

## Keynote Address

# Biomarkers in nutritional epidemiology

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### Abstract

*Objective:* To illustrate biomarkers of diet that can be used to validate estimates of dietary intake in the study of gene–environment interactions in complex diseases.

*Design:* Prospective cohort studies, studies of biomarkers where diet is carefully controlled.

*Setting:* Free-living individuals, volunteers in metabolic suites.

*Subjects:* Male and female human volunteers.

*Results:* Recent studies using biomarkers have demonstrated substantial differences in the extent of measurement error from those derived by comparison with other methods of dietary assessment. The interaction between nutritional and genetic factors has so far largely gone uninvestigated, but can be studied in epidemiological trials that include collections of biological material. Large sample sizes are required to study interactions, and these are made larger in the presence of measurement errors.

*Conclusions:* Diet is of key importance in affecting the risk of most chronic diseases in man. Nutritional epidemiology provides the only direct approach to the quantification of risks. The introduction of biomarkers to calibrate the measurement error in dietary reports, and as additional measures of exposure, is a significant development in the effort to improve estimates of the magnitude of the contribution of diet in affecting individual disease risk within populations. The extent of measurement error has important implications for correction for regression dilution and for sample size. The collection of biological samples to improve and validate estimates of exposure, enhance the pursuit of scientific hypotheses, and enable gene–nutrient interactions to be studied, should become the routine in nutritional epidemiology.

**Keywords**  
Diet  
Cancer  
Biomarkers  
Epidemiology

Epidemiology has traditionally been regarded as a hypothesis-generating exercise, but modern methods aim to test hypotheses and establish relative risks. In the absence of satisfactory animal models of human chronic degenerative diseases, such as cancer, nutritional epidemiology provides the only direct approach to the assessment of risk from diet in man.

There is a large body of evidence to show that the marked international differences in the occurrence of most of these chronic diseases, such as cancer, are mainly due to environmental factors, such as diet. Many dietary factors are associated with disease prevention or causation; these factors range from traditional nutrients such as antioxidant vitamins, fat and plant polysaccharides, to foods such as vegetables, meat and fruit, to phytochemicals such as glucosinolates, phyto-oestrogens and carotenoids, and to contaminants such as heterocyclic amines and aflatoxins. However, causal associations require evidence of individual risk from exposure to particular items of diet. This entails accurate measures of the habitual diet

of very large numbers of free-living individuals, one of the most difficult and challenging problems in nutrition. Furthermore, the complexity of the subject has increased markedly in recent years with the need to assess risks from dietary items, such as phytochemicals and contaminants, for which no databases of food levels are published. The past 15 years have seen a marked increase in the recognition of the need to improve, and quantify the errors involved in, dietary assessment. The success of the four International Conferences on Dietary Assessment Methods, at St. Paul, Boston, Arnhem and Arizona, and earlier European meetings, is a testament to the importance with which nutritional epidemiology is now viewed.

Despite strong international associations between diet and cancer, and high estimates of attributable risk from diet in Westernised societies, within-population estimates of relative risks between dietary factors and chronic disease such as cancer are rarely greater than 1.5 for most foods or dietary items<sup>1,2</sup>. It is difficult to ascribe causality to these low estimates, in comparison with relative risks of

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the order of 15 for smoking and lung cancer, for example. Two main factors may account for these low within-population estimates: genetic susceptibility and measurement error in dietary assessment.

### Gene–environment interactions in nutritional epidemiology

Genetic factors are important in influencing individual susceptibility to environmental factors, including the effects of diet. Studies of common variants of genes alone have generally proved disappointing in elucidating strong risks of sporadic cancer, for example in studies of polymorphisms of genes controlling xenobiotic metabolism<sup>3,4</sup>. However, there are examples where the interactions between dietary factors and single nucleotide polymorphisms have markedly increased risk estimates. In individuals with the fast alcohol-to-aldehyde metabolising form of alcohol dehydrogenase (ADH) – ADH<sub>3</sub><sup>1</sup> – genotype, the odds ratio for oral cancer in moderate alcohol drinkers is only 1.2. In heavy drinkers it rises to 40 in those with the fast genotype ADH<sub>3</sub><sup>1</sup> but to only 4.4 in those who are ADH<sub>3</sub><sup>2</sup>. These associations provide strong causal evidence for the carcinogenicity of acetaldehyde<sup>5</sup>. There are numerous examples emerging in the literature of the interaction between disease risk, common polymorphisms and dietary factors, including *N*-acetyl transferase polymorphisms in relation to meat consumption and cancer risk; apolipoprotein E in relation to saturated fat and coronary heart disease; methylene tetrahydrofolate reductase in relation to alcohol, folate, pyridoxine status and adenomatous polyps; and glutathione-S-transferase status in relation to DNA adduct levels and diet<sup>6–9</sup>.

In general, however, the interaction between nutrition, biomarkers and genetic factors has so far only been studied in comparatively small populations, so that the reported elevated risks associated with an interaction may have been based on very small numbers of subjects. Findings might thus have arisen by chance. Furthermore, misclassification error in dietary assessment will increase sample size requirements markedly; with only a 20% loss in sensitivity in dietary assessment, the required sample size for interactive investigations may more than double<sup>10</sup>. In addition, the preferred method of investigation is a prospective approach, rather than a case–control study, to avoid the introduction of bias in the assessment of the environmental exposure.

Very large sample sizes are required for prospective studies of gene–environment interactions in cancer, and they can be studied only in epidemiological cohorts that have measured environmental exposures, collections of biological material and a sufficient number of disease endpoints. The European Prospective Investigation of Cancer (EPIC) is one such study, with a cohort size of 400 000. Over 10 000 cases of cancer are expected within only five years of follow-up<sup>11</sup>.

### Measurement error in nutritional epidemiology

There are a variety of methods in use for the measurement of diet in cohort, cross-sectional and intervention studies, where the aim is to assess contemporaneous diet. Details together with practical advice on equipment, timing and protocols are given elsewhere<sup>12–15</sup>. Methods generally involve either the collation of observations from a number of separate days' investigations, as in records, checklists and 24-hour recalls, or attempts to obtain average intake by asking about the usual frequency of food consumption, as in the diet history and food-frequency questionnaire (FFQ). In all methods of dietary assessment, some estimate of the weight of food consumed is required and, for the determination of nutrient or other food component intake, either an appropriate description for use with food tables or an aliquot for chemical analysis is necessary.

Methods of measuring diet are associated with both random and systematic error. These errors arise from the use of food tables, assessment of the frequency of food consumption, portion size, daily variation and failure to report usual diet, due to either changes in habits whilst taking part in an investigation or misreporting of food choice or amount. The presence of these errors has generated much controversy and discussion as to the most 'accurate' method of dietary investigation. Numerous validation studies have been conducted comparing results of one dietary assessment method with another, presumed more accurate, method on the same individuals. However, unless food consumption can be observed independently, the 'true' value for assessing validity is unknown because all traditional methods, even weighed records, rely on food consumption as reported by the individuals. Furthermore, errors associated with the method under investigation may be correlated with those of the reference method, so that correction for regression dilution is substantially underestimated<sup>16</sup>.

### Biomarkers of nutrient intake

Beaton *et al.*<sup>17</sup> stated that: 'There will always be error in dietary assessments. The challenge is to understand, estimate, and make use of the error structure during analysis.' The ability to do this, however, has only become possible with the advent of biological markers in biological specimens such as blood, urine or hair, which reflect intake sufficiently closely to act as objective indices of true intake. These biomarkers could also replace estimates of intake based on traditional methods. Biochemical markers of intake are potentially independent of the errors associated with dietary survey methods. Their usefulness is illustrated in a follow-up study of markers of aflatoxin exposure in relation to liver cancer. The range of aflatoxin contamination of foods is very great, so that use of food tables of average levels of contamination is unlikely to reflect individual exposure.

Relative risks of cancer from aflatoxin consumption were only 0.9 and insignificant (confidence interval 0.4–1.9) for individuals classified to have had high dietary exposure, as assessed by an interview of the frequency of consumption of 45 foods. However, aflatoxin exposure biomarkers in urine samples obtained from individuals in the cohort were able to detect substantial and significant relative risks for liver cancer of the order of 6–10. The estimated relative risk was 59.4 (16.6–212.0) in individuals positive for urine biomarkers of both aflatoxin and hepatitis B<sup>18</sup>.

Biomarkers may also be used to validate the accuracy of dietary assessment methods, but prior calibration studies under controlled conditions, for example in a metabolic suite, are necessary to ascertain that the predictability of the biomarker in humans consuming varying diets is at least as good as the dietary intake method that is being validated. Few biomarkers of dietary intake have been studied in this way.

### **Fatty acids**

Subcutaneous adipose tissue samples are reputedly easy to obtain and their fatty acid composition can be related to estimates of fatty acid intake. Plakke *et al.*<sup>19</sup> demonstrated quite close relations between polyunsaturated fat intake as assessed from adipose tissue and a 2-day food record in 321 individuals ( $r = 0.5$ ), although the agreement was less good for monounsaturated fat and saturated fat ( $r = 0.22$  and  $0.24$ , respectively). More recently, the utility of pentadecanoic acid (15:0) as a marker for dairy fat has been investigated; this fatty acid is synthesised by the bacteria in the rumen and is not produced endogenously by mammalian cells. The correlation between total dairy product intake from 4-week food records and 15:0 in subcutaneous tissue was 0.63, and between total dairy product intake from an FFQ and 15:0 in subcutaneous tissue, 0.40<sup>20</sup>. There were also significant associations between the total amount of fat from milk products assessed by a 7-day record and 15:0 in serum cholesterol esters ( $r = 0.46$ )<sup>21</sup>. Biomarkers of fat intake are discussed elsewhere in this supplement<sup>22</sup>.

### **Doubly labelled water**

The doubly labelled water method is an important advance in the measurement of energy expenditure since it can be used on free-living individuals with virtually no interference with everyday life, in contrast to previous procedures. Subjects are given a carefully weighed oral dose of  $^2\text{H}_2^{18}\text{O}$  and are then required only to donate timed urine samples over the next 15 days. Carbon dioxide production is measured as the difference in the water pool (measured by  $^2\text{H}_2$ ) and the bicarbonate plus water pool (measured as  $^{18}\text{O}$ ) and energy expenditure estimated. The energy expenditure should be equal to energy intake, taking into account changes in body weight<sup>23</sup>. This method has been used to validate dietary assessment methods intended for surveillance of UK

population samples<sup>24</sup>. In early reports, energy expenditure assessed from this method was unexpectedly low, 1.4 times the basal metabolic rate (BMR) on average in a small group of sedentary women. In women of normal weight, the mean energy intake from weighed dietary records agreed with mean energy expenditure data. But in obese women, mean energy intake assessed from 7-day weighed records was about 2 MJ (465 kcal) lower than mean energy expenditure, suggesting that overweight women do not report their habitual food intake<sup>25</sup>. In a later study, energy expenditure also exceeded energy intake measured from 7-day records in 31 normal individuals, on average by 20%. As a ratio to BMR, energy intake was  $1.46 \pm 0.31$  MJ, and energy expenditure was  $1.82 \pm 0.24$  MJ<sup>26</sup>. These and other studies have been summarised and show that, in general, self-reported energy intake tends to be less than energy expenditure as measured by the doubly labelled water method<sup>27</sup>. Published results from dietary surveys show that, using the ratio of energy intake to calculated BMR (see below), 24-hour recalls tend to give low results, many below limits compatible with normal energy expenditure with no loss in weight, whereas diet histories give higher and records give intermediate values<sup>28</sup>.

The doubly labelled water method is too expensive for routine use by most investigators. BMR can be calculated from body weight, using equations<sup>29</sup>. The BMR then has to be multiplied by a factor to allow for total energy expenditure, which has been derived from the difference between total energy expenditure and BMR in different population samples, and ranges from 1.2 to 4.5. In sedentary men and women, the factor to allow for physical activity is about 1.6<sup>30</sup>. Individuals are said to ‘underreport’ when the ratio of energy intake to BMR is less than this. However, the measurement of both energy intake and the calculated estimate of BMR and total energy expenditure are imprecise, so that ‘cut-off’ limits for underreporting are quite wide; for example, 0.90 if a single 24-hour recall is used and 1.35 for ‘normal circumstances’<sup>31</sup>.

### **24-Hour urine nitrogen**

24-Hour urine nitrogen is the most well known biological marker, with results from published metabolic studies, where individual dietary intake is kept constant over prolonged periods of time, showing a fair correlation between daily nitrogen intake and daily urine nitrogen excretion. Its use depends on the assumption that subjects are in nitrogen balance, there being no accumulation due to growth or repair of lost muscle tissue, or loss due to starvation, slimming or injury. This was appreciated as early as 1924, when it was suggested that actual protein intake, as assessed from 24-hour urine excretion, was far lower than the recommended level<sup>32</sup>. The apparent accuracy of 24-hour urine nitrogen as a biological marker has led to the suggestion that it be used to validate estimates of protein intake from various dietary survey methods<sup>33</sup>. In 1980, Isaksson<sup>33</sup> summarised a number of

studies carried out by his group and showed that estimates of protein intake obtained from 24-hour recalls of food intake were low when compared with urinary nitrogen, but those estimated from diet histories and records were in good agreement with the urine values. Van Staveren *et al.*<sup>34</sup> also found good agreement between 24-hour urine and diet history estimates of protein intake. However, these comparisons were investigated only on a group basis because each individual contributed only a single or two 24-hour urine collections. Other early comparisons between average urine nitrogen and dietary intake have been summarised<sup>12</sup>.

To investigate the applicability of using 24-hour urine nitrogen to validate estimates of protein intake on an individual basis, four men and four women were given their usual varying diet over a 28-day period whilst living in a metabolic suite<sup>35</sup>. Duplicates of diets were made up each day for each individual, 24-hour urine and faecal collections were also made over this period, and diets, urine, faeces and skin losses measured for their nitrogen content. Urine nitrogen underestimated intake at higher levels of protein intake and overestimated at lower levels, but a constant factor for faecal and skin losses can be used to counteract this, and output from urine can be expressed as a percentage of intake, 0.81<sup>35</sup>. Although this study was based on results from a comparatively small group, a later meta-analysis of a large set of data has confirmed that urine nitrogen should be approximately 80% of dietary intake on average<sup>36</sup>. However, less good correlations between individual estimates of usual protein intake and the 24-hour urine nitrogen output will be obtained if fewer observations on each individual are made, and if the collections are not verified for their completeness with *p*-aminobenzoic acid (PABA)<sup>37</sup>. PABA is now in routine use and has been used extensively in methodological studies. Individuals who are judged to underreport by the 24-hour urine nitrogen method also tend to be classified as underreporters by the doubly labelled water method<sup>38</sup>.

To assess the validity of several different methods of dietary assessment, 160 women were asked to complete 16 days of weighed food records over one year, as four repeated 4-day records. The volunteers were also asked to provide eight 24-hour urine collections, as four repeated 2-day collections, and completeness of the urine collections was assessed using the PABA check method. Different methods of dietary assessment were completed during the year by the volunteers and it was shown that correlations were greater between the biomarker 24-hour urine nitrogen and estimates of nitrogen intake from records, than from estimates of intake with other methods including the FFQ<sup>39</sup>.

Using the ratio of urine to dietary nitrogen, it was also possible to distinguish between those individuals who provided valid records of food intake and the 20% who underreported their food intake. Individuals who underreported were heavier, with a lower energy intake to BMR

ratio than the others, and their intakes of energy and all energy-yielding nutrients calculated from weighed records were significantly lower than those from individuals who did not underreport. On average, there was an 18 g difference in reported fat consumption, and a 27 g difference in reported sugar consumption, between the values reported by the underreporters according to the urine to dietary N ratio and the rest of the population. Mean consumption of cakes, breakfast cereals, milk, eggs, fats and sugars was also significantly lower in those individuals classified as underreporters. However, there was no difference in reported consumption of meat, fruits, vegetables and potatoes between these underreporters and the other 80% of the population who gave valid records, nor in vitamin C or carotene<sup>39</sup>. Hence, bias in reports of food intake obtained from methods of dietary assessment can be assessed from 24-hour urine nitrogen. Only some, not all, nutrients and foods may be underreported, and differences in means are reduced by energy adjustment. Overweight individuals in particular are likely to underreport the amount they eat<sup>17</sup>. There are also age effects; whereas there was good agreement between intake from records and expenditure by the doubly labelled water technique in 8-year-old girls, there was a marked trend towards worsening agreement with age, so that approximately 20% of energy was underreported by 12- to 16-year-olds<sup>40</sup>.

This problem is not confined to weighed dietary records, since it has been documented with all methods of dietary assessment, including diet histories, FFQs and 24-hour recalls<sup>25,33,39,41–46</sup>.

#### **24-Hour urine potassium**

In healthy persons, urine is the major route of excretion of potassium. Faecal excretion of potassium constitutes from 5 to 13 mmol per day in Western populations, or 11–15% of the dietary intake<sup>47–49</sup>. The correlation between intake and excretion of potassium can be high, even when dietary intakes are calculated from food tables rather than analysed, provided that sufficient 24-hour urine samples are obtained. Studies that have obtained at least eight 24-hour urine collections, validated for their completeness, have shown correlations of at least 0.7 between calculated intake and excretion<sup>50,51</sup>. Potassium has an advantage as a biomarker of diet because a greater variety of foods are good sources of potassium than those containing protein, for example vegetables and fruits.

#### **Use of biomarkers for calibration and to assess measurement error**

To meet the sample size requirements for studies in gene–nutrient interactions in chronic disease, pooling of data from multiple cohorts is becoming common practice. This also increases the heterogeneity of dietary habits, which is useful for overcoming measurement error in individual

dietary assessments. However, since each participating centre may have used different methods of dietary assessment, all with different measurement errors, calibration is then necessary to correct for any bias in mean intakes associated with these methods<sup>52</sup>. Within the main EPIC study for example, a standardised computerised 24-hour recall method, EPICSOFT, has been developed and administered to representative sub-samples within each cohort<sup>53</sup>. One hundred to 350 participants within each sub-sample have also provided 24-hour urine samples, verified for completeness using PABA, in order to assess the validity of the EPICSOFT method. Initial results suggest a high correlation between mean nitrogen intake and nitrogen excretion levels across the populations studied, allowing confidence to be placed in the validity of the calibration method (Slimani *et al.*, unpublished results).

In large epidemiological studies, it is now common practice to correct for measurement error in the assessment of relative risk by regression calibration, and the correction factors are derived by comparison of the method in use, such as an FFQ, with a 'reference' method, such as a record. However, this practice relies on the assumptions that errors in the reference instrument are uncorrelated with both 'true' intake and errors in the method in use. These assumptions can be examined if repeat estimates of intake and repeat biomarker comparisons are available. Kipnis *et al.*<sup>36</sup> have re-examined the work comparing repeat measures of intake of nitrogen using different methods of dietary assessment and with repeat 24-hour urine excretion values. Using a new measurement error model that allows for individual bias, for example in the tendency to underreport food intake, they showed that neither of these assumptions was true, leading to greatly underestimated attenuation factors and consequently under-powered studies. The National Cancer Institute is now initiating large studies of the measurement errors of dietary assessments using doubly labelled water and 24-hour urine nitrogen and potassium from urine samples, verified for completeness using PABA, as underlying validation measures, for example in the Observing Protein and Energy Nutrition (OPEN) study (Subar *et al.*, unpublished results).

In a recent study to assess the accuracy of methods in the EPIC UK cohorts, repeat biomarker estimates were also obtained from EPIC participants over a 9-month period. Urinary nitrogen, potassium and sodium were estimated from two to six complete 24-hour urine collections in 134 subjects and plasma ascorbic acid from two or three fasting blood samples in 118 subjects. PABA was used to verify the completeness of the 24-hour urine collections. Subjects completed two FFQs and two 7-day food diaries, and the second diary and FFQ were sent at varying times over the course of the study. 24-Hour urine samples were not collected during the time that subjects were recording their dietary intake, making it more likely that any errors

between the dietary method and biomarker were completely independent of each other. In both men and women, results calculated from the 7-day food diary were much closer to estimates of output from urinary biomarkers than those calculated from the FFQ. The agreement between plasma vitamin C and vitamin C intake was of the same order no matter which method was used<sup>54</sup>.

The design of this study also allowed error variance analysis to be conducted from the repeated dietary intake measures and the repeated urine collections. Marked differences in error variances associated with the different dietary assessments were shown. The most accurate method, the 7-day food diary, had substantially less error variance than the FFQ. Using the urine biomarkers as indices of 'true' intake, the correction factors for measurement error of relative risk estimates from the dietary assessment methods could be estimated. Correction factors for regression dilution from the food diary were only 1.8 to 2.0, whereas those for the FFQ were too large to use with confidence (4.8 for potassium and 9.0 for nitrogen). Furthermore, the confidence limits around these estimates for the FFQ became impossibly wide, 1.7 to 16.2 for nitrogen for example<sup>55</sup>.

EPIC within the UK thus will utilise dietary data from different methods. The FFQ data are associated with a greater degree of measurement error, as described above, so that correction factors for regression dilution are substantially greater than those required for the food diary. Findings in the UK, where dietary variation between individuals is smaller and hence the need to use a more accurate individual method greater, will be derived from the 7-day diary information on a nested case-control basis. All subjects within EPIC-Norfolk have dietary information from two 24-hour recalls that can be used in the event that diary information should not have been forthcoming from some eventual cases. However, if between-individual variation is increased, correction factors become smaller. Hence the FFQ is to be used particularly in pooled analyses of risk from diet in relation to cancer incidence within the larger European EPIC study, where measurement error is more likely to be overcome by large dietary heterogeneity on an international basis.

## Summary

Some biomarkers for the validation of methods for assessing dietary intake have been developed. There is a need for a greater variety of dietary biomarkers to be developed to reflect wider aspects of diet. At present, the doubly labelled water technique and 24-hour urine nitrogen and potassium are in routine use for validation studies. Using these biomarkers, it has been shown that there could be substantial attenuation of diet effects and loss of statistical power in epidemiological studies.

Attenuation and loss of power, together with genetic variation in response, could account for the inability of existing studies to show causal links between diet and chronic disease such as cancer. Some of this measurement error can be overcome by studying populations whose dietary habits are more heterogeneous than those of single populations. But biomarker studies suggest that improved, more detailed, methods of dietary assessment will be necessary if causal associations between diet and disease are to be established in future large-scale epidemiological studies.

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