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ORIGINAL COMMUNICATION

The use of biomarkers in multicentric studies with particular consideration of iodine, sodium, iron, folate and vitamin D

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Objective: To determine which biomarkers should be used in a European monitoring system to assess intake of iodine, sodium, iron, folate and vitamin D. These nutrients are relevant for human health but difficult to assess in the diet in a comparable way among countries.
Results: Iodine and sodium are mainly excreted in urine. Mean population intakes of iodine and sodium can be estimated by casual urine samples. However, habitual individual intake assessments require multiple 24 h urine collections. Serum ferritin is a sensitive test of iron status (mobilizable storage iron) and continues to be the leading single determination for individual iron status, but with serum transferrin receptor as a promising alternative because it is more specific than ferritin. Erythrocyte folate and homocysteine in blood are markers of folate status which are able to discriminate between normal status and levels of insufficiency in an individual. Serum 25-hydroxyvitamin D is a good marker for vitamin D deficiency in the individual, and can distinguish between overt deficiency and marginal deficiency, especially if combined with serum parathyroid hormone.
Conclusions: Specific and sensitive markers for the dietary intake of iodine, sodium, iron, folate and vitamin D are available, which have the advantage of being more accurate than intake estimations derived from dietary surveys. However, the use of biomarkers also has disadvantages such as extra burden in terms of logistics, budget and ethical approval, and can moreover reduce the participation rate. More basic data for the validity of cross-country comparisons are needed and ideas in terms of study design and sampling frame.
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Introduction

Not all dietary components considered as highly relevant indicators for health in European countries (Steingrímsdóttir *et al*, 2002) can be collected through dietary intake studies assuming valid, reliable and comparable information on dietary supply across populations. An alternative to obtaining probably biased data by dietary assessment methods in the multinational context would be collection of biological specimens and subsequent laboratory analysis of such material. Such biological specimen-based dietary information will be subsequently denoted biomarkers.

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Biomarkers of dietary intake can generally be categorized into two types (Kaaks *et al*, 1997): (1) those biomarkers which provide an absolute quantitative measure of dietary intake related to a time dimension such as 24 h; and (2) those biomarkers which measure the concentration of a given factor, ie concentration in blood or other tissue, but without relation to a time dimension.

In this paper we describe the status with respect to biomarkers for micronutrients that should be part of a pan-European survey but are not considered measurable by dietary assessment instruments. This includes iodine, sodium, iron, folate and vitamin D. Information on vitamin D in food composition tables is often incomplete and folate values are not comparable in European databanks due to different laboratory methods for determination. The use of iodized salt and iron-fortified foods makes intake

measurements difficult for these nutrients, and for iron there is the added problem of varied bioavailability depending on its chemical form (eg haem iron *vs* non-haem iron) and the effect of many dietary enhancers and inhibitors on iron absorption. Finally, sodium intake is difficult to measure from food intake data, as sodium content of otherwise similar foods varies greatly according to the amount of salt used in preparation and at the table.

Iodine

Since more than 90% of dietary iodine is excreted in the urine, urinary iodine is a widely used biochemical marker of iodine intake. However, large within-person (within-day and day-to-day) variation is a characteristic for urinary iodine and iodine intake.

The easiest urinary samples to collect in epidemiological studies are casual urine specimens (spot urines). To reduce variation, it has been suggested that first morning urine is collected, and iodine excretion expressed as a concentration or as a ratio between urinary iodine and urinary creatinine (Bourdoux, 1998; Thomson *et al*, 1996). The reliability of the different estimates has been discussed extensively, and comparisons have been made, mostly in favour of iodine concentration, especially when used in areas of undernutrition where creatinine excretion is low. Recently, age- and sex-adjusted iodine excretion, corrected for creatinine, in casual urine has been shown to allow a better estimate of iodine excretion compared to non-adjusted iodine concentration and iodine/creatinine ratio (Knudsen *et al*, 2000).

Sampling of 24 h urine is considered to be the most reliable method of assessing the iodine intake of an individual (Hetzel & Dunn, 1989). However, 24 h urine collections are inconvenient for the subject and difficult to collect accurately. Because of the significant day-to-day variation in iodine excretion, several 24 h urine samples should be used to determine the habitual iodine intake of an individual (Rasmussen *et al*, 1999).

Thyroglobulin concentration in serum is inversely associated with urinary iodine excretion, and may turn out to be a sensitive marker of iodine status in a population (Knudsen *et al*, 2001).

The determination of thyroid size by palpation or by ultrasound has been used as a marker of iodine status in field studies. However, palpation is difficult with small goitres and ultrasound requires skilled operators.

Sodium

The intake of sodium (salt = sodium chloride) is the sum of the natural content in foods, sodium added in manufactured foods, and salt used in the kitchen and at the table. Dietary intake studies tend to overestimate true intakes (Pietinen, 1982), primarily attributed to the inability to precisely account for added salt (Caggiula *et al*, 1985), and the fact that much salt, about 75%, is discarded in the cooking water (James *et al*, 1987). Salt may also be lost when manufactured foods are cooked.

More than 90% of dietary sodium is recovered in the urine with sodium output in sweat and faeces amounting to a few percent (in temperate climates and without heavy sweating). Consequently, in theory total intake of sodium can be estimated by measuring urinary sodium excretion. However, there are large individual day-to-day variations in 24 h urinary sodium excretions, as in iodine excretion (Dyer *et al*, 1997). Shortt *et al* (1988) have calculated that a sample size of at least 30 subjects would be required to estimate group mean sodium intake with a standard error of less than 5% assuming the availability of a single 24 h sample. Subsequently, several 24 h urine collections are necessary to characterize an individual's usual sodium intake (Liu *et al*, 1979; Schachter *et al*, 1980).

Information obtained from 24 h urines will, provided they are complete, be more accurate than figures estimated from spot samples, samples from the first urination in the morning or timed overnight collections. However, as for iodine, all sampling methods can probably be used to estimate group mean intakes in larger population studies if related to urinary creatinine.

Discretionary sodium intake can be measured with lithium-labelled salt via its excretion in urine (Leclercq *et al*, 1990).

Iron

A continued negative iron balance will result in depletion of iron body stores, eventually leading to a state of iron-deficient erythropoiesis, and finally the most severe form of iron deficiency with overt anaemia (microcytic hypochromic).

Serum ferritin has been found to correlate well with body iron stores in healthy individuals (Walters *et al*, 1973). Serum ferritin is of value throughout the range of iron stores, including the earliest stages of iron depletion, however an optimal level has not been defined (Cavill, 1999). Ferritin is an acute phase reactant protein, which increases in response to inflammation and infection. Serum transferrin receptor could be a good marker for tissue iron availability (Ahluwalia, 1998), especially in the clinical situation; however it awaits simple and reliable analytical methods to be found. The level of receptor in serum is not confounded by infection and inflammation, or by pregnancy, and hence is more specific for iron depletion than ferritin. Further, it is a sensitive indicator for iron availability, increasing progressively in response to iron deficiency — and to enhanced erythrocyte production.

Further progression of iron deficiency results in changes in transport iron variables (serum iron and transferrin). Serum iron is considered an unreliable iron status parameter, primarily because it exhibits pronounced within-day and day-to-day variations. Serum transferrin is relatively stable during the day; however, it increases with oral contraceptive use and decreases with infections and inflammations.

Indirect measures of transferrin concentration like transferrin saturation and total iron binding capacity, which decreases and increases, respectively, in iron deficiency, vary with changes in serum iron, and have low sensitivity and specificity. Free erythrocyte protoporphyrin accumulates with iron deficiency. The analysis method is simple and rapid, and has a high reproducibility, and only requires a drop of blood and minimal technical expertise.

Decreased mean corpuscular erythrocyte volume, mean cell haemoglobin concentration and serum haemoglobin concentration are late stages in iron deficiency, and are non-specific, and have low sensitivity as iron status indicators.

The examination of bone marrow aspirates for iron content (total body iron stores) is considered the gold standard for assessing iron status, but is evidently impractical for use in field studies.

Several recent comprehensive papers are available on the topic of iron status determination (Worwood, 1997; Cook, 1999). It has to be stressed that there are several causes for negative iron balance apart from low dietary iron, eg blood loss, parasites and gastro-intestinal diseases.

Folate

Intracellular folates exist predominantly as polyglutamates, incapable of cellular exit, while circulating folates are monoglutamyl derivatives. With increasing folate deficiency there is a progression of changes in laboratory tests prior to the development of clinical functional deficit, ie megaloblastosis and anaemia (Bailey, 1990). Decreased serum folate is the first indicator of negative folate balance, but does not provide information regarding body stores. Serum folate is very sensitive to recent intake, and therefore may be less suitable for use as a marker of folate status in epidemiological studies (Stites *et al*, 1997). Erythrocyte folate is a marker of the content of the vitamin intracellularly and its determination can be used as a measure of status. While the level of erythrocyte folate discriminates between normal folate status and deficient body stores, it may be less suitable for diagnosis of incipient folate deficiency (Ueland *et al*, 1993). Further, erythrocyte folate is reduced in vitamin B_{12} deficiency (Selhub & Rosenberg, 1997).

Homocysteine in serum seems promising for the evaluation of folate status, and even a marginal folate deficiency increases homocystein levels in blood (Jägerstadt & Pietrzik, 1993; Ueland *et al*, 1993). However, levels of homocysteine are dependent on several other dietary factors than folate intake (Rasmussen *et al*, 2000), including intakes of vitamin B_6 and B_{12} (Selhub *et al*, 1993).

An abnormal deoxyuridine suppression test provides biochemical evidence of folate depletion; however the test is tedious to perform and not practical to use in population studies. Other indices of folate deficiency, such as increased mean cell volume of red cells in bone marrow and blood, and hypersegmentation of blood neutrophils and anaemia, are the final stages of folate deficiency.

Smoking, alcohol and many drugs increase folate requirements. Although studies have shown good correlations between folate intake from diet and the level of folate biomarkers, it has to be stressed that biomarkers are an expression of folate status rather than folate intake.

Vitamin D

For assessment of the vitamin D nutritional status the concentration of 25-hydroxyvitamin D [25(OH)D] in serum is considered as an accurate, integrative measure reflecting an individual's dietary intake and cutaneous production (Parfitt, 1998). Dietary vitamin D intake alone correlates poorly with 25(OH)D (Takeuchi *et al*, 1995; Thomas *et al*, 1998). Degree of long-term solar exposure and time spent outdoors are better predictors of serum 25(OH)D than is dietary vitamin D intake (Thomas *et al*, 1998). However, the optimal level of serum 25(OH)D and the supply of vitamin D from either source to attain that level in children and adults is debated (Marriott, 1997; Vieth, 1999). Since vitamin D deficiency tends to decrease calcium level in blood with consequent secondary hyperparathyroidism, the measurement of intact parathyroid hormone (PTH) in serum has proven to be a valuable indicator of vitamin D status that can give additional information concerning the degree of compensatory changes. The level of serum 25(OH)D above which no further alteration in serum PTH occurs has to be defined but could define optimal levels of 25(OH)D (McKenna & Freaney, 1998).

The concentration of 1,25-dihydroxyvitamin D, the biological active form of vitamin D, will usually be normal or even slightly elevated in vitamin D deficiency, and therefore provides no information with respect to nutritional status (Hollis, 1996; Parfitt, 1998). Blood concentration of native vitamin D reflects recent intake of vitamin D and/or exposure to sunlight, and therefore may vary greatly over a short time in an individual.

Several other markers for vitamin D status have been used including: (1) urinary calcium excretion (decreases with low status), and urinary excretion of pyridinoline crosslinks and other bone metabolism biomarkers (increases with low status); (2) radiographic bone development of rachitis in children and osteomalacia in adults; (3) histpathologic changes in bone biopsies; and (4) alkaline phosphatase activity in adults (increases with low status). However, these methods are cumbersome and costly, or only indicative of severe vitamin D deficiency.

Discussion

Generally, quantitative measurements of dietary intake are not considered to be identical to the estimate of biomarkers derived from body compartments regarding physiology and disease aetiology. Molecular epidemiologists consider the

step from intake measurements to biomarker measurements as obtaining a more refined estimate of the biological active dose (Perera & Weinstein, 2000). In the nutritional literature concentration values are usually labelled as status measurements.

For the purpose of intercountry comparisons the easiest biomarkers to measure are those that measure intake directly, preferentially in the same unit as the intake itself. However, there are not many biomarkers with this attribute. A good example is urinary nitrogen from 24 h urines, which is believed to reflect 80% of the ingested nitrogen in a subject in nitrogen balance (Isaksson, 1980). Urinary as well as dietary nitrogen is measured in the same unit and might therefore reflect the same factor. In general, biomarkers based on excreted material such as urine seem to have a higher potential to reflect intake than concentration or other measurements in body compartments.

Biomarkers obtained in body compartments are usually not directly related to intake and follow saturation curves. Many of the biomarkers obtained in specific body compartments are not only influenced by the actual intake but also by the long-term exposure. In addition other exposure pathways may exist.

The relation between dietary intake and a biomarker obtained in body compartments shows, in general, nonlinear relationships and are measured in other dimensions than intake such as mol/l instead of g/day. It is often not easy to define exactly the relation between dietary intake and the concentration of a biomarker in a body compartment due to the many influencing variables, which may prevail. A nice example is recently given by Brubacher *et al* (2000) in their meta-analysis on the relation between vitamin C intake and its concentration in plasma. They analysed 36 studies published in the literature on this topic. The data allowed a common overall saturation relationship to be derived between the two variables. However, smoking and age were important determinants changing the curve. This meta-analysis also highlights that in a human study large interindividual variations of concentration exist despite standardized intake conditions. Thus, measurements in body compartments have to go along with measurements of other variables such as climate, lifestyle variables and genetic background.

For the micronutrients iodine, sodium, iron, folate and vitamin D, biomarkers exist that have the potential to generate comparable information across national dietary surveys in Europe. For these nutrients substantial problems with respect to validity exist if measured by dietary intake methods. The biomarker approach is assumed to generate more reliable information on the provision of these nutrients in a population than the use of any dietary assessment instrument.

Quantitative estimates of dietary intake on the basis of urinary output are possible for iodine and sodium. For iodine and sodium casual urine samples might be sufficient to give a first estimate of intake in a population. Accuracy is increased if adjustments are made for sex and age. Several 24 h urine collections are required for an accurate estimation of habitual sodium and iodine intake in an individual.

Blood specimens can be used to evaluate the population dietary status in respect to iron, folate and vitamin D. In healthy individuals serum ferritin is a sensitive test of iron status (mobilizable storage iron) and continues to be the leading single determination. Serum transferrin receptor is a promising alternative. It is more specific than ferritin and can be used in population-based surveys including elderly (inflammations) and pregnant women. For long-term folate status erythrocyte folate is the method of choice. Homocysteine in blood can probably be used as an alternative method. Both methods are able to discriminate between normal status and levels of insufficiency in a single individual. Serum 25(OH)D is a good marker for vitamin D deficiency in the individual, and can distinguish between overt deficiency and marginal deficiency, especially if combined with serum PTH. However, the level of 25(OH)D which defines hypovitaminosis D is not known. In the case of vitamin D, the biomarker approach includes vitamin D supply from sunlight. This might be important considering different levels of sunshine between south and north of Europe.

Biomarkers of intake have the advantage of not being reliant on the subject's memory or on the accuracy of recording food intake, and they are not dependent on the accuracy of food data tables (Crews *et al*, 2001). Ideally, biomarkers should be specific and sensitive, and they should not be too invasive for human studies. The inclusion of biomarkers in dietary surveys gives some problems such as extra burden in terms of logistics, budget and ethical approval, and can moreover reduce the participation rate. It was found that for the nutrients being considered urine collections as well as blood specimens are required. Thus, future surveys considering these parameters have to deal with new management problems because time-consuming urine collections as well as invasive blood drawing procedures need to be conducted on a representative scale.

The sampling frame for biomarkers might differ from those being proposed for dietary measurements depending on the statistical precision of the estimate. For the general design of biomarker studies that aim to describe populations we would propose to follow the philosophy of conducting multi-centric dietary surveys. In multicentric dietary surveys, detailed and flexible short-term measurements such as 24 h recalls conducted over a certain time period such as a year are converted into habitual population distributions (Hoffmann *et al*, 2002). This approach requires at least two measurements per individual in order to remove statistically the intra-individual variation. Intra-individual variation will not affect the mean of the measurement but other characteristics of the distribution. Since the distribution of a biomarker within a population is an important information the same principle of collecting the biological specimens as for dietary surveys might apply.

Actual experiences in using biomarkers for direct comparisons of populations are rare in the literature (van Kappel *et al*, 2001). In addition, aside empirical experiences in conducting representative biomarker studies and comparing actual data, more theoretical work is needed to develop the biomarker approach further. It is not clear yet which of the other influencing variables need to be taken into account when the population distribution is derived. It is also not obvious according to which principles a biomarker need to be validated. If, as in the above-mentioned examples, no valid dietary assessment method exists, do we need clinical trials with different levels of definite dietary exposure in order to derive the proper relation between exposure and the biomarker response? How can we validate a parameter such as serum 25(OH)D, which is influenced from diet as well as from sunshine, for cross-cultural comparison of diet? Probably, some of the questions have already been addressed in the literature. However, systematic reviews on such questions are still needed to convert the principle idea of using biomarkers within a pan-European survey into a well-developed strategy. Proof needs to be given that a biomarker value contains identical information across populations taking their diversity into account.

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