

# Role and limitations of epidemiology in establishing a causal association

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## Abstract

Cancer risk assessment is one of the most visible and controversial endeavors of epidemiology. Epidemiologic approaches are among the most influential of all disciplines that inform policy decisions to reduce cancer risk. The adoption of epidemiologic reasoning to define causal criteria beyond the realm of mechanistic concepts of cause-effect relationships in disease etiology has placed greater reliance on controlled observations of cancer risk as a function of putative exposures in populations. The advent of molecular epidemiology further expanded the field to allow more accurate exposure assessment, improved understanding of intermediate endpoints, and enhanced risk prediction by incorporating the knowledge on genetic susceptibility. We examine herein the role and limitations of epidemiology as a discipline concerned with the identification of carcinogens in the physical, chemical, and biological environment. We reviewed two examples of the application of epidemiologic approaches to aid in the discovery of the causative factors of two very important malignant diseases worldwide, stomach and cervical cancers. Both examples serve as paradigms of successful cooperation between epidemiologists and laboratory scientists in the pursuit of the understanding of cancer etiology.

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## 1. Introduction

The purview of cancer epidemiology has increased in recent years to reflect the discipline's ever expanding role to multiple fronts of research and professional practice in evidence-based oncology. However, of all practice domains of epidemiology cancer risk assessment remains with little doubt one of the most visible and controversial endeavors from the public's viewpoint. The role and limitations of epidemiology as a methodological discipline concerned with the identification of carcinogens in the physical, chemical, and biological environment is the focus of this overview. We reviewed herein the value of epidemiology in the context of all scientific disciplines concerned with risk attribution in cancer, underscoring the fact that despite its limitations, epidemiologic approaches remain a well-respected line of inquiry to inform policy decisions. The advent of molecular epidemiology further expanded the armamentarium of cancer risk assessment to allow more accurate exposure

assessment, improved understanding of the intermediate endpoints in the natural history of cancer, and enhanced prediction of inter-individual risk by incorporating knowledge on mediating genetic factors. It is essential, however, that the limitations of molecular epidemiology be well recognized, before we harvest its dividends in cancer prevention. Finally, we examined two relatively recent examples of the application of epidemiologic approaches to aid in the discovery of the causative factors of two very important malignant diseases worldwide, stomach and cervical cancers. Both examples serve as paradigms of successful cooperation between epidemiologists and laboratory scientists in the pursuit of the understanding of cancer etiology, however tortuous that pursuit may be, as was the case of the relation between papillomaviruses and cervical cancer.

## 2. Epidemiology and other approaches for evaluating carcinogenicity

Public health and regulatory agencies such as the World Health Organization's International Agency for Research on

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Table 1

Epidemiologic and other approaches<sup>a</sup> considered by regulatory and public health agencies in assessing the totality of the evidence concerning the carcinogenicity of a suspected chemical, physical, or biological exposure or its circumstances (adapted from Ref. [1])

Approach	Type of scientific evidence	Level of inference	Type of study	Features
Mechanistic	Analogy	Molecular structure	Structure–activity relationships	Useful to identify potentially carcinogenic compounds based their molecular similarity to known carcinogens
Toxicology	Experimental	DNA, cellular, organ	In vitro short-term genotoxicity assays	Rapid screening system for candidate compounds or exposures
		Organ, whole organism	In vivo animal studies	Provides proof of principle and insights into dose-response effects
Epidemiologic	Observational	Non-inferential, descriptive Population	Case reports	Suggestion of association
			Surveillance of incidence and mortality	Documentation of baseline disease burden, exploratory hypotheses
			Ecologic (correlation or aggregate) studies	Coarse verification of correlation between exposure and disease burden
			Cross-sectional studies	Correlation between exposure and disease (or marker) without regard to latency
	Individual	Case-control studies	Correlation between exposure and disease (or marker) with improved understanding of latency; suitable for rare cancers	
Experimental	Individual <sup>b</sup>	Individual	Cohort studies	Correlation between exposure and disease (or marker) with improved understanding of latency; suitable for rare exposures
			Randomized controlled trials of preventive intervention	Most unbiased assessment of correlation between exposure and disease (or marker)

<sup>a</sup> Other supporting in vivo and in vitro data relevant to evaluation of carcinogenicity and its mechanisms can also be used, particularly if they provide insights into mechanisms of absorption, metabolism, DNA binding or repair, hormonally-mediated effects, genetic damage, altered cell growth, loss of euploidy, cytopathic changes, and related effects.

<sup>b</sup> On occasion, randomized controlled trials may target communities or health care providers as units of randomly allocated intervention. However, this is done for convenience of study design; in practical terms inference is at the individual level.

Cancer (IARC), the US Environmental Protection Agency (EPA), and the US National Toxicology Program (NTP) consider the body of evidence from epidemiologic studies a key component in the complex decision making process in evaluating the carcinogenicity of suspected exposures. The epidemiologic evidence is merged with the body of evidence from animal and experimental studies. The types of studies that are typically considered by the above agencies are described in Table 1 [1]. On occasion, candidate agents are selected for review based on anticipated carcinogenic properties because of their chemical analogy to proven mutagens. Relatively rapid toxicology assessment via genotoxicity screening followed by rodent assays can provide the proof of principle that a particular agent exhibits a dose-response carcinogenic effect under a variety of experimental conditions that may include the contribution of other substances believed to act as modifiers of the tumorigenic process [2,3]. Long-term rodent assays are very useful in this regard. Most established human carcinogens for which there is adequate experimental data have been shown to exhibit carcinogenicity in one or more animal species [4]. The value of animal studies is also underscored by the fact that, for many agents, experimental carcinogenicity in animal models was proven before epidemiologic data became available [5].

On the other hand, empirical evidence from animal studies cannot be guaranteed in very case, particularly for occupational or environmental exposures in which the putative carcinogenic insult cannot be readily identified. Likewise, the assessment of carcinogenicity for some microbial agents that only infect humans cannot be made in animal models; e.g., human papillomavirus (HPV) infection, an established cause of cervical cancer [6]. In these circumstances, however imperfect, epidemiologic studies contribute the main component of the evidence base to assess carcinogenicity, aided by other pertinent data (Table 1). Moreover, sometimes the evidence concerning carcinogenicity in animal studies is equivocal or cannot be readily extrapolated to humans. Therefore, in the judgment of agencies such as the IARC, EPA, and NTP, despite the lack of controlled experimental conditions in most observational studies, the evidence from consistent and unbiased epidemiologic evidence tends to take precedence over the knowledge base from animal studies or other laboratory investigations. This is not to say that policy decisions are always free of misgivings. There are many controversial issues surrounding the weight of evidence that regulatory agencies assign to epidemiologic studies relative to that from animal experiments, particularly if substantial disagreement exists between these two lines of evidence [7].

### 3. Causal criteria in cancer epidemiology

Risk assessment agencies consider the totality of the published epidemiologic evidence for a given candidate carcinogen. Germane to this discussion is the definition of cause. The operational epidemiologic definition is a factor that alters the risk of disease occurrence. In the infectious disease realm, the definition has been more mechanistic; a cause is either a factor that must exist for disease to occur (necessary) or always produces disease (sufficient). This definition is applicable to the study of infectious diseases, where a microbial agent is a necessary and sometimes a sufficient cause of disease, depending on the interplay between agent, host, and environmental factors. On the other hand, the situation is less clear for cancer, a group of diseases of multi-factorial etiology, which ultimately result from the interaction between environmental (external) causes and the genetic (internal) make-up of the individual. Few of the accepted causes of human cancer are deemed necessary (e.g., HPV in cervical cancer; see below) or sufficient (e.g., possibly some of the high penetrance cancer genes, as discussed below). Unlike most infectious diseases, cancer has a long latency period, which underscores the succession of time-dependent events that are necessary for normal tissue to develop into a lesion with malignant potential and ultimately to progress into invasive cancer. Carcinogenesis is a multi-stage process where the final risk of disease development is a function of the combined probabilities of relatively rare events occurring in each stage. These events depend on myriad factors related to carcinogen absorption and delivery to target cells, metabolic activation, binding with relevant gatekeeper or caretaker genes, and to the ability of the affected tissue to reverse these initiating processes. Also to be considered is the contribution of promoters, which will favor cell proliferation with consequent selection of clones with increasingly malignant traits that confer upon the affected cells a selective growth advantage within the surrounding tissue. Eventually, other factors will facilitate progression of the precursor lesion or minimally invasive cancer to the point when it becomes a detectable malignant neoplasm that has fully invaded the adjacent connective tissue. At this stage, detection of the tumor may contribute to the incidence statistics collected by a population-based tumor registry and the affected patient could end up being enrolled in an epidemiologic study. Each one of the factors influencing the passage from one step to the next in the above sequence contributes a causal role to cancer development and, at least in theory, it should be possible to measure its epidemiologic effect on the overall risk of that particular cancer.

This is easier said than done, however, as there are considerable hurdles in the way of designing, conducting, analyzing, and interpreting epidemiological studies of a chronic disease such as cancer. The standards of scientific logic into what constitutes the criteria for judging whether or not a given risk factor is a cause of cancer have changed little in the last 40 years but to date they continue to be a matter of in-

tense public health and even legalistic debate [8–10]. What is practiced today concerning evaluation of environmental carcinogens is based on Bradford Hill's criteria [11], a subset of which are referred to as the Surgeon General's criteria [12] (Table 2). These criteria were first proposed at the time of a vigorously debated health issue of the early 1960s, namely, the interpretation of the accrued scientific data concerning the role of tobacco smoking as a cause of lung cancer. Hill's nine criteria were: strength of the association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experimental evidence, and analogy (Table 2). In his seminal paper, he downplayed the importance of specificity, plausibility, and analogy, which are viewed today as non-essential and can be even considered counter-productive distractions to the discussion of any possible cause-effect relationship in cancer. Unfortunately, however, he also concluded that "none of my nine viewpoints can bring indisputable evidence for or against the cause-and-effect hypothesis and none can be required as a *sine qua non*" [11]. If published today, the second part of that statement would have been disputed immediately. Clearly, temporality is a necessary causal criterion, and biological gradient, consistency, and strength of the association are among the most frequently used by those involved with cancer risk assessment (reviewed in [13]).

Although highly persuasive in establishing causality, the availability of experimental evidence from randomized controlled trials is a rare commodity. More typically, we observe the change in prevalence of a disease after the prevalence of a causal determinant has been modified, after allowing for sufficient latency. The best examples in North America are the decline of lung cancer mortality following by about 20 years the onset of smoking cessation programs [1], and the decline of endometrial cancer incidence following by 5 years the reduction in prescriptions of unopposed estrogens for hormone replacement therapy [14]. More often, epidemiologists derive evidence from observational studies such as those described in Table 1. In cohort studies, we observe groups with different exposures to or prevalences of a purported causal factor, and measure cancer incidence, prospectively. In case-control studies, the prevalence of a potential causal factor is compared among cases and non-cases, also offer important causal evidence. All of these study designs have a potential for bias. If sufficient evidence is obtained from epidemiologic studies in which the likelihood and magnitude of bias is deemed unlikely to account for an observed effect, then we need not await laboratory evidence before public policy can be implemented with the aim of reducing or abolishing the harmful exposure.

Although useful for environmental, occupational, and lifestyle determinants, Hill's criteria do not capture very well the evidential foundation of causal claims for microbial agents and their respective malignant diseases. Historically, causal relationships in infectious diseases have been assessed using the mechanistically based Henle-Koch's postulates, which are based on the expectation that the

Table 2

Criteria or guidelines used in cancer epidemiology to help in attributing causality to candidate environmental, occupational, lifestyle, and biological risk factors or their respective exposure circumstances

Hill [11]: general purpose	Surgeon general's [12]: general purpose	Evans [15]: Infections and cancer	Evans and Mueller [16]: infections and cancer	Fredricks and Relman [17]: infections and cancer
Strength of the association: magnitude of the relative risk association between factor and incident disease or mortality	Consistency	Antibody to the agent is regularly absent prior to the disease and exposure to the agent	Geographic distributions of viral infection and tumor should coincide	Nucleic acid belonging to putative pathogen should be present in most cases and preferentially in organs known to be diseased
Consistency: findings are consistent across studies in different populations	Strength (implies also a dose-response gradient)	Antibody to the agent regularly appears during illness and includes both immunoglobulins G and M	Presence of viral marker should be higher in cases than in controls	Few or no copy numbers should occur in hosts or tissues without disease
Specificity: exposure to factor tends to be associated with only one cancer outcome	Specificity	Presence of antibody to the agent predicts immunity to the disease associated with infection by the agent	Incidence of tumor should be higher in those with the viral marker than in those without it	Copy number should decrease or become undetectable with disease regression (opposite with relapse or progression)
Temporality: onset of exposure should precede with sufficient latency the occurrence of tumor	Temporal relationship	Absence of antibody to the agent predicts susceptibility to both infection and the disease produced by the agent	Appearance of viral marker should precede the tumor	Detection of DNA sequence should predate disease
Biological gradient: dose-response relation between factor and rate of tumor development	Biological coherence	Antibody to no other agent should be similarly associated with the disease unless a cofactor in its production	Immunization with the virus should decrease the subsequent incidence of the tumor	Microorganism inferred from the sequence should be consistent with the biological characteristics of that group of organisms
Plausibility: does the association make sense in relation to the existing knowledge of likely mechanisms that could be affected by exposure				Tissue-sequence correlates should be sought at the cellular level using in situ hybridization
Coherence: does the association conflict with other knowledge about the tumor				Above evidence should be reproducible
Experimental evidence: data from randomized controlled trials of exposure or intervention				
Analogy: is there a comparable exposure-tumor association that seems analogous?				

microbial agent must be necessary, specific, and sufficient for the disease to occur. These postulates are only of indirect help in assessing cancer etiology since they imply the causation of the immediate infectious disease or condition that originated from the agent and not the final malignant process at the end of a lengthy chain of events triggered by the infection itself. A case in point is the causal pathway represented by the acquisition of infection with the hepatitis B virus (HBV) in non-immune individuals, followed by the development of acute hepatitis and then chronic hepatitis,

and finally, many years later the onset of hepatocellular carcinoma. Each step in succession affects smaller proportions of patients than the previous. Henle–Koch's postulates are useful up to the first or second steps of this pathway but are of no guidance for the imputation of a causal link between the pathways' beginning and terminal events. Sets of useful guidelines for causal attributions involving infectious agents have been proposed by some investigators [15–17] (Table 2). These causal criteria take into account the knowledge about the timing, specificity, and level of

immune response against putative viruses or the advances in nucleic acid detection methodology as used in modern molecular epidemiologic investigations.

In summary, what prevails today is an operational definition of cause, which incorporates the criteria required in different settings, depending on the type of carcinogenic mechanism being studied and its particular set of circumstances to ascertain exposure and intermediate endpoints. Decisions concerning carcinogenicity of specific exposures must entertain both scientific and public health issues as a dynamic process that is constantly updated as new knowledge from more insightful and more valid epidemiologic studies becomes available.

#### **4. Role and limitations of molecular epidemiology in exposure assessment**

There now exists a wide array of biomarkers used in molecular epidemiology studies that have the potential to assist in the early identification of human carcinogens. These markers monitor effects along the continuum from exposure to early response to disease. They are as diverse as measurement of agents in the body, of DNA or protein adducts, of mutations in blood or other cells, and of chromosomal damage (reviewed in [18]). DNA damage is now well established as a critical step in the process of cancer development. Lack of repair of damaged DNA before cell replication can lead to mutations, translocations, amplifications, etc in critical genes controlling cell growth and differentiation. Thus, the demonstration that a chemical results in the formation of adducts in humans can be an important component of the evaluation of its carcinogenicity.

A number of methods have been developed to measure DNA adducts in humans including immunoassays, <sup>32</sup>P-postlabeling, fluorescence, and gas chromatography/mass spectroscopy (GC/MS) [19]. Each method has its strengths and limitations. They vary in sensitivity, specificity and cost but, for establishing causality, more specific methods are preferable. For example, immunoassays can be readily used on large numbers of samples but may lack specificity when antibodies that recognize multiple adducts are used. In contrast, GC/MS allows absolute identification of adducts but requires expensive instrumentation. To identify whether a specific exposure causes DNA damage, the particular adduct it might form needs to be accurately determined.

A number of studies have found elevated levels of DNA adducts in cases compared to controls using samples collected at the time of diagnosis. There are several limitations to these studies. One is that the biomarker may reflect the disease rather than its etiology. In addition, most studies to date have used the presence of the biomarker in a surrogate tissue, usually blood, instead of the less readily accessible target tissue. Thus, white blood cell DNA adducts have been most frequently measured but albumin adducts have some-

times been measured as a surrogate for DNA adducts. There are only a handful of studies that investigated the relationship between adduct levels measured in a target tissue such as lung with those in blood. These studies are also limited to cases since it is usually not possible to get target tissue (the exception being oral cells and skin) from healthy subjects. Another limitation is that most case-control studies have also measured adducts at a single time point. Yet we now know that there are large intra-individual variations in most biomarkers, including DNA adducts, over time. The modulating effect of polymorphisms in carcinogen metabolism and DNA repair genes influences DNA damage levels as do dietary factors such as antioxidant consumption. Thus, individuals with the same external exposure will have different levels of damage, complicating interpretation of the results.

While animal studies can administer a single agent at high doses, humans are exposed to many agents in the diet and environment at relatively low levels. Certain occupational exposures may be sufficiently high to allow detection of elevated levels of DNA damage from a particular chemical. However, the situations where this will be possible in the general population are likely to be limited. Low and multiple exposures in combination with dietary and genetic factors that modulate adduct formation in individuals make it difficult to identify individual carcinogens. One study looked at multiple exposures in liver tissue in relation to hepatocellular cancer risk using immunologic methods to measure the DNA adducts of aflatoxin B1, polycyclic aromatic hydrocarbons, and 4-aminobiphenyl [20]. While this study is limited by the small number of liver tissues obtained from controls, it demonstrated dramatic increases in risk among subjects that had detectable adducts of increasing numbers of chemicals.

Further complicating the issue of using DNA adduct measurement for the identification of human carcinogens is the presence of background or endogenous adducts in all samples. These adducts result from normal metabolism, oxidative stress, and chronic inflammation. DNA adducts resulting from alkylating agents have been well studied in animals as a result of treatment with certain classical carcinogens such as nitrosamines. However, these adducts are common in unexposed animals possibly as a result of endogenous methylating agents [21]. Adducts formed by lipid peroxidation products have also been demonstrated in humans. These background levels of damage will make it difficult to determine if exposure to a potential carcinogen leads to increased levels of these types of DNA damage.

The major limitation of using most biomarkers to determine the relationship between exposure and cancer development is that they usually measure only relatively recent exposure, whereas most cancers take decades to develop. While there are some chemicals, such as organochlorine compounds, that have relatively long half-lives in vivo, most studies looking at blood or urine levels of a chemical or its DNA or protein adducts can detect exposure only in recent days or months. For this reason, case-control studies



nested in long-term cohort investigations are required to demonstrate the predictive value of biomarkers measured in biological specimens collected at baseline. While much progress has been made in this area, there are still only a handful of studies that have demonstrated significant relationships between the biomarker and risk for cancer development. The best data demonstrating that biomarkers can be used to predict risk from a chemical carcinogen are the studies carried out on the dietary mold contaminant, aflatoxin [22,23]. Nested case control studies in China and Taiwan have demonstrated that elevated levels of exposure to aflatoxin, measured as either albumin adducts in blood or urinary excretion of metabolites or the excised guanine adduct were predictive of subsequent risk. These studies also demonstrated a synergistic interaction in subjects who were both carriers of the hepatitis B virus and exposed to aflatoxin. It should be stressed that these studies demonstrate the biomarker's utility in populations but not for predicting individual risk. However, from the perspective of determining causality, they can be considered definitive. Their major limitation, in addition to cost, is the long time lag between sample collection and development of sufficient numbers of cases to enable estimating relative risks with adequate precision. This limits their utility for early identification of human carcinogens.

Mutational spectra in oncogenes and tumor suppressor genes have been suggested to be an alternate biomarker that can provide some indication of the etiologic agent. The best example is p53 where a G to T mutation at codon 249 has been associated with liver cancers from regions with high aflatoxin exposure and where mutations resulting from thymine dimer formation have been found in skin cancers resulting from UV exposure [24]. Variations in mutation spectra by tumor type, such as those between colon and lung, also suggest causation from endogenous processes and exposure to bulky carcinogens, respectively. However, it is unlikely that there will be sufficient specificity to provide clues to etiology except in very rare cases such as the UV-induced skin cancers or aflatoxin-induced liver cancers. The problem is that many agents produce the same type of DNA damage and mutations. For example, many bulky carcinogens as well as endogenous damage such as 8-oxodeoxyguanosine produce the same G to T mutations.

Cytogenetic assays including chromosomal aberrations and sister chromatid exchanges (SCE) are also useful biomarkers since aberrations are associated with specific cancers, indicating a mechanistic relevance, and SCEs have been shown *in vitro* to be caused by DNA damaging agents [25]. Chromosomal aberrations in peripheral blood lymphocytes is the only other marker, using a nested case-control design, that has been shown to predict cancer risk. The risk associated with higher aberrations was not affected by the inclusion of occupational exposure or smoking in the statistical model [26]. This suggests that they are an intermediate end point in the pathway leading to disease, perhaps reflecting inherent genetic susceptibility. Because chromosomal

aberrations are not chemical specific and are not as sensitive to chemical exposures as are SCEs, it is unlikely that they will be as useful as DNA adducts in establishing a causative association. Nevertheless, the demonstration of elevated levels of aberrations or SCEs in subjects with specific exposures compared to those without should be taken into consideration in the evaluation of chemicals.

Despite the above limitations, there is already one example where biomarkers have provided important information in the classification of a chemical carcinogen. Ethylene oxide was found to increase hemoglobin adducts, chromosomal aberrations and SCE in those occupationally exposed and this information was used by the IARC to classify it as a human carcinogen.

In summary, the major advantages of biomarkers are (i) their discriminant value, since abnormalities are more frequent among those destined to develop cancer, and (ii) their use as intermediate endpoints, as alterations in biomarkers can be found earlier than waiting for cancer to develop. Findings from biomarker studies have already been used for evaluation of human carcinogenicity. As methods for DNA adduct detection become more sensitive, the problem of background levels of adducts and their relevance will become more critical. Numerous large sample banks have been established around the world that will dramatically expand the number of nested case-control studies that can be carried out. However, the long-term nature of nested studies are problematic for the quick identification of human carcinogens.

## 5. Genetic susceptibility issues: pitfalls in Determining risk in population subsets

There is substantial evidence for the existence of host or genetic susceptibility to cancer, and for variation in the nature of this susceptibility. Subsets of the population may be susceptible through carrying inherited mutations of known cancer-related genes, or they may be susceptible as carriers of genetic variants that affect their ability to manage carcinogen exposure. Identification of these genetic factors and characterization of their interaction with environmental exposure in the development of cancer is of paramount interest [27,28]. A framework for approaching genetic susceptibility to cancer has been proposed by Knudson, who defined four categories of cancer causation or "oncogenes": (i) spontaneous or background, (ii) hereditary, (iii) environmental, and (iv) interactive (gene-environment); the latter accounting for the majority of cancer occurrences in most populations [29].

Until recently, classic epidemiologic approaches have utilized a "black box" approach to sort out cancer risks that makes it difficult to identify gene-environment interactions in cancer causation. Traditional approaches restricted the evaluation of host susceptibility factors to age, sex, family history, and ethnicity in studying exposure-cancer associations. More recently, however, the advent of molecular

epidemiology [30] has led to incorporation of tools from molecular genetics, cytogenetics, biostatistics, and bioinformatics, while maintaining the proven study design features of classic and genetic epidemiology. This has allowed researchers to measure biologically effective doses of carcinogens, analyze host susceptibility factors, and elucidate mechanisms involved in carcinogenesis. Though molecular epidemiology mandates the collection and analysis of biological specimens, technical innovations have enabled rapid, precise analysis of small volumes of DNA on hundreds or thousands of individuals. The advantages of molecular epidemiology include: (1) improved accuracy in ascertaining exposure, (2) defining surrogate endpoints to predict disease outcome, thereby reducing the time interval between exposure and the recognition of a relevant cancer effect, (3) identifying individuals with varying degrees of genetic susceptibility to cancer, and (4) identifying intermediate endpoints and targets for risk assessment and intervention.

The above promises of molecular epidemiology notwithstanding, many issues need to be resolved before genetic predisposition factors can be fully incorporated into the validation of a causal association with specific exposures. First, in terms of genetic predisposition, there are two broad categories: high penetrance and low penetrance genes, implying strong and weak predisposition, respectively [31]. High penetrance mutations of known cancer genes are rare events and development of cancer by carriers of these mutations is less dependent on environmental exposure. The inherited mutation is likely sufficient for cancer development although the attributable risk is generally low. Low penetrance genes are typically represented by genetic polymorphisms that are associated with sporadic cancers, and may involve multiple, distinct genes that aggregate with disease. In such cases, environmental exposure is likely to be necessary. Since these polymorphisms are frequent in the population, their attributable risk is high. As a corollary, low penetrance polymorphisms can be used to assess carcinogen associations. However, one needs to carefully consider gene–environment interactions by assessing susceptibility as a function of specific exposure dose and circumstances, when validating causal associations with a given agent.

A second limitation is represented by the overly simplistic scenarios of gene–environment interactions that have been contemplated to date in the molecular epidemiology literature. Most cancer molecular epidemiology studies are able to study a single or at most only a few genetic factors. However, carcinogenesis is a multifactorial and multistage process that is dependent on myriad mechanisms and pathways that regulate absorption, metabolic activation and excretion, DNA repair, control of mitotic cycle, hormonal stimulation of cell growth, inflammatory responses, and many other local or distant events that themselves are under genetic control. Genetic variability in any of these pathways can alter an individual's inherited predisposition. Therefore, in cancer risk assessment, consideration of a single marker is not sufficient to capture the full extent of the mediating effects of

susceptibility genes. Future studies should take a more comprehensive approach to the selection of candidate genetic markers to be investigated in the context of specific exposures. Such a selection should be made by carefully considering the putative genetic and epigenetic pathways involved for the relevant agent and organ system.

A third issue is represented by the choice of assays to capture the variability in genetic susceptibility. There are numerous such assays and they can be broadly divided into genotypic assays and phenotypic assays, depending on whether they directly probe the genome (DNA sequence for a target gene) or gene expression and cellular activity, respectively. Some phenotypic assays measure effects from a combination of genes, some of which are unknown. Recently, new phenotypic assays and proteomic technologies have appeared, permitting studies of cell cycle checkpoints, DNA damage/repair (mutagen sensitivity, comet assay, host cell reactivation assay), and detection of DNA adducts [32–35]. For the field of molecular epidemiology, the search for relevant single nucleotide polymorphisms (SNPs) in relevant cancer genes has become an important pursuit. Although the number of SNPs is huge, the vast majority do not lead to changes in amino acid sequence relative to the prototypic gene variant, and thus no discernible phenotypic variability is present. However, a small minority of SNPs that lead to amino acid changes in the encoded protein, affect regulatory sequences, or cause mRNA alternative splicing, etc. are worthy of investigation. One major difficulty faced by molecular epidemiologists is how to select SNPs for costly large-sample studies. Many questions remain to be answered. For example, how can computational algorithms (SIFT, POLYPHEN, etc), which prioritize target non-synonymous SNPs, be applied in the molecular epidemiological setting [36]? Are non-coding or intronic genetic variants important? How does one interpret associations with silent SNPs (those not leading to amino acid substitutions)? How do we deal with multiple SNPs within a single gene or in linkage disequilibrium? What is the joint effect of multiple SNPs within a pathway, or in the context of a specific exposure? How do we deal with statistical multiple comparison issues? Is it useful to study single DNA repair gene polymorphisms with respect to certain phenotypes (DNA damage/repair) instead of analyzing pathways? Are phenotypic assays practical for large-scale epidemiology studies? Is DNA repair capacity more important in the etiology of cancers associated with environmental insults than those that are originated via hormonal influences? These are only some of the complex questions facing those in the field [37].

A fourth issue, and one directly related to the above discussion on the multitude of assays, is the fact that few markers have been validated to the point where they can be applicable to risk assessment. There is a need to develop exposure pathway-specific biomarkers and validate them for population studies. Validation studies will need to keep pace with technological progress in assay develop-

ment. Robust study designs, access to large, representative populations, conservative control of confounding factors, and state-of-the-art laboratory procedures are essential for biomarker validation [30,38].

## 6. Recent paradigms in the identification of carcinogens: *Helicobacter pylori* and gastric cancer

Mortality and incidence statistics show an uneven distribution of gastric cancer throughout the world. The highest rates are found in Japan and Korea followed by China and Eastern Europe and the Andean populations of the Americas. The rates have been decreasing steadily in most countries following a birth cohort pattern. Cultural dietary patterns, rather than geography or race seem to determine the uneven distribution.

The present consensus that *Helicobacter pylori* infection is a cause of gastric cancer has a very short history. The events leading to that conclusion reached by the IARC in 1994 [39] are summarized below as an example of a coherent body of knowledge emerging from the contributions of epidemiology and other disciplines. The first suggestion of that association in the medical literature was made only 11 years before the “official” conclusion by the IARC [40,41]. The process started with the original observation by an astute surgical pathologist, Robin Warren. Together with his gastroenterologist colleague Barry Marshall, these Australian clinical investigators set in motion an extraordinary flurry of clinical and scientific activity, which persists at the present time. Warren first called attention to the fact that a spiral organism was seen adjacent to the gastric mucosa in the increasing numbers of gastric biopsies sent to him because of the generalized use of flexible gastroscopes. It stained positively with silver nitrate, as spirochetes usually do, and its presence was consistently associated with chronic gastritis.

### 6.1. Contributions of epidemiology

Ecologic studies have shown a positive correlation between gastric cancer rates and prevalence of *H. pylori* infection across different countries [42]. There have been numerous international case-control studies of the association between gastric cancer and *H. pylori* infection. Similar numbers of studies have shown either a positive association or no association. Most gastric cancers are diagnosed after the fifth decade of life. On the other hand, *H. pylori* infection usually starts during childhood. It usually persists for life if not treated. However, in advanced precancerous stages such as gastric atrophy and intestinal metaplasia, the gastric microenvironment becomes unfavorable for bacterial colonization and the infection may resolve. Thus, some case-control studies may have been affected by exposure misclassification among cases due to this temporal bias. Case-control studies which take into account these temporal differences in the biology of the two events, clearly

showed a positive association [43]. Several historical cohort studies stored frozen serum samples at entry and followed the participants for decades. They have clearly shown that subjects whose serum had anti-*H. pylori* antibodies at entry into the cohort, have a significantly elevated risk of gastric cancer years later [44–46]. The longer the exposure to the infection, the higher the relative risk [47]. Recall bias in these circumstances can be ruled out.

The strength and consistency of the epidemiologic studies led to the classification of *H. pylori* infection as a type I human carcinogen by the IARC in 1994. At that time, experimental support for the conclusion was lacking. That support was later provided when an appropriate animal model was identified: chronic infection of Mongolian gerbils with *H. pylori* induces gastric carcinoma, preceded by the precancerous stages as previously described in humans [48].

Although *H. pylori* infects approximately one half of the world population, a very small fraction of infected individuals develop gastric cancer. Molecular epidemiology studies have contributed greatly to predict cancer risk among infected subjects via the identification of genetic polymorphisms of the bacteria and of the host.

High cancer risk bacterial genotypes include virulence-associated genes: *cagA* and *vacA*. Subjects infected with *cagA*-positive, *vacA*<sub>1m1</sub> genotypes display the highest cancer risk [49,50]. High-risk human genotypes have been found associated with inflammatory cytokines induced by the bacterial infection: interleukin 1 beta (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 10 (IL-10). Polymorphic variants of IL-1 $\beta$  and TNF- $\alpha$ , which inhibit gastric acid secretion, are associated with higher cancer risk [51,52]. Expectedly, a synergistic effect is observed between bacterial and host genetic polymorphisms; high virulence bacterial genotypes infecting individuals more susceptible to cancer development because of their cytokine genes are associated with relative risks that are much higher than those conveyed by each genetic factor alone [50,52].

### 6.2. Etiologic model

The causes of gastric cancer can be described following the classical epidemiologic model as the interaction between the agent, the host and the environment, as outlined in the diagram shown in Fig. 1. The agent is *H. pylori*. It infects the gastric mucosa inducing chronic active gastritis. The damage it inflicts on the mucosa can vary from minimal to severe, depending on the virulence genes. Bacterial genotypes possessing the *cagA* gene convey cytotoxicity. The *vacA* gene induces cell vacuolization. The *babA* gene disrupts cell adhesion. The outcome of the infection is characterized by the presence or absence of cell loss (atrophy) as well as by the topographic distribution of the lesions. Multifocal atrophy involving the antrum and the corpus of the stomach increases cancer risk. On the other hand, non-atrophic gastritis is predominant in the antrum and does not increase the risk of cancer [53,54]. The type of gastritis is also modulated by



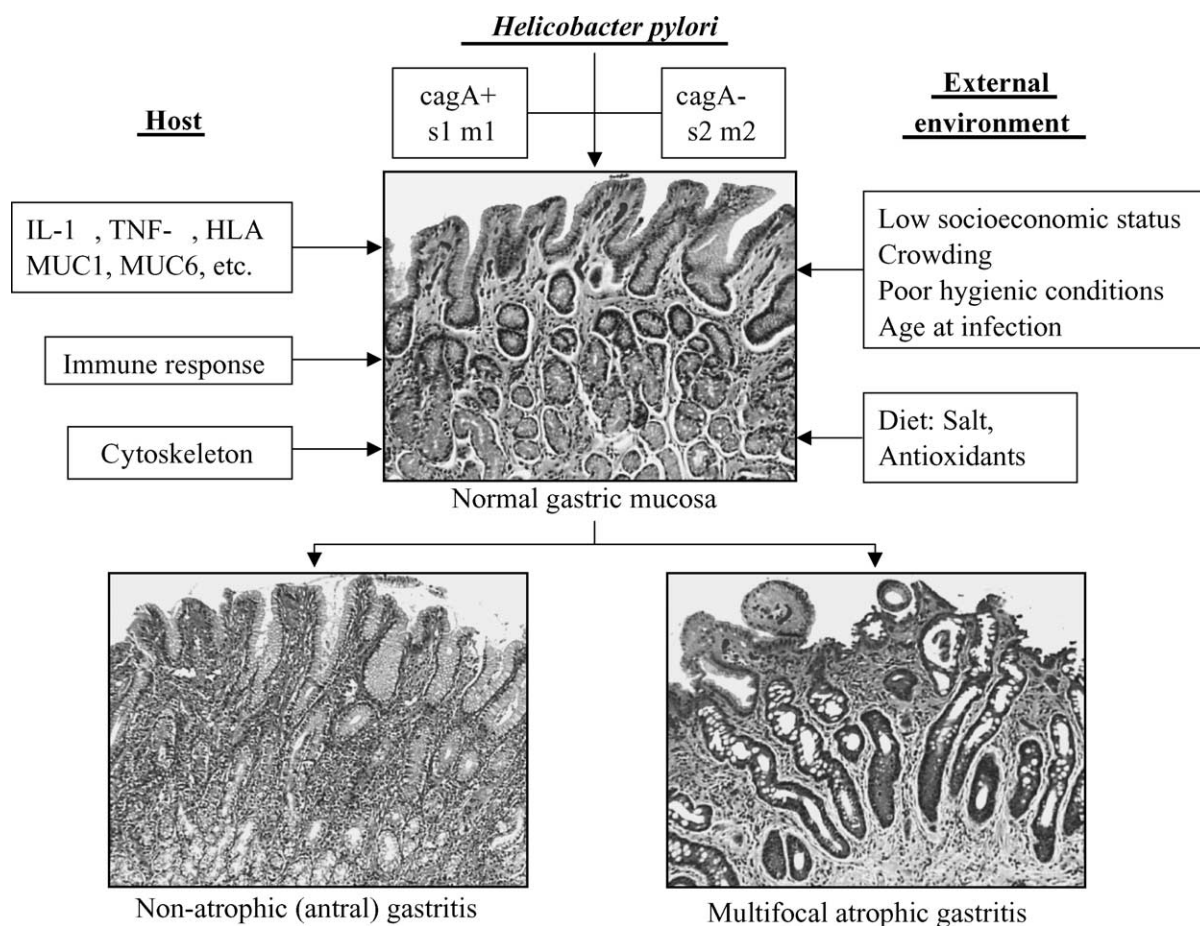


Fig. 1. Etiologic model of *Helicobacter pylori* infection in gastric cancer showing the contribution of host and environmental factors.

host polymorphisms, such as those associated with the induction of inflammatory cytokines, especially IL-1- $\beta$ . The mucin molecules, which protect the gastric epithelium from external injuries, are also polymorphic: larger molecules are more protective than smaller molecules. It is suspected that immune mechanisms modulate the inflammatory response but at this point they are not well understood. Most malignant gastric neoplasms are of epithelial origin, but the genotypic and phenotypic changes in the epithelial cells are far from clarified at this point. The normal gastric cells undergo intestinal metaplasia as a cancer-precursor lesion. Finally, the external environment also plays an important role in gastric cancer causation: overcrowding during childhood and low socioeconomic conditions can lead to early and severe infection, which increase cancer risk. Dietary factors have been identified which either increase (excessive salt) or decrease (fresh fruits and vegetables) the cancer risk.

Although the ultimate mechanisms of malignant cell transformation are unknown, coherent epidemiologic and molecular pathology findings point to oxidative damage as the final pathway of carcinogenesis. *H. pylori* gastritis results in the expression of inducible nitric oxide synthase, which leads to the presence of several nitric oxides in the gastric mucosa. Some are potent mutagens. At the same

time, antioxidant enzymes (catalase and superoxide dismutase) are expressed in the mucosa in chronic active gastritis. After infection, gastritis persists for decades. It thus appears that the *H. pylori*-infected gastric mucosa is exposed for a long time to both oxidant and antioxidant microenvironments, which either damage DNA or protect the host from such damage. The net effect of such influences may determine whether or not infection will be followed by neoplasia.

The knowledge generated by the molecular epidemiology of gastric cancer has permitted progress in the prevention front. Several chemoprevention trials are being conducted and a few have been completed. For instance, a trial conducted in a high-risk population of Colombia reported that curing the infection increases the probability of regression of precancerous lesions [55]. Similar effects were obtained with dietary supplementation for 6 years with ascorbic acid and/or beta-carotene.

## 7. Recent paradigms in the identification of carcinogens: human papillomavirus and cervical cancer

Cervical cancer is the second most common malignant neoplasm of women worldwide, representing nearly 10% of

all female cancers [56]. The highest risk areas are in Central and South America, Southern and Eastern Africa, and the Caribbean, with annual incidence rates around 40 per 100,000 women. Cervical cancer has been shown to have a central causal agent, HPV infection [6]. HPV infection is now considered to be a necessary intermediate step in the genesis of cervical cancer [57–59]. This conclusion is unique in cancer research; no human cancer has yet been shown to have a necessary cause, so clearly identified. Some of the well-studied paradigms of cancer prevention, such as tobacco smoking in lung cancer and chronic hepatitis B in liver carcinoma, are among the strongest epidemiologic associations, but they do not represent necessary causal relations. Lung cancers can occur in people who never smoked and had only minimal exposure to environmental tobacco smoke and liver cancer may occur in individuals who never had hepatitis B. The establishment of the HPV-cervical cancer link spawned approaches to preventing cervical cancer on two fronts: via screening for HPV infection as the biological surrogate that reveals asymptomatic cervical cancer

precursor lesions, and via immunization against HPV infection to prevent the onset of such lesions.

7.1. Emergence of HPV infection as the main etiologic factor in cervical cancer

The etiologic model of cervical carcinogenesis is shown in Fig. 2. The most “upstream” antecedents in the natural history are represented by variables related to the sexual behavior of the woman and of her male partners (reviewed in [60,61]). During much of the 1960s and 1970s, the consistency of findings from early epidemiologic studies pointing to a sexually-transmitted disease model for cervical neoplasia inspired research efforts to identify the putative microbial agent or agents acting as etiologic factors. The evidence available at the time indicated that genital infection with the Herpes simplex viruses (HSV) was the most likely culprit. This information was incorporated into textbooks of cancer epidemiology published until the mid-1980s (e.g., Ref. [62]). Although HSV was proven to be carcinogenic

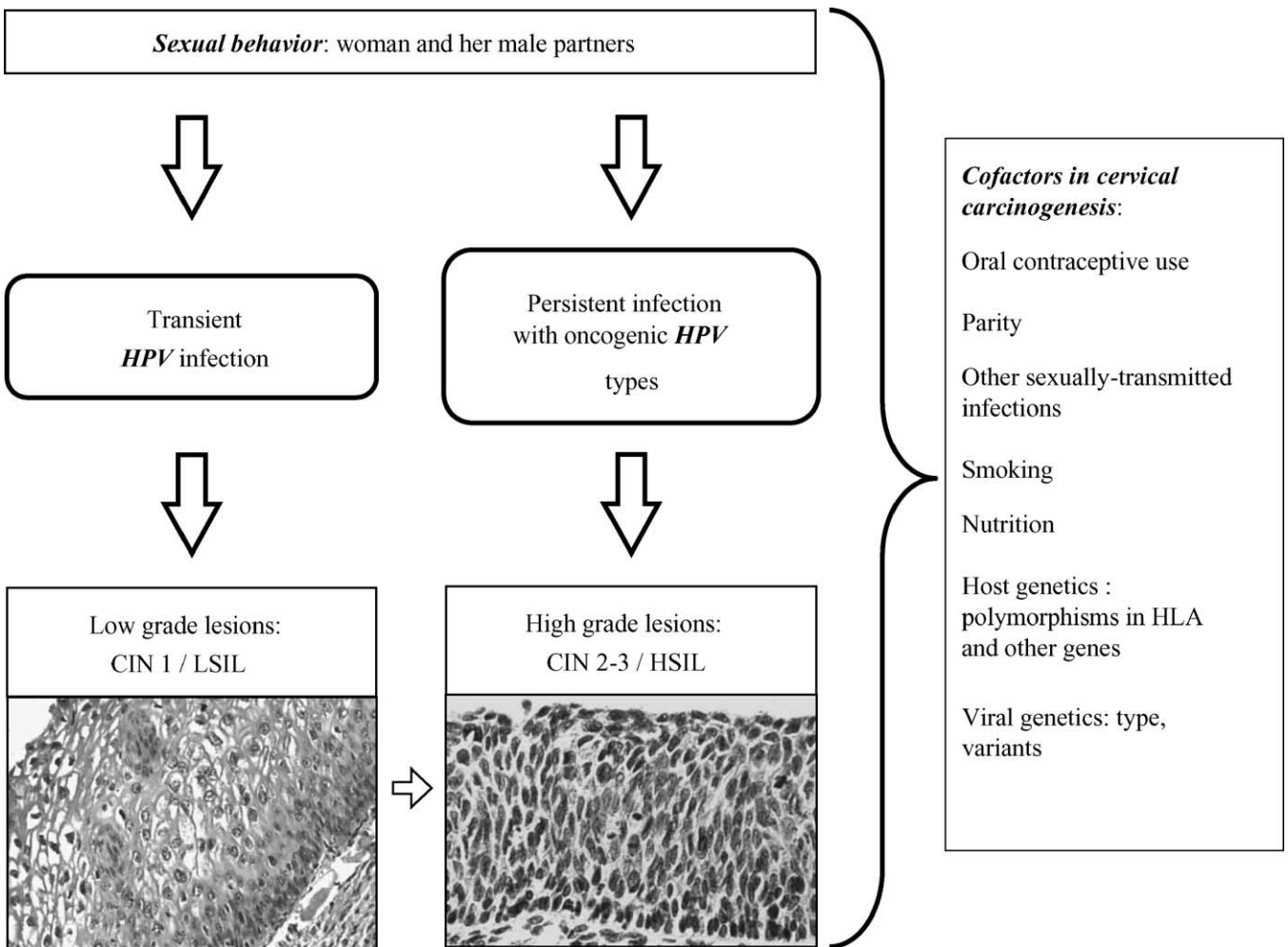


Fig. 2. Etiologic model of human papillomavirus (HPV) infection as a necessary cause of cervical cancer incorporating the role of host, reproductive, lifestyle, and viral co-factors.

in vitro and in vivo, the evidentiary link to cervical cancer was mostly indirect [63]. During the 1980s, the attention gradually turned to a new candidate, HPV, with the emergence of a consistent evidence base from molecular biology that implicated infection with certain types of this virus as the central sexually transmitted intermediate.

Unfortunately, the emergence of supporting epidemiologic evidence was delayed because the initial large-scale molecular epidemiology investigations launched in the mid-1980s employed HPV testing methods with insufficient sensitivity and specificity to detect viral DNA. The resulting misclassification of HPV exposure status in these studies led to relative risk estimates for the two putatively intermediate causal links, i.e., sexual behavior-HPV infection and HPV-cervical cancer that were disappointingly low and inconsistent with the postulated cervical cancer model in which HPV infection played the central role (reviewed in [64]). With the advent of polymerase chain reaction (PCR) protocols in the early 1990s the molecular epidemiology of HPV and cervical cancer became more congruent with the evidence from molecular biology [65]. In 1995, the IARC classified HPV types 16 and 18 as carcinogenic to humans and HPV types 31 and 33 as probably carcinogenic [6]. This classification was conservatively made on the basis of the available published evidence until 1994. A monograph revision planned for 2005 is likely to label up to 13 genital HPV types, in addition to HPVs 16 and 18, as carcinogenic, in the wake of recent research in the field [58,59,66].

The possibility that the first IARC monograph on HPVs was delayed because of the incoherent results of early epidemiologic studies has been debated by some authorities [67,68]. Germane to this discussion was the belief that the epidemiology-bred skepticism about the HPV-cancer link may have delayed the biotechnology sector in deciding to invest in HPV-based initiatives, such as HPV testing and immunization to screen and prevent cervical cancer precursors, respectively [67].

## 7.2. Etiologic model

HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are considered to be of high oncogenic risk because of their frequent association with cervical cancer and cervical intraepithelial neoplasia (CIN), the precursor, pre-invasive lesion stage [66]. The remaining genital types, e.g., HPV types 6, 11, 42–44, and some rarer types are considered of low or no oncogenic risk and generally cause subclinical and clinically evident genital warts, also known as condylomata.

The expression of two oncogenes of high-risk HPVs, E6 and E7, is responsible for cervical carcinogenesis. E7 complexes with the retinoblastoma (Rb) protein causing uncontrolled cell proliferation [69]. Binding of E6 to the p53 protein degrades the latter, leading to loss of DNA repair function and prevents the cell from undergoing apoptosis [70]. The cell can no longer stop further damages and

becomes susceptible to additional mutations and genomic instability.

Clinical, subclinical, and latent HPV infections are the most common sexually transmitted infections today. Latent genital HPV infection can be detected in 5–40% of sexually active women of reproductive age [6]. HPV prevalence is highest among young women soon after the onset of sexual activity and falls gradually with age, as a reflection of accrued immunity and adoption of a monogamous lifestyle. Most women who engage in sexual activity will probably acquire HPV infection during their lifetime. The vast majority of these infections will clear and will be no longer detectable [71–73]. The concern is with long-term persistent infections with oncogenic HPV types, which substantially increase the risk of CIN and cancer [74–76].

Today, it is well established that persistent infection with high oncogenic risk HPV types is the central, necessary causal factor in cervical cancer [6,59]. Relative risks for the association between HPV and cervical cancer are in the double to triple-digit range, which is among the strongest statistical relations ever identified in cancer epidemiology. HPV infection satisfies nearly all of standard causal criteria in chronic disease epidemiology. That not all infections with high risk HPVs persist or progress to cervical cancer suggests that HPV infection alone is not sufficient to induce the disease; other factors are also involved (Fig. 2). Among these environmental and host-related co-factors are: smoking, high parity, use of oral contraceptives, nutrition, and genetic polymorphisms in the HLA and other genes conferring susceptibility to infection and lesion progression (reviewed in [77]).

Research on prevention has already begun in several populations in the form of trials assessing the efficacy of HPV vaccines and studies of the value of HPV testing in cervical cancer screening. Progress on both counts is very promising. While the benefits of vaccination against HPV infection as a cervical cancer prevention tool are at least a decade into the future, the potential benefits of HPV testing in screening for this disease can be realized now in most populations.

## 8. Conclusions

Although epidemiologic approaches have become more robust with the advent of molecular methods to better ascertain exposure and examine gene–environment interactions they remain prone to the vicissitudes of learning via controlled observations of the joint distribution of exposure and disease in human populations. Only occasionally are epidemiologists able to use experimentation; most of the time their studies have to include design maneuvers or statistical analysis strategies to mitigate the effects of confounding biases and other issues that impair interpretability of causal relations. The core set of scientific criteria that help in deciding about the quality and quantity of the epidemiologic evidence in support of or against a causal relation in cancer

has changed little over the last 40 years. On the other hand, causal criteria have become more eclectic to incorporate specific circumstances related to the study of infectious agents in cancer and the advances in laboratory detection made in recent years. The application of these criteria at a single point in time may not always result in the appropriate policy decisions, as exemplified by the pitfalls of the first large scale molecular epidemiologic studies of HPV and cervical cancer. Constant updating of the knowledge base, replacing obsolete elements of the accrued evidence by more recent findings with enhanced validity and depth is a dynamic and open process that ensures timely discovery of carcinogens and appropriate preventive actions.

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