

Application of biomarkers in cancer epidemiology

Workshop report*

In epidemiology, a biological marker (commonly abbreviated, for convenience, to biomarker) is any substance, structure or process that can be measured in the human body or its products and may influence or predict the incidence or outcome of disease. Biomarkers can be broadly classified into markers of exposure, effect and susceptibility. Biomarkers may include the following: xenobiotic agents and their metabolites in tissues or body products; normally occurring body constituents whether in physiological or pathological amounts; endogenous compounds that are not present under normal conditions; and inherited and acquired abnormalities of body chemistry, structure or function, including pathological manifestations of precursors to disease. Biomarkers should be distinguished from biomarker assays, specific laboratory tests aimed at measuring particular biomarkers, and biomarker measurements, the amounts of particular biomarkers present in specified units of tissues or body products as measured by biomarker assays.

Biomarkers have been used in epidemiological studies of cancer for many years. Early examples include the classic studies of B. MacMahon and colleagues on the geographical correlation of urinary estrogen concentrations with cancer of the breast (MacMahon *et al.*, 1974; Dickinson *et al.*, 1974), subsequent studies of the relationship between urinary and blood estrogens and breast cancer in case-control studies (Cole & McMahon, 1969; MacMahon *et al.*, 1982, 1983) and analyses of the relationship of cancer mortality to serum cholesterol concentrations in the Framingham and other cohort studies (McMichael *et al.*, 1984). Two recent, major contributions of biomarkers to epidemiological studies are the demonstration of the carcinogenicity of aflatoxins in combination with hepatitis B in humans through a cohort study of liver cancer, including measurements of urinary metabolites and nucleic acid adducts of aflatoxin (Ross *et al.*, 1992; Qian *et al.*, 1994), and the identification of the major role of human papillomaviruses (HPVs) in causing cancer of the cervix worldwide (Muñoz *et al.*, 1992; Bosch *et al.*, 1995). In spite of the quite extensive current use of biomarkers in cancer epidemiology, methodological aspects of their use have not been extensively elucidated or described. While there have been important contributions to this subject, including textbooks (Hulka *et al.*, 1990; Schulte & Perera, 1993) and comprehensive meeting reports (Mendelsohn

et al., 1995; Institute for Environment and Health, 1996) on the use of biomarkers in epidemiology, there remains a substantial need for relevant methodological research, wider discussion and understanding of the methodological issues, and their incorporation into the formal and informal training of cancer epidemiologists and other scientists pursuing careers in biological research on cancer.

The general objective of the use of biomarkers in cancer epidemiology is the same as that of cancer epidemiology itself: to gain knowledge about the distribution and determinants of disease occurrence and outcome that may be applied to reduce the frequency and impact of disease in human populations. There are, however, several specific objectives for the use of biomarkers in cancer epidemiology which should guide the evaluation of proposals to develop or apply biomarkers. Principally, they are to increase the accuracy of measurements of genetic or other acquired susceptibility to disease; of exposures that may cause or prevent disease; of exposures that confound or modify the associations between risk and other exposures; of disease itself; and of factors that may determine the outcome of disease, such as disease

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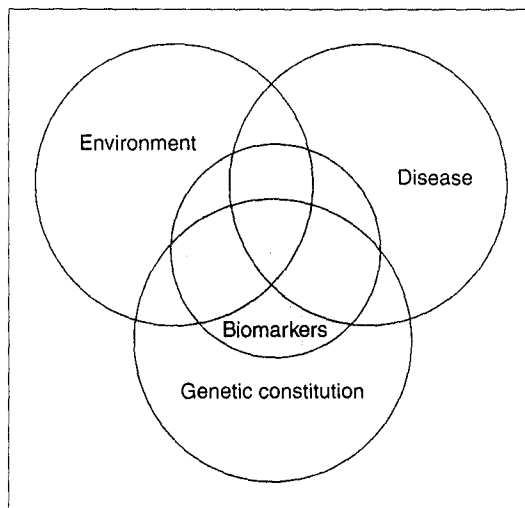


Figure 1. Schematic representation showing how biomarkers may be used to measure phenomena that reflect the amount or effects of agents that influence or predict disease incidence or outcome.

precursors and stages. Biomarkers may also be used to reduce, in the proposed study, the time interval between the relevant exposure and measurement of the putative effect and to increase the yield of information on disease pathogenesis. Ultimately, they should serve to increase the cost-effectiveness of epidemiological studies, in the sense that, as a result of their use, more information is gained per unit cost.

Because of their origin in the structure and function of the human body, biomarkers may provide more complex measures of the underlying exposure, process, etc. that they represent than other measures that are common in epidemiology. This complexity is illustrated in Fig. 1. The three outer circles, *genetic factors*, *environment* (broadly defined) and *disease*, represent the universe of phenomena that can be measured to reflect the amount or effects of exogenous or endogenous agents that may influence or predict the incidence or outcome of disease. The inner circle, *biomarkers*, represents the subset of these phenomena that can be measured in the human body. That almost all of the intersections between the three outer circles fall within the inner circle illustrates pictorially that biomarkers, more than any other epidemiological measure, may measure elements of any two, or all three, of genetic factors, environment

and disease. For example, the activity of an inducible, carcinogen-metabolizing enzyme is influenced by both exposure to inducing agents and the host genetic constitution; blood cholesterol concentration, an indirect indicator of fat nutrition, is almost certainly influenced by the presence of early cancer (International Collaborative Group, 1982; Sherwin *et al.*, 1987); presence of CC→TT mutations in the *p53* gene in normal skin, a plausible marker of lifetime exposure to UV radiation (Nakazawa *et al.*, 1994), is also influenced by DNA repair enzymes (genetic susceptibility) and may be influenced by the pathological consequences of *p53* gene mutation (e.g. a proliferative advantage for the mutated cells). The practical consequences of this complexity have not yet been elucidated.

In many cases, as measurements get closer to the biologically effective dose of an external agent at its target tissue, the relationship between the biomarker and disease will be less likely to reflect the relationship between the amount of *external* exposure to the agent and disease. However, exposures from multiple sources, such as nitrate, which may include exogenous pathways, may be better evaluated using biomarkers.

Because of the complexity of the interrelationship between biomarkers, their potential for increasing knowledge about cancer epidemiology and pathogenesis is now emerging. Research is needed to explore the methodological characteristics and biological functions of specific biomarkers and how they relate to one another. Most particularly, their application in epidemiological studies should be undertaken after thorough preparation and interdisciplinary collaboration. Even then, interpretation of results must be undertaken with appropriate caution.

Epidemiological study design and analysis

The use of biomarkers of exposure or dose, disease or individual susceptibility aims to contribute to the elucidation of the causal relationships in human populations between diseases and factors such as external exposures (via personal habits, occupation and the ambient environment), genetic traits and interventions.

The types of epidemiological studies used in biomarker research closely parallel the types used in other fields of epidemiology; biomarker studies,

however, raise specific methodological issues which are addressed in the following sections.

Transitional studies

Transitional studies provide a bridge between the use of biomarkers in laboratory experiments and their use in cancer epidemiology studies (Schulte & Perera, this volume). They have as their outcome the characterization of biomarkers and of the problems in their use; thus, they yield preliminary results rather than end results about cancer etiology and prevention. Their intent should be to lay the groundwork for the use of a marker in full-scale epidemiological studies by addressing the following aspects: intra- and intersubject variability; the feasibility of marker use in field conditions; potential confounding and effect-modifying factors for the marker; and mechanisms reflected by the biomarker. Transitional studies can be divided into three functional categories: developmental, characterization and applied studies.

Developmental studies involve determining the biological relevance, pharmacokinetics, reproducibility of measurement of the marker, and the optimal conditions for collecting, processing and storing biological specimens in which the marker is to be measured.

Characterization studies involve assessing inter-individual variation and the genetic and acquired factors that influence the variation of biomarkers in populations. This includes assessing the frequency or level of a marker in populations, identifying factors that are potential confounders or effect modifiers, and establishing the components of variance in the biomarker measurement, laboratory variability, intra-individual variation and interindividual variation. The ratio of intra-individual variation to interindividual variation has important implications for study size and power.

Applied transitional studies assess the relationship between a marker and the event that it marks, namely exposure, pre-clinical effects, disease or susceptibility. Applied studies are often of cross-sectional or short-term longitudinal design, and are not intended to establish or refute a causal relationship between a given exposure and disease. Rather, they are intended to assess whether relevant correlations exist and if they are strong enough to be useful in full epidemiological studies.

Like other studies using biomarkers, transitional studies raise ethical issues when the meaning of the biomarker results is not known. The objectives of the research generally are not to identify health risks but to identify characteristics of the biomarker or distribution by population subtypes. There is a need to anticipate the impact of transitional studies on study participants and plan to address their concerns (Hunter, this volume).

Cohort and case-control studies

Cohort and case-control studies represent the two most widely used types of observational study (Potter, this volume). They can be population-based or based on patients at a hospital, clinic or practice. For simplicity, studies based on patients will be referred to throughout as clinic-based studies.

In clinic-based cohort studies, of treated patients or screened populations, the inclusion of biological measures of exposure and susceptibility is both methodologically sound and logistically feasible. In population-based studies, collection of biological material for such markers is feasible but logistically more complex. For early outcome markers, collection of material (e.g. precancerous lesions) is logistically feasible in a hospital setting, but becomes more difficult in the population setting. End-points assessed routinely at a variety of institutes may produce major problems of standardization of methods (e.g. histological diagnosis). Except for some routinely registered biological characteristics (e.g. receptor status in breast cancer), even greater problems attend the issue of identification of biologically or pathologically defined disease subsets.

Most of the considerations that make prospective cohort studies an attractive study design apply to all methods of exposure assessment, including biomarkers. The strengths of such studies include the fact that exposure is measured before the outcome, that the source population that gave rise to the cases is explicitly defined, and that participation can be as high as 100% if specimens are available for all subjects and follow-up is complete. Weaknesses of prospective cohort studies involving biomarkers include the usually small number of cases of each of many types of cancer, the lack of specimen if the biomarker requires large amounts of specimen or unusual specimens, degradation of the biomarkers during long-term storage,

and the lack of detail on other potentially confounding or interacting exposures. Cohort studies are, in principle, the preferred method for studying the temporal sequence of intermediate end-points; however, the costs and complexity of repeated screening may often make this difficult.

Important issues in prospective cohort studies involving biomarkers, especially of exposure, involve the frequency and timing of specimen collections. A major concern in cohort studies of short duration (as in case-control studies) is the possibility that the disease process has influenced the biomarker level among cases diagnosed within 1-2 years of the specimen being collected. In studies of longer duration, there may be considerable misclassification of the aetiologically relevant exposures if specimens have been collected only at baseline. This misclassification occurs not only because an individual's exposure level may change systematically over time, but also because there may be considerable intra-individual variation (from day to day or even hour to hour) in biomarker levels. The effects of intra-individual variation can be reduced by taking multiple samples, but this greatly increases the expense of sample collection and storage and the burden on study subjects; similar considerations apply to taking samples at several points in time in an attempt to estimate time-integrated exposures or exposure change. An alternative approach is to estimate the extent of intra-individual variation, and the misclassification involved in taking single specimens, by taking multiple specimens in a sample of the cohort. This information can be used to correct for bias to the null introduced if the misclassification is non-differential, and therefore de-attenuate observed relative risks.

Use of biomarkers of disease susceptibility in cohort studies raises significant ethical issues, particularly if there is repeated contact with study subjects. In particular, informing the cohort members of their biomarker level is problematic if the biomarker is not considered to be sufficiently predictive of disease, and if there are no known preventive steps cohort members can take to reduce their risk of the disease.

A nested case-control study or a case-cohort design can be used to reduce the expenses of data collection and sample analysis. In a nested case-control study, biological samples may be col-

lected from all cohort members and stored at the beginning of the study; the controls are then selected throughout the course of the study, ideally at the time that each case is diagnosed. An advantage of the nested case-control approach is that biomarkers can be measured in specimens matched on storage duration and case-control sets can be analysed in the same laboratory batch, reducing the potential for bias introduced by sample degradation and laboratory drift. On the other hand, a case-cohort design may be used when sample collection from the entire cohort is not feasible, or when the cost of storage or analysis of each sample is prohibitive. This design involves collecting biological samples from the cohort at the beginning of follow-up and then collecting samples from cases as they occur. However, as samples are being taken at different times for cases and 'controls' (i.e. the reference sample from the entire cohort), bias will be introduced if sample degradation or laboratory drift occurs over time. Furthermore, the case-cohort approach may lead to laboratory personnel being unblinded to case and control status.

In case-control studies, biomarkers of internal dose or effective dose are appropriate when they are stable over a long period of time (e.g. carrier status for infectious agents, such as HBsAg) or when exposures have been constant over the relevant exposure period; however, it is essential that they are not affected by the disease process, diagnosis or treatment (except in those circumstances when it is possible to collect specimens prior to the commencement of treatment). For genetic susceptibility markers, case-control studies are highly appropriate. Clinic-based case-control studies are particularly suitable for studies of intermediate end-points, as these end-points can be systematically measured. Population surveys (or screening) are a prerequisite for the identification of cases in population-based case-control studies of intermediate end-points.

Clinic-based case-control studies are of particular value for studying the etiology of precancerous lesions (e.g. early cervical intraepithelial neoplasia). None the less, the relation between these intermediate disease markers and the cancers of which they are precursors is usually difficult to study for both ethical and logistical reasons. In case-control studies involving cancer as the end-

biomarkers of exposure (though not of any inherited genotype) are of limited value, as the disease may alter its therapy, or behaviour changes in patients, may alter any exposure marker or biological process thought to be a precursor of disease.

Case-case comparisons can be useful in the study of biologically defined disease subsets when differences in the etiology of such subsets are being examined. However, if a control group is not included, any heterogeneity in the exposure associations among disease subsets may be the result of an increased risk in one subset as opposed to a decreased risk in another.

Intervention studies

Intervention studies (trials) assess prospectively whether an intervention is efficacious in changing the frequency of a marker of exposure or dose (e.g. measures to encourage smoking cessation and whether serum cotinine or protein or DNA adducts) or pre-clinical condition or disease (e.g. *p53* mutation; dysplasia and cell proliferation in trials of antioxidants to prevent second tumours). Biomarkers can be particularly valuable in assessing compliance with the intervention (e.g. serum β -carotene in a randomized trial of β -carotene). Biomarkers of susceptibility (e.g. *GSTM1* polymorphism) could be used to determine whether groups in various intervention arms are comparable. Intervention studies may also be conducted as transitional studies when there are still questions about the response of biomarkers following an intervention. Critical in all types of intervention studies is the need to address those same issues that are important in cohort studies, including laboratory drift, loss to follow-up, and intervening exposures that may affect the biomarker.

Ecological studies

Ecological studies use groups rather than individuals as units of observation. They include studies of geographical differences and time trends in disease incidence and prevalence. While ecological studies provide useful information on exposure, disease and modifying factors, they are, in most cases, inadequate to establish causal relationships. Although, in general, ecological studies based on biomarkers have the same advantages and limitations as the other types of ecological studies, it is possible to conduct carefully planned ecological

studies, in particular in the control of confounding. Such studies often do not seek to make causal inferences about exposure-disease relationships; rather, they are aimed to elucidate plausible mechanisms (Kuopio *et al.*, 1995).

Cross-sectional studies

Cross-sectional study designs, with single or repeated sampling, have been frequently used in the validation of markers of exposure and of disease. They can provide a useful snapshot of the relationship between exposure and susceptibility factors, on the one hand, and the selected biomarker, on the other, in populations whose exposure can be well characterized. Frequently, the studies have involved industrial populations, chemotherapy patients and smokers—all groups exposed to 'model' carcinogens—and have compared them to appropriate non-exposed groups. Such studies can also be used in healthy individuals to establish the possible range of measurements of protective factors, e.g. monitoring of vegetable intake by measurement of carotenoids or phenolic compounds in plasma and urine. This design can be used to establish an association between the biomarker and the exposure under study.

Family-based studies

Family studies are intended to address scientific questions related to four general areas of research:

1. Does familial aggregation exist for a specific disease or characteristic?
2. Is the aggregation due to genetic factors or environmental factors, or both?
3. If a genetic component exists, how many genes are involved and what is their mode of inheritance?
4. What is the physical location of these genes and what is their function?

Using linkage methods, biomarkers that characterize known DNA haplotypes have been instrumental in locating a number of cancer-related genes. These studies require relatively large amounts of germline DNA (usually represented by DNA extracted from white blood cells). This is because there are multiple marker loci that require evaluation. Once a locus is identified, further DNA is necessary to identify the gene. Often, fibroblast or

Epstein-Barr virus (EBV)-transformed cell lines are required to provide adequate DNA for these studies.

Special concern must be paid to ethical and counselling issues in family studies in which biomarker results for an individual may also have predictive value for other members of the family.

General issues in study design and analysis

As with other epidemiological studies, the design and statistical analysis of a study involving biomarkers involves in general: (1) relating a particular disease (or marker of early effect); (2) to a particular exposure; while (3) minimizing bias; (4) controlling confounding; (5) assessing and minimizing random error; and (6) assessing interactions (Pearce & Boffetta, this volume).

Many studies using biomarkers of disease are of cross-sectional design and measure the prevalence of the disease state, which is dependent on both its incidence and its duration. Thus, in a study examining markers of cell damage as an effect of exposure to known or suspected carcinogens, the results would depend on factors such as the turnover of the cells in which the marker is measured and the capacity to repair the damage.

When deciding whether to measure exposure with biomarkers, it is important to consider how useful and informative the biomarkers are in relation to the study hypothesis. This is a particular issue for exposures that change over time; for example, a biomarker may be more valid than a questionnaire in assessing exposure to HPV, whereas a questionnaire will be more valid than a biomarker in assessing cigarette smoking 10 years previously. It should be stressed that the concept of 'useful and informative' depends on: (1) whether, in principle, it is most appropriate to study external exposure or internal dose (i.e. which hypothesis is being tested); (2) what information is available for the etiologically relevant time period; (3) whether the exposure or dose measurement is subject to intra-individual variation; and (4) whether it is desirable and possible to obtain (with a biomarker) information on interindividual variation in exposure or biological response.

When exposure or disease is measured with biomarkers, it is important to ensure that any misclassification is non-differential (i.e. it applies equally to the groups being compared) and is as small as possible. However, because the relation-

ships between exposure, marker and disease are, in most cases, obscure, the capacity to establish the presence or absence of misclassification in the interpretation of the findings of biomarker-based studies is limited.

The use of biomarkers of exposure does not reduce the need to control for confounding and, in some instances, the use of biomarkers may actually introduce confounding into a study (Pearce & Boffetta, this volume). Confounding can be controlled in the analysis by stratifying the data into subgroups according to the levels of the confounder(s), or by the use of mathematical modelling. However, problems of multicollinearity can occur when variables that are highly correlated (e.g. serum levels of various micronutrients) are entered simultaneously into a model; this will make the model unstable and can lead to invalid effect estimates.

Some biomarkers may be surrogates for intermediate stages in the disease process. For example, when the relationship between reproductive risk factors and cervical cancer is adjusted for HPV status, the relative risks for number of sexual partners are reduced. This suggests that HPV is a mediator of the relationship between the reproductive risk factor and cervical cancer, helping to substantiate the causal role for HPV. In cases such as this, the biomarkers should not be treated as potential confounders, i.e. they should not be adjusted for in the analysis. None the less, analyses alternatively including and excluding the putative intermediate can be used to help determine causal pathways.

When choosing the method of exposure or dose assessment, it is important to consider their implications on study size, particularly when an expensive or invasive method may be used. An additional consideration in study size estimation is the ratio of the number of assays per individual and the number of individuals in the study. Many biomarkers show marked variation from day to day within the same individual (in part from problems of repeatability of the laboratory tests, but also from genuine differences), and in some cases the intra-individual variation may be greater than the interindividual variation (e.g. 24-h urinary sodium measurements). Thus, it is important to take into account the trade-off between including more participants, on the one hand, and gathering multiple samples from each participant, on the other. It is essential that

the relative magnitude of intra-individual variation of a biomarker is adequately characterized.

Biomarkers present better opportunities for assessing interactions between genetic and environmental factors. In particular, to infer interaction, biomarkers of genetic susceptibility should show a higher disease risk in exposed susceptible groups than in exposed non-susceptible and in non-exposed groups. None the less, such testing for interaction usually requires a substantial increase in study size.

As is the case for other aspects of epidemiological research, if the relative risk between a biomarker and exposure or outcome is not very strong (e.g. less than 10), then results from multiple studies will be required before agreement on the existence of an association can be reached. It is critical, therefore, that the results from all existing studies are available. Unfortunately, the tendency for investigators and journals to publish only 'positive' results may bias the literature in favor of positive studies, a phenomenon often called 'report bias' or 'publication bias'. Biomarker research may be even more prone to this bias than other fields of epidemiological research, as multiple biomarkers may be assessed, sometimes at relatively low cost. It is important that investigators publish, or make available in abstract form, all their results from studies in which the results are reliable, even if these results are 'null'. Publication bias can be particularly relevant when several analyses of the data are done on subgroups of the study population, defined according to characteristics such as tumour markers or susceptibility gene polymorphisms. Such multiple analyses increase the probability to obtain significant results by chance only. Although these analyses may provide useful information on effect modifiers, the selective reporting of significant or 'positive' results should be discouraged.

Incorporation of biomarkers into epidemiological studies

Biological markers undergo a process of discovery, characterization and refinement before use in epidemiological studies. Animal studies contribute to the development and validation of biomarkers and provide insights into the mechanisms of the multistage process of carcinogenesis. Transitional studies assist in this process by optimizing sample

processing, evaluating intra-individual and inter-individual variation in the biomarker in target populations, determining laboratory assay variation, and studying the impact of exogenous and endogenous influences on the biomarker.

Regardless of the specific protocol used for the collection, processing and analysis of a specific biomarker, two important issues to consider are the storage of biological samples and the evaluation of sources of variation in biomarker measurement. This section will therefore review storage and variation issues following the discussion of individual biomarkers.

The following biomarkers have been selected to demonstrate the wide range of assays available for incorporation into epidemiological studies, and to comment on their utility for various study designs relevant to the study of cancer. This is not meant to be a comprehensive list; other important categories of biomarkers in blood (e.g. immunological markers) and in normal and preneoplastic tissue are not addressed here.

Biomarkers of biological agents

Biological agents associated with chronic infection and subsequent development of cancer are measured using serological or nucleic acid markers (Muñoz & Bosch, this volume). An example of nucleic acid-based biomarker is HPV DNA detection where the presence of type-specific DNA at a given time is measured by PCR-based assays. Cohort studies having high-grade squamous intraepithelial lesions as the end-point have shown that the infection preceded the disease. HPV DNA infections are often transient, especially in young women. Therefore, repeated sampling is required to assess persistent HPV infections. Accurate serological assays aimed at distinguishing transient from persistent infections need to be developed. Another example is the hepatitis B virus (HBV), for which there are serological markers that distinguish between past and persistent infections. HBV DNA detection in sera further refines the assessment of exposure. These markers have been used in all types of epidemiological design (cross-sectional, case-control, cohort and intervention studies).

Biomarkers of internal dose for chemical exposures

Biomarkers of internal dose of external chemical exposures are measurements of a parent compound

or its metabolite(s) in an accessible biological matrix, such as serum or urine (Institute for Environment and Health, 1996). They have potential applications in several types of epidemiological studies (Coggon & Friesen, this volume). These include studies to establish the importance of different sources of exposure as determinants of total dose; studies to validate other methods of exposure assessment such as the use of questionnaires; studies to establish that a chemical reaches a suspected target tissue; cross-sectional studies relating exposure/dose to biomarkers further downstream in the exposure-disease continuum; and case-control and cohort studies relating exposure/dose to disease.

The utility of an internal dose marker in case-control and prospective cohort studies depends, in part, upon the half-life of the external agent or its metabolites in the body; the pattern of the exposure it is measuring (e.g. regular, daily exposure versus infrequent, episodic exposure); whether secular trends have occurred in that exposure (e.g. smoking cessation); and direct or indirect influences of the disease process. The information that a biomarker of internal dose provides must be compared to the availability and quality of other sources of data (e.g. questionnaires, environmental measurements, medical records). Essentially, all exposure measures misclassify some subjects on their usual pattern of exposure; it is the relative ability of different sources of data to place individuals correctly into exposure categories that is important. In case-control studies, questionnaires remain the primary source of exposure data. There are, however, a few instances where a measure of internal dose may be a more suitable measure of cumulative exposure, particularly in the study of cases with preneoplastic lesions or early disease. The ideal biomarker should persist over time (e.g. fat-soluble substances such as DDT metabolites) and should not be affected by disease status. Markers of internal dose may be useful in prospective cohort studies as long as components of variance of the biomarker are well characterized, since the problem of reverse causality (i.e. disease status affects the level of the biomarker) is minimized.

Biomarkers of dietary intake and nutritional status

Dietary intake is usually assessed by various types of questionnaire and other methods (e.g. diaries); however, there are many sources of error involved

in using this approach. Biomarkers can be useful in nutritional epidemiological studies at two different levels (Kaaks *et al.*, this volume). First, biomarkers may be of interest as potentially more precise, more specific or more objective measurements (or correlates) of the intake levels of specific foods or food constituents, compared to measurements obtained with questionnaire and interview methods. Secondly, most biomarkers can also be seen as indicators of a nutritional/metabolic status, which may be intermediate between 'exogenous' nutritional lifestyle factors (e.g. the composition of diet, physical activity) and disease risk. When used as an indicator of intake, they are useful in four different types of study: validation (comparison of a measurement against a gold standard), calibration (comparison of two measurements), observational epidemiological and intervention studies. They are especially important in validation or calibration studies where biomarkers represent an additional category of measurement, the 'random' errors (i.e. variations that are independent of individuals' true habitual intake levels) of which can be assumed to be statistically independent of those of the questionnaire and other methods. The factors that are important in deciding whether to use a given biomarker in a particular study include whether it is a good indicator of intake; whether it is a long- or short-term marker; whether there is need for multiple measurements; whether it is acceptable to the researcher and the subject; and whether it is compatible with the design of the study itself (case-control of early disease, case-control of late disease, or a cohort study). Biomarkers can also be informative if they provide an integrated biological measure of intake, lifestyle and metabolic processes. For example, measurements of red cell folate coupled with serum homocysteine would offer a picture of medium-term intake of folic acid, which is distinguishable from transient fluctuations in dietary intake (Green & Jacobsen, 1995).

Biomarkers for endogenous hormones

Biomarkers are available to measure specific endogenous hormones (Lemaster & Schulte, 1993; Hulka *et al.*, 1994). Cross-sectional studies, where the biomarker is the outcome measure, are important for the assessment of differences in marker levels between subjects with different characteristics (e.g. sex, race, anthropometry, geographical

location). A case-control study is not an appropriate study design to assess associations between metabolic and hormone markers and disease outcome, since marker sampling occurs after the clinical appearance of disease. This problem may be less significant or insignificant if the outcome is a pre-clinical condition or a very early lesion. Cohort studies, including case-control studies nested within cohort studies, are ideally suited for evaluating the association between hormone biomarkers and disease. Sampling issues important in cohort studies are the frequency of sampling and the timing of collection in relation to events which may influence measurement (e.g. stage of menstrual cycle, menopause, oophorectomy, ageing, medications).

Macromolecular adducts as biomarkers of exposure to reactive chemicals

Chemicals can bind covalently to cellular macromolecules such as nucleic acids and proteins (Wild & Pisani, this volume). The product of this addition of a chemical moiety to a macromolecule is termed an 'adduct'. The adduct may be highly specific for the carcinogen of interest, but not necessarily specific for a given exposure because of multiple sources of the carcinogen within the environment. Adduct formation normally occurs after the metabolic activation of the carcinogen; DNA repair may follow adduct formation. As a result, measured adducts represent an integration over time of carcinogen exposure and interindividual variations in carcinogen metabolism, DNA repair and other host factors. The persistence of adducts is determined by the chemical stability of the adduct itself and the turnover of the macromolecule to which the chemical is bound. In practice, this gives a half-life of adducts on proteins (haemoglobin and albumin) of a few weeks to months, while DNA adducts may have half-lives of a few hours to several years depending on the cell type concerned. Adducts of more remote exposure (such as modified amino acids in histone proteins from non-dividing cells) would represent a major advance in the utilization of these markers in epidemiological studies. Adduct measurements can be made in blood and exfoliated cells, and metabolites of adducts can be measured in urine. The quantity of material required is dependent on the assay sensitivity for a given adduct.

Cross-sectional studies evaluate exposure-adduct relationships in populations currently exposed to agents of concern. Elevated adduct levels in an 'exposed' versus 'unexposed' population may suggest that the exposure is associated with a higher cancer risk, given the increasing evidence that DNA damage represents a primary mechanism of carcinogenesis. In this instance, the adduct is not being used as a dosimeter, but rather as evidence of a potentially harmful response *in vivo*. Such studies should be regarded as providing supporting evidence only, until the association of adducts with subsequent development of cancer has been demonstrated. It is relevant to note, however, that in the presence of limited epidemiological evidence of a chemical's carcinogenicity, the demonstration in humans that the chemical causes a dose-dependent increase in macromolecular adducts or in other biomarkers that reflect genotoxic damage provides supporting evidence that the chemical is carcinogenic to humans. This type of evidence has been used, for example, in the recent IARC evaluation of carcinogenicity of ethylene oxide within the Monographs programme (IARC, 1994).

The utility of using adducts as markers of biologically effective dose is limited in case-control studies due to the relatively short half-life of most adducts evaluated to date. They may have utility in prospective cohort studies, again with the caveats previously described for all exposure markers.

Biomarkers of somatic cell mutations

Somatic mutations provide evidence of irreversible genetic damage (Albertini & Hayes, this volume). Furthermore, specific mutations (mutation spectra) may, in principle, identify exposures to specific agents or mechanisms; however, measurements of mutations are usually less sensitive in this regard than are other biomarkers of exposure (e.g. metabolites, adducts). Somatic mutations should have their greatest utility in epidemiological studies when it is possible to establish a qualitative association between exposure and specific mutations, thus identifying the agents of concern. They also have promise as surrogate markers of outcome. It must be emphasized, however, that for this last purpose, mutations must initially be assumed to be associated with increased risk of cancer. In-vivo mutational response may also be used

to define interindividual differences in sensitivity to mutagens or carcinogens.

Somatic mutations have potential application in case-control studies when the exposures of concern are chronic and stable, and the disease does not alter mutagenic responses in the test cells. Their greatest utility, however, may be in studies that use mutations as an intermediate end-point to evaluate genetic responses under various exposure conditions and in studies concerned with prevention. There is a need in the future for rapid, inexpensive and sensitive molecular assays that may increase the range of genes and tissues amenable for study; this, in turn, should facilitate the evaluation and potential use of those biomarkers as surrogates of disease-relevant genotoxic responses.

Biomarkers of cytogenetic damage

Chromosomal aberrations are commonly found in tumour cells and, in some cases, there is very good evidence that they play a causative role in carcinogenesis (Tucker *et al.*, this volume). Two prospective cohort studies have shown that chromosomal aberrations measured in peripheral lymphocytes were associated with an increased risk of cancer (Hagmar *et al.*, 1994; Bonassi *et al.*, 1995). Non-target cells may be appropriate tissue for this marker. Translocations, in particular, are useful for quantifying certain types of acute and chronic exposure, as well as exposure that occurred many years previously. Translocations are therefore a reasonable and appropriate biomarker for use in cross-sectional studies because they provide a direct and quantifiable indication of DNA damage. In case-control studies, the analysis of stable translocations in early, local disease may provide an insight into accumulated cytogenetic damage and its link with cancer. It is possible, however, that the frequency of translocations is affected by advanced disease, but this remains to be established. The use of translocations in prospective cohort studies could have substantial utility but is limited by the logistic constraints of culturing or cryopreserving lymphocytes from a large number of subjects.

The analysis of translocations by fluorescent in-situ hybridization (FISH) with probes that paint whole chromosomes represents an important advance in cytogenetics; FISH is a rapid, sensitive and highly reproducible technique which relies on the detection of colour 'junctions' between

painted and unpainted chromosomes. Improvements in the techniques of molecular cytogenetics continue to be made, and new DNA probes are being developed on a regular basis. Cytogenetic assays have been performed on metaphase cells, but the more recent applications of FISH to interphase cells means that cell culture is no longer required and that relevant target tissues can be examined directly for chromosome rearrangements. The analysis of interphase cells appears to have significant promise for use in future epidemiological studies and may make the banking of the relevant tissues from a large numbers of subjects feasible.

Biomarkers of genetic susceptibility

Biomarkers have increasingly played a role in studies that evaluate the role of genes in cancer (Caporaso & Goldstein, this volume). Family-based studies have led to the mapping of several cancer-related genes characterized by high penetrance, high absolute and relative risk, but low attributable fraction for the most common cancers. The environment plays a variable but arguably small role in the manifestation of these genes. Other genes (e.g. metabolic polymorphism genes that lie in the metabolic pathways of carcinogens and therefore have a mechanistically plausible role) are characterized by modest relative risks, low absolute risks and high attributable risks. The environment is crucial in determining the effects of these genes. A major contrast between these two is the low prevalence of the former and the high prevalence of the latter.

For a given genotype, the phenotype is determined by the penetrance. The use of phenotype or genotype in a specific study depends on numerous factors. In general, the methodology of genotyping has increased because of rapidly improving technology. Recent advances in the analysis of genetic polymorphisms using DNA present in serum, in formalin-fixed, paraffin-embedded pathology samples and in material collected non-invasively by buccal swabs and oral rinses will provide new opportunities for incorporating biomarkers of genetic susceptibility into epidemiological studies.

Tumour biomarkers

Tumour markers include characteristics of tumours at the anatomical, histological, serum, chromosomal

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and molecular levels (Zhang *et al.*, this volume). Using tumour markers in the assessment of etiological heterogeneity represents a progression from these previous studies and may have greater specificity for particular patterns of exposure. Tumour markers can readily be incorporated into case series and case-control studies, as well as into prospective studies that study the relationship between biomarkers in an initial tumour and the risk of developing second primary tumours or the relationship between precursor lesions and subsequent malignancies. There is a rapidly increasing ability to evaluate a range of tumour markers in formalin-fixed, paraffin-embedded tissue. Frozen tissues are easier to assay, but more difficult and costly to obtain and store for epidemiological purposes.

Evaluating sources of variation in biomarkers

Before embarking on an epidemiological study that uses a biomarker, it is important to understand the potential measurement error in the biomarker (Gompertz, this volume; Vineis, this volume; White, this volume). The researcher's first concern should be to prevent or rule out, as far as is practicable, differential measurement error. Because differential measurement error can bias the odds ratio in either direction, its presence to any appreciable degree in a biomarker will invalidate its use in an epidemiological study. Differential measurement error is a particular concern in case-control studies and among the early cases in cohort studies when the marker may be influenced by pre-clinical disease, by the effects of the disease after diagnosis or by treatment.

When differential measurement error is not likely to be present, the researchers should focus on assessment of the non-differential error, or at least some of the major components of error in the biomarker. Error components include laboratory variation, variation from specimen collection and storage, and biological variation. The latter may be short-term (e.g. day to day), medium-term (e.g. seasonal), or long-term (e.g. variation over an etiological period of years). Ideally, one would conduct a validity study in which the biomarker to be used was compared to a perfect (true) measure. However, much of the total error can be measured in a well-designed reliability study which requires the collection and analysis of two (or more) specimens from a group of subjects in a

way that the error in one measure is not repeated in another, e.g. the two specimens are collected at different times over the relevant etiological time period, and handled, stored and analysed with the variation in specimen collectors, laboratory technicians or batches that would occur in the parent epidemiological study.

Similarly, reliability studies could be designed to partition the specific components of error: the effects of handling and storage; laboratory variation; and short-, medium- and long-term biological variation. In any case, researchers should not assume that small laboratory error implies that a measure is good, because these other sources of error introduced by the design and needs of the epidemiological study can be far greater than the laboratory measurement component of error.

Storage of biological samples

The use of biological markers in epidemiological studies often requires storage of the relevant biological samples for a period of time, which may vary from a few weeks up to years or even decades, depending on the study design (i.e. cross-sectional, prospective) and the timing of the laboratory analysis (Landi & Caporaso, 1997, this volume). Factors that can be considered in the choice of storage method include the type of biological material; the type of laboratory analysis planned; the duration of storage and spread over time of laboratory analysis; and the logistical and practical conditions. For long-term storage of a large collection of blood samples, the safest and, in most cases, the most efficient method of storage is at liquid nitrogen temperature.

Storage temperature should be as low as possible, depending on the biological material, and should ensure stability of all potential analyses over long periods of time. For very long-term storage, it is worthwhile to store pools of biological specimens containing known concentrations of the analytes of interest in order to be able to monitor possible degradation over time. A stable isotope standard may be useful in such cases.

Application of biomarkers

Biomarkers may be used in epidemiological studies to increase the information obtained from classical study designs and to expand the areas of scientific inquiry to which epidemiology can contribute. Use of

biomarkers in epidemiological research has grown significantly in recent years and can be expected to increase rapidly in the years ahead. The areas of application include research on etiology of disease; correlation between external exposure and internal dose; susceptibility and gene-environment interaction; clinical trials; disease mechanisms and pathogenesis; and cancer prevention.

Etiological epidemiological research

In etiologic research, biomarkers of exposure represent the independent variable and are used to predict disease occurrence. Research of this type has been limited in the past because of the need for transitional studies to develop, characterize and evaluate biomarkers in their application. When biomarkers are tested as outcome variables they are useful in often being much more prevalent in the population than cancer itself and they occur much closer in time to the exposure than does cancer; exposure will therefore be measurable more accurately by whatever method is used. The majority of epidemiological biomarker research reported to date has been from transitional studies. As this body of literature has accumulated, it is now becoming possible to use selected biomarkers in the context of classical etiologic research.

At current levels of development of biomarkers, there will still be many circumstances in which questionnaire methods of assessing exposure are complementary or preferable to technically sophisticated biomarkers of the same exposure. Assays of urinary levels of metabolites of tobacco components such as nicotine, for example, reflect only short-term consumption. Such assays tell us about smoking habits in the relatively short time before measurement; questionnaires are still preferable to determine long-term exposure.

Etiological studies using biomarkers generally employ case-control or cohort epidemiological designs. Modifications of these are the case-case design and the applied transitional study. The case-control study nested within a cohort offers distinctive opportunities by using blood or tissue collected in the past.

The use of biomarkers in case-control studies may necessitate greater use clinic-based (or hospital-based) studies (Potter, this volume). Clinic-based studies of biomarkers may be more feasible, practical and economical. They also have a higher

response rate with respect to collection of biological samples than community-based studies, and differential participation by cases and controls is less of a problem. For case-control studies of precursor lesions, no population-based registries exist from which to identify cases, whereas such cases can be identified in the health care setting and, given appropriate survey conditions, in random samples of the population. In cohort studies, where the biomarker is the exposure of interest, unexposed subjects, defined as people not carrying the biomarker, may be identified through the health care setting. Tissue specimens may be available only through pathology departments associated with hospitals. Opportunities to obtain specimens may be enhanced if health care providers are co-investigators in the research. The method for selecting study subjects is an important potential source of bias in clinic-based studies. In studies based on biomarkers, however, it may be less important than in other types of epidemiological studies, because the characteristics of the subject measured by the biomarker (e.g. genetic polymorphism status) may not be linked to the probability of inclusion in the study. Similarly, it is unlikely that markers of exposure and effect are affected by referral and detection, since the individual's biomarker status prior to the study is not known. In most cases, however, it is unknown how subjects are selected with respect to the biomarker, and the possibility of selection bias should always be considered.

Exposure-internal dose and exposure-biological response correlations

Transitional studies of exposure frequently correlate external exposure measured in a traditional fashion (e.g. interview or ambient monitoring) with internal dose measured in body fluids or tissues. These studies are generally cross-sectional or short-duration longitudinal in design. The exogenous measure is considered the independent variable, and the internal dose the dependent variable. Rather than an internal dose measure, the dependent variable can be a biological response marker, e.g. a *p53* signature mutation associated with aflatoxin exposure (Links *et al.*, 1995). Such transitional studies frequently focus on heavily exposed (and unexposed) persons in order to minimize sample size and maximize the opportunity

of biological studies, and controls is good precision, future studies may be able to employ the more easily obtained and inexpensive exposure measurement.

Susceptibility and gene-environment interaction
 Studies of susceptibility and gene-environment interaction are emerging as an important component of epidemiological research (Garte *et al.*, this volume). This represents a significant development in epidemiology; it emphasizes the importance of individual differences and the combined influence of genes and environment in determining disease risk. Although relevant genes lie in a continuum with respect to prevalence and expressivity, a distinction should be made between single gene mutations of large effect and polymorphisms of small effect. *BRCA1* exemplifies the former, and metabolizing enzymes for substrates that form carcinogenic metabolites illustrate the latter. Intermediate situations exist in individuals heterozygous for some genes, such as ataxia telangiectasia, which may affect a large proportion of the population, and for which there is some evidence of increased risk of various cancers, including breast cancer (Easton, 1994). Because estimates of cancer risk in relation to single gene mutations have been derived from high-risk families, it is not known what the cancer risk is for gene carriers within the general population. There is a need for epidemiological studies of cancer in the general population to study risk in single gene carriers who are not members of high-risk families.

Several methodological issues have arisen in the context of these studies. Lack of concordance between genotype and phenotype has been reported and it is not easy to discern which is more informative with respect to disease effects. However, some advantages of studies of susceptibility and gene-environment interactions are the potential for case-case studies (Begg & Zhang, 1994) and for studying 'exposed' subjects only.

Khoury *et al.* (1988) has described six models for gene-environment interaction. These concepts may be simplified by considering the possibility of an additive effect and effect modification for both the susceptibility marker and the exposure. For some cases, the susceptibility marker can behave as an

'exposure', increasing risk of disease in the entire study population. Usually, however, susceptibility is manifested as an effect modifier of exposure. If the population is stratified into two groups, based on presence or absence of the susceptibility gene, the effect of the exposure variable may be more evident in the susceptible group. It may also be useful to stratify on levels of the exposure variable, e.g. smoking or age groups, to identify qualitative and quantitative aspects of the association between the susceptibility marker and disease across levels of the exposure.

Cancer prevention

In cancer epidemiology, biomarkers can be used to offer quantitative insights about biological events occurring at different stages of the pathologic process (McMichael & Hall, this volume). The improved knowledge of the natural history of cancer should have application to research aiming at cancer prevention and control. At least three aspects of cancer prevention or cancer prevention research may make use of biological markers. They are screening, community-based intervention trials and the monitoring of biomarkers as risk factors for disease. Screening may concentrate on the identification of precursor lesions or early stage disease (e.g. cervical cytology), high-risk individuals (e.g. prostate-specific antigen, PSA) or susceptibility markers (e.g. *BRCA1*). Cervical cytology is well established as an effective tool to reduce mortality from invasive cervical cancer. PSA is controversial because it is not specific to invasive prostatic cancer or its precursors. It cannot distinguish between precursor lesions that will invade and the majority that will not, resulting in a significant amount of over-treatment and morbidity. Furthermore, and crucially, there is no evidence that screening reduces mortality from prostatic cancer. Screening for *BRCA1* became possible only recently when the gene was cloned and sequenced (Futreal *et al.*, 1994; Miki *et al.*, 1994). Currently, testing for *BRCA1* has been suggested only for women in high-risk families in whom the gene is known to segregate, or for women with multiple first-degree relatives who have had an early age at diagnosis of breast cancer. Even in these situations, screening is not universally accepted because of the limited risk and disease management options, which markedly reduce the utility of the genetic information. Even

in the cases of high penetrance cancer genes, however, there is the possibility to contribute to cancer prevention by modifying the environmental factors that interact in the carcinogenic process.

Community-based intervention trials for risk reduction may benefit from biological markers. For example, smoking cessation trials have found that urinary cotinine levels may be a better indicator of smoking status, when measuring the trial outcome, than reports on interview or questionnaire.

Monitoring biomarkers of exposure to carcinogenic agents in populations may reveal trends and assist in risk assessment in high-risk groups.

Clinical prevention trials

The use of biomarkers in chemopreventive trials raises important methodological issues. The testing of chemopreventive agents in clinical trials using the end-point of cancer incidence requires a study period of many years, very large sample sizes and great expense. Therefore, short-term, smaller clinical trials that use surrogate end-point biomarkers (SEB) have had to be developed. A SEB may be defined as an early change during the intraepithelial, pre-invasive phase of neoplastic progression, at the molecular, cellular or tissue level, whose response to a chemopreventive agent predicts the effectiveness that the agent would have in a large clinical trial using the end-point of cancer incidence reduction (Boone & Kelloff, this volume). Examples of SEBs include computer-assisted quantitative image analysis of pathological specimens, which measures nuclear features (altered size, shape, and chromatin texture), and cytological features by binding to chromagen-antibody conjugates. Markers and indices of proliferation (e.g. Ki-67, PCNA), oncogene mutation or amplification, and allelic loss and other alterations may also be useful SEBs.

Studies are needed, however, to evaluate the validity of SEBs. To be suitable for use as an SEB, there should be evidence that the marker is a necessary step on the pathway to cancer, or at least very highly correlated with cancer occurrence, i.e. the attributable fraction for the marker in relation to cancer must approach 1. This evidence may be obtained primarily from observational epidemiological studies and clinical trials that incorporate the marker and have explicit cancer end-points.

Certain laboratory and pathology studies may also yield pertinent evidence, as in the case of the colon adenoma to carcinoma sequence. With markers having attributable fractions substantially less than 1, there may exist alternative pathways to cancer that bypass the marker in question. An exposure or intervention may operate through the alternative pathway(s) in a way that offsets the cancer effect mediated by the original marker. For such markers, inferences to cancer are problematic (Schatzkin *et al.*, 1990, and this volume).

Although there are well-recognized difficulties in extrapolation of carcinogenesis and chemoprevention data from animal experiments to humans, animal models provide opportunities for biomarker research. Linkages between mechanistic pathways in different species may ultimately be useful in resolving problems in inter-species extrapolation.

Ethical aspects of biomarkers in cancer epidemiology

The use of biological markers presents potential ethical issues because biomarkers are obtained from an individual's unique tissues and can be used to provide important data about exposures, biological effects and susceptibility to cancer (Schulte *et al.*, this volume). The ethical issues arise from the possibility of abuse or misuse of biomarker data and failure to respect the rights of persons participating in research. Biomarker data can be misused by failing to keep data confidential and by using it, or allowing it to be used, to stigmatize research subjects. Research subjects have a right to privacy as well as a right to be told of risks of participating in biomarker studies and of any clinically important findings. There remains a range of opinion whether banked specimens collected for one study or purpose may be used for another. Generally, the degree of risk in participation and the extent to which the results are clinically important should be considered when using banked specimens and determining whether subjects need to be notified.

Recommendations for future studies of cancer epidemiology involving biomarkers

The Workshop considered the main issues regarding the use of biomarkers in future studies in cancer epidemiology, and agreed a set of recommendations. These are presented in Box 1.

Box 1. Recommendations for future studies

Biomarkers should be chosen because of their biological relevance to the question and on the basis of the science, not the technology.

Biomarkers should be evaluated by transitional studies before use in full-scale studies. Regulatory and funding agencies should become aware of the importance of transitional studies for the conduct of epidemiological studies based on biomarkers.

In addition to etiological research, appropriately validated biomarkers should be used in prevention: in screening, biomarkers of neoplasia or susceptibility, in clinical trials (biomarkers of dietary modification and compliance, biomarkers of surrogate end-points), and in monitoring exposure to carcinogenic agents (e.g. trends in biomarker levels in general populations and subgroups, exposure status and risk assessment in high-risk populations, and exposure-susceptibility interactions).

Epidemiological studies based on biomarkers should meet the requirements of good epidemiological research design, including identification and minimization of potential sources of bias and confounding. Research should be conducted on methodological aspects to clarify advantages and disadvantages of different options in the design of biomarker-based studies, in particular to determine whether, in the community (or hospital-based) setting, studies of biomarkers of exposure or susceptibility are less subject to selection bias than traditional studies of behavioural and environmental risk factors of disease.

Studies should be designed with appropriate statistical methods to minimize the problem of random error and maximize the capacity to establish the presence and strength of associations, particularly between markers of susceptibility and exposure.

It is important to define the components of variance in order to establish the capacity of the biomarker to measure, and, where possible, the relevant exposure, process and outcome. For continuous measurements, it is highly desirable to estimate the amount of random error due to within-person variability in the measurement in a subset of the study population. If this cannot be estimated directly in the

study population, a similar population should be used because the ratio of within-person to between-person variance may vary substantially between populations due to differences in the range of exposure, or fluctuations in exposure.

7. There should be strong and open communication between laboratory scientists and epidemiologists with regard to identification, investigation and control of specific sources of measurement error in the biomarker assay. Such issues would include the need for replicate measurements and standardized non-variable experimental protocols during the study; internal controls; operator blindness; monitoring experimental drift; sample handling; and development of study design by both epidemiologists and laboratory scientists to deal with these issues.

8. Epidemiologists should work closely with laboratory investigators when developing protocols for the collection, processing and storage of biological samples to ensure that the samples can be analysed for the main biomarkers of interest and that the requisite quality control procedures are implemented.

9. Standardized procedures should be developed for the collection, handling, processing and storage of biological samples. A biospecimen documentation sheet should be generated for each biological sample collected, documenting critical variables that may affect biomarkers.

10. Storage of blood samples in multiple aliquots, and ideally in separate freezers, is advisable whenever long-term storage and multiple measurements spread over long time periods are planned. The samples should be stored at the lowest feasible temperature. Assessment of sample stability would be facilitated by storage of aliquots of a pool prepared specially for this purpose.

11. Samples should be collected not only for the main biomarkers of interest in a particular study; rather, they should be processed and stored in a way that allows a wide range of biomarkers to be tested in the future. In particular, consideration should be given to collecting a source of genomic DNA in epidemiological studies to allow the evaluation of genetic markers. While this is optimally obtained from peripheral blood samples, various non-invasive methods for

Box 1. (Contd) Recommendations for future studies

collecting genomic DNA are available and continue to be developed, including the use of buccal swabs or oral rinses to collect exfoliated epithelial cells.

12. Where feasible, tumour pathology samples should be collected for analysis of tumour characteristics in addition to histological confirmation. This generally requires collecting more material than is usually obtained for histological evaluation alone. If tumour blocks are available, collection of multiple slides from formalin-fixed, paraffin-embedded blocks is desirable. Availability of frozen fresh tumour and normal tissue should be encouraged when relevant and feasible.

13. In studies of the natural history of malignant disease and its prevention, it is recommended that molecular and protein alterations be studied in normal tissues, benign lesions, premalignant precursor lesions and the surrounding unaffected tissue. This evaluation should also be extended to non-target tissues and to the other organ, in the case of paired organs. Assays that can be applied to paraffin-embedded, formalin-fixed tissues are of particular importance to epidemiological research.

14. Special emphasis should be given to improving correlation between animal studies and human in-vitro studies designed to further develop, refine and allow for better bio-

logical interpretations of results obtained from epidemiological studies using biomarkers. An iterative process is envisaged in which epidemiological and laboratory components collaborate in efforts to define pathogenesis.

15. Care should be taken to consider the possible ethical issues when using biological markers. Biological marker data should be kept confidential and not released in ways that will allow for identification of participants in research.

16. When using banked specimens, the need for informed consent and reporting back results to subjects should be based on the degree of clinical risk associated with the biomarker.

17. Investigators should be encouraged to publish, or make publicly available, data involving biomarkers even if no associations are observed. This applies in particular to transitional studies.

18. More opportunities for joint training in both laboratory and epidemiological methods should be developed. Encouraging effective collaboration between laboratory-based scientists and epidemiologists and the creation of interdisciplinary programmes would foster research in this area.

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