Modern molecular methods are rapidly becoming an integral part of many epidemiological studies. While approaches to evaluate genetic factors were described in another chapter, this one deals with the use of molecular methods to measure exposure, early evidence of malignant transformation, and individual susceptibility. As we will see, such epidemiologic studies are subject to problems of design and analysis similar to those encountered in more traditional epidemiologic investigations.

The use of biological measurements to assess variables of interest in epidemiological studies is not new. In the areas of infectious and cardiovascular diseases, research that would nowadays be viewed in the context of biomarkers has been conducted for decades. As an example, Figure 5–1 shows the results linking elevated serum cholesterol levels to the risk of ischemic heart disease in the Framingham prospective study (Truett et al, 1967). During the last decade, however, the use of biomarkers in cancer epidemiology has greatly increased.

Several reasons may explain this expansion. The search for carcinogens, characterized by complex exposure circumstances and possibly weak effects, has become increasingly difficult with traditional epidemiological approaches. An example is the investigation of the role of diet, in particular early in life, in breast carcinogenesis (Okasha et al, 2003). In parallel, increasing knowledge of mechanisms of carcinogenesis led to the proposal of models involving genetic and epigenetic events, as well as cellular and histological alterations. These models, which need to be tested in human studies, represent a theoretical framework for molecular epidemiological research. Furthermore, developments in molecular biology and genetics, such as the use of robots and the increasing throughput of automatic analytical equipments, allow the large-scale application of assays that would otherwise be very resource intensive.

It is useful to consider biomarkers in general and molecular epidemiology tools in particular within the larger framework of epidemiological studies. Epidemiology aims
at identifying determinants of disease and quantifying their role, while taking into account sources of random and systematic error (bias and confounding), as well as factors that modify the effect of the determinant(s) of interest (Figure 5-2). To a large extent, biomarker-based epidemiological studies fit into the same framework: They represent epidemiological studies, in which risk factors, outcomes, confounders, or effect modifiers are measured with biomarkers. Similarly, the same arguments should be applied to the design, analysis, and interpretation of biomarker-based and more traditional epidemiological studies.

In practice, there is a continuum from the development of biomarkers to be applied in human studies, to their characterization in early field studies, to their application in full-scale epidemiological investigations (Garcia-Closas et al, 2006). However, these logical steps are often bypassed, with promising but yet unvalidated biomarkers being applied in human studies. While this pattern reflects the vivacity of a young discipline, a more cautious approach is needed in order to avoid misuse of research resources.

In the context of epidemiological studies, a biomarker has been defined as a substance, structure, or process that (1) can be measured in the human body or its products and (2) may influence the incidence or outcome of disease in human populations (Workshop Report, 1997). It is important to bear in mind the distinction between marker, assay, and measurement. While the marker is the variable to be measured, the assay is the test used to measure it. Measurement is an ir marker.

A distinction has been made between markers of exposure, disease, and susceptible (Figure 5-1). This distinction is not arbitrary. For example, in the context of exposure, biomarkers that can monitor exposure to exogenous agents can be considered markers of exposure. However, points toward a role as biomarkers for prediction (bias and confounding) or effect modifiers in the study of disease. When one looks at the later steps relevant to this respect, they cannot be considered biomarkers. Further still, it is worth noting that any scheme presented in Figure 5-1 can be used not only for the understanding of the phenomenon of carcinogenicity but also for the measurement of carcinogenicity.

In other words, the scheme presented in Figure 5-1 can represent “boxes” within which biomarkers can be divided, depending on their role. For example, in the scheme for carcinogenicity, the markers of exposure are considered to be those that can be measured in the human body or its products, while the effect modifiers are considered to be those that can influence the incidence or outcome of disease in human populations.

**Figure 5-1.** Incidence of ischaemic heart disease and serum cholesterol in Framingham cohort—12-year follow-up of men. (Source: Truett et al, 1967)

**Figure 5-2.** Identification of exposure-disease relations with epidemiology.

**Figure 5-3.** Schematic representation of the biological process.
the test used to measure the marker, and the measurement is an individual value of the marker.

A distinction has been made between biomarkers of exposure, intermediate events, disease, and susceptibility (Figure 5–3; Table 5–1). This distinction, however, is somewhat arbitrary. For example, chromosomal aberrations have been used for decades to monitor exposure to environmental carcinogens (Tucker et al, 1997). From this point of view, they can be classified as biomarkers of exposure. However, growing evidence points toward a role of chromosomal aberrations for prediction of cancer risk, irrespective of exposure (Norppa et al, 2006). In this respect, they can be seen as intermediate biomarkers. Furthermore, it is important to notice that any scheme, such as that represented in Figure 5–3, reflects our current understanding of the complex biological phenomenon of carcinogenesis and our ability to measure events that are considered relevant to it. In other words, the steps in the carcinogenic process depicted in Figure 5–3 represent “boxes” where we allocate available biomarkers: In fact, more emphasis is given in the scheme to the early steps (internal dose, biologically effective dose, etc) than to the later steps simply because of the larger availability of markers—and their more straightforward interpretation—to measure the former as compared to the latter events. The increase in the understanding of the late steps in carcinogenesis, and the development of relevant and valid biomarkers, represents a main challenge to molecular cancer epidemiology.

**EXPOSURE BIOMARKERS**

In many instances, epidemiologic research is hampered by misclassification of exposure ascertained, for example, by means of questionnaires, interviews, or job histories. The rationale for using biomarkers is to measure the biologically relevant exposure more precisely. In some instances, there is an obvious improvement in using an exposure biomarker. Aflatoxin provides a good example in which exposure biomarkers represent a step forward in the identification of the human cancer hazard. The fungus Aspergillus flavus is a contaminant of foodstuffs, in particular cereals and nuts. Exposure is common in West Africa and East Asia. Depending on storage conditions, A. flavus may produce a toxin, called aflatoxin, with strong hepatotoxic and carcinogenic properties in animal models. It is difficult to...
Table 5-1. Classes of biomarkers and examples of application to cancer epidemiology

<table>
<thead>
<tr>
<th>Class</th>
<th>Biomarkers</th>
<th>Examples of application to cancer epidemiology (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure</td>
<td>Environmental pollutants</td>
<td>Dioxins and breast cancer risk (Warner et al, 2002)</td>
</tr>
<tr>
<td></td>
<td>Nutrients</td>
<td>Folate and cancer risk (Ross et al, 2006)</td>
</tr>
<tr>
<td></td>
<td>Infectious agents</td>
<td>HPV infection and oral cancer risk (Mork et al, 2001)</td>
</tr>
<tr>
<td></td>
<td>Endogenous compounds</td>
<td>Hormones and breast cancer risk (Kaaks et al, 2001)</td>
</tr>
<tr>
<td></td>
<td>DNA adducts</td>
<td>Aflatoxin adducts and liver cancer risk (Ross et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Protein adducts</td>
<td>Ethylene oxide adducts (Schulthe et al, 1992)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Cytogenetic abnormalities</td>
<td>Chromosomal aberrations and cancer risk (Boffetta et al, 2007)</td>
</tr>
<tr>
<td></td>
<td>DNA adducts</td>
<td>PAH-DNA adducts and lung cancer risk (Tang et al, 2001)</td>
</tr>
<tr>
<td>Disease</td>
<td>Gene mutation</td>
<td>TP53 mutations in lung cancer (Le Calvez et al, 2005)</td>
</tr>
<tr>
<td></td>
<td>Epigenetic alteration</td>
<td>Promoter methylation and bladder cancer risk (Marsit et al, 2006)</td>
</tr>
<tr>
<td></td>
<td>Genomic instability</td>
<td>Microsatellite instability in colorectal cancer (Slattery et al, 2000)</td>
</tr>
<tr>
<td>Susceptibility</td>
<td>Single nucleotide polymorphism</td>
<td>Polymorphisms in DNA repair genes and lung cancer risk (Hung et al, 2005)</td>
</tr>
<tr>
<td></td>
<td>DNA repair capacity</td>
<td>DNA repair capacity and lung cancer risk (Wei et al, 2000)</td>
</tr>
</tbody>
</table>

Table 5-2. Relative risk of biomarker

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>No aflatoxin biomarker</td>
<td>1.0</td>
</tr>
<tr>
<td>Any aflatoxin biomarker</td>
<td>1.5</td>
</tr>
<tr>
<td>AFB1-\N7-guanine</td>
<td>2.4</td>
</tr>
</tbody>
</table>


know whether the food humans have consumed has been contaminated by aflatoxin. Studies on the carcinogenic effect of aflatoxin are therefore limited by the difficulty of determining exposure status at the individual level, although ecological analyses have indicated a higher incidence of hepatocellular carcinoma in areas with frequently contaminated food than in neighbouring areas with less frequent contamination.

The identification of serum and urine biomarkers of aflatoxin exposure—namely urinary metabolites of aflatoxin itself and of its adducts formed with DNA—paved the way for important developments. Table 5-2 reports an increased risk of hepatocellular carcinoma in the first study using exposure biomarkers in subjects with samples collected and stored prospectively. Individuals with any urinary marker of exposure had a 2.4-fold increased risk of liver cancer relative to individuals without markers; the relative risk was as high as 4.9 among individuals positive for the urinary adduct degradation product AFB1-\N7-guanine (Ross et al, 1992). These results, replicated in other populations, provide strong evidence of a causal association between aflatoxin and liver cancer in humans (IARC, 2002).

Exposure markers measure the presence or level of exogenous agents (eg, pollutants), agents formed endogenously (eg, hormones), the metabolites of exogenous and endogenous agents, the products of the interaction of the agents or the metabolites with macromolecules (eg, DNA adducts), and physiological responses elicited by the exposure (eg, antibodies).

The use of biomarkers to measure exposure is not a panacea; the performance of exposure biomarkers should be compared to that of other exposure-assessment methods, such as medical records, questionnaires, and environmental monitoring. Main concerns are the relevance of the biomarker to the exposure of interest, its specificity (eg, chemicals often share common metabolites), and the characteristics of the assay, including sensitivity, kinetics, source of variability, and effect modifiers (Rothman et al, 1995). For example, the half-life of blood and urine markers of exposure to chemicals varies from a few hours (carbons) to years (eg, Friesen, 1997).

Most biomarker-prospective and retrospective single biological sample approaches as compared to exposure assessment and questionnaire or interviewer approaches, history variations in exposure matrices—feasible. Assessment of tobacco-related cancer exposures in cancer time-related variables is strongly point of induction and latency occurrence, the implication and intensity need to assess changes in the history of exposure periods. Different assessment (eg, biomarker-based) should be well as they accomplish how well they accomplish.

While most biomarker exposure, there are temporal changes and variety to collect repeated...
from a few hours (eg, chlorinated hydrocarbons) to years (eg, dioxins) (Coggon and Friesen, 1997).

Most biomarker-based studies, of both prospective and retrospective design, rely on a single biological sample. This represents a drawback as compared to traditional exposure assessment based on, for example, questionnaires or interviews. With the latter approaches, historical reconstruction of variations in exposure is—at least in some instances—feasible. An example is the assessment of tobacco smoking. Biomarker-based approaches, such as the measurement of nicotine or its metabolites in plasma, provide a precise indication of recent exposure. However, no biomarkers are currently available to assess cumulative tobacco consumption, or other time-related aspects of exposure that are important predictors of tobacco-related cancer risk.

Exposures in cancer epidemiology are often time-related variables. Moreover, both carcinogenesis models and empirical evidence strongly point toward the importance of induction and latency periods in cancer occurrence, the importance to evaluate both duration and intensity of exposure, and the need to assess changes in disease risk after cessation of exposure. The goal of exposure assessment is the reconstruction of a full history of exposure during relevant time periods. Different methods for exposure assessment (eg, biomarker-based versus questionnaire-based) should be evaluated on how well they accomplish this objective and on how well they complement each other.

While most biomarkers measure recent exposure, there are ways to use them to assess temporal changes. One approach is to collect repeated samples from subjects enrolled in prospective studies. While theoretically excellent, this option is often financially and logistically prohibitive. A compromise is the collection of repeated biologic samples from a fraction of the original cohort. If the sample is representative and large enough, it becomes possible to identify predictors of temporal changes in the biomarker and apply them to the whole study population. A more serious problem exists in retrospective studies, in which measurement of exposure biomarkers at the biologically relevant time (eg, several years before onset of the disease) is generally not possible.

When biological samples are available on a subset of the study population, it is possible to model exposure in a way similar to that described previously for repeated samples. For example, in a study assessing cancer risk from dioxin exposure among 1167 chemical workers from the Netherlands, recent serum dioxin measurements were available for 1.44 individuals, while a detailed occupational history was available for all workers (Hooiveld et al, 1998). In an analysis of the results from 144 workers, three factors—namely years in main production, employment before 1970, and exposure during an industrial accident—explained 85% of the variance in serum dioxin level. The application of these factors to the occupational histories, and the incorporation of terms accounting for the kinetics of dioxin accumulation during exposure and release after cessation of exposure, generated an estimated maximum level of exposure for all workers. In other instances, a biomarker can be used on a subset of the study population to validate exposures estimated by other means.

### Table 5-2. Relative risk of hepatocellular carcinoma and biomarker of exposure to aflatoxin

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cases</th>
<th>Controls</th>
<th>Relative risk</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>No aflatoxin biomarker</td>
<td>9</td>
<td>87</td>
<td>1.0</td>
<td>Reference</td>
</tr>
<tr>
<td>Any aflatoxin biomarker</td>
<td>13</td>
<td>53</td>
<td>2.4</td>
<td>1.0-5.9</td>
</tr>
<tr>
<td>AFBl-N7-guanine</td>
<td>6</td>
<td>11</td>
<td>4.9</td>
<td>1.5-16</td>
</tr>
</tbody>
</table>

A special group of exposure markers are adducts formed by carcinogens with DNA and proteins (Farmer, 2004). DNA adducts are directly relevant to carcinogenesis, since they reflect the interaction of the active compound with the relevant cellular target. They also integrate internal dose and repair capacity, thus providing a relevant indicator of biologically active dose (Rundle, 2006). However, the drawbacks in their use in epidemiology are the low levels detected in most exposure circumstances and the relatively short half-life. Some techniques to measure adducts are more sensitive, although this is often obtained by losing specificity. The half-life of white blood cells, which are often used to measure DNA adducts, is variable, but in general rather short. On the other hand, the detection of adducts to proteins—mainly albumin and hemoglobin—allows a measure of internal dose integrated over several weeks or months. In general, DNA and protein adducts represents a useful complement to other approaches of exposure assessment, but in very few circumstances have they provided critical novel information, in either qualitative or quantitative terms, to epidemiological research.

The use of exposure biomarkers poses additional methodological problems. As discussed in the following, epidemiological results based on exposure biomarkers are potentially subject to bias and confounding, as are other types of observational studies. An additional problem exists when exposure markers are used in retrospective case-control studies: their possible dependence on the disease process. For example, lipid metabolism might be altered in breast cancer and other hormone-dependent neoplasms (Demark-Wahnefried et al., 2001), resulting in possible bias in the measurement of compounds stored in adipose tissue, such as many organic contaminants. Despite the potential limitations, exposure biomarkers represent an important tool in cancer epidemiology. Technological developments are rapid in the field, improving the accuracy, sensitivity, and precision of the assays and facilitating the application of the markers to large-scale population studies.

INTERMEDIATE BIOMARKERS

Intermediate biomarkers measure early—in general nonpersistent—biological events that take place in the continuum between exposure and cancer development. These events include measure of cellular or tissue toxicity; chromosomal alterations; changes in DNA, RNA, and protein expression; and alterations in functions relevant to carcinogenesis (eg, DNA repair, immunological response). Similar to exposure markers, these markers are generally measured in easily accessible biological samples, typically blood components. Several of these assays offer a potentially important contribution to molecular epidemiology, because of their direct relevance to carcinogenesis and the parallel development of intermediate- or large-throughput technical platforms (eg, microarrays).

The use of DNA and RNA expression arrays in large-scale population studies has started, and their contribution is expected in the next few years (Gunn and Smith, 2004). As proteins and peptides are more stable and relatively easier to measure than RNA and DNA, proteomics and metabolomics represent interesting new approaches to the investigation of intermediate events in molecular cancer epidemiology. Also in this instance, however, the application to large-scale population-based studies is only starting, and the characteristics and performances of the biomarkers are not fully understood. An additional challenge brought by microarray and proteomic analysis is the complexity of data, since several hundreds or even thousands of data points are generated for each sample, which poses novel statistical challenges, including an increased likelihood to produce false-positive results (see following).

Chromosomal alterations measured in peripheral lymphocytes have been used for monitoring exposure to mutagens and carcinogens. In recent years, several cohort studies have beenestablishing the occurrence of carcinogens who underwent aberration testing (Norppa et al., 2005). DNA adducts have been associated with a higher levels of chromosomal aberrations, and the need to study the role of DNA adducts in cancer risk (Table 5-3)—the highest excess risk in digestive system.

Despite the fact that they primarily biomarkers of obvious, DNA adducts have predict cancer risk in retrospective studies (Phillips, 2005). A role of DNA adducts in risk has still to be elucidated, but in the need to study the disease relationship as a case-control study. As in the case of exposure of biomarkers to measure an epidemiological study has the idea to increase measurement—that is, toxicity and the sensitivity of the outcome. For example, based on techniques to mes...
BIOMARKERS

BIOMARKERS measure early—biological events continuum between development. These changes of cellular or tissue alterations; changes in protein expression; and s relevant to carcinogen, immunological re-posure markers, these measured in easily ac-quisition, typically blood of these assays offer a contribution to mo-because of their direct enesis and the paral-intermediate- or large-platforms (eg, micro-

Table 5-3. Meta-analysis of studies of chromosomal aberrations and cancer risk

<table>
<thead>
<tr>
<th></th>
<th>Relative risk</th>
<th>95% Confidence interval</th>
<th>Test for heterogeneity p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total chromosomal aberrations</td>
<td>1.0</td>
<td>Ref.</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1.3</td>
<td>1.1, 1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Medium</td>
<td>1.4</td>
<td>1.2, 1.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Chromatide-type aberrations</td>
<td>1.0</td>
<td>Ref.</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1.1</td>
<td>1.0, 1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>High</td>
<td>1.3</td>
<td>1.1, 1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Chromosome-type aberrations</td>
<td>1.0</td>
<td>Ref.</td>
<td>-</td>
</tr>
<tr>
<td>Low</td>
<td>1.1</td>
<td>1.0, 1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>High</td>
<td>1.3</td>
<td>1.1, 1.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Meta-analysis of the studies by Hagmar et al, 1994; Bonassi et al 1995 (these two studies were combined in Hagmar et al, 2004); Rossner et al, 2005; Boffenb et al, 2007.

studies have been established by measur-ing the occurrence of cancer among indi-viduals who underwent chromosomal ab-erration testing (Norppa et al, 2006). Four such cohort studies have been reported: A meta-analysis of their results suggests that higher levels of chromosomal aberrations are associated with a modest increase in cancer risk (Table 5-3)—the cancers with the highest excess risk were those of the digestive system.

Despite the fact that they are considered primarily biomarkers of exposure (see pre-vious), DNA adducts have been used to predict cancer risk in several prospective studies (Phillips, 2005). Although the exact role of DNA adducts in predicting cancer risk has still to be elucidated, this is an ad-ditional example of the artificial distinction between “exposure” and “effect” biomark-ers, and the need to study the exposure—disease relationship as a continuum.

DISEASE BIOMARKERS

As in the case of exposure markers, the use of biomarkers to measure the outcome of an epidemiological study (typically cancer) has the aim to increase the validity of the measurement—that is, to increase the specificity and the sensitivity in the definition of the outcome. For example, microarray-based techniques to measure the expression of a large number of genes has led to the discovery that cases of breast cancer indistinguishable according to traditional histo-

Alterations (eg, mutations, deletions, epigenetic modifications) in genes with a role in carcinogenesis or a characteristic cytoge-netic alteration, rather than the tumor itself, might become the outcome of a molecular epidemiological study. Studies based on bio-markers of disease are best suited in a pro-spective design, since the identification of early events relevant to carcinogenesis would hopefully impinge on preventive strategies. For example, mutations typical of tobacco-related cancers have been found in sputum samples of heavy smokers, suggesting that they can be used as markers of lung cancer (Kersting et al, 2000).

Disease biomarkers can also be used in retrospective designs, in which only dis-eased individuals are enrolled. In such case-only studies, comparisons are made among subgroups of cases with differences in the profile of genetic mutations (or other molecular characteristics). For example, different frequencies and patterns of mutations in TP53 have been detected in lung cancer, in correlation with tobacco smoking and exposure to other carcinogens (Pfeifer et al, 2002).
BACKGROUND

As in the case of exposure markers, the time coordinates of early effect markers are crucial for their application in molecular epidemiology. While adequate knowledge of the natural history is lacking for most human neoplasms, models of carcinogenesis developed for various tumors, eg, colon cancer (Fearon and Vogelstein, 1990), stomach cancer (Correa, 1992), and head and neck cancer (Sidransky, 1997), provide a framework for the application of effect markers.

SUSCEPTIBILITY MARKERS

The broad interindividual variability at both the genetic and the epigenetic levels provides a framework to explain the inherited susceptibility to cancer. Susceptibility markers can be measured at the genotypic level and at the functional (phenotypic) level. The advantages of genotypic markers are their stability across tissues and time, and the growing throughput of genotyping technologies. Phenotypic markers, on the other hand, integrate the effect of multiple genes, epigenetic phenomena, and post-translational modifications with respect to given characteristics such as DNA repair. Their implementation in large-scale population studies, however, remains limited.

The most commonly studied genetic variants are single nucleotide polymorphisms (SNP); other types of variants include microsatellites, deletions, insertions, gene amplifications, and variations in the number of gene copies (Redon et al, 2006). Early studies of susceptibility markers considered only one or few variants in candidate genes; technological developments have later conferred the ability to look at hundreds or even thousands of variants in a panel of genes. In the last years, the analysis of a large number of variants has become possible (current microarrays include several hundreds of thousands of SNP, but this number is expected to grow in the near future). In such genome-wide association studies any effort to select variants and genes based on a priori functional knowledge has been abandoned.

While a detailed discussion of genetic cancer epidemiology is beyond the scope of this chapter, it should be stressed that few variants conferring modest, at most, increases in cancer risk have been consistently found. It is likely that most of the genetic susceptibility to cancer arises from a combination of deleterious variants in different genes, each providing only a marginal excess risk. Furthermore, it is plausible that most of the effect occurs from the combination of genetic make-up and exposure to endogenous or exogenous factors (so-called “gene-environment interactions”; see following) (Hunter, 2005). The high-throughput genotyping approaches developed in recent years have started to be applied to genetic cancer epidemiology. It is expected that they will contribute substantially to the understanding of mechanisms underlying genetic susceptibility, and eventually mechanisms of human carcinogenesis.

Epigenetic alterations, particularly changes in promoter methylation status, are increasingly used as markers of carcinogenesis. Methylation status in lymphocytes and other surrogate tissues may reflect inherited characteristics that are relevant to individual susceptibility, possibly in relation with lifestyle and environmental exposures.

Functional (phenotypic) markers can be used to complement the information provided by the analysis of genetic variations. In particular, the individual ability to repair DNA damage has been investigated using different types of assays—such as host-cell reactivation assay, mutagen sensitivity, Comet assay—in case-control settings (Spitz et al, 2003). In general, cancer cases have shown a decreased DNA repair capacity as compared to controls, but the interpretation of these results is hampered by the small sample size and the use of lymphocytes as surrogate cells. The technical complexity of these assays has so far prevented their application in large-scale prospective studies.

BIAS

Three main types of bias are recognized in epidemiology, and all three may operate in biomarker-based studies (Rothman and Greenland, 1996). Selection bias arises from lack of comparability of the study. For example, be more (or less) likely than exposed cases to bias involves different environmental factors. Finally, confounding by factors other than the following).

Selection bias can be identified by modifying the study design to optimize the response of the biomarker. Unfortunately, many logical studies pay too little attention to the definition of source population. Common in studies of genetic factors under prospective studies are less bias than retrospective

BIOMARKER

Sources of variation measurements might arise

Table 5-4. Sources of variation measurements might arise

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Sources of variation measurements might arise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormones</td>
<td>More important source of variation in biomarker-based studies (Rothman and Greenland, 1996). Selection bias arises from lack of comparability of the study. For example, be more (or less) likely than exposed cases to bias involves different environmental factors. Finally, confounding by factors other than the following). Selection bias can be identified by modifying the study design to optimize the response of the biomarker. Unfortunately, many logical studies pay too little attention to the definition of source population. Common in studies of genetic factors under prospective studies are less bias than retrospective.</td>
</tr>
</tbody>
</table>
BIOMARKERS IN CANCER EPIDEMIOLOGY

It is recognized that few variants are consistently found. It is genetic susceptibility to a combination of genetic factors in different genes, in particular, is responsible for most of the combination of genetic factors that contribute to genetic risks (so-called "gene-by-environment" interactions) and genetic cancer risk. Exposed cases might be more (or less) likely to participate in a study than exposed controls. Information bias involves differential or even non-differential misclassification of participants with respect to disease or exposure status. In biomarker-based studies, information bias encompasses the issues of inherent validity, reproducibility, and stability of markers. Finally, confounding is a special form of bias, generated by co-exposure to causal factors other than those under study (see following).

Selection bias can be avoided by properly identifying the study population, and by optimizing the response rate. Furthermore, it can be controlled in the analysis by identifying factors that are related to selection and by controlling them as confounders. Unfortunately, many molecular epidemiological studies pay too little attention to the definition of source population and selection of participants. This is particularly common in studies of genetic factors, since it is considered that any selection of participants is unlikely to be associated with the genetic factors under study. In general, prospective studies are less prone to selection bias than retrospective studies.

BIOMARKER VARIATION

Sources of variation in biomarker-based measurements might arise from intergroup variability. How-ever, other sources of variation exist that generate misclassification. Interindividual variability might be due to genetic or environmental factors affecting the biomarker under study. Intraindividual variability refers to components of variation such as diurnal variation in hormonal level. Finally, measurement error might arise from sampling and laboratory variation. Table 5-4 provides some examples of sources of variation for selected biomarkers used in molecular cancer epidemiology (Vineis, 1997).

Proper precautions should be taken to minimize the sources of variation other than intergroup variability. Such sources are numerous: the circumstances under which biological samples are taken, processed, stored, and analysed; the technical aspects of the assays; etc. It is important to ensure that, if all sources of variation cannot be controlled (as it is often the case), they should apply equally to the groups being compared. Therefore, if long-term storage of samples might affect the measurement, it is important to match cases and controls in the study by duration of sample storage. In this situation, misclassification is said to be "non-differential" (meaning acting equally on the groups being compared). Nondifferential misclassification generally produces bias toward the null value—that is, it obscures an existing association, but it does not generate one when none exists, nor does it accentuate an existing positive or inverse one.

Table 5-4. Sources of variation for selected biomarkers used in cancer epidemiology (modified from Vineis, 1997)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Inter-individual</th>
<th>Intra-individual</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormones</td>
<td>+</td>
<td>+</td>
<td>(-)</td>
</tr>
<tr>
<td>Organochlorine compounds</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DNA adducts in white blood cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chromosomal aberrations</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SNP (genotyping)</td>
<td>-</td>
<td>-</td>
<td>(-)</td>
</tr>
<tr>
<td>DNA repair capacity</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ More important source of variation
- Less important source of variation
[ ] No or few data
On the other hand, a misclassification that is "differential" with respect to case/control (or exposed/unexposed) status generates a bias in an unpredictable direction. For example, if there is substantial interbatch (or interreader) variability in the measurement, the inclusion of samples of cases and controls in different batches would generate differential misclassification, while a proper mix of samples in each batch would at worst result in nondifferential misclassification.

TRANSITIONAL STUDIES

The issue of variation in biomarker-based measurements impinges on the need to validate biomarkers before application to large-scale studies. This is the domain of so-called transitional studies, which aim to characterize the biomarker itself rather than the underlying biological phenomenon. The aspects assessed by transitional studies include intra- and intersubject variability, feasibility of application of a biomarker in field conditions (and optimization of its use), identification of determinants with confounding and effect-modifying potential, and exploration of biological mechanisms underlying the variation of the marker.

Transitional studies may involve healthy individuals, patients, or subjects with specific exposures (eg, groups of workers). Three types of transitional studies have been described in the continuum between development of a new assay and its large-scale application to human populations (Table 5-5) (Schulte and Perera, 1997; Rothman et al, 1995).

Developmental transitional studies have several goals. They aim to identify the biological phenomena measured by the marker and their relevance to the exposure, the disease, or the host variables of interest. In addition, developmental studies address the reliability (reproducibility) of the newly developed marker, by blindly measuring replicate samples. These samples should be representative of the values likely to be found in populations that the marker has to be applied to (Rothman et al, 1995). Assessment of reliability encompasses both random laboratory variation and nonrandom (systematic) error. Principles for assessment of marker reliability have been proposed (Vineis et al, 1993; Droz, 1993). Developmental transitional studies should also address aspects of relevance to field applications such as kinetics and stability of the marker. They should contribute to the clarification of the temporal relevance of the biomarker in relation to the underlying biological phenomenon (Droz, 1993).

The main aim of characterization-type transitional studies is to assess interindividual variation in the marker and to reveal genetic and acquired factors that contribute to such variation. When applied in the field, interindividual variation of the marker will be studied in conjunction or the outcome of interindividual variability and, to a certain extent, the temporal relevance of the marker itself. The identification of the level of the marker in tissues, since field studies involve samples of sur- living subjects and, to a certain extent, the temporal relevance of the marker itself. The identification of the level of the marker in tissues, since field studies involve samples of sur- living subjects...
Biomarkers in Cancer Epidemiology

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be studied in conjunction with the expo-

ure or the outcome of interest. Sources

of interindividual variation should then be

known and, to a certain extent, controlled

for. One particular aspect of interindividual

variability is the difference in presence or

level of the marker in different organs and

tissues, since field studies often have to rely

on samples of surrogate tissues, typically

blood. The identification of factors affecting

the level of a marker goes beyond the char-

acterization of the marker and impinges on

the interpretation of the results of molecu-

ar epidemiological studies. For example,

the finding that a metabolic polymorphism

is a modifying factor for a marker of expo-

sure provides important information on the

metabolic pathways relevant to the expo-

sure of interest.

Applied transitional studies aim to assess

the relationship between a marker and the

phenomenon that it is considered to indicate,

ie, the relationship between exposure and

marker or between marker and disease. For

example, the association between exposure
to butadiene and several markers, including

mutations in the HRPT gene, has been stud-

ied, in order to assess the usefulness of these

markers in predicting cancer risk among

exposed workers (Albertini et al, 2001).

CONFOUNDING

The use of biomarkers does not prevent

confounding. For example, an association

between tobacco smoking and cancer of the

uterine cervix has been observed in many

populations but it is likely to be confounded

by infection with the human papilloma vi-

rus (HPV), a cause of cervical cancer. In

many populations, smokers are more fre-

quently positive for HPV than nonsmokers.

Hence, HPV would be a confounder no

matter how smoking, infection, and cervi-

cal cancer are assessed (via questionnaires,

medical records, biochemical methods, or

molecular techniques). Furthermore, to use

biomarkers might introduce confounding.

If, for example, workers occupationally ex-

posed to polycyclic aromatic hydrocarbons

( PAH) have a higher consumption of to-

bacco (an important source of PAH) than

other workers, then the assessment of oc-

cupational exposure with a biomarker of

PAH is confounded by tobacco smoking

(Figure 5–4) (Pearce et al, 1995). This would

have not been the case if occupational ex-

posure were assessed without biomarker

methods.

INTERACTION

Biomarkers have been widely applied to

studies of gene–environment interactions

and gene–gene interactions in the pathogen-

esis of cancer and other chronic diseases

(Hunter, 2005). Table 5–6 provides an ex-

ample from a lung cancer study that ad-

ressed dietary intake of cabbages and

other cruciferous vegetables. Such vegetables


![Figure 5-4. Example of confounding](image-url)
Table 5-6. Example of gene-diet interaction: Relative risk of lung cancer for dietary intake of cruciferous vegetables, stratified by GSTM1 and GSTT1 polymorphism (Brennan et al., 2005)

<table>
<thead>
<tr>
<th>Dietary cruciferous vegetable intake - RR (95% CI)</th>
<th>High (reference)</th>
<th>Medium</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1+ and GSTT1+</td>
<td>1.00</td>
<td>0.87 (0.62, 1.22)</td>
<td>0.88 (0.65, 1.21)</td>
</tr>
<tr>
<td>GSTM1+ and GSTT1-</td>
<td>1.00</td>
<td>0.82 (0.60, 1.12)</td>
<td>0.80 (0.60, 1.08)</td>
</tr>
<tr>
<td>or GSTM1- and GSTT1+</td>
<td>1.00</td>
<td>0.26 (0.10, 0.63)</td>
<td>0.28 (0.11, 0.67)</td>
</tr>
</tbody>
</table>

RR, relative risk; CI, confidence interval

contain the potentially chemopreventive isothiocyanates. Polymorphism for genes encoding for the enzymes glutathione-S-transferase (GST) M1 and T1, are implicated in the metabolism of isothiocyanates (Brennan et al, 2005). The apparent protective effect of high intake of isothiocyanate-rich diet was stronger among carriers of the null genotypes in the GST enzymes than in carriers of the wild-type genotype in one or both genes.

Molecular epidemiology studies address other types of interaction between two or more factors may be discussed following a similar approach. For example, in the study of aflatoxin exposure and liver cancer mentioned previously, the investigators addressed the possible interaction of aflatoxin with hepatitis B virus (HBV) (Ross et al, 1992). When compared to HBV-negative subjects exposed to aflatoxin, the relative risk in HBV-positive subjects also positive for aflatoxin markers was 60, which was greater than the product of the relative risks for the two factors separately (4.8 for HBV and 1.9 for aflatoxin). Thus a supermultiplicative synergism between aflatoxin and HBV in liver carcinogenesis is suggested. The wide confidence interval in the group with both exposures (6.4–560) precludes, however, rejections of the null hypothesis of no interaction according to a multiplicative model (4.8 x 1.9 = 9.1). This wide interval also stresses another methodological concern in molecular epidemiological studies, namely the need for a large sample size.

RANDOM ERROR

From several of the examples quoted previously it is clear that an important problem in biomarker-based epidemiological research is the insufficient number of subjects included in each study. The main reasons for a small study size are logistical and financial constraints. Indeed, any biomarker-based measure introduced in epidemiology should be compared with traditional measures, and the possible gain in sensitivity and specificity of the biomarker measure should be considered in the light of the possible decrease in the number of study subjects.

Authors have proposed formulas to calculate the sample size needed to detect main effects and interactions among risk factors (Garcia-Closas and Lubin, 1999). Molecular epidemiology studies often do not include a sufficient number of individuals, and this has been the reason for unstable and conflicting results. For example, many studies have been published on the possible association between slow acetylation polymorphism and bladder cancer risk. The biological rationale is that individuals with variants leading to reduced acetylation might have a reduced capacity to detoxify environmental bladder carcinogens, including aromatic amines. It is unlikely, however, that the relative risk would be higher than, say, 1.5, and, in fact, recent meta-analyses confirmed the presence of increased risk of the order of 40% (Garcia-Closas et al, 2005). Given that the frequency of the relevant polymorphic variant in European populations is a and the same number to achieve an 80% practically significant relative goal is to detect an in polymorphism and an (eg, tobacco smoking sure to aromatic amines required cases and co larger (between 2- ar on the expected stren

As shown in Figure 29 studies of NAT2 polymorphism included in had an adequate size fect of the polymor| the power to doc action with an envir recent years, large-s set up, in order to pr|sults (eg, Slattery et al et al, 2005; Hung et i approach is the pooling ducted studies (Ioann
BIOMARKERS IN CANCER EPIDEMIOLOGY

The line indicates the number of cases needed for 80% power to detect a statistically significant relative risk of 1.4 (prevalence of slow acetylation 0.5).

Figure 5-5. Number of cases in studies of NAT2 slow acetylation polymorphism and bladder cancer (Garcia-Closas et al, 2005)

Since lack of statistical power is an important problem in molecular and genetic epidemiological studies aimed to detect weak associations, the independent conduct of small-scale studies that address several hypotheses is a reason for the generation of many false-positive results (Ioannidis et al, 2006a). This problem has been mainly addressed in the context of genetic association studies, and in particular as a consequence of the growing ability to measure a large number of genetic variants; but it may affect other areas of molecular epidemiological research. Guidelines for the reporting of results and the interpretation of “positive” associations have been proposed (Ioannidis, 2006).

PUBLICATION BIAS

A problem related to the generation of false-positive results is the tendency to selectively report significant results, in particular when they show an effect in the expected
direction. The net result is a biased underreporting of null results. As an example, several studies have been conducted on polymorphism of the CYP2D6 gene, which encodes for an enzyme possibly involved in the activation of lung carcinogens, and lung cancer risk. Figure 5-6 shows the results of the 18 studies available for a meta-analysis (d’Errico et al, 1999) reported in terms of the logarithm of the relative risk for high-risk CYP2D6 polymorphism, and its standard error. Each study is identified by one dot, studies to the right of the figure are smaller than those to the left, and studies at the top are more positive than those at the bottom of the figure. If no publication bias existed, the pattern of such results should resemble a triangle (or a funnel), with larger

Table 5-7. Publication bias in studies of selected associations between polymorphisms in genes encoding for metabolic enzymes and cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer site</th>
<th>N of risk estimates</th>
<th>Publication bias, p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>Lung</td>
<td>18</td>
<td>0.6</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Lung</td>
<td>18</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Urinary bladder</td>
<td>7</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Lung</td>
<td>50</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Urinary bladder</td>
<td>28</td>
<td>0.3</td>
</tr>
<tr>
<td>NAT2</td>
<td>Lung</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Urinary bladder</td>
<td>29</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Colorectal</td>
<td>14</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Based on d’Errico et al, 1999, except for GSTM1 and lung cancer (ad-hoc search), GSTM1 and bladder cancer (Garcia-Closas et al, 2005) and NAT2 and bladder cancer (Garcia-Closas et al, 2005).

Publication bias is assessed according to Begg and Mazumdar, 1994.
studies converging on the left side around the central ("true") value, and smaller studies symmetrically dispersed on the right side. However, the empty side at the bottom right corner of the graph suggests that smaller studies were more likely to be reported if they showed a positive effect, and a formal analysis revealed publication bias for CYP2D6 and lung cancer (Table 5-7), that was due to studies published before rather than after 1993.

It may be argued that such an initial report of false-positive results should be considered no major scientific problem, since subsequent studies, aimed to replicate the early positive results, will eventually establish the truth. However, this approach is inefficient and represents an important waste of resources, more so when it comes to expensive molecular epidemiological studies. A preferable approach is to critically evaluate and report results on the basis of criteria other than—or including but not limited to—statistical significance (Ioannidis, 2006). Biological plausibility, possible sources of bias and confounding, and numbers of tested associations are among such criteria. Statistical approaches have been proposed to take into account the possibility that significant results are generated by chance when many comparisons are made (Greenland, 1994). In addition, authors should be encouraged to systematically report their results, even those that are "negative" or "null."

CONCLUSIONS

Since the term molecular epidemiology was proposed in 1982 (Perera and Weinstein, 1982), molecular techniques have dominated biomarker research and have found an important and growing role in epidemiological studies. In several instances the application of a molecular approach has represented an important step beyond the evidence brought by traditional epidemiological methods. Assessment of exposure to aflatoxins, enhanced sensitivity and specificity of assessment of past viral infection, and detection of protein and DNA adducts in workers exposed to reactive chemicals such as ethylene oxide, are among the examples in which molecular epidemiology has contributed to the understanding of human cancer. In many other cases, however, initial promising results have not been confirmed by subsequent, usually methodologically sounder, investigations. They include in particular the search for low-penetrance genetic variants leading to modest increases of cancer susceptibility (Ioannidis et al, 2006b).

If biomarkers are to offer new opportunities to overcome some of the limitations of epidemiology, then their added value over traditional approaches should be systematically assessed. Biomarkers should be validated and consideration of sources of bias and confounding should be no less stringent than in other types of epidemiological studies. Similarly, other aspects of the study such as determination of required sample size, statistical analysis, and reporting and interpretation of results should be approached with the same rigor as in epidemiology in general.

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