Schematic of proposed toxgnostics within personalized medicine regimen.

proportional hazards model, the chance of a false positives is roughly 1 in 4. If the study would be expanded to include 1000 patients, with all other study parameters remaining constant, the false positive gene identification would be 4%.

An RCT testing anticancer treatment will include the assessment of adverse treatment effects in the protocol. These side effects will be recorded using a standardized protocol, the CTC Grading, minimizing any subjective observer bias.

We propose that all cancer treatment RCTs (and other significant studies) store a sample of peripheral blood for future DNA analysis, and by doing so essentially form biobanks (Figure 18.1).

18.4.2 Biobanking and Future-Proofing Samples

Biological material can be stored from patients in repositories referred to as biobanks. The samples from within the banks should be accessible to researchers from within the community, with the ultimate aim of advancing treatment.

Research commissioned by Breast Cancer Campaign in 2008 identified the possibility that the lack of biobanks may be a limiting factor in the translation of laboratory findings to clinical practice [29]. As a result, over recent years the number and type of biobanks have been increasing. In particular, a "super" bank has been created – collaboration between four centers within the United Kingdom (Barts Cancer Institute, Universities of London, Leeds and Dundee) and the NHS. This is the United Kingdom's first specialist breast cancer biobank; hopefully, biobanks will be established for other cancer sites in due course (Breast Cancer Campaign Tissue Bank) [30].

To advance research and facilitate the translation from benchside to bedside, researchers should have access to up-to-date, accurate, and detailed data, preferably from large, multicoated randomized controlled trials [31]. Standard information that currently accompanies biological tissue samples includes patient demographics such as age, gender, and survival status. It is now becoming routine that disease-specific information is provided such as tumor type, grade, and, for certain cancer types, hormone receptor status.

To really achieve the full potential of such far-reaching research programs, the patient demographic information collated could be significantly expanded through the use of an extensive patient questionnaire. Patients could be asked to provide detailed information regarding any possible exposure to potentially toxic material through lifestyle choices such as diet, living conditions, and exposure in the workplace. Indeed, it has been estimated that nearly a quarter of all diseases, accountable for approximately 13 million deaths annually, are the result of environmental exposure that could be avoided, or at least minimized [32].

The patient data collated could be used to facilitate a retrospective epidemiological study to try and identify any causative links between patient exposure and disease development, allowing the further characterization of known risks and raising the possibility of identifying novel carcinogens. Such compounds would, of course, need further characterization and confirmation of a truly causative relationship, not just confounding. It is, of course, important to remember that any information recorded could be prone to recall or social desirability bias.

With such large repositories of patient information and correlating disease characteristics, it is feasible that complex relationships could be identified. For example, not only the fact that the exposure to a specific agent increases the risk of developing a certain cancer could be recognized, but also the further associated disease characteristics could be determined. Such details could include cancer tumor type, grade, and, for certain cancer types, hormone receptor status along with responsiveness to particular treatment regimens. Within the realm of toxgnostics, it could also be possible that certain genotoxic agents could not only influence the patient's response to treatment, but could also potentially modulate the presence or absence of treatment-associated adverse effects. Therefore, one feasible proposition would be that patient information recorded is extended to include specifics of patient adverse effects.

It is reasonable to hypothesize that any disease states and treatment responses may result from epigenetic changes. Epigenetics refers to heritable alterations in gene expression that occur without changes in the DNA sequence, and thought to occur through two main mechanisms: DNA methylation and histone modification. It has been previously demonstrated that epigenetic changes, which may have the possibility to influence disease state, can occur as a result of environmental changes [33,34]. Excitingly, the epigenome is emerging as the interface between the environment and the genome [35].

Of significance is that epigenetic modifications may be stable, and therefore alter gene activity state from one generation to the next, that is, it can be transgenerational. Therefore, the consequences are that any environmental exposure in generation one could be passed on to future generations. Bearing this in mind, should the patient questionnaires extend to cover any parental exposure?

In addition, with the development of next-generation, high-throughput sequencing, the databases could contain the sequenced genome of the tumor and the patient. These sophisticated techniques also contain high-resolution investigation of the epigenome, including the detection of any posttranslational histone modifications and genome-wide methylation [35].

In addition to relying upon detailed patient information, could it be possible to identify any genotoxic exposure by performing molecular assays on the tissue samples? Could experiments be designed to assay for known carcinogens and developed for any novel genotoxins identified? This obviously needs more careful thought but would provide many benefits including minimizing any possible recall bias as previously discussed.

The development of an assay, which could identify any previous exposure either through detection of the compound itself, or perhaps through its downstream molecular effects, could permit a targeted screening approach for patients who are identified as being at an increased risk. Of course, any screening programs would have to undergo rigorous testing, before being proposed. Furthermore, it would be necessary to consider whether the new screening program would fulfill Wilson and Jungner's screening criteria [36]. This classic set of criteria has been thought of as the gold standard tool of decision-making when considering the introduction of a screening program [37,38]. With the advances in genetic technology, is it time to modify the criteria to make it more applicable [39]?

One breast cancer biobank is forwarding research by ensuring that all tissues taken from their bank are returned accompanied with the data generated [31]. A policy has been generated, in accordance with the Independent Cancer Patient's Voice, the UK patient advocate group, which requires researchers to return all data generated from the tissue to the bank, in its unprocessed form, within 2 years (Breast Cancer Campaign Tissue Bank) [30,40].

It can take significant time to see the fruits of labor from basic research, but by "putting the work in now," including data on adverse effects, we could have an invaluable and complete database. Such data repositories could be used by physicians to make fully informed decisions regarding personalized treatment, maximizing treatment benefit, and minimizing adverse effects.

The donation of tissue by a patient should be thought of as an altruistic act. Researchers have a moral responsibility to ensure that the most is made from donated tissue.

18.4.3 Data Protection and Full Consent

As previously mentioned, correlation is necessary between clinical and genetic data, to elucidate any genotype–phenotype correlations. One important consideration for such work, and in particular for the future of biobanks and clinical data as a whole, is that of data protection. With increasing banks of electronic data, along with new technology such as cloud-sharing, comes the possibility of privacy infringement. As such, new legislation is needed to protect against this, and this comes in the form of the General Data Protection Regulation (GDPR). The cancer research community, as a whole, welcomes legislation protecting patient's data; however, the community is concerned regarding one particular section of this proposed guideline. Amendments 191 to Article 81 of the guideline have been interpreted by some to suggest that there exists a requirement to ask patient's consent for every individual piece of research performed on their data, rather than a blanket consent [41]. A further concern is that population-based disease registries will be affected, and that consent will be required for patient data to be stored in such databases. By their very nature, such consortia of data need to be all-inclusive for any meaningful correlations to be identified. If these databases are affected by this new regulation, population genetics and epidemiology studies could be detrimentally affected, thus losing a deeply powerful weapon in the arsenal of cancer research methodologies [42].

Lessons have been learned from the past. HeLa cells are a familiar name among cellular biologists and cancer research scientists. The cell line was initially created in 1951, in Baltimore in the United States. The cervical tumor material was taken from Henrietta Lane, a working-class African-American patient. The cells were the first to be successfully grown in a laboratory, but, infamously, were taken without the consent of the patient or her family. The cell line has, without question, advanced modern medicine in countless ways – arguably one of the most important discoveries was the characterization of human telomerase, the key to the aging cell. However, injustices in Henrietta Lane's treatment are only now being rectified. In recent time, the National Institute of Health (NIH) has been working with the Lane family to try and make amends for years of mistreatment. As a result, the Lane family has endorsed a new approach – case-by-case release of information – that is

subject to approval by a committee that includes members of the Lane family. This agreement has permitted the release of the full HeLa genome along with previous data that had been withdrawn from public access due to the family's concerns. The legacy of Henrietta Lane and her family have benefited cancer research in the six decades since they were taken. Without question the Lane family has been treated erroneously in the past, but hopefully these wrongs are now being corrected [43].

Oncologists and cancer research scientists have respect for patient's information and for the principle of valid consent. However, there is an urgent need for access to disease registries and collected human tissue. Unfortunately, due to its very nature, a significant proportion of information and human tissue currently stored was obtained from patients with valid consent; however, these patients are no longer alive so more detailed prospective consent could not be gained. We need to ensure that the future consent process covers any potentially incidental findings thus future-proofing our resources. If we fail to do so, we risk losing a valuable tool in modern cancer research.

18.4.4 The Need for a Collaborative Approach

A truly collaborative approach between entire cancer networks, spanning local, national, and international cancer centers, is required to maximize the potential to identify true toxgnostic polymorphisms.

The geographical nature of research occurring across national and international centers has further benefit. The enrolment of patients from a worldwide pool ensures that there is maximum heterogeneity of both genotypes and disease burdens, thus maximizing the possibility of true and significant associations that will benefit future patients.

One prime example of how a truly collaborative, and geographically wide-reaching, approach can ultimately benefit patient care is the IPAS study (Iressa Pan-Asia Study) [44]. This study was a phase 3, multicenter, randomized, open-label, parallel-group study. The trial compared gefitinib (Iressa, AstraZeneca) with carboplatin (Paraplatin, Bristol-Myers Squibb) plus paclitaxel (Taxol, Bristol-Myers Squibb) as a first-line treatment for advanced non-small cell lung cancer. The study was undertaken across an extensive geographical area – between March 2006 and October 2007, 1217 patients were recruited from 87 centers across Hong Kong, other centers in China, Indonesia, Japan, Malaysia, the Philippines, Taiwan, Thailand, and Singapore. Furthermore, the two groups were stratified with respect to demographic and baseline characteristics. The primary endpoint of the study was progression-free survival.

During the study, the patients also provided biological samples for DNA analysis, in particular the identification of mutation status at epidermal growth factor receptor (*EGFR*), the locus encoding epidermal growth factor. Inhibitors of the EGFR tyrosine kinase have been previously shown to have clinical efficacy

compared with best supportive care [45] or standard chemotherapy [46] as second- and third-line protocols for advanced non-small cell lung cancer.

The study identified a significant correlation between treatment response and *EGFR* mutation status. Indeed, progression-free survival was significantly longer for patients receiving genfitinib than carboplatin–paclitaxel in the mutation-positive subgroup, and significantly shorter among patients receiving genfitinib than carboplatin–paclitaxel in the mutation-negative subgroup.

At the time of the study publication, platinum-based combination chemotherapy, such as carboplatin–paclitaxel, one of the study arms, was the gold standard treatment for advanced non-small cell lung cancer. The IPAS study demonstrated that genfitinib alone is superior to the gold standard of carboplatin–paclitaxel in a selected population of East Asian patients, specifically nonsmokers or former light smokers, with pulmonary adenocarcinoma. Pertinently, the study identified that the overall treatment benefit with genfitinib in the subgroup was associated with *EGFR* mutations.

In addition to prolonged progression-free survival, genfitinib treatment was also associated with increased objective response rate, reduced toxic effects, and improved quality of life. Applicable to this chapter, genfitinib, as compared with carboplatin–paclitaxel, was associated with a lower rate of grade 3 or 4 adverse events, lower rate of adverse events leading to discontinuation of treatment, and a lower rate of dose modification due to toxic side effects.

Overall, this large-scale and geographically far-reaching study identified that the presence of an *EGFR* mutation was a reliable predictor of improved overall outcome with gentifinib.

18.4.5 Open Access to Results

To maximize efficiency of research between all cancer networks, we propose the use of open-access platforms for data sharing. The ultimate aim would be for raw data and basic results to be available freely and at real time [47]. We suggest the National Institute of Health Research (NIHR) Clinical Research Network (CRN)'s new platform, the Open Data Platform, to be used as a working example. Open access to real-time results should facilitate a more streamlined and efficient research effort. Centralized registries of research activities could minimize duplication of research efforts, important in the resource-critical economic climate we are currently experiencing.

Furthermore, we believe that results should be accessible prior to publication, allowing researchers to direct their projects or interpret results in a time-critical manner. Questions remain regarding the extent of the access to real-time results – Should raw data be available or should it be confined to those that involve patient data only? With the prospect of open-access results arises the issue of digital security. Only appropriate people – that is, members of a specific research community – should have access to the results, especially important if

accessed results involve patient data. All data uploaded to open access would have to remain anonymous with no potentially identifiable features. Administrators of the open-access platform would have to ensure not only that the platform is robust and could tolerate expected Internet traffic but that it is also a secure site.

One obvious proviso to real-time data platforms that requires further clarification is that any intellectual property or ownership of results would still need to belong to the original authors. By not ensuring rightful ownership, we risk that the most promising results remain "under the radar" until publication and in doing so undermining the collaborative nature of the research.

18.4.6 Translation from Bench to Bedside

The benefits of any scientific research are primarily derived from appropriate access to the results and subsequent positive changes to best medical practice. Research should direct medical management, so the most effective, efficient, and up-to-date treatments are delivered patients. Furthermore, with public investment in research there is an increasing responsibility that research has a demonstrable benefit to patient care, all the more relevant in the current economic climate. Purely academic research is becoming an intellectual luxury that we may increasingly find difficult to socially accept.

For continuing excellence in clinical care, we need to overcome any potential reasons why research findings may be slow to influence practice. Such causes may include obstructed access to information at the pertinent moment; individual and institutional barriers to change; and a lack of uniform medical practice within a professional body. Perhaps above all else, the major block is the increasing time pressure health care professionals face, confounded by increasingly distant targets and seemingly never-ending paperwork. To overcome such blocks, research results need to be easily accessible, up to date, and relevant. With today's ever more sophisticated technology, it should be possible for information to be available at a click of a button. Automatic reminders on clinical systems could and should provide clinicians with the most up-to-date, research-driven guidelines. We are increasingly using technology in our everyday lives, and this continues into our clinical workplace. Smart phones are increasingly becoming a go to resource – traditional sources of information for clinicians are now accessible as phone 'apps' such as the British National Formulary and the Oxford handbook of clinical care – a well-thumbed resource for all junior doctors.

Advances in technology have also been shown to allow patients to have more ownership of their medical management. This dovetails with the recent emphasis that has been placed on patients to be more responsible for their own medical management and health needs. A feasibility study by Dr Andrew

Weaver and others demonstrated the successful self-management of patient's chemotherapy side effects with home monitoring of their symptoms via mobile phone. During this study, six patients undergoing adjuvant chemotherapy for colon cancer entered their symptoms onto a mobile phone, twice daily. This facilitated real-time self-assessment of common side effects, such as nausea, vomiting, and diarrhea. This information was then sent, via a secure network, to a remote computer. If the computer receives information regarding severe symptoms, a designated nurse was immediately contacted via pager to further manage the patient. The patients involved in the study confirmed that they felt secure that their symptoms were being closely monitored and were satisfied that they were actively involved in their own management [48].

18.5 Fiscal Matters

In today's health care marketplace, few recommendations can be made without financial considerations. It has yet to be demonstrated whether the use of toxgnostics in clinical practice will result in any financial gains in real terms. The more next-generation sequencing and GWAS is used, the more costs should continue to fall, increasing any positive margins. Currently, initial outlay costs for necessary laboratory equipment heavily skew start-up costs, something that needs consideration when calculating study costs in real terms. This positive margin will continue to grow as in hospital care expense increases, which would make the aftermath of anticancer therapy side effects more expensive to manage. Detailed health economics are required to carry out accurate cost–benefit analysis, but the hope is that toxgnostics, with its preventative rather than reactive approach, will be cost-effective in the long term.

18.6 The Future of Toxgnostics

In the field of oncology, as with other specialties, there exists an abundance of practical guidance as to how best the clinician can deliver patient care. Chemotherapy prescription, due to its nature of prescribing highly toxic chemicals, has perhaps more guidance than most inhospital treatments. To maximize safety, the majority of chemotherapy protocols include detailed dose modifications, which are based upon any previous adverse effects the patient has experienced in previous treatment rounds. By its very nature, this modification occurs after any adverse effects have already occurred. It would be preferable if any necessary modifications could occur before the first round of treatment, therefore not exposing the patient to any risk more than necessary. This approach would enable the Hippocratic fundamental to be adhered to, *primum non nocere.*

The aim of toxgnostics is to be able to apply any necessary modifications to a personalized prescription in a *prospective* manner, therefore mitigating any risks before the patient is exposed. As with most elements of medicine, there are advantages and disadvantages to treatment. One consideration, which would need careful thought, is to ensure that any risk reducing modifications do not reduce the effectiveness of treatment. Physicians strive for the ultimate balance of maximum effectiveness and minimal toxicity; we hope that toxgnostics will facilitate this process.

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19

Ethical Considerations in Developing Strategies for Protecting Fetuses, Neonates, Children, and Adolescents from Exposures to Hazardous Environmental Agents

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19.1 Introduction

Previous chapters in this book have addressed research on how environmental exposures have differential adverse health effects at various stages of human development. For quite some time, scientists have known that exposure to alcohol, drugs, and tobacco smoke *in utero* can adversely impact the health of the fetus and future child [1–3] and exposure to lead *in utero* and during childhood can adversely impact neurological development and learning [4]. More recent studies have shown that children, adolescents, neonates, and fetuses may be particularly susceptible to exposures to pesticides [5], air pollution [6,7], and endocrine disrupting chemicals [8,9]. Researchers are also investigating the health effects of many other environmental agents, including industrial chemicals, allergens, foods, hormones, ionizing radiation, and pathogens at different stages of the lifecycle and attempting to develop strategies and interventions intended to mitigate or reverse the adverse health effects of such exposures [10,11].

The genesis of this new knowledge creates ethical issues for health care professionals, public health officials, employers, as well as society as a whole, because competing fundamental values may be at stake in formulating and implementing strategies (i.e., practices and policies) designed to prevent or mitigate disease and advance human health. For example, potential strategies for minimizing prenatal exposure to alcohol and illegal drugs range from educating pregnant women about how lifestyle choices impact the health of their babies to incarcerating women who abuse alcohol or drugs during pregnancy. Deciding which strategy to use requires one to resolve an ethical conflict between respecting the pregnant woman's autonomous choices and

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preventing harm to fetuses and children [12]. The question of whether the government should ban the use of the endocrine disrupting compound bisphenol A (BPA) in some types of products involves a conflict between protecting the health of infants or children and increasing consumer costs [13]. In this chapter, we will examine the ethical considerations for developing and implementing strategies for protecting fetuses, neonates, children, and adolescents from exposures to harmful environmental agents.

19.2 What Is Ethics?

Before beginning our analysis, it will be useful to say a few words about ethics. Ethics can be understood as standards of conduct that distinguish between right/wrong, good/bad, and so on [14]. Ethics (or morality) includes general standards of conduct for all people as well as special standards for people in specific social roles, occupations, or professions (i.e., applied ethics). Ethics can be studied from a normative or descriptive (or empirical) viewpoint. Normative ethics attempts to evaluate and justify conduct. It examines questions concerning what we ought to do, how we ought to live our lives, and how we should make choices that profoundly impact ourselves and others [14]. Descriptive ethics attempts to describe and explain conduct. It examines the social, cultural, psychological, neurobiological, and evolutionary underpinnings of ethical behavior, judgment, and reasoning [15]. This chapter will focus on normative ethics issues.

19.2.1 Some Fundamental Ethical Values

Ethical controversies usually involve conflicts of fundamental values. We will not attempt to cover every value that may come into play in ethical controversies but will highlight some of those that seem most relevant to the issues at hand.

19.2.1.1 Benefits and Costs

Many ethical dilemmas involve balancing benefits and costs. Benefits that need to be considered include individual life, health, happiness, and well-being; social welfare (e.g., economic prosperity, public health); animal welfare, and environmental welfare (e.g., biodiversity, ecosystem stability). Costs may include individual pain, suffering, or disease; economic disruption and social decay; and harms to animals or the environment. Various philosophical theories and religious traditions hold that we should avoid causing harm and that we should promote good consequences for ourselves, our family, and society at large [14].

19.2.1.2 Individual Rights and Responsibilities

Many ethical dilemmas involve conflicts between protecting individual rights (e.g., rights to autonomy, property, privacy, and freedom of speech and religion) and promoting individual, social, or other goods [14]. For example, in the case involving limiting fetal exposure to alcohol and drugs (already mentioned), the mother's right to autonomous control over her own body may conflict with the goal of protecting the welfare of the fetus. Individual responsibility is the flipside of individual rights. People want to take responsibility for their own conduct and they expect others to do the same. Some ethical issues, such as the scope of liability for negligence, may involve conflicts between promoting individual responsibility and social goods [16].

19.2.1.3 Justice

Ethical dilemmas often involve conflicts between different conceptions of justice (or fairness). Justice includes distributive justice (the distribution of benefits and costs in society), procedural justice (fairness in governmental and societal decision-making), and retributive justice (fairness in decision-making pertaining to crime and punishment). Distributive justice is an especially contentious concept. For example, libertarians hold that benefits and costs should be distributed according to a fair procedure that protects human rights but does not redistribute wealth, whereas egalitarians hold that benefits and costs should be distributed to promote equality of opportunity, and utilitarians hold that benefits and costs should be distributed in a manner that promotes the net good of society. Egalitarians and other theorists hold that society should protect the welfare of vulnerable citizens, such as children, mentally disabled adults, and individuals who are socioeconomically disadvantaged or chronically ill [16]. Questions concerning the distribution of health in society and access to health care raise issues of justice [17].

19.2.2 Value Conflicts and Ethical Decision-Making

Ethical disputes may arise when fundamental values conflict and one must decide which values should take priority in particular circumstances [18]. We have already mentioned some types of conflicts but other examples abound: animal experimentation involves a conflict between animal and human welfare; the clearing of land to build a housing development involves a conflict between human welfare and rights and environmental and animal welfare; and taxation of wealth and income involves a conflict between different conceptions of distributive justice. In this chapter, we will not attempt to resolve conflicts among fundamental values that may arise in developing strategies that protect fetuses, neonates, children, or adolescents from exposures to hazardous environmental agents. Instead, we will discuss some of the values at stake and describe a process for ethical decision-making (see Box 19.1).

Box 19.1: Process for Ethical Decision-Making

1) Define the question, problem, or issue.
2) Gather or obtain relevant information concerning the facts and circumstances pertaining to the problem, question, or issue.
3) Explore realistic options for addressing the problem, question, or issue.
4) Describe how ethical values apply to the different options and how they may conflict.
5) Resolve value conflicts.
6) Make a decision and act.

For an illustration of how this process can work, consider the Environmental Protection Agency's (EPA's) decision-making concerning ambient air quality standards. The EPA develops national standards for acceptable levels of different pollutants, which the states enforce with their own rules [16]. As of May 6, 2015, the standard for ozone was to not exceed 0.075 ppm for the annual fourth-highest daily maximum 8-h concentration, averaged over 3 years [19]. On November 14, 2014, the EPA proposed lowering the standard to 0.065–0.070 ppm, with implementation of the new standard to occur in 2016 [20]. The ethical question for setting a new air quality standard for ozone can be defined as "What should be the national ambient air quality standard for ozone?" Relevant information could include the health impacts of ozone on the general population as well as vulnerable populations (e.g., children, asthmatics), the protective effects of the current and proposed standard, and the economic costs of the new standard as compared to the old. The options would include keeping the standard as it is, lowering the standard to 0.065–0.070 ppm, and lowering it to a different range. The ethical values that apply to the different options include individual welfare, social welfare, animal welfare, environmental welfare, and justice. A potential conflict arises between economic prosperity, which is part of social welfare, human health, animal welfare, environmental welfare, and justice. The lower standards could promote health, especially the health of vulnerable people as well as the welfare of animals and other species, but this change could come at some economic cost, since communities, electric power companies, and businesses may need to make adjustments to meet the lower standards, which could negatively impact economic development. In resolving this potential conflict, one would need to decide whether the health gains (and potential benefits for animals and the environment) are worth the economic costs, considering both short- and long-term costs and benefits.

Resolving value conflicts can be the most difficult part of the decision-making process because people often have fundamental disagreements about what is most important [18,21]. Sometimes value conflicts can be resolved as people

acquire new information. For example, if information indicates that the economic impacts of the lower ozone standard would be minimal, then this might convince parties who oppose the new standard to accept it. Because acquiring new information can help to resolve value conflicts, scientific research can have a direct bearing on ethical disputes. This does not mean, however, that science provides answers to ethical questions, only that it can supply the information needed for rational decision-making [16]. In many cases, scientific research may also create new dilemmas by detecting risks that were previously unknown. For example, people did not have to face any ethical dilemmas concerning protection of pregnant women from exposures to lead until scientists discovered the risks of lead exposure to the fetus. When new information does not lead to conflict resolution, the best option may be to seek a reasonable compromise between competing positions. To achieve a compromise, it may be necessary to consider options that both sides of a dispute find acceptable though not necessarily preferable. For example, for setting EPA ozone standards, a reasonable compromise might be to expand the upper range of standards and give states additional time to implement them. A compromise such as this might allow the EPA to enhance protections for human health and the environment without creating harmful economic disruption.

19.3 Ethical Considerations for Strategies Used to Protect Fetuses, Neonates, Children, and Adolescents from Exposures to Harmful Environmental Agents

We will now discuss ethical considerations for some potential strategies for protecting fetuses, neonates, children, and adolescents from exposures to harmful environmental agents. Many of these strategies also protect adults but we will focus on protection of nonadults in this chapter.

19.3.1 Education

Education involves communicating information about health risks and benefits to relevant parties, such as pregnant women, parents, teachers, children, employers, and others. Communication can occur directly, for example, when an obstetrician informs an expectant mother about the risks to the fetus of smoking and alcohol or drug use during pregnancy; or indirectly, for example, through public service advertisements on television, radio, or in print; informational websites; community outreach activities; classes taught in K-12 schools or colleges; or consumer product labeling. One of the strengths of education is that it treats recipients of information as autonomous decision-makers who can make responsible choices to protect their own health or the

health of others. It can empower people to make healthy choices and promotes individual and social welfare, individual rights and responsibility, and justice [22]. One of the ethical issues that may arise in education is how best to present information to recipients. Informers of the public must decide whether to present information at face value, or whether to adopt a paternalistic approach involving manipulation of information to promote specific choices deemed to be beneficial [23,24]. For example, in the United States and many other countries, cigarette manufacturers are required to print warning labels on packages and advertisements that inform consumers about the health risks of smoking, such as lung cancer and emphysema. There is an ongoing controversy whether it is legal (and ethical) to require cigarette manufacturers to include graphic images, such as pictures of diseased lungs or cancer patients on warning labels. In 2009, the Food and Drug Administration (FDA) required manufacturers to include graphic warning labels on cigarette packages but a federal appeals court struck down the requirement on the grounds that it violated Constitutional protections for free speech because the government had not provided enough evidence that the warnings would reduce smoking [25]. In the United States, cigarettes are legal, but the government was attempting to control consumer choices by manipulating how information is presented. Public health advocates argued that the manipulation was justified as a means of promoting health, but advocates of free speech and individual autonomy argued that the government's attempt to control corporate speech and consumer choices was not justified. Controversies have also arisen concerning the presentation of information pertaining to nutritional guidelines [23]. For nearly two decades, there has been a dispute among researchers about the recommended daily sodium intake. In the 1970s, numerous studies demonstrated a relationship between high sodium intake and increased blood pressure, which is a risk factor for cardiovascular disease (CVD). During the 1990s, numerous health organizations recommended restricting dietary sodium intake to less than 2.4 g per day (or about 6 g of table salt). In 2003, the World Health Organization reviewed the best available evidence and recommended that adults should restrict sodium intake to less than 2 g per day. Since then, evidence has emerged showing that people without existing CVD probably do not benefit from restricting sodium to 2 g or less per day, and that most people will find it difficult to achieve this goal. Moreover, some studies have suggested that less than 2 g of sodium per day is associated with increased CVD risk [26]. Some public health advocates have argued against discussing all of these facts with the public on the grounds that it could lead to uncertainty and defeat efforts to convince people to reduce their sodium intake. Others have argued that the best strategy is to present all of the information to the public to allow people to make their own choices [23]. The preceding controversies that concern with presenting the public with health information illustrate a potential conflict between promoting health and respecting autonomy [23]. Some have

argued that health care professionals, public health officials, educators, and others should present consumers with the relevant information so they can make informed choices [24]. Providers of information may recommend particular choices, but they should not manipulate information or its presentation in order to control consumer decisions. While this approach makes a great deal of sense when communicating with adults, one might argue that it may not be an appropriate way of communicating with children and adolescents, because they are not fully autonomous and may be susceptible to exploitation or undue influence from private companies. For example, one might argue that the government should regulate the content of advertising that specifically targets children in order to promote their health [27].

19.3.2 Testing/Screening/Monitoring

Testing, screening, and monitoring are other useful strategies for protecting fetuses, neonates, children, and adolescents from harmful environmental exposures. Testing includes performing laboratory assays on biological samples (e.g., blood, urine) or environmental samples (e.g., household dust) to detect the presence of chemicals, allergens, pathogens, or other harmful environmental agents. Tests could also be performed to determine whether individuals have immunologic, metabolic, or genetic characteristics that increase their susceptibility to adverse effects from specific environmental exposures. Test results could be used to diagnose, prevent, or treat diseases. For example, if children are living in a home that is suspected of containing lead paint, the home could be tested for lead in household dust, or the children's blood could be tested for lead. If dangerous levels of lead are detected, the children could be treated for lead exposure and the home could be remediated to reduce lead exposure [16].

Screening involves testing populations or groups that may be exposed to dangerous agents or may have unique susceptibilities. For example, houses in an area where homes were built prior to implementation of lead regulations could be tested for lead. The homes of workers who are exposed to dangerous substances in the workplace could be tested to protect them or their children from exposure. Monitoring involves periodic testing to detect new or ongoing exposures to dangerous environmental agents. For example, if children in a household have asthma or allergies to environmental agents in the air (such as dust mites or mold), the parents could purchase allergy test kits to monitor the home for levels of allergens and use that information to take steps to reduce exposures. Also, an employer could monitor employees for exposures to hazardous substance in the workplace (such as lead or mercury) and use this information to reduce exposures.

Tests that are used should be reliable and accurate [28]. Tests that are not reliable or accurate could yield false positive results (detecting an agent when none is present) or false negatives (failure to detect an agent when one is

present). Tests with a high false positive rate could cause recipients needless worry or lead them to unwise choices based on faulty information. For example, a false positive test for dangerous levels of lead in a home could cause the owners considerable anxiety and might lead them to sell the home. Conversely, tests with a high false negative rate might lull recipients into a false sense of security, which could prevent them from taking effective action to promote their health. To promote accuracy and reliability tests should be performed by a certified laboratory. If a test kit is used, the kit should have undergone appropriate testing, validation, and quality control to ensure reliability and accuracy and include user-friendly instructions for proper use. Recipients of test results should receive appropriate counseling and advice [28]. They should be informed of the meaning of their results and any actions they could take to reduce or mitigate their health risks, such as receiving medical treatment or making dietary or lifestyle changes. When testing is conducted in homes to detect potential exposure to harmful environmental agents, recipients should be informed if their exposure levels are classified as safe or unsafe by appropriate authorities (such as the EPA), and how their exposure levels compare to the other homes. Recipients should also be informed if there are no recommended exposure levels and no well-established comparators [29,30]. Recipients could use this information to decide whether to take steps to reduce their exposures.

There is an ongoing ethical dispute concerning the extent of testing and reporting of results. Some argue that results with only clinical value should be tested for and reported [28]. A test result has clinical value if it can be used to diagnose, prevent, manage, or treat a disease. If a test is designed to detect the presence of an agent for which there is no known safe or normal level of exposure, or no effective strategy for mitigating adverse health effects, the test results may provide recipients with information that is confusing, worrisome, and potentially harmful. Recipients may become unnecessarily alarmed about exposure levels and make inadvisable choices based on uncertain information. For example, phthalates are a group of chemicals used as plasticizers in various industrial applications. Phthalates are an important public health concern because some of these compounds are known to be endocrine disruptors that can modify or alter the endocrine system, potentially leading to adverse health effects. Fetuses, neonates, children, and adolescents are especially susceptible to the effects of endocrine disruptors [8]. Children are also commonly exposed to phthalates through their diet and use of plastic toys. Although the EPA has established safe exposure levels for some phthalates, it has not established safe levels for all of them [31]. Providing parents with test results concerning their child's exposure to a phthalate for which there is no established safe exposure level could cause them needless worry, since they might not know what to do with this information. There may also be no known effective strategy for reducing the child's exposure to this chemical. Others argue that the extent of testing and the reporting of results should be determined by the

recipients. Proponents of this view argue that recipients have a right to receive all of their results. Recipients may be curious about their results and may want to use them to make health-related decisions [29,30]. Advocates for this view also argue that most people who receive results with uncertain clinical value will not experience needless worry or confusion. If they are concerned about their results, they can ask a health care professional for more information or clarification. Additionally, recipients who are concerned about receiving potentially useless results can always request that the extent or testing and/or reporting of results be limited to what they want to know. The dispute about the extent of testing and reporting of results involves a conflict between respecting the autonomy and harm avoidance. Those who favor extensive testing and reporting of results emphasize the importance of recipient autonomy, while those who favor testing for and reporting only clinically useful results emphasize the importance of avoiding harm [32]. The cost of testing and counseling also raises ethical issues. Though some tests, such as tests for PKU, are relatively inexpensive, others, such as genetic testing for predispositions to cancer, can be expensive. While some people may be able to afford to pay for testing and counseling out-of-pocket, others will require financial assistance. Insurance companies may be reluctant to pay for tests with questionable clinical value, and they may set a very high bar for providing insurance coverage even for tests with clinical value. For example, some insurance companies do not cover tests for BRCA1 and BRCA2 genetic mutations, even though these tests provide reliable and accurate information pertaining to the lifetime risk of breast or ovarian cancer. Other companies provide coverage only under limited conditions. BRCA1 and BRCA2 testing is expensive – as high as $2000 – and some companies may decide that the benefits of testing are not worth the costs [33]. Government health care agencies, such as Medicaid and public health departments, face similar dilemmas concerning financial support for testing. Testing for the presence of hazardous environmental agents may present unique challenges concerning payment, because many of these tests are currently under development and their clinical value has not been well established. Some of these tests may also be expensive. While some tests, such as tests for lead in the home, have minimal costs and well-established clinical value, others may be expensive or may have questionable clinical value. It may be difficult to convince insurance companies or government agencies to pay for these tests until evidence clearly shows that their benefits outweigh their costs. The cost of testing and counseling also raises issues pertaining to justice, since socioeconomically disadvantaged individuals are more likely to have difficulty affording testing and counseling than well-to-do individuals. Problems with access to testing and counseling could, therefore, exacerbate existing health disparities [17]. Egalitarians would argue that the government should pay for testing and counseling for those who cannot afford it to counteract health disparities or protect the welfare of children and other vulnerable groups.

Libertarians might oppose government subsidization of testing and counseling as an illegitimate form of wealth redistribution, while utilitarians would accept government subsidization as long as the benefits to society outweigh the costs. One final issue related to testing is whether it should ever be mandated by the government. Most states pay for and mandate PKU testing and several other types of newborn screening [34]. The rationale for newborn screening programs is that they are cost-effective methods for preventing disease, promoting health, and reducing health disparities [35]. However, some parents might object to certain types of screening for philosophical or religious reasons. Many states include religious exemptions from newborn screening programs [36]. Mandatory screening or testing of neonates, children, or adolescents, therefore, creates a potential conflict between promoting health and respecting freedom of religion and parental rights.

19.3.3 Worker Protection

Before the enactment of child labor laws in the early to mid-1900s, children often toiled long hours in factories, mines, and other places that exposed them to toxic substances and workplace hazards. Today, most nations have laws that protect children from dangerous working conditions, but many developing nations do not [37]. Adolescents may still encounter dangerous working conditions in countries that have child labor laws. These laws may permit adolescents to work on farms and restaurants, where they may be exposed to pesticides, fertilizers, cleaning solutions, hot grease, and other dangerous substances [38]. Fetuses, neonates, children, and adolescents may also be indirectly exposed to dangerous environmental agents via parental exposures in employment. For example, if a pregnant woman works in a battery factory, her fetuses may be exposed to lead. A man who works as a pesticide applicator for an industrial farm may bring pesticides into the home on his clothing.

Most employers have implemented programs to protect employees and their family members from occupational exposures. For instance, employers may modify the work environment to contain or limit occupational exposures, and they may provide employees with protective equipment, clothing, and training on workplace safety. Employers may also provide employees with the education and resources needed to avoid exposing family members to hazardous substances when they return home [39].

Employers who take steps to protect employees and their families from harmful exposures usually have three sources of motivation. First, some may genuinely care about the health of employees and their families. Healthy employees tend to have better productivity and morale than sick ones [39]. Second, employers may want to avoid lawsuits from employees who are injured in the workplace or develop diseases related to occupational exposures. Third, employers may be required by federal or state laws to implement occupational

health and safety measures. The United States and developed nations have developed extensive regulations that set maximum allowable exposures to hazardous substances in the workplace and establish safety standards [39].

Employers have also implemented safety measures that protect female and adolescent workers from dangerous workplace exposures, some of which may raise ethical issues. Perhaps the most controversial of these are fetal protection policies. In a landmark case, the US Supreme Court found that Johnson Controls' fetal protection policy was illegal because it violated the Civil Rights Act of 1964 by discriminating against women [40]. Johnson Controls manufactures batteries, which contain hazardous chemicals, including lead. Johnson Controls had allowed women to work in its factory but had monitored their blood for lead levels deemed unsafe by the Occupational Safety and Health Administration (OSHA) and had warned them about potential risks to the fetus if they become pregnant. In 1982, after discovering that eight women became pregnant while maintaining dangerous levels of lead, the company decided to exclude all women, except those who were medically infertile, from working in jobs that exposed them to high levels of lead. The company implemented this policy to protect itself from lawsuits from children who might be harmed as a result of exposure to lead *in utero*. In 1984, female workers at Johnson Controls brought a class action lawsuit against the company. Johnson Controls argued that its policy was justified as a matter of business necessity since there was no other way to protect fetuses from dangerous exposures to lead. A federal district court ruled in favor of the company. After the Seventh Circuit Court upheld the district court's ruling, the plaintiffs appealed the case to the US Supreme Court, which reversed the lower court's ruling. The Supreme Court held that the company's policy was discriminatory because it only applied to fertile women, even though scientific evidence shows that men who are exposed to lead may also risk harming the fetus if they impregnate a woman [40].

Although the Supreme Court's ruling makes it illegal for companies to adopt fetal protection policies that discriminate against women, the ethical dilemmas concerning protecting fetuses from harmful workplace exposures still remain [41]. Companies may decide to use other strategies for minimizing fetal exposures, such as warning women (and men) about risks to the fetus from dangerous exposures, monitoring employees for exposure levels, and implementing other safety measures to protect all employees from potentially harmful exposures. Moreover, women are likely to bear the brunt of the ethical quandaries concerning fetal protection, due to the difference between male and female biology. Women must decide whether they want to work at jobs that may expose their fetuses to risks if they become pregnant. To a lesser extent, men must also take these concerns into account in their decision-making. Fetal protection policies present a conflict between protecting fetuses and children, and minimizing legal costs to businesses on the one hand, and promoting justice and protecting worker's rights on the other [41]. These conflicts are

likely to continue to arise as scientists learn more about how hazardous agents in the workplace can impact the health of the fetus and future child.

Establishing appropriate exposure levels for adolescents also raises ethical issues. The values of promoting health and protecting vulnerable people from harm would tend to favor the lowest possible exposure to hazardous substances in the workplace for adolescents. However, setting the lowest possible exposure levels would prevent adolescents from working at jobs that offer them important benefits, such as money, experience, and self-esteem. For example, a rule that mandated that minors have no pesticide exposures in farmwork would prevent them from working on farms that use pesticides, which would deny them employment, and so on. To establish appropriate exposure levels to hazardous substances in the workplace for adolescents, one must, therefore, balance competing values, that is, adolescent welfare versus the right to work.

19.3.4 Government Regulation

Governments create and enforce many different types of laws including statutes (laws drafted by legislatures), the common law (laws developed by judges through legal rulings), and regulations (laws developed by administrative agencies to enforce statutes or other types of laws). Numerous regulations protect citizens from exposures to dangerous environmental agents, such as those pertaining to air and water quality; domestic, agricultural, and hazardous waste disposal; occupational health and safety; pesticides and industrial chemicals; drugs, biologics, and food additives; building safety; and food safety and quality [16]. Regulations may establish acceptable (or safe) exposure levels to a substance or restrict its use [42]. For example, the EPA sets acceptable exposure levels for air pollutants (such as ozone and sulfur dioxide), pesticide residues on foods, and drinking water contaminants (such as arsenic and microorganisms). The EPA has significantly restricted the use of asbestos and PCBs (polychlorinated biphenyls), and many countries have banned DDT (dichlorodiphenyltrichloroethane) [16]. Some of these laws specifically address the need to provide additional protection for vulnerable groups, such as children and people with chronic illnesses. For example, the Food Quality Protection Act (FQPA) increased by 10-fold the protection for children from exposure to pesticide residues in food, and US courts have interpreted the Clean Air Act (CAA) as providing protection for vulnerable groups such as asthmatics [16]. Since there is not sufficient space in this chapter to discuss the ethical dilemmas related to all of these laws, we will focus on some recurring themes.

All government regulations have some type of economic impact [43]. Though business leaders often view government regulation as harmful to the economy, regulations often have positive economic impacts. For example, regulations designed to prevent companies from acquiring monopolistic control over certain sectors of the economy can promote fair competition and regulations

that protect intellectual property rights and can promote innovation. Regulations that protect public health and safety can save considerable expenses related to health care costs and loss of life. However, regulations can also have negative impacts on businesses and the economy. Companies may need to change their business practices and hire administrative and legal staff to comply with regulations [43]. For example, an electric power company might need to spend millions of dollars to modify its operations so that its emissions meet air quality standards [16]. Companies may pass on the added costs of regulation to the consumer, which has a negative impact on the economy. The economy can also be harmed when companies reduce their labor force or operations or go out of business as a result of regulations [43].

Coming to terms with the costs of regulations that protect citizens from dangerous environmental exposures raises the ethical issue: are the benefits of regulation worth the costs? The benefits may include benefits to human health and welfare, and animal and environmental welfare (e.g., pesticide regulations that protect nontarget species) [42]. Most of the costs of regulation have to do with their negative impacts on businesses and the economy. Proposed changes to the EPA's air quality standard for ozone are likely to cost industry as much as $15 billion per year [44]. The EPA has also developed proposed regulations aimed at cutting carbon dioxide emissions from coal-burning electric power plants to 30% of 2005 levels by 2030. While environmentalists have championed this plan as an important step toward reducing greenhouse gas emissions responsible for climate change, business representatives and economists have argued that it will cripple the coal industry and drive up the price of electricity, which will harm the economy [45]. Both of these regulations could help protect the health of fetuses, neonates, children, and adolescents, but they will also have some economic costs. On the positive side, they could increase the economic viability of sustainable energy investment, which could provide a long-term win–win effect.

Regulations also involve enforcement costs. Government agencies and officials must oversee regulatory compliance, which includes educating the public about rules, monitoring compliance, investigating and prosecuting cases of suspected noncompliance, and administering sanctions or fines for noncompliance. Enforcement activities can expand existing government bureaucracies or create new ones, adding to the cost of government. For example, the EPA, which enforces a variety of federal environmental regulations, has a proposed budget for fiscal year 2016 of $8.9 billion [46]. State and local governments also have their own agencies in charge of enforcing environmental laws. For example, the California Environmental Protection Agency had a budget of $3.6 billion for fiscal year 2015 [47]. Since government revenue is limited, money spent on enforcing regulations reduces the government's ability to fund other activities. Regulations may also have noneconomic costs. For example, during the 1970s, states passed laws requiring some domestic

products, such as furniture and children's sleepwear, to include flame retardants. These regulations were motivated by a desire to protect health and safety, since flame retardants could help prevent death or severe burns if a fire were to occur in a home. Since then, evidence has emerged that some types of flame retardants, such as polybrominated diphenyl ethers (PBDEs), increase the risk of cancer, have adverse effects on fetal and child development, and can disrupt endocrine, immunologic, and reproductive functions [48]. Some manufacturers have stopped putting PBDEs in their products and have begun using other flame retardants. However, there is a concern that flame retardants substituted for those found to have health risks might also have adverse impacts on human health. For example, brominated and chlorinated organic flame retardants (BFRs and CFRs) also pose health risks [49]. Some organizations have lobbied governments to ban the use of certain types of flame retardants [50]. One could argue that the fire safety benefits of PBDEs, BFRs, and CFRs for human health do not outweigh their health risks, because the risk of fire in most homes is low and most people die from smoke inhalation rather than burns [48,51]. Of course, the balance could shift the other way if the risk of catching on fire were much higher. For example, since the risks of catching on fire is high for firefighters or race car drivers, the fire safety benefits of flame retardants associated with adverse health effects might outweigh their other health risks. However, this argument would not apply to children, who usually are not exposed to a high risk of catching on fire.

Sometimes regulations raise issues of social justice. As mentioned earlier, some theorists hold that justice requires that society take steps to protect its most vulnerable members from harm. As mentioned earlier, the FQPA and the CAA provided additional protections for vulnerable groups (children and people with respiratory problems, respectively). While few people would challenge the notion that society should protect its most vulnerable members, these protections may have economic costs and practical limitations [52]. For example, suppose that 500 people out of the approximately 319 million people in the United States have a rare respiratory disease that makes them unable to tolerate ozone at a level of >0.035 ppm, or about half the EPA's proposed air quality standard for ozone. The economic costs of lowering ozone level to 0.035 ppm throughout the United States would be tremendous. Indeed, it might not even be possible to achieve an ozone level this low without radically altering our culture, technology, and economy. Should the EPA lower the ozone standards to 0.035 ppm or less to protect these vulnerable people? Would the social and economic costs of protecting this small minority be worth the benefits to them? Clearly, there need to be some practical and economic limits on protections for vulnerable groups, but it is difficult to say what those limits might be, or how much society should be willing to pay to protect its most vulnerable members [16,52].

Regulations may also raise issues concerning government restrictions on human freedom because they seek to control the behavior of business or

individuals. Some regulations have been controversial because they interfere with freedoms that are important to people. For example, in 2012, the New York City Health Department adopted a regulation that prohibited food establishments from selling sugared drinks greater than 16 oz. The soft drink ban, which applied to restaurants but not convenience stores, exempted some types of fruit juices and milk products. Industry representatives and critics argued that the ban was unwarranted state paternalism, and polls showed that 60% of residents opposed the ban. Courts have blocked the ban on the grounds that the city has not produced enough evidence that the regulation would promote public health [22]. Although public health advocates have defended the soft drink ban as an effective strategy for preventing obesity and diabetes [53], others have argued that the ban failed to respect an important human right – the right to decide what one eats [54].

Although most regulations designed to protect people from harmful environmental exposures probably do not raise significant issues concerning human freedom, some might. For example, human freedom will be an important concern in regulations that seek to control the human diet [22]. Freedom may also be an important consideration in regulations that govern occupational health, since people may want to be free to choose to work in dangerous conditions in order to earn money. When freedom is an important ethical concern pertaining to a regulation, society must decide whether the regulation's benefits justify its restrictions on conduct [16].

19.3.5 Taxation

Taxation is another strategy that society can use to protect fetuses, neonates, children, and adolescents from exposures to harmful environmental agents. Taxation can generate revenue for the government and discourage a particular activity by increasing its costs. One of the virtues of taxation is that it does not interfere with human freedom since it allows consumers to make choices, albeit at a higher cost. Governments may be able to use taxes to discourage consumers from making choices that lead to harmful environmental exposures to themselves or others. For example, for many years state governments have levied high taxes on tobacco products to reduce smoking and tobacco use. Evidence indicates that tobacco taxes can be very effective at reducing tobacco use, especially among people of lower socioeconomic status (SES), who are more sensitive to prices [55]. Evidence has shown that high tobacco taxes can significantly reduce the youth smoking rate because they cannot afford the higher prices that are passed on to them when they buy these products legally or illegally [56]. Using taxation to reduce the smoking rate among pregnant mothers and parents can also reduce the second-hand smoke exposures of fetuses, neonates, children, and adolescents [55]. Some states have raised taxes on sugar-sweetened soft drinks and other foods high in calories or fat and low

on nutrition (i.e., junk foods) to help prevent obesity, CVD, and diabetes. To date, the evidence concerning the effectiveness of these taxes is mixed, although it suggests that people of lower SES may be more likely to change their behavior in response to higher prices [57,58]. Taxes on sugared soft drinks and "junk foods" could be an effective strategy for preventing obesity and diabetes among children and adolescents.

Taxes on consumer products often raise issues of fairness. While few people would object to the fairness of high tobacco taxes as a means of reducing tobacco use, taxes on foods and beverages raise justice concerns because they tend to be regressive. As a result, these taxes can undermine access to food among people of lower SES, which can exacerbate socioeconomic inequalities [22]. Also, food and beverage taxes may have unintended adverse consequences. For example, the Danish government levied a tax on foods high in saturated fats in 2011 as a means of preventing obesity. However, the government rescinded that tax after one year because it impacted not just "junk" foods but also "healthy" ones, such as cheese. Consumers also traveled across the border to purchase foods high in saturated fat [22]. Policymakers need to think carefully about issues such as these before adopting food and beverage taxes.

Another ethical concern with taxes used to control behavior is that they may not be effective enough. For example, suppose that instead of adopting regulations prohibiting lead in paint, toys, gasoline, and other products, the US government had imposed a tax on lead in consumer goods. While a tax might have been able to reduce the use of lead in consumer goods, it probably would not have reduced exposures among children and adults to safe levels. Taxation may be appropriate when the goal of government action is to reduce but not eliminate a harmful exposure. If the goal is to eliminate or substantially reduce an exposure, regulation may be a more prudent option.

19.3.6 Civil Liability

Countries have various civil laws that may impose legal duties on individuals and corporations to reduce harmful exposures to affected parties, such as employees, neighbors, the public, and so on. Some of these include tort law, which deals with legal duties to avoid causing harm to others; contract law, which addresses the duties of contracting parties; and child custody law, which allocates parental rights based on the best interests of the child [59–61]. For example, if a company does not take reasonable steps to protect its employees from exposure to a toxic substance in the workplace and the employees are injured as a result, they can sue the company for negligence. If someone has a rubbish heap in their backyard that leeches dangerous chemicals into his neighbor's yard that interferes with the neighbor's use of his property (e.g., it poisons his plants), the neighbor may be able to sue under nuisance laws. If a food manufacturer fails to take reasonable steps to oversee the quality of its

food, and consumers are harmed as a result of eating the food, they can sue the company for negligent manufacture. If an automobile manufacturer fails to take steps to prevent exhaust from entering the passenger compartment, and a consumer is injured as a result, the consumer can sue the company for making a defective product. If the seller of a house knows that the dwelling contains lead paint and he fails to disclose this to the buyer before they sign the contract, the buyer may be able to avoid purchasing the house because the contact would be invalid [62]. Finally, evidence that a parent has been exposing a child to secondhand smoke could be used against the parent in a child custody dispute [63].

As one can see, civil law includes a variety of strategies for holding individuals or corporations accountable for protecting people from dangerous exposures. Although judges decide liability in particular cases, legislators can pass laws that increase or reduce liability. For example, since the 1970s, many states have adopted statutes that impose liability on doctors for failing to disclose to their patients information that a reasonable person would want to know [64]. The National Childhood Vaccine Injury Act is a federal law that shields vaccine manufacturers from legal liability for vaccine injury claims. The law was enacted to encourage manufacturers to invest in vaccine research and development by lowering the legal risks. Injured parties can receive compensation by filing a claim with the National Vaccine Injury Compensation Program, which is funded by a tax on vaccines [65].

Most of the ethical issues discussed in the previous sections also arise when considering whether civil liability should be used as a strategy for protecting fetuses, neonates, children, and adolescents from harmful environmental exposures. One must consider the benefits and costs of imposing (or not imposing) liability. While imposing legal liability can benefit human health and welfare, it can increase costs for businesses and adversely impact the economy. According to one study, the United States spends $865 billion annually on civil lawsuits, or about 2% of its gross domestic product [66]. Litigation expenses increase the costs of doing business and can impede economic growth. Of course, not imposing liability has costs as well, since liability can also deter individuals or corporations from engaging in behaviors that expose people to risks.

Using civil liability as a strategy to prevent harmful environmental exposures also raises issues of justice, because wealthy people and large corporations can afford competent legal representation, whereas poor people and small businesses usually cannot, unless an attorney agrees to be paid a percentage of the winnings from the case (contingency fee). Unlike criminal law, civil law does not provide indigent litigants with free access to an attorney. A result of the disparity of legal representation in civil law is that wealthy individuals and large corporations can often manipulate civil law to promote their own interests. This does not always happen, of course, since poor people and small

businesses may sometimes achieve significant legal victories over wealthy people and large corporations. For example, thousands of workers with mesothelioma have won lawsuits and legal settlements from companies that exposed them to asbestos [67]. However, this type of result tends to be the exception rather than the rule. None of the foregoing implies that civil law should not be used as a strategy to protect fetuses, neonates, children, or adolescents from harmful environmental exposures; only that strategies other than litigation, such as regulation or education, may be better at promoting justice. Using civil liability as a strategy to prevent harmful environmental exposures also raises human rights issues, because litigation, or the threat of litigation, may restrict conduct. For example, consider cases where one parent has accused the other of smoking around a child in a child custody dispute. While exposing a child to secondhand smoke is not healthy for the child, is this harm significant enough to take away parental rights? Is a child better served by living with a loving mother who smokes than with an uncaring father who does not? Furthermore, allowing parents to use evidence of smoking in child custody cases opens the door to a huge list of possible home exposures (such as BPA, "junk food', etc.) that could be used as evidence, and there does not appear to be an easy way of deciding which ones should (or should not) be admitted into legal proceedings. The implications for this type of litigation for parental rights are unsettling, to say the least. Finally, using civil liability as a strategy to prevent harmful exposures also raises issues of personal responsibility, since it shifts legal responsibility away from the individual who is harmed toward the defendant(s). Since the 1950s, smokers who have developed respiratory problems have sued tobacco companies for damages. Smokers have filed a number of different torts against tobacco companies, including product liability, negligence, false advertising, and fraud. Smokers have won many of these cases, which often resulted in awards of millions of dollars. In 1998, four of the largest tobacco companies entered into a settlement agreement with 46 states. Under the terms of the settlement, the companies agreed to pay the states $206 billion to compensate them for health care costs related to smoking and to refrain from certain types of advertising. Smokers have continued to sue tobacco companies despite this wide-ranging settlement [68]. In many cases, most would agree that defendants should be held legally responsible, but in other cases one might question the extent of their responsibility. For example, in 1994, a jury awarded Stella Liebeck, a 79-year-old woman, $2.86 million dollars for the severe burns she suffered as a result of spilling hot coffee sold by McDonald's in her lap. The award included compensation for medical bills (a small fraction of the money), pain, and suffering, as well as punitive damages [69]. A judge later reduced the award to $500,000. Many commentators criticized the judgment in the Liebeck case on the grounds that the plaintiff was responsible for her burns because she should have known about the risks of holding a cup of hot coffee in her lap [70]. Since then, other plaintiffs have sued restaurants for hot coffee burns. Some

have been successful, while others have not. For example, in May 2015 a jury refused to award any money to Matthew Kohr, a police officer who suffered burns after spilling free hot coffee served by Starbucks in his lap [71]. Since the prospects for lawsuits against companies that expose consumers to dangerous environmental agents are virtually unlimited, one might ask whether some limits on liability should be imposed to promote personal responsibility and deter costly litigation. For example, inspired by successful lawsuits by smokers against tobacco companies, some plaintiffs with health problems related to obesity have sued fast-food restaurants for making them fat. Some of these cases have alleged that the restaurants engaged in deceptive advertising and failed to warn plaintiffs about the risks of eating their food. So far, these lawsuits have been very unpopular, and over 20 states have adopted "personal responsibility" laws that limit liability in cases such as these [72]. As attorneys continue to develop creative ways of suing companies for the risks they impose on the public, other states may decide to take action to limit liability.

19.3.7 Criminal Liability

Finally, criminal liability is another strategy that might be used to protect fetuses, neonates, children, and adolescents from harmful environmental exposures. As mentioned previously, prosecutors in some states have brought criminal charges (e.g., delivering drugs, corrupting a minor) against mothers who have endangered their fetuses by using drugs and alcohol during pregnancy. Many commentators have argued that this strategy unethically and illegally infringes on the women's autonomy and is ultimately counter-productive because alcohol and drug-abusing women may avoid prenatal care out of fear that they could be arrested. The best way to handle these cases, according to many, is to offer pregnant women drug and alcohol counseling and treatment [12].

Pregnant women may not be the only targets of criminal prosecution. Most states have laws that make it a crime for any adult to contribute to the delinquency of a minor. For example, a father who provides his fourteen-year-old son with alcohol or drugs for a party could be charged with such an offense [73]. Parents (or other responsible parties) could also be charged with criminal child abuse if their behavior causes serious harm to their child or creates an imminent risk of serious harm. Though most child abuse cases involve physical, sexual, or emotional abuse, it is conceivable that prosecutors could charge parents with child abuse for exposing their children to dangerous environmental agents. For example, some commentators have argued that charges of child abuse should be considered for parents who allow their children to become morbidly obese. The rationale for such charges is that morbid obesity represents an imminent threat to the health of a child. Children with morbid obesity could be placed in foster care and returned to their parents when their

health improves [74]. Others have argued that such charges are not warranted because the child's obesity may be due to factors beyond the parents' control and removing the child from the home will do more harm than good [75]. Criminal liability is by far the most coercive strategy considered in this chapter, since defendants who are found guilty may face hefty fines, imprisonment, or other serious consequences. Because criminal prosecution is highly coercive and often harmful, we do not recommend its use as a strategy for protecting fetuses, neonates, children, or adolescents from harmful environmental exposures unless evidence clearly demonstrates that they have been seriously harmed by their parents (or other responsible parties) or face an imminent risk of serious harm, and other strategies are not likely to be effective at dealing with the situation.

19.4 Research with Human Participants

Before concluding this chapter we will briefly review some of the ethical issues raised by scientific research on the effects of environmental agents on the health of fetuses, neonates, children, and adolescents. Scientific research plays a key role in developing strategies to protect these groups from harmful environmental exposures, but it also raises a number of ethical issues [76]. In the following discussion, we will focus on some of the ethical issues concerning environmental health research involving human participants. While a great deal of our knowledge concerning the health impacts of environmental exposures has come from animal and cell studies and chemical experiments, human studies are important to better understand human health effects and develop safe and effective strategies for minimizing the impact of harmful environmental exposures. Numerous regulations and ethical guidelines apply to research with human participants, including the Common Rule [77], which applies to research supported by 17 different federal agencies; the FDA regulations [78], which apply to FDA-regulated research; the EPA regulations [79], which apply to research supported or regulated by the EPA; the Nuremberg Code (1947) [80], the first international human research ethics guideline; the Helsinki Declaration [81], an influential international guideline; and the Belmont Report [82], an influential report on the basic ethical principles of research with human participants. These and other documents support the following ethical principles for research with human subjects [83]:

- *Sound scientific design:* The research is well-designed to answer an important scientific question
- *Social value:* The research is expected to produce knowledge that is valuable to society
- *Risk minimization:* The research minimizes risks to human participants and others

- *Risk acceptability:* The risks of the research are acceptable in relation to anticipated benefits to the participants or society
- *Informed consent:* Informed consent from the participants or their representatives is sought and appropriately documented
- *Confidentiality/privacy:* The research includes measures to protect the confidentiality and privacy of the participants.
- *Equitable participant selection:* The selection of research participants is scientifically appropriate and equitable
- *Protection of vulnerable subjects:* The research includes additional protections for participants who may be vulnerable to exploitation or harm, such as children, prisoners, and fetuses
- *Independent oversight:* The research is reviewed and overseen by an independent body, such as an institutional review board (IRB)

There are several different research designs that investigators might use to better understand the health impacts of environmental exposures and develop strategies to minimize their impact. These include the following [16]:

- *Observational studies:* These studies do not involve any experimental manipulation of human participants but collect data and/or samples from participants for analysis. Observational studies include prospective cohort studies, in which investigators collect data and/or biological samples from a group with a common characteristic (such as an environmental exposure or occupation); and retrospective, case–control studies, in which investigators collect data and/or samples from a group (i.e., cases) with an adverse outcome (such as a disease) for comparison to control group.
- *Interventional studies:* These studies involve comparison of an experimental group with one or more control groups to determine the effectiveness of the experimental intervention at preventing, mitigating, or treating a disease. To minimize bias, participants may be randomly assigned to different groups and blinded with respect to the nature of the intervention. In some cases, participants in the control group may receive a placebo. While clinical trials in medical research investigate the safety and effectiveness of treatments (such as drugs or medical devices), interventional studies in environmental health research often investigate the safety and effectiveness of environmental interventions, such as lead remediation (discussed further) or drinking water purification.
- *Intentional exposure studies:* These studies involve exposing human subjects to environmental agents under controlled conditions to better understand their effects. For example, investigators might ask human participants to breath air containing ozone under controlled conditions to better understand its effects on the human respiratory system [84]. Research with human participants raises a host of ethical issues that we cannot hope to cover in this chapter [85]. In the following sections, we will focus only on some key issues for environmental health research.

19.4.1 Return of Individualized Research Results

Investigators may collect and analyze many types of data, which may be of interest to human participants, such as blood pressure, pulse, body mass index, blood tests, urinalysis data, exposures to environmental agents, the results of genetic/genomic tests. Section 19.3.2 includes a detailed discussion of the ethics of returning individualized results, so we will not say much more about these issues here, except to note that environmental health research often involves the collection of data that may not have established clinical value. Additionally, testing may often be done by uncertified research laboratories. For example, a study of home exposures to environmental chemicals may yield results that are useful to investigators but not very useful to research participants and are potentially harmful [86]. As noted earlier, there is an ongoing debate between those who favor return of all results that participants choose to receive [30] and those who favor a more cautious approach [32]. In some cases, significant community harms could occur if researchers return results to participants [86]. For example, if researchers are studying DDT exposure in a community that is spraying the chemical indoors to control malaria, informing participants about their DDT exposures could lead members of the community to stop using DDT, which could do more harm than good, since the human health risks of malaria (e.g., death, chronic disease) are likely to be greater than the risks of DDT exposure.

Although the return of research results without clinical value is controversial, there is little disagreement about the importance of returning results with well-established clinical value in a timely fashion. In some cases, researchers may need to make emergency treatment available to participants with dangerous conditions, such as high blood pressure or blood sugar. In other cases, researchers may need to make a referral for follow-up medical care [28]. A case that illustrates the importance of returning results with clinical value was a study of lead abatement methods conducted by the Kennedy Krieger Institute (KKI) at Johns Hopkins University in Baltimore, MD, in the early 1990s [87–89]. The goal of the study was to determine whether less expensive levels of lead abatement are as effective at preventing dangerous exposures to lead from interior house paint as full lead abatement, which can be very expensive ($10,000 or more per home at that time). Most of the homes in the Baltimore neighborhoods targeted for the study contained lead paint because they were built before lead was banned in interior paint (1980). Officials from the city of Baltimore were concerned that landlords would abandon the homes because lead abatement would be too costly. The study, which was sponsored by the Environmental Protection Agency (EPA) and the Maryland Department of Housing and Community Development, included three experimental groups consisting of homes with $1650, $3500, or $6000–$7000 worth of lead abatement; and two control groups, one consisting of homes with full lead abatement

and another consisting of homes without lead paint. Twenty-five homes participated in the study, five in each group. KKI worked with landlords to obtain public funding and grants for lead abatement, with the understanding that they would rent homes to families with young children. The investigators recruited families with young children who were already living in these homes to take part in the study. Investigators measured levels of lead in dust, soil, and water samples, and in the children's blood over a 2-year period. To remain in the study, families had to have a young child living in the home. The investigators paid families between $5 and 15 to participate in the study and also provided them with small gifts. The consent document signed by the families did not inform them that their children might accumulate dangerous levels of lead in their bodies as a result of continuing to live in these homes. The consent document also did not inform the families of the risks of lead exposure to children, which include neurological damage and problems with learning and behavior. The investigators planned to warn parents in a timely fashion of dangerous lead levels, but this did not happen in at least two cases. Viola Hughes, the mother of Ericka Grimes, was not warned of dangerous levels of lead in her daughter's blood until 9 months after these levels were detected. Catina Higgins, the mother of Myron Higgins, was not informed that dust samples contained dangerous levels of lead until about 5 months after these levels were detected [87].

These two families sued KKI and the investigators for negligence, arguing that the researchers failed to warn them of dangerous lead levels in a timely fashion [87]. The district court dismissed the case on the grounds that the investigators had no legal duty toward the research participants because they did not have a physician–patient (therapeutic) relationship with the participants. The Maryland Appellate Court overturned this decision, ruling that the investigators had a legal duty toward the participants, including a duty to warn them of dangerous lead levels in a timely fashion. The court found a basis for a legal duty in the informed consent document, which it treated as a contract, and the federal research regulations, which it said established legal duties. Since the defendants did not warn the families of dangerous lead levels in a timely fashion, they could be sued for negligence [87].

The Kennedy Krieger case established two important points concerning returning individualized research results: (1) investigators may have legal duties toward research participants even when there is no physician–patient relationship and (2) there is a legal duty to share clinically significant information (such as lead exposure) with research participants in a timely fashion [16,90].

19.4.2 Protecting Privacy and Confidentiality

The KKI study involved the collection of data and samples in private homes. Many other environmental health research studies also involve data or sample

collection in private homes or in private workplaces [32]. Collecting data or samples in a private home or workplace raises ethical dilemmas related to the protection of confidentiality or privacy. For example, suppose that investigators are collecting samples and data in a home and they happen to observe evidence of child abuse, elder abuse, domestic violence, or other illegal activity, such as possession or sale of illegal drugs. Should they report any of these suspected problems to the relevant authorities (i.e., social services or the police)? One could argue that there is a legal and ethical obligation to report suspected child or elder abuse. Most states in the United States have laws that mandate reporting of suspected child abuse for certain professions (e.g., health care, education, social work) or for the general public. Fewer states have laws requiring reporting of elder abuse [32]. One might argue that at minimum, researchers have an obligation to follow relevant state laws. Additionally, one might argue that researchers have ethical duties to report suspected child or elder abuse, since children and elderly people under someone's care are vulnerable to harm or exploitation [32].

Data and sample collection in private workplaces raise similar issues. For example, a researcher who is studying occupational exposures in a factory might observe evidence of OSHA violations, workplace violence, sexual or racial harassment, or other illegal or hazardous activity. The researcher would need to decide which (if any) of these problems to report and to whom. A key question for the researcher would be whether reporting problems – and therefore violating confidentiality or privacy – would do more harm than good. Although problems observed in the workplace may cause potential harm to many employees, researchers may not have a legal obligation to report these problems. Reporting could also alienate employers and jeopardize the research. Research participants must be adequately informed of plans to report suspected child or elder abuse or other problems during data or sample collection in the home or workplace. Some potential research subjects might decide not to participate if they know that these problems may be reported. Employers may be unwilling to allow researchers to collect data or samples at the worksite if they face potential legal liability or government audits as a result of reporting of problems.

19.4.3 Interventional Studies

As mentioned earlier, environmental health researchers sometimes conduct controlled experiments to determine whether interventions can prevent or mitigate adverse environmental exposures. The KKI study (discussed earlier) is an example of this type of research. Other examples include studies of mold remediation, asthma mitigation, water purification, sanitation techniques, and even the use of transgenic mosquitoes to prevent mosquito-borne illnesses [16,88,89,91]. One of the key issues in an interventional study is whether

it is ethical to include a group that receives an intervention thought to be ineffective or substandard. For example, a clinical trial of a new drug might include a group that receives a placebo to control for bias due to the placebo effect. A clinical trial might test an intervention thought to be substandard because it is likely to be more affordable for the population than the standard therapy but still significantly effective [89,92]. Some have argued that it is unethical to include a placebo control group in a clinical trial if a safe and effective intervention is available when the trial begins, because this would be denying human participants medical therapy, and physicians have an obligation to provide their patients with effective medical therapy [93]. One might extend this line of reasoning to testing therapies thought to be substandard (when standard therapies exist) because physicians have duties to provide their patients with the best available therapy [93]. Some have argued, however, that patients can receive ineffective or substandard therapies if they understand the risks and potential benefits of the study and they are not significantly harmed or treated unfairly as a result of their participation [92,94]. The Helsinki Declaration states that placebos can be used in clinical trials when there is no proven intervention for the disease or when subjects who receive a placebo will not be "subject to additional risks of serious or irreversible harm [81]."

While environmental health investigators are usually not physicians who have therapeutic obligations to their patients, some of the concerns related to providing people with ineffective or substandard environmental interventions may still arise because these researchers still have obligations to promote participants' welfare and treat them fairly [89]. Some have argued that the KKI study design was unethical because it included three experimental groups that would receive substandard lead abatement [95]. Since full lead abatement was known to be effective at preventing lead exposure, enrolling participants in groups that received less than full lead abatement would be like giving patients a substandard medical treatment when a standard treatment is available. Others have defended the KKI study design on the grounds that the participants who lived in homes that received less than full lead abatement were provided with the significant benefit of partial lead remediation, and were supposed to be informed about dangerous lead levels. Furthermore, the study benefited the community, as well as other people living in houses with lead paint, because it showed that less than full lead remediation can be an affordable and effective alternative to full lead remediation [92]. An important factual issue is whether the families that received partial lead abatement were already living in homes with lead paint when the study began, since it appears that some families may have moved into partially abated homes when they joined the study. One could argue that the risks of the study for participants in the experimental groups would be unacceptable if the research design required participants to move into homes with interior lead paint, since the participants would face the risks of living in partially abated homes [96]. In this view, the risks would be acceptable

if the participants in the experimental groups were already living in homes with interior lead paint, since the risks of lead exposure would be part of their environment and not risks imposed on them by the study [96].

For an interventional study less controversial than the KKI study, consider a mold remediation study conducted by Kercsmar *et al.* [97] in which 62 families with asthmatic children living in homes with indoor mold were randomly assigned to receive mold remediation and education about controlling mold in the home (the experimental group) or just education about controlling mold in the home (the control group). The investigators found that the combination of mold remediation and education was significantly more effective at controlling asthma exacerbations in the children living in homes with indoor mold than education alone [97]. Was this study unethical? Even though mold remediation was thought to be effective at reducing asthma exacerbations when the study began, one might argue that the study was not unethical because participants in the control group were not harmed by the study and they still received the benefit of education about controlling indoor mold [16,89].

19.4.4 Intentional Exposure Studies

Intentional exposure studies raise concerns about imposing risks on healthy human subjects. As noted earlier, one of the key principles of research ethics is that the risks should be acceptable in relation to the benefits. Although clinical trials often impose significant risks on participants, most people regard these risks as acceptable as long as the participants have a reasonable chance of benefiting medically. However, participants who are healthy volunteers may be exposed to risks without any compensating personal benefits [84]. For example, the EPA's National Exposure Research Laboratory sponsors studies in which human participants are exposed to air pollutants, such as ozone or diesel exhaust [84]. In a typical experiment, subjects first undergo a physical examination, complete a health survey, perform a breathing test, and provide biological samples. Next, they go into a sealed chamber in which they breathe purified air that has been mixed with a predetermined amount of a pollutant. Although the air contains a pollutant, it is safe to breathe for a limited time, since it is generally no more polluted than the air found along the side of highway [84]. Investigators monitor participants' heart rate and other vital signs and may ask them to perform some activities that involve moderate physical exertion (such as riding an exercise bike). After the participants complete their time in the chamber, investigators perform another breathing test and collect additional data and biological samples. They may also perform a bronchoscopy on participants to collect bronchial cells [84]. The most significant risks of studies like these are those associated with the bronchoscopy. These risks include coughing, respiratory irritation, or mild bleeding. In rare cases, a bronchoscopy may cause a pneumothorax, a condition in which gas collects

around the lungs. This problem usually goes away on its own or can be easily treated [98]. Participants who have a cardiac or respiratory problem may be at risk of breathing the air in the chamber and exercising, but investigators usually exclude such participants from the study to protect them from harm [84,99]. However, some studies have included cardiac patients in order to determine the impact of air pollution on cardiac function. These studies have included appropriate measures to protect the patients from harm [99].

The most significant issue raised by intentional exposure studies on healthy volunteers is whether the risks are acceptable in relation to the benefits. Although the participants do not benefit, society may benefit from the knowledge gained. For example, studies of the effects of breathing polluted air on the human respiratory and cardiovascular system may provide information that can be used to develop air pollution regulations or measures for mitigating adverse health impacts of air pollution [84,99]. Some might object to such experiments on the grounds that we already know a great deal about the adverse health effects of air pollution from animal experiments and human observational studies. There is, therefore, no need to conduct experiments that expose human beings to harmful pollutants. One might respond to this objection by noting that there are important gaps in our knowledge that can only be filled by conducting human experiments. Animal experiments can tell us a great deal about how air pollution impacts mammalian physiology but it cannot identify the precise effects on human beings, because there are important differences between the animals used in experiments (such as rodents) and humans. Observational studies can provide general information about human health impacts, but they cannot provide us with information concerning causal mechanisms or specific outcomes [84]. One might argue, therefore, that the risks of these experiments are acceptable, provided that investigators take appropriate steps to minimize risks and the studies comply with other ethical and legal requirements, such as informed consent [84].

Some of the most controversial intentional exposure studies were privately funded experiments in which human participants were exposed to pesticides [88,100]. During the 1990s, several pesticide companies conducted studies that exposed human participants to small quantities of pesticides orally or dermally. Investigators measured pesticide metabolite levels in the participants' blood and urine to better understand how the human body metabolizes and eliminates these chemicals. Some of the compounds tested included organophosphates, phenoxy herbicides, phenol derivative fungicides, pyrethrins, or pyrethroids [100]. In some cases, the participants were company employees. The goal of these experiments was to determine whether pesticide exposures at these levels are safe. The companies hoped to be able to submit data to the EPA that would convince the agency to allow higher exposures to pesticide residues on food than were permitted under the Food Quality Protection Act (FQPA). The FQPA mandated an additional safety factor of 10 for allowable pesticide residues on food in order to provide additional protections for children. As a result of the

FQPA, the EPA enacted regulations mandating that pesticide residues on commercial foods be not greater than 1/1000 the exposure level that causes no adverse effects in rodents [100]. Farmers and pesticide companies were concerned that this additional safety factor would interfere with the effective use of pesticides in commercial agriculture [88]. Many commentators objected to these experiments on the grounds that the risks to the human subjects were unacceptable because the research would benefit private companies, not society. Critics also objected to the studies because the sample sizes were too small to yield significant results and the companies used employees as research participants, which was potentially coercive [101,102]. Others argued, however, that well-designed human pesticide experiments would be justifiable if they were likely to yield important public health benefits and complied with ethical and legal rules [88,103].

One of the consequences of the public debate concerning human pesticide experiments is that the EPA revised its human research regulations in 2006, with additional revisions in 2010 and 2013. The regulations govern privately funded research submitted to the EPA for regulatory purposes as well as research conducted or funded by the EPA. The regulations are similar to Common Rule, except they prohibit intentional exposure studies involving children or pregnant or nursing women. They also create a human studies board that reviews all research funded by the EPA or submitted to the EPA for regulatory purposes [79]. Prior to 2006, the EPA had no regulations concerning privately funded research submitted to the agency and followed the Common Rule for EPA research [16]. The main reason that the regulations prohibit intentional exposure studies involving children or pregnant or nursing women is that Congress required the agency to include these protections because of public concern about pesticide research involving children. The study that created this public uproar, the Children's Environmental Exposure Research Study (CHEERS), was an observational study, not an intentional exposure study. However, critics of the study and the media portrayed it as an intentional exposure study, and EPA scientists were unable to correct this misperception [104].

CHEERS was sponsored by the EPA, the Centers for Disease Control and Prevention, and the Duvall County, Florida Health Department. The study, which was approved by three IRBs and peer review committees at the EPA and CDC, would have taken place in the fall of 2004. The American Chemistry Council also contributed funding to the study but had no role in its design. The goal of the study was to better understand how children are exposed to pesticides and other chemicals in their homes. Knowledge of children's actual pesticide exposures is important for protecting their health. The investigators originally planned to recruit 60 families with young children who lived in homes with high pesticide use and then later added a control group of families with low or no pesticide use in response to critiques of the study design. The investigators would visit the homes 30 times during a 2-year period to collect blood and urine from the children and soil and dust samples from the homes. Home visits would

last approximately 3 h. The parents would keep a journal of their pesticide and chemical use and videotape their children's activities. Parents would receive a free video camera and $970 to compensate them for their time and inconvenience. To determine whether the families qualified as high pesticide users, the investigators planned to go to the homes immediately after enrollment to verify the level of pesticide use in the home. This procedure was included to prevent families from becoming high pesticide users (and thus exposing their children to pesticides) in order to qualify for the study. The investigators also planned to instruct families on proper (safe) use of pesticides and inform them of unsafe levels. Families could withdraw from the study at any time and could remain in the study even if they reduced their pesticide use [104]. The study was revised in the fall of 2004 but was never initiated, due to the public controversy. Indeed, California Senator Barbara Boxer threatened to derail Stephen Johnson's nomination as EPA Administrator if the agency did not cancel the study, which it did in the spring of 2005 [104].

CHEERS was controversial for several reasons. First, as noted earlier, it was portrayed as an intentional exposure study, but as we have seen, it was designed to be an observational study. Critics of the study claimed that investigators would ask parents to expose their children to pesticides as part of the study, but, as noted above, families would be enrolled in the high pesticide use group only if they were already using pesticides. The study did not require children to be exposed to any more pesticides than they were already being exposed to. Indeed, the IRBs classified the study as no more than minimal risk, that is, the risks of the research are not greater than the risks of routine medical or psychological tests or exams or the ordinary risks of daily life [77]. The main risk of the study to the children was the blood draw [104]. Second, critics argued that the study was targeting low-income, minority families. However, the research protocol listed neither race, nor ethnicity, nor income as participation criteria. Investigators selected Duval County as the study site because of evidence of high home pesticide use in homes in the county. Critics also claimed the financial incentives were too high and could constitute undue inducement. However, since the study involved more than 150 h of the participants' time (including home visits, videotaping, and journal writing), the rate of payment would be less than the federal minimum wage [104]. CHEERS investigators certainly could have done more to enhance the public perception of the study – for example, they could have consulted with community members on study design and recruitment – but the study itself was ethically and scientifically sound [104].

19.4.5 Protecting Vulnerable Participants

The discussion of the CHEERS study brings us to the last topic of this section – protecting vulnerable participants. The CHEERS study subjects were children, who are classified as vulnerable participants due to their compromised ability to

provide informed consent and their susceptibility to exploitation [105]. As noted earlier, the KKI study also focused on children. Other types of vulnerable participants include fetuses, neonates, prisoners, and mentally disabled adults. Participants may sometimes be regarded as vulnerable due to their socioeconomic condition, their status as students or employees, or lack of proficiency in a language used to conduct research (such as English). The Federal research regulations include additional protections for children, pregnant women and fetuses, and prisoners [77]. Other influential documents, such as the Helsinki Declaration [81] and the Belmont Report [82] include guidelines for protecting vulnerable participants. Additional protections for vulnerable populations frequently include the following:

- *Limitations on exposure to risks.* For example, the Federal research regulations limit the risks the children, neonates, pregnant women, fetuses, and prisoners may be exposed to in research [77]. As noted already, the EPA regulations prohibit intentional exposure studies involving children or pregnant or nursing women.
- *Sound scientific rationale for including vulnerable participants.* Vulnerable participants should be included in a study only if there is a legitimate scientific reason for including them; they should not be included as a matter of convenience [81,82].
- *Procedures for obtaining valid consent.* If a research participant is not capable of providing consent, then consent should be obtained from a legally authorized representative, such as a parent or guardian. Research studies that enroll mentally disabled adults should include procedures for evaluating their ability to consent to research participation and locating legally authorized representatives if they cannot consent [76].
- *IRB representation.* The Federal research regulations require that IRBs that review research on prisoners include a member who can represent the interests of prisoners [77]. Other guidelines recommend that IRBs include members who are knowledgeable about the needs and interests of vulnerable populations if they review research on those populations [76].

There are two rationales for including additional protections for vulnerable research participants. First, while autonomous adults can decide whether they want to participate in studies that expose them to risks, vulnerable participants may not have the ability to make such choices. Those who make choices for vulnerable participants, therefore, have an obligation to protect their interests. For instance, a parent who provides consent for a child to participate in research should protect the child's interests. If a study exposes a child to significant risks without any compensating benefits to the child, then it may not be in the child's interests to participate [106]. IRBs and investigators also have obligations to protect the interests of vulnerable participants. Second, one might argue that it is unfair (or exploitative) to include vulnerable participants in research that

exposes them to risks if they are not likely to personally benefit from the research or the research is not likely to benefit other members of their group. For example, during the 1960s and 1970s, prisoners were often used as research subjects for Phase I drug studies. However, these studies offered no significant benefits to the participants or the prison population [76]. Additionally, safeguards for vulnerable subjects are, therefore, required to protect them from exploitation [82]. In this chapter, we have focused on strategies for protecting fetuses, neonates, children, and adolescents from exposures to harmful environmental agents. To develop and implement these strategies, it is often necessary to conduct research on fetuses, neonates, children, or adolescents so that we can better understand how environmental exposures impact their health. However, additional protections for vulnerable participants may limit scientists' ability to conduct research on these populations. For example, the EPA regulations prohibit EPA-funded intentional exposure studies on children or pregnant or lactating women, regardless of the level of risk or the potential for benefit. One implication of this policy is that the EPA cannot conduct or sponsor clinical trials involving children or pregnant or lactating women that expose them to medical treatments or environmental agents [107]. The EPA also cannot collaborate with institutions that are conducting such research. The policy also implies that the EPA cannot conduct or sponsor minimal risk intentional exposure studies involving children or pregnant or lactating women. For example, children are frequently exposed to sunscreens and insect repellants as part of their daily life. A child on a camping trip may use both at the same time. It would be important to know how these products impact children's health and interact with each other, but the EPA regulations do not permit research that would involve intentionally exposing children to the levels of sunscreens or insect repellants that they normally encounter in daily life [107]. The Common Rule would permit such research on children if the IRB determined that it would be minimal risk or a minor increase over minimal risk [106,107]. The Common Rule and FDA regulations limit the risks that pregnant women, fetuses, or children can be exposed to in research. Although these regulations permit pregnant women to participate in clinical trials that have the potential to benefit the health of the mother or the fetus, researchers often exclude pregnant women from these studies due to concerns about legal liability if the fetus were harmed [108]. Because pregnant women are frequently excluded from clinical research to protect the fetus, we lack knowledge about how drugs affect pregnant women and their fetuses, which is an important concern for women's and children's health [108]. Likewise, children and adolescents are routinely excluded from clinical studies due to liability or marketing issues. As a result, most pediatric medications are prescribed on an off-label basis due to lack of knowledge about how children respond to drugs [76]. Because overprotecting vulnerable participants prevents researchers from obtaining knowledge that can be of use to promote the health of the

groups to which they belong, investigators and oversight committees must deal with the issue of overprotecting versus underprotecting these participants. In the past, most of the ethical and regulatory emphasis was on protecting vulnerable participants from harm and exploitation [109], but, as we have seen, too much protection can exclude vulnerable participants from studies that may benefit the health of the groups to which they belong [106–108]. Investigators and oversight committees must, therefore, strike a reasonable balance between protecting vulnerable participants from harm and including them in studies that can promote their health [76].

19.5 Conclusion

As we have seen in this chapter, society can use a number of strategies for protecting fetuses, neonates, children, and adolescents from exposures to harmful environmental agents, including education, testing/screening/monitoring, worker protection, government regulation, taxation, civil liability, criminal liability, and scientific research. These different strategies often create ethical dilemmas because they may involve conflicts among basic values, such as human health, economic prosperity, individual rights and responsibility, justice, and animal and environmental welfare. Additional scientific research may help to resolve some of these dilemmas by providing decision-makers with information about how environmental exposures affect the health of fetuses, neonates, children, and adolescents. However, research often creates new dilemmas by detecting risks that were previously unknown. In any case, it is important for researchers, health care professionals, policymakers, and the public to engage in thoughtful discussion about the ethical issues in light of the best available scientific evidence.

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