Biologic and Methodologic Issues for Nutritional Biomarkers

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ABSTRACT Nutritional biomarkers are used for a variety of purposes in large-scale population surveys and epidemiologic studies as well as smaller clinical studies. The main reasons for using nutritional biomarkers are to provide measures of nutritional status that have less error than dietary data, nutrient status for nutrients with inadequate dietary data, to obtain a more proximal and integrated assessment of nutrient status that incorporates metabolism, to assess dietary change and compliance in intervention studies, and dietary intake for the validation of dietary questionnaires. However, often there is oversight by the investigators regarding biologic and laboratory issues, which have implications for the utility of nutritional biomarkers. This article reviews some of the physiologic issues that contribute to between-person variability in nutrient status and the utility and meaning of specimens from various body compartments. Issues related to the collection and storage of biologic specimens are addressed, although it is recommended that investigators contact laboratory colleagues at the beginning of any study for updated information. The necessity for blind quality surveillance of laboratory analyses beyond the normal procedures employed by collaborating laboratories also is addressed. The advantages and disadvantages of nutritional biomarkers are reviewed, especially in comparison with using dietary methodology. J. Nutr. 133: 875S–880S, 2003.

KEY WORDS: • biomarkers • diet assessment • epidemiology • nutrition • methodology

There are three main reasons for using nutritional markers. First, biochemical markers of nutrient status can have less error than dietary assessment of nutrient status (1). In addition to issues related to measurement errors in dietary assessment (i.e., errors in completion of the instrument and food-composition tables), there are additional issues such as the combination of foods eaten together and the extent of cooking of foods that influence nutrient content and absorption. These issues add error to our estimates of nutritional status that are obtained from dietary instruments. Second, for some nutrients, dietary data are inadequate because of limitations in food-composition data, whereas biomarkers of nutritional status related to these nutrients are available. For example, the selenium content of cereals and grains is determined by the soil content of selenium where the grain was grown (2). Because of food distribution and processing strategies, it is not possible to produce an accurate estimate of selenium content for dietary data. In one study (3), the toenail selenium content did not correlate with the dietary intake measure. Vitamin E is also particularly difficult to quantify for the general public from food-composition data (4).

The main source of vitamin E is fats and oils (5), and the content of vitamin E varies depending on processing procedures (6–8), type of oil, shelf life and addition of antioxidants to restore oxidized vitamin E (6,9). None of these issues can be addressed with dietary assessment instruments. In addition, many processed foods that are major contributors to total vitamin E intake, such as doughnuts, cookies and cakes (5), may provide vitamin E depending on the source of fat, and this information is also unavailable using standard dietary assessment techniques. Third, biomarkers provide a more proximal measure of nutrient status than dietary intake data for disease outcomes and population nutritional status measures. The biomarker serves as an integrated measure of metabolism of the nutrient of interest.

The nutritional biomarker can be used as a measure of internal dose, which is an indication of the amount of nutrient available to the tissues after absorption and metabolism. The marker can also be used as a measure of dietary change in studies of dietary interventions or for compliance with a new dietary regimen (10). Nutritional biomarkers are often used to “validate” dietary questionnaires, but they are not a gold standard and can be more useful for comparisons across dietary instruments. The limitations of the markers must be understood for them to be used for validation purposes. For some purposes, dietary data can be more appropriate than biomarker data.

It is often assumed that a biochemical marker of a nutrient is closely related to the amount of nutrient present in the diet. For most nutrients, there are a variety of reasons why the relation may not be simple and why other factors need to be considered when one is trying to relate biomarkers to dietary intake.

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Physiology

There is variation between individuals in physiology and nutrient metabolism. To a large extent, much of the interindividual variability is not well understood. Nonetheless it must be appreciated that these differences between individuals exist.

Absorption. Many factors influence the absorption of nutrients. Some effects are well known, but for others, large unexplained differences exist among individuals. For example, in a carotenoid feeding study, absorption of pharmacologic doses of \(\beta\)-carotene had a coefficient of variation of 61% in 79 male volunteers (11). Although there appears to be large between-person variability in this study, there is some evidence of within-person consistency in response to a given load (12). We do know that the prior nutrient status at the tissue level provides feedback control to increase or decrease efficiency of absorption of some nutrients such as calcium (13). If the prior status was low, the individual would have great efficiency in absorption of a nutrient. Large interindividual variations in transit times can influence absorption (14). These differences are not well understood. The concentration of the intraluminal contents at the time of nutrient presentation also influences absorption. For example, the fiber content of a meal appears to decrease the availability of carotenoids from foods (15). It is thought that the food forms, matrix and interaction of carotenoids and other fat-soluble dietary constituents influence absorption (16–20). Some carotenoids are absorbed with greater efficiency than others (21), which is due to structural or lipophilic differences that influence incorporation of the nutrient into the micelle and bioavailability (22).

For some foods, cooking can diminish the content of nutrients such as vitamin B-6 (23) and vitamin C (24) depending on the exposure to light and the extent of cooking. Nutrient-antinutrient interactions can be present in the gut. For example, phytates, which are present in wheat, inhibit the absorption of divalent cations (14) such as calcium and zinc, and the level of oxalic acid in green leafy vegetables influences the absorption of calcium (13). Finally, whether or not the nutrients are delivered as a bolus or in smaller quantities over time can also be an influence (19).

Some of the absorption issues are estimated and incorporated into the food-composition data table, but the influence of food combination in the lumen cannot be addressed. Dietary questionnaires rarely include the details of food preparation and currently cannot capture information about foods eaten together. Therefore these factors may influence the absorptivity of nutrients in question and blur any relationship between dietary estimates and biologic measures.

Tissue turnover and excretion. After the dietary constituents have been absorbed and have entered the blood system, there are variations in how the constituents are processed. There are influences on tissue distribution and the rates of transfer between compartments. Much of the variability is not well understood, but could relate to age and gender. Certainly tissue and renal saturation levels of nutrients provide feedback regarding absorption and excretion of these nutrients (24). Transfer of nutrients from one compartment to another occurs, such as fatty acid transfer from the red cell membrane to adipose tissue to muscle (6,25). Evaluating red blood cell fatty acids could indicate a steady-state equilibrium or a transient state with new fatty acids that are yet to be delivered to a body compartment. When observing a nutritional biomarker in the serum, it should be kept in mind that what is being viewed is a snapshot in time, similar to a 24-h recall.

The kidneys are a point of control for the body load of many dietary constituents, particularly the water-soluble nutrients. Because excretion is a point of control, urinary markers of nutrients are often not useful. For example, typically all of the extracellular pool of calcium is reabsorbed by the kidney, and none would appear in the urine (13). In addition, after saturation of the glomerular filtration rate, excess vitamin C spills into the urine, but this does not necessarily correlate with intake (24). Interindividual variation in degradative pathways exists, which could influence any assumptions of the bodily load or dietary intake. Also, numerous xenobiotics, medications and medical conditions interfere with metabolism and excretion of nutrients. Alcohol, diuretics, antibiotics and inducers of cytochrome P450 enzymes could affect excretion rates (23,26–30). Additional research in understanding the relationships of these factors on metabolic pathways is clearly needed.

Purpose of the study

These issues of metabolism need to be considered in the context of the purpose of the study and the reason that the nutrient is being studied. Sometimes investigators are only interested in the amount of nutrient reaching the tissues, although ultimately they may wish to make some statement about dietary intake. In general, epidemiologists only measure a nutrient from one of the many compartments where the nutrient can be found. For example, zinc is mostly stored in muscle and liver, but we are unable to obtain specimens from these sites. A small percentage of zinc is found in the serum, which is generally not a sensitive marker of zinc status. There is a lack of an established functional or enzymatic marker of zinc status (31). Plasma \(\beta\)-carotene is routinely evaluated in blood.

This nutrient is also found in adipose tissue, cell membranes, liver and tissues throughout the body. Flux across the tissues occurs (e.g., from plasma to adipose tissue) and in some cases, there exists a preferential concentration of a nutrient in a tissue, such as the concentration of lutein and zeaxanthin in the retina (32,33). Depending on other factors such as oxidative stress, the serum concentration may be altered owing to the use of the nutrient as an antioxidant. Although some of these oxidative stresses such as smoking status can be controlled for statistically, other factors such as radical formation from macrophages cannot (34). Such issues diminish correlations between diet and serum levels.

The purpose of the study must be considered when the investigator is choosing a biomarker. The current state of knowledge should be evaluated not only for selecting an appropriate biomarker, but also in assessing how to evaluate the biomarker appropriately. In some cases, dietary data can be more appropriate than biologic data. Much of the work that has been done in cross-sectional surveys and case-control epidemiologic studies suffers from the potential effects of the disease on the biologic marker of interest. An indirect method of evaluating this effect is to consider the mean values of the nutrient by the severity of the disease. Although such studies cannot preclude the possible disease effects, some studies have revealed the potential problems of using currently ill individuals (35–37). The influence of the disease process is less of a concern in prospective cohort studies, although often investigators exclude the first 2 y of data after specimen collection to avoid any preclinical effects of the disease on the biomarker of interest.

Temporal relation with dietary intake

It is important to think about the biologic relationship of the biomarker with dietary intake. If one is interested in short-term
markers, which are defined as those that respond to dietary intake within hours, then the studies can be conducted easily within a defined setting. Such markers might be the hydrogen breath test (38) or the $^{13}$C-lactose digestion test (39) for lactose intolerance. In most large-scale studies, researchers are interested in the average status of the population or the usual long-term status of the study population. For such studies, it is important to know the biology of the nutrient’s absorption and metabolism to know whether one is evaluating very recent intake, because the main interest may be long-term intake. For example, serologic vitamin C or triglycerides respond with postprandial spikes for several hours. In such cases, one would prefer to have fasting samples for indicators of longer-term status. In some settings it may not be possible to obtain fasting samples, so one would want to ask individuals about recency of intake and types of foods consumed and then use these data in the laboratory or statistical analysis phase of the study.

Medium-term markers are those that respond over weeks or months. For example, the use of red blood cells for essential fatty acid composition or folate content can provide an average of the previous 120 d of intake of these constituents. Because the lifetime of each red blood cell is 120 d, some of the cells will be nascent, some will be at the end of their lifetime and the rest will reflect all gradations in between, which creates an average of recent intake. White blood cells concentrate zinc and vitamin C, which makes them less likely to correlate well with intake. In addition, these cells respond to biologic stress, which also reduces any correlation with dietary intake.

Epidemiologic studies in general are focused on long-term intake, and so markers that respond over months or years are useful. Hair or toenail samples have been used to measure trace-element content, which should represent long-term intake. Fatty acids are metabolized such that palmitic acid is mainly synthesized and then further metabolized so that oleic acid is more predominant in vivo compared with its content in the diet (6). This metabolism of fatty acids diminishes any relation to their dietary intake. Although essential fatty acids and (n-3) fatty acids are metabolized to prostaglandins, thromboxanes and leukotrienes (6), adipose levels of these fatty acids were shown to have some relationship with their dietary intake (25). Adipose levels of carotenoids tend to be less stable than those of fatty acids, which suggests that adipose carotenoids may not be good markers of long-term intake (25). More research is needed in terms of stability of fat-soluble dietary constituents in adipose-tissue samples. A recent intake of high doses of fat-soluble constituents would saturate tissues, and would be present in the adipose tissue. Again, questionnaire data would be useful to differentiate those with long-term steady intakes versus those with recent short-term high intakes.

For most biomarkers, questionnaire data related to intake and changes in intake are essential for evaluation of most biomarkers, especially with current trends for intakes of dietary supplements that can influence correlations between dietary intake and a biologic marker of a nutrient. In some instances, dietary data can provide better information than biologic samples; for example, for fatty acid intake. For other nutrients, the biologic specimen could be more relevant to the disease outcome if the population being studied commonly consumes dietary supplements and the research question only involves the internal dose to the tissues. For common nutrients, it can be desirable to know whether an association with disease is related to diet only or instead to diet plus supplement levels, such as the relation of vitamin E and heart disease (40). Questionnaire data combined with biologic data can address this issue better than dietary data alone.

New dietary supplements present major problems for nutritional epidemiologists. The extent of the problem and some of the solutions depend on the purpose of the study. Recent problems include estimation of the intake for some antioxidants, individual carotenoids, phytoestrogens and other constituents that now can be consumed in a variety of foods from beverages to breakfast cereals to pills. If the intent of the study were to validate a food-frequency questionnaire, then expensive efforts would need to be made to estimate intake of these new food items. If the intent of the study were to estimate usual levels in the population via 24-h recalls, then efforts would be necessary to include specification of the exact brand name and item being consumed. However, if the intent of the study were to estimate nutritional status from food and supplements, then biomarkers for known dietary constituents could be the appropriate approach. The intake of vitamin and mineral pills has always presented problems for epidemiologists because of the intermittent usage, variety of formulations and lifestyle characteristics of the users (41). With new dietary constituents available in pill form and widely used by the population, new efforts must be considered to estimate the intake of these constituents. Again, biologic measures of some of these single constituents that are available in foods and in pill form (e.g., tofu constituents) may be the best alternative at this time. A combination of dietary and biologic measures could also be desirable.

**Choice of specimen**

A variety of biologic specimens can be obtained to evaluate the nutritional status of the individual or population. Most of the commonly used biologic samples in nutritional sciences do lend themselves to large-scale studies. It is not practical to collect some types of specimens for epidemiologic or surveillance studies owing to the subject burden and logistic considerations.

Studies that require fecal or urine samples are intuitively informative for the evaluation of nutritional issues. Studies of fecal components can be important for measuring fibers or bile acids that are relevant to colon cancer and other diseases, but may only be amenable to small-scale studies. For the urinary content of nutrients or their degradative products, a 24-h collection can be required, which is impractical in epidemiologic studies. Urine may be useful for investigating water-soluble nutrients, but the urinary output depends on nutrient saturation of tissues and dietary intake, so this measure may only be relevant for nutrients with a consistent intake. Urine may require acidification and cold storage to prevent degradation of some nutrients [e.g., vitamin C (42)]. In addition, some dietary constituents do not pass through the urine [e.g., iron (42)]. With recent interest in a variety of phytochemicals, the urinary metabolites of these compounds may be useful in the future. The development of methods that use spot urines would be beneficial for large-scale studies of populations.

A variety of other biologic specimens are amenable to large studies but may have limited utility for some research questions. Breast milk is easily accessible and has many known components, but it is probably not relevant to many of the chronic diseases studied with epidemiologic designs. Although having been breastfed has been related to some childhood (43) and adult (44) cancers, it is probably not appropriate at this time to discern which constituents in the breast milk confer the protective effect. Hair and nail samples are easily accessible and can be evaluated for trace elements. The validity of these markers has been questioned, however (42). The utility of adipose-tissue specimens is reviewed with fatty acids and...
fat-soluble constituents (45,46). Buccal cells increasingly are being used in epidemiologic studies that involve DNA, but these cells tend to have limited utility for nutritional factors, because the specimens are often contaminated with food. Research is currently being pursued to investigate new methods for buccal cell collection.

Venipuncture blood samples are the biologic specimen of choice for most large-scale studies that involve hundreds, thousands and hundreds of thousands of individuals. Blood samples are accessible, present minimal subject burden and are logistically feasible on a large scale. Systems can be worked out to have samples shipped on ice overnight or prepared and stored in low-temperature freezers at the site of collection. Knowledge of the probable assays to be conducted at the end of the study dictates which serologic components to collect: plasma, white cells and red cells or serum. Investigators often do not know all of the potential assays and simply store additional aliquots of the serologic samples in the hope that the blood will be adequately stored for new hypotheses that will emerge. Laboratory personnel conducting the assays of interest should be contacted before initiation of the study to determine the best serologic component (e.g., serum versus plasma) for the assay and any possible problems to be avoided in the processing and storage of the samples.

Emerging areas of interest for large-scale studies include the use of spot blood samples, perhaps on filter paper or in capillary tubes, for characterizing nutrients such as vitamin A (47,48) or folate (49). Diabetic patients currently evaluate insulin levels at home via a spot test, which suggests that this could be a more acceptable procedure for some individuals compared with venipuncture. Given the continuing problem of low response rates in many large-scale studies, which become even lower with the inclusion of biologic components, noninvasive procedures could improve response rates of population-based research. However, the reliability of these new techniques needs to be evaluated, because some methods were developed as screening tools and may not be appropriate for research that requires highly reliable and valid nutrient estimates. White cells are being used for evaluation of DNA and RNA, but new technologies could have utility for some nutrients. Nonetheless, genetic variation identified through DNA testing can be used in combination with nutritional information to better understand some variations in metabolism and perhaps identify subpopulations that are at risk of disease. Another emerging area involves biologic measures of phytochemicals. Measurement of enterolactone in blood is a promising technique that could be very useful in classifying individuals on their exposure to isoflavonoids. The complex issues related to the utility of enterolactone are discussed in another article (50).

Storage and laboratory issues

Consultation with laboratory personnel before beginning the fieldwork will dictate optimal processing and storage conditions for the analytes. Issues such as aliquot processing, cryovials, volumes and long-term storage conditions should be addressed. Many vitamins are unstable when exposed to heat, light or oxygen and will decay in storage. Analysis for vitamin C is particularly problematic for population studies, because a buffer must be made in the field and added to each aliquot before storage, and the processing of the sample must be conducted in a timely manner (42). Riboflavin is sensitive to light exposure, and folate may require vitamin C as a preservative for long-term storage (42). Many other nutrients do not require additional procedures and can be stored without special handling or preservatives. New technology continually emerges that can influence how specimens are prepared and stored, so consultation with laboratory specialists is beneficial before any large-scale study.

There are several issues related to the quality-control (QC) procedures employed by the laboratory that will assay the specimens from a study that should be appreciated by the researcher. The investigator should inquire about in-house QC procedures, how well the assay has been validated, what manufacturers are involved, etc. These issues may be particularly important for newly emerging dietary constituents or metabolites of interest such as phytochemicals or homocysteine. Even for an established nutrient such as folate, a large variation within and between laboratories has been observed with a two- to ninefold difference between laboratories testing the same material (51). Such variation makes it difficult to compare absolute values among studies, and imprecision diminishes the ability to observe differences between groups. In general, clinical laboratories focus on concentrations of substances outside of the normal range, whereas the interest in research studies is often on concentrations within the normal range. This focus within the normal range requires a high level of precision in the laboratory and must be appreciated. Although laboratories have internal QC procedures, it is always beneficial to include blinded QC specimens that are provided by the investigator. These QC samples can be obtained from a variety of sources and are typically from donors that are similar to study participants. Samples from study subjects are generally deemed too valuable to be used for QC purposes. Investigators can use pooled serologic specimens from volunteers, or subjects who were deemed ineligible for the study and other sources. It may be necessary to test a laboratory with only QC blood sample for a week or two to ascertain measures of variability in the assay of interest.

It is noteworthy that much of the work conducted by epidemiologists involves measures of nutrients within the normal range; however, some population studies do evaluate deficiency levels. Alternatively, if study subjects report vitamin supplement usage for a nutrient of interest, then the biomarker would be far outside of the normal range. The laboratory should be apprised of studies involving extremely low or high concentrations of a nutrient so that the assay can be adjusted accordingly. Finally, the assay methodology must be highly reliable and valid within a variety of ranges, and, certainly, for the range expected for the population being studied.

Specimens from subjects should appear identical regardless of case status, intervention status, gender, etc. Likewise, if possible, it is desirable to have the QC samples also be identical to the study subjects’ samples (i.e., same type of vial, type of label, sample volume). Blind QC samples should be inserted into every batch with the subjects’ samples. In general, 10% QC samples should be adequate to monitor the laboratory performance during and after the assays have been completed. Any problems identified through the blind QC data should be immediately reported to the laboratory, and efforts should be made to identify problems and make appropriate modifications to the procedures. Problem batches can also be removed from statistical analyses to evaluate their influence on the findings.

Advantages and limitations

Biomarkers provide accurate measures that can be correlated to dietary intake and can have less error than dietary intake estimates (1). The estimates are objective measures that are independent of memory, capacity to describe foods (1), capacity to estimate average intake over a period of time, and
social desirability issues. In addition, errors associated with biologic variables are independent of those associated with questionnaire data, which are important for statistical analyses involving measurement-error correction (1,52). Combining nutrient estimates from questionnaire data with serologic measures of the same nutrient can provide a powerful tool for estimating the exposure of interest and assessing risk (53). This combination may eliminate some of the errors associated with each of the methods of nutrient estimation (Table 1). For example, a large study obtained both serum and dietary data and evaluated the risk of disease related to β-carotene. The data were divided into two categories of low and high β-carotene status within the dietary data and within the serum data. If these data were used together, the comparison of individuals low in diet and serum levels with those individuals high in diet and serum levels would yield stronger results than would be obtained using either diet or serum data alone. The individuals who were classified in discordant cells (Table 1, cells B and C) were removed from the comparison of concordant cells (Table 1, cells A and D), thus yielding a more valid comparison. Two measures of an exposure provide a more powerful analysis of the true exposure compared to only one measure (52,54). Methods have been developed for statistical analyses of repeated biologic samples or repeated dietary assessments, but typically these two types of data are not used together. Methodologic work that uses multiple measures of an exposure would be useful.

Many of the limitations of nutritional biomarkers have been discussed, and they are not unique to nutritional research. Three other issues that are distinctive to nutritional biomarkers also warrant mention. Although we have identified many dietary constituents in foods, there are more constituents yet unknown or unidentified. For example, we focus on select phytochemicals, lignans and isoflavones that have been identified; research into these compounds is currently burgeoning with newly identified constituents each year. Relying on food-group analyses from questionnaire data may be more informative than biomarkers in terms of associations with risk of disease or risk factors for disease. This issue of unknown dietary constituents is a limitation of nutritional biomarkers. Perhaps in time we can identify biomarkers that are good markers for a dietary food group. For example, it has been shown that serum α-carotene and total carotenoids are good markers for fruit and vegetable intake that perform better than other individual carotenoids (55). In a related issue, because there are constituents of foods that have yet to be identified, it is unwise to make statements that risk is related to one dietary constituent as if it were the sole risk factor. It is particularly easy with biomarkers to believe that a single entity is having an effect because the easily measured, well-characterized constituent may have less error associated with it than its dietary counterparts, yielding stronger findings. Sometimes the effect may truly be related to that one dietary constituent [e.g., lycopene and prostate cancer (56)], but more likely it is representative of a food or a food group. For example, genistein may be a good indicator of soy intake, but it may not be the sole player in any effect observed in an epidemiologic study. Clinical intervention studies provide important roles for differentiating single constituents from foods themselves as being influential in a disease process.

Many issues related to the biology of nutrients or methodologic issues related to their biology, storage or laboratory analyses have been briefly reviewed. In most cases, there is no quantification of the magnitude of the problem in relation to large-population studies. More work needs to address the impact of these issues, in particular, to relate the magnitude of the between- and within-person variabilities in metabolism to epidemiologic studies. Issues related to storage and laboratory performance should be incorporated into every study, and updated information should be obtained before fielding any study. Finally, it is the responsibility of the investigator to properly use QC information and the interpretation of bio-marker results in the context of the whole diet and current literature.

LITERATURE CITED


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**TABLE 1**

<table>
<thead>
<tr>
<th>Cross tabulation of dietary and biologic estimates of a nutrient</th>
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<tbody>
<tr>
<td><strong>Diet nutrient estimate</strong></td>
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<tr>
<td>Low</td>
</tr>
<tr>
<td>May include some hyperabsorbers or underreporters of diet (A)</td>
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<td>High</td>
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