Metabolomics in human nutrition: opportunities and challenges[1–3]

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ABSTRACT
Metabolomics has been widely adopted in pharmacology and toxicology but is relatively new in human nutrition. The ultimate goal, to understand the effects of exogenous compounds on human metabolic regulation, is similar in all 3 fields. However, the application of metabolomics to nutritional research will be met with unique challenges. Little is known of the extent to which changes in the nutrient content of the human diet elicit changes in metabolic profiles. Moreover, the metabolomic signal from nutrients absorbed from the diet must compete with the myriad of nonnutrient signals that are absorbed, metabolized, and secreted in both urine and saliva. The large-bowel microflora also produces significant metabolic signals that can contribute to and alter the metabolome of biofluids in human nutrition. Notwithstanding these possible confounding effects, every reason exists to be optimistic about the potential of metabolomics for the assessment of various biofluids in nutrition research. This potential lies both in metabolic profiling through the use of pattern-recognition statistics on assigned and unassigned metabolite signals and in the collection of comprehensive data sets of identified metabolites; both objectives have the potential to distinguish between different dietary treatments, which would not have been targeted with conventional techniques. The latter objective sets out a well-recognized challenge to modern biology: the development of libraries of small molecules to aid in metabolite identification. The purpose of the present review was to highlight some early challenges that need to be addressed if metabolomics is to realize its great potential in human nutrition. *Am J Clin Nutr* 2005;82:497–503.

KEY WORDS Metabolomics, metabonomics, nutrigenomics, metabolic pathways, pattern recognition, metabolic profiling

INTRODUCTION

Since the discovery of essential nutrients and key metabolites, nutritionists have been conducting nutrition profiles of mankind. Such profiling has been targeted at specific ranges of plasma nutrients and metabolites, depending on the hypothesis being tested. Today, with rapid advances in analytic chemistry technologies such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), the capacity exists for a far wider collection of data on nutrients, metabolites, and other compounds in various human biofluids. This approach to human assessment can be either open-ended through total data capture (1) or highly targeted, such as measuring the full spectrum of lipids (2). Of course, the assessment can also be both, and this comprehensive spectrum of metabolites and nutrients is known as the metabolome. Whereas the potential of metabolomics in pharmacology and in toxicology has been well recognized (3), its role in human nutrition is in its infancy. *Metabolomics* and *metabonomics*, 2 terms that in effect mean the same thing, have emerged from the fields of plant science and pharmacology, respectively. The former term is now more widely accepted (4), but readers are warned to always conduct literature searches with both terms. Metabolomics will be central to biology in the coming decades because it has been highlighted for funding in the recently published roadmap of the US National Institutes of Health (NIH) (5). Note that this review focuses on the study of human nutrition and excludes studies of animal models, which were extensively reviewed previously (6).

The 2 biggest challenges for metabolomics in human nutrition center around the vast output of spectral data on compounds in

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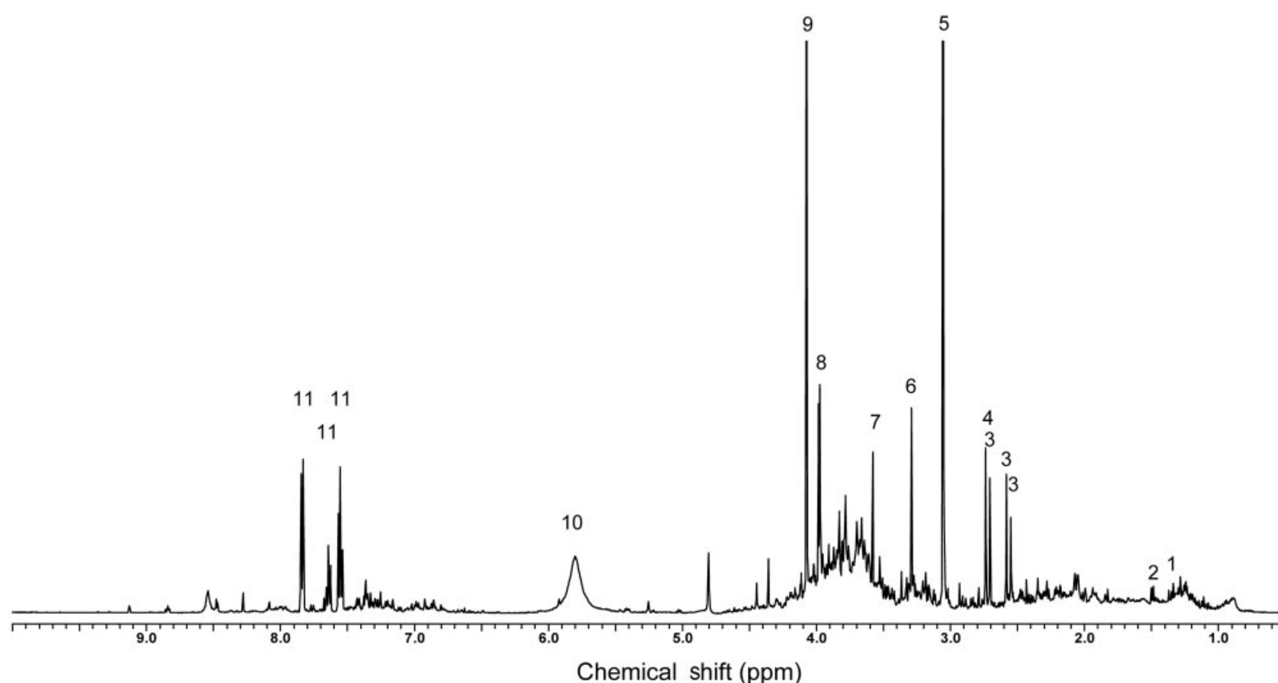


FIGURE 1. A typical 500-MHz ^{1}H nuclear magnetic resonance spectrum of human urine. The identification of the major metabolites is highlighted. 1, lactate; 2, alanine; 3, citrate; 4, citrate and dimethylamine; 5, creatinine; 6, trimethylamine-*N*-oxide; 7, glycine; 8, hippurate; 9, creatinine; 10, urea; 11, hippurate.

biofluids, which are generated by advanced 1- or 2-dimensional MS and NMR technologies (**Figure 1**). The first challenge must be to identify all the chemicals in different biofluids that are linked to the human nutrition metabolome, and the priority must be to gain a consensus for the definition of a metabolome in human nutrition. The second biggest challenge associated with the large NMR and MS outputs is how to work with these large total data-capture data sets in which many compounds remain unidentified. Pattern-recognition techniques can be used to work with these partially resolved data sets and, thus far, have been very successful in identifying the metabolic signatures of many phenotypes. The extension of this technology to human nutrition offers enormous potential. These are the core issues of this review, which also includes discussions on other related areas such as the value of different biofluids in nutritional metabolomics, the issues of nonnutrient chemicals and large-bowel metabolites, and the linkage of metabolomics with the wider elements of nutrigenomics.

Creating the human nutrition metabolome

Metabolomics is about small molecules, and one of the key aims of metabolomics is to identify those small molecules that make the difference between the effects of different diets and, in so doing, deepen our knowledge of human health and the interacting and regulatory roles of nutrition. Comparable goals exist for the use of metabolomics in toxicology and pharmacology. For traditional nutrition, analytic standards are used to identify compounds in chromatograms. However, only a limited capacity to identify compounds exists for metabolomics. The problem is that a comprehensive library of small molecules for NMR and MS spectra is not yet publicly available. Although some compounds can be identified, the complete identification of all compounds will require considerable additional analyses, in many instances beyond the scope of the average researcher. For example, in a detailed study of deproteinized plasma, 38 compounds were identified with the use of ^{1}H NMR but 14 (25%) were unidentified (7). Because MS is a far more sensitive method than NMR, identification of compounds is likely to be harder with that technology. Thus, it is not surprising that the application of gas chromatography–time-of-flight–MS technology to understand the metabolome of *Corynebacterium glutamicus* led to the identification of only one-half of the metabolome (8).

Thus, a major initiative of the NIH roadmap is the construction of libraries of small molecules and their chemistry (5). As part of this initiative, the Molecular Libraries Screening Center Network was established, a new chemoinformatics database was constructed (9), and a plan for the development of better analytic platforms was established. Initially, PubChem will build up data on 500 000 chemicals. Many of these chemicals will be used in the rapidly expanding field of small-molecule microarrays for drug discovery (10). This technology allows for small molecules to be immobilized onto a variety of slides, which can then be used to sequester compounds that have a binding affinity with the small molecule. In the plant sciences, a new initiative to create a publicly accessible library of indexes on mass spectra and retention times has been established (8). The Standard Reference Database of the National Institute of Standards and Technology will also be valuable in this regard (11). The challenges for the nutrition sciences will be to create a consensus of small molecules that are important for the study of metabolomics and then to create the standards needed for their identification with MS, NMR, and other emerging technologies.

This then begs the question of how we might create a list of nutrients and metabolites that might populate the ideal metabolome. Recently, the enzyme classification number mapping of

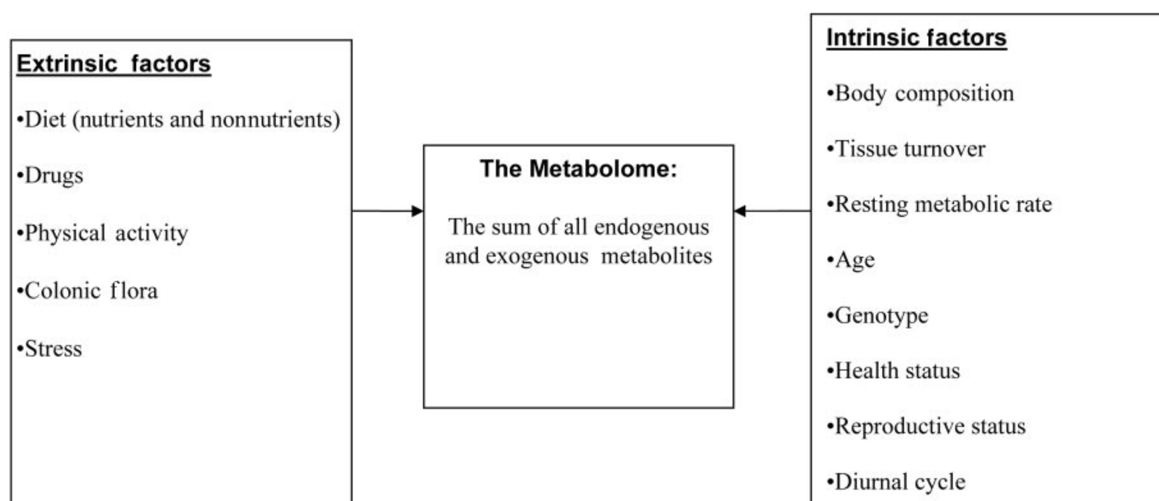


FIGURE 2. Exogenous and endogenous factors likely to influence the human nutritional metabolome.

metabolically active enzymes to metabolic pathway and to genome data (12, 13) was carried out. In one study, the HumanCyc database was used to assign 2709 human enzymes to 135 predicted metabolic pathways (12). Many metabolites will exist in signaling, receptor binding, translocation, and other reaction pathways. However, it must be possible to begin to list the key metabolites of the various metabolic pathways that nutrients are involved in and to begin to build up a library of compounds that particularly interest nutritionists. A first priority must be to analyze the carbohydrate, fat, protein, and energy metabolism pathways along with the mineral, trace element, and vitamin metabolism pathways. These pathways will involve anabolic and catabolic pathways as well as transport and transformation pathways. Subsequently, we will need to address reproductive, inflammatory, satiety, and other such pathways as well as tissue-specific pathways, signaling pathways, and cell regulatory pathways. In all these endeavors, consideration must be given to their relevance in human nutrition.

Pattern-recognition techniques and their application to human nutrition

The large data sets produced with the use of metabolomic analyses in pharmacology and toxicology have been used to identify compounds that differ between 2 treated groups, similar to the uses described in the previous section, and they have also been used for the recognition of an overall pattern of NMR or MS spectral output but not for the recognition of specific compounds. In metabolomics, this pattern recognition is achieved through the use of principal component analysis, which is unsupervised, and with the use of partial least-squares discriminate analysis, which is supervised and separates classes of individuals or animals. To date, pattern-recognition techniques have been used in metabolomics research to successfully separate case and control subjects for cardiovascular disease (1), for multiple sclerosis (14), for hypertension (15), for epithelial ovarian cancer (16), for the detection of inborn errors of metabolism (17), for species of animals (18), for strains of animals within a species (19), for animals treated with drugs (3) or fed different diets, for humans fed different diets (20), or for humans from disparate geographic locations (21, 22). This application of metabolomics may have great potential in nutrition research, but the issues raised in ensuing parts of this review that relate to the nonnutrient elements of human foods will need to be factored in when comparing different diets. If these effects can be either eliminated or controlled for in some way, then pattern-recognition approaches offer enormous opportunities for the identification of the metabolic signatures of different diets. If a protocol for linking NMR or MS metabolomics to phenotypes can be established and annotated to an international standard, and if corresponding databases are created and made publicly available, then the science of human nutrition will experience a giant leap. So great is that potential leap, that testing the validity of this hypothesis is worth thorough and collaborative efforts. Thus, any expert group that sets out to define a consensus on the nutritional metabolome, as described in the previous section, should also be charged with setting up the standards that will allow the creation of databases that link metabolomes to phenotypes.

In pharmacology and toxicology, a major international collaborative project (the Consortium on Metabonomics in Toxicology) is underway to fully characterize the NMR-derived metabolomes of selected rat and mouse strains that were exposed to 150 drug-development compounds of interest (19). A similar initiative in human nutrition is clearly worth exploring. Notwithstanding the fact that the pattern-recognition element of metabolomics works with both the knowns and unknowns in the large NMR and MS outputs, in ensuing sections we discuss that in human nutrition we must ensure that pattern recognition does not confuse the strong effects of nonnutrients in the diet with those of the nutrients we wish to study and that the significant effect of all exogenous and endogenous factors that may influence the metabolome under question are taken into account (**Figure 2**).

Metabolomics and food

The numbers of different molecules in the food supply that are not nutrients outweigh the numbers that are nutrients by orders of magnitude. For example, plants accumulate secondary metabolites for defense, reproduction, and so forth; however, none of these are essential nutrients. In traditional nutrition, these phytochemicals were mostly ignored until recently, when the potential metabolic effects of plant compounds were noted: flavones in

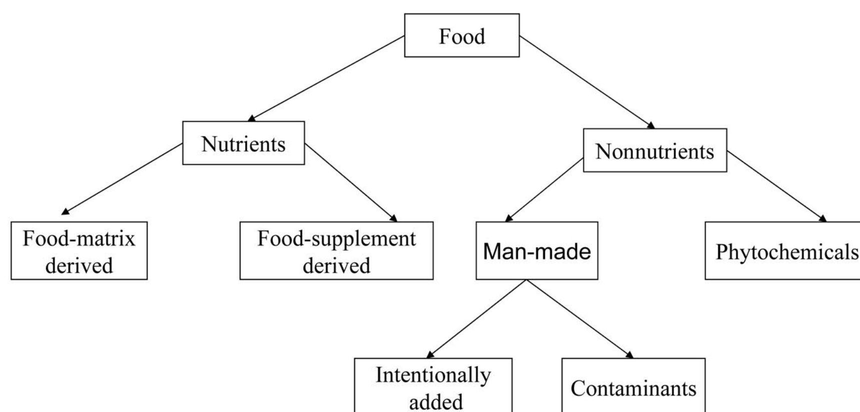


FIGURE 3. Nutrients and nonnutrients in the human food supply.

heart disease, stannols in cholesterol metabolism, and soy-based estrogen analogues in cancer (23). These nonnutrients with potential metabolic effects, along with the hundreds of thousands of food compounds that do not have metabolic effects but that make food a gastronomic delight, must all be factored into the metabolome. In addition to the thousands of compounds in foods such as red wine, coffee, fruit, fish, and vegetables, nonnutrients also exist in the food supply, some of which are man-made and are present either intentionally or accidentally (**Figure 3**).

A few examples of the effects of nonnutrients are worth considering. Salicyluric and salicylic acids, which are generally found in fruit and vegetables, are excreted in urine at higher concentrations in vegetarians than in omnivores (24). Allylmercapturic acid, found in garlic, is recovered in urine in high concentrations after garlic ingestion (25). This study also showed that the half-life for the disappearance of this compound in urine is 6 h, which indicates the potential of nonnutrients from plant foods to exert a significant acute effect on metabolomic profiles, a fact borne out by other examples cited in this review. Certain foods are known to produce obvious changes in urine in some, but not all, individuals, which indicates a genotype interaction. In some individuals, beetroot produces red urine; in others, asparagus gives rise to malodorous urine (26). Metabolites of coffee are detected in urine collected 4–5 h after coffee ingestion (27). In that study, the concentrations of the compounds that were metabolized by the cytochrome P450 1A2 pathway were increased by as much as 13-fold over baseline. The appearance of 23 quercitin metabolites in blood and urine after the ingestion of 270 g fried onions has been studied (28), and 18 of the quercitin metabolites were found in urine collected 0–4 h after the test meal. Pesticides have been frequently reported both in urine (29, 30) and in saliva (31); epoxy resins from food-packaging material have also been detected in urine (32).

Finally, we need to consider the chemical transformation of the food matrix after foods are cooked or digested. This brings animal food into consideration as sources of significant nonnutrient signals. In one study, concentrations of heterocyclic amines, which are produced when meats are grilled, were found to increase 14–38-fold in urine on the day after grilled beef was eaten and returned to baseline concentrations within 48–72 h after the cessation of meat intake (33). These compounds have also been detected in the urine of human volunteers who were fed a single meal of grilled chicken; in this study, most of the target metabolites were excreted within 12 h of the test meal, and very low concentrations were found at hour 18 (34). Clearly, careful chronic dietary interventions could be undone by the acute ingestion of different foods the evening before final biofluid samples are taken. Thus, dietary nonnutrients, which may not be important in pharmacology or toxicology, may be critically important in human dietary studies that seek to use metabolomics. A major consensus decision for the field of nutritional metabolomics will be how to address endogenous human metabolites and exogenous components of food that coexist at least transiently in human biofluids.

The microbiome

The gut microflora is often associated only with the large bowel, but, depending on the biofluid in question, the roles of the oral microflora and of gastric colonization by *Helicobacter pylori* may also need to be factored into nutritional metabolomics. Notwithstanding that caveat, most of our data relate to the large-bowel microflora. Healthy humans have ≥400–500 microbial species in their large bowel that can directly deliver compounds from their metabolome, which are absorbed and either contribute to human metabolism (such as amino acids, vitamins, and energy substrates) or are not considered metabolically important. Regardless of their diverse origin, metabolites can be broadly classified as being either endogenous (from directly regulated reactions) or xenobiotic (not directly involved in metabolic function). However, because of the various interactions from entities such as the gut microflora, intermediate categories of metabolites have been proposed (35). These intermediate classes of metabolites have been categorized as symendogenous compounds, symxenobiotic compounds, and transxenobiotic compounds. The microflora can change constituents in food and make them available to themselves or to the host for additional metabolism. For example, microbial enzymes hydrolyze soy isoflavones to release aglycons, daidzein, genistein, and glycitein. These compounds may be absorbed as such and contribute to the metabolome or may enter the microbial metabolome for conversion to the following other compounds: daidzein to equol or to *O*-desmethylangolensin and genistein to *p*-ethyl phenol (35). These in turn can then enter the host metabolome. Perhaps these less defined and facile reactions are partly responsible for idiosyncrasies that are observed in response to a diet. It has been proposed that regulated metabolic pathways do not truly exist for xenobiotics, and this can result in various metabolic fates or endpoints. Major metabolites stem from reactions that have a

high probability of occurring whereas micrometabolites stem from reactions that have a lower probability of occurring (35). Metabolomic studies in rat urine have shown very marked differences between rats with a germ-free status and rats with a conventional status (36). However, whereas large differences between the total absence of a gut microflora and its presence might be expected in urinary metabolomes, exactly how diet-related changes in the composition of the gut microflora of humans influence the metabolomic profiles of his different biofluids remains to be determined.

Which metabolome?

Having considered these various potential confounding factors in human nutrition metabolomics, the available biofluids and what role they might play in the field are worth considering. Blood, urine, and saliva are the most likely sources of biofluids for human metabolomics. Fecal water offers an opportunity to study gut microflora metabolomics but must be treated cautiously because this biofuluid cannot indicate the metabolites from the large-bowel microflora that are actually absorbed by the host. Obtaining other metabolomes (eg, cerebrospinal fluid, liver, gut, or muscle biopsy specimens) is more invasive, but we can anticipate the use of such tissues, as well as the use of cultured human cells such as peripheral blood mononuclear cells or fibroblasts, for metabolomic studies. Nonetheless, the 3 main biofluids that will probably be used in nutritional metabolomics are saliva, blood, and urine.

Saliva is not widely used in human nutrition research, but a case for its inclusion in nutritional metabolomics can be made. Saliva is a readily attainable biofluid that is rich in hormones such as 17-OH progesterone, testosterone, estradiol, and free cortisol (37). Its fatty acid composition has been used as a biomarker of plasma arachidonic acid (38), and it has been extensively studied for its antioxidant capacity (39). Although saliva has not been used in metabolomic studies, its potential for distinguishing between metabolic profiles and for monitoring changes in metabolic profiles induced by diet would be worth exploring. Both serum and plasma will undoubtedly be used for nutritional metabolomic analyses, but they will yield very different NMR and MS spectra because of the large number of small molecules that are released in the clotting process, which gives rise to serum. The nature of the anticoagulant used when the plasma samples are obtained may also have an effect on the metabolomic analysis.

A major difference between urine and plasma is the ratio of metabolites (signal) to nonmetabolites that are derived from plant food phytochemicals and chemicals that arise from cooking (noise); urine has a higher level of noise than does plasma. Blood is a rich source of nutrients and metabolites that are in transit from one organ to another. These metabolically active compounds are retained in blood as much as possible and only spill over into urine when their concentrations in plasma rise and exceed the relevant renal threshold. In contrast, the diet-derived nonnutrient compounds that are not involved in metabolism are rendered more polar to decrease their renal threshold, which favors their entry into urine. The major function of urine is to dispose of unwanted compounds in the body; consequently, the concentration of nonnutrient compounds is usually higher in urine than in plasma. In the study of the acute effects of onion ingestion on quercitin metabolism, 11 quercitin metabolites unique to urine were found, whereas only 5 quercitin metabolites were unique to plasma (28). Thus, if the objective is to study the direct effect of dietary intervention on the urinary metabolome, then a relative enrichment of urine in nonnutrient compounds represents an increase in noise. A second major difference is that lipid-soluble compounds can exist in plasma but not in urine. Urine, however, has become a major biofluid of choice in pharmacologic and toxicological metabolomics and, thus, is also likely to be of major importance for many nutritionists.

These examples point to the necessity of standardizing the application of metabolomics in nutrition studies, at least in terms of sample collection and preparation and of standardization of fluids, times, volumes, and processing aids. The use of databases for comparison of dietary or other treatment groups and the identification of discriminating metabolites makes sense only if certain minimal criteria are met for all elements of the data collection. Several initiatives are being undertaken to standardize approaches (40–42). Such standardization has been established for the application of metabolomics to plant sciences (43).

Adjusting metabolomic profiles for the experimental input

Toxicological and pharmacologic studies apply an external compound, drug, or toxin and then measure the effects on metabolomic profiles. However, the drug or chemical and their metabolites should not, as signals, be confused with the metabolic consequences of the signal and are normally deleted from the metabolic profile. From the limited number of animal studies that have used single nutrients as metabolic inputs (signals), such as ascorbic acid in metabolomics research, a similar approach of signal correction has been applied (44). Although the principle of correcting for the spectral effect of the test nutrient is possible for compounds such as vitamin C or folic acid, this correction will not be possible in nutritional studies that involve complex mixtures of nonnutrient small molecules. For example, a study of the differences between the effects of soy protein and the effects of cow milk protein will show very different urinary metabolomes, and the frequently used statistical techniques, which involve megavariate analyses, will show a significant separation of the 2 treatment groups. Will this difference be due to the metabolic consequences of differences in amino acid compositions, to differences in the metabolic effects of soy- or milk-derived peptides, or simply to the appearance of soy phytochemicals in biofluids?

Another example of the problems or challenges we face in nutrition is when removing the direct effect of the input is not feasible. The addition of fatty acids to a diet will lead to their incorporation into a metabolic pool of fixed size, such as in a pool of phospholipids, and will lead to the displacement of some fatty acids that are already present therein. In other instances, metabolic pools will resist change, eg, pools of ionizable calcium in plasma or pools of any mineral or trace elements in plasma. Finally, for complex dietary interventions, such as altering the intake of fruit and vegetables, the deletion of signal noise will be impossible with nonspecific techniques such as NMR and will only be possible with selective techniques such as MS.

Linking metabolomes with cell regulatory processes

The tendency exists to think that the connection of one gene to one transcript to one protein to specific metabolites can be universally applied and that through a systems biology approach, which integrates all connections, we will eventually obtain a

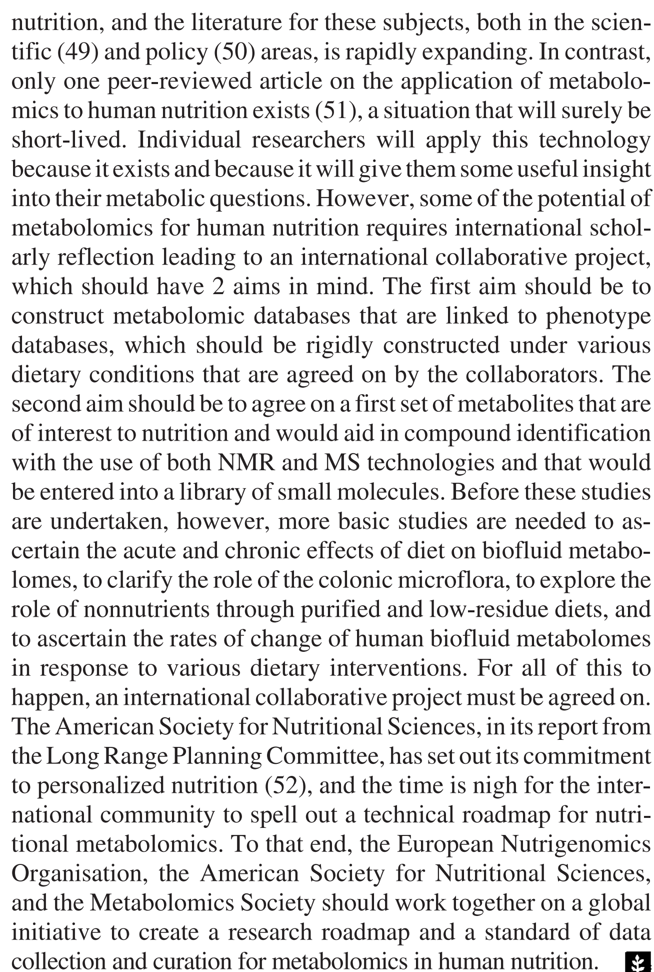
qualitative, quantitative, and probabilistic overview of biological processes. Metabolism, however, is dynamic, and measurements of the flux of metabolites through metabolic pools, perhaps for very narrow or focused metabolomes (eg, the folate metabolome), will somehow need to be measured with the use of stable isotopes (45–47). Even with a comprehensive set of transcriptomic and proteomic data with some elements of dynamic measures, linking metabolites back to proteins and genes will not be simple. Cells operate many sensory, regulatory, and compensatory systems that regulate the flux of metabolites through pathways without involving hormonal or endocrine signals, and although these pathways are known, the exact sensor remains unclear (48). AMP-activated protein kinase is uniquely sensitive to the ratio of AMP to ATP in cells, whereas amino acids are positive regulators of mammalian target of rapamycin kinase, which regulates cell size. Recently, a direct effect of metabolic cofactors on gene expression has been discovered, but this effect does not involve any of the normal signal transduction pathways (12). A series of metabolic-related enzymes, which are named metabolic transcription factors, act independently of their catalytic properties and in direct association with enzyme cofactors such as ATP, NAD, NADP, FAD, and *S*-adenosylmethionine and appear to be key in the regulation of gene expression. For example, *S*-adenosylmethionine in association with histone methyltransferases regulates histones, and arginine 82 requires ATP binding to modulate the arginine- and phosphate-responsive gene transcription factor. Clearly, metabolic function does not necessarily lead to gene expression through hormones or through signal transduction pathways, which is an important fact for systems biology.

Metabolomics—nutrition compared with pharmacology and toxicology

Experimental pharmacology and toxicology differ from human nutrition in 3 major respects with regard to metabolomics. First, much of the research in pharmacology and toxicology is conducted in laboratory animals that are genetically and nutritionally more homogeneous than are humans. Second, experiments in both pharmacology and toxicology involve the direct administration of a xenobiotic at a dose that is intended to have an effect on metabolism. Finally, major metabolic signals that act in concert on the pathologic regulation of the disease have a profound effect on the human metabolome and will affect the application of metabolomics in clinical medicine for the detection of diseases, such as cardiovascular disease or multiple sclerosis. Because of these differences, the signal-to-noise ratio will be higher in pharmacology and toxicology research than in human nutrition research. Thus, it is clear that, in human nutrition research, a great effort should be made to maximize the accuracy and precision of metabolite measurements to ensure that the data obtained maintain the biological information that underlies the phenotype variations of interest. The field will need this level of accuracy to understand the separate effects of drugs, food supplements, stress, physical activity, body composition, age, sex, colonic flora, and reproductive factors.

CONCLUSIONS

Nutrigenomics and nutrigenetics dominate the diet-gene and gene-diet responsiveness research in the field of personalized nutrition, and the literature for these subjects, both in the scientific (49) and policy (50) areas, is rapidly expanding. In contrast, only one peer-reviewed article on the application of metabolomics to human nutrition exists (51), a situation that will surely be short-lived. Individual researchers will apply this technology because it exists and because it will give them some useful insight into their metabolic questions. However, some of the potential of metabolomics for human nutrition requires international scholarly reflection leading to an international collaborative project, which should have 2 aims in mind. The first aim should be to construct metabolomic databases that are linked to phenotype databases, which should be rigidly constructed under various dietary conditions that are agreed on by the collaborators. The second aim should be to agree on a first set of metabolites that are of interest to nutrition and would aid in compound identification with the use of both NMR and MS technologies and that would be entered into a library of small molecules. Before these studies are undertaken, however, more basic studies are needed to ascertain the acute and chronic effects of diet on biofluid metabolomes, to clarify the role of the colonic microflora, to explore the role of nonnutrients through purified and low-residue diets, and to ascertain the rates of change of human biofluid metabolomes in response to various dietary interventions. For all of this to happen, an international collaborative project must be agreed on. The American Society for Nutritional Sciences, in its report from the Long Range Planning Committee, has set out its commitment to personalized nutrition (52), and the time is nigh for the international community to spell out a technical roadmap for nutritional metabolomics. To that end, the European Nutrigenomics Organisation, the American Society for Nutritional Sciences, and the Metabolomics Society should work together on a global initiative to create a research roadmap and a standard of data collection and curation for metabolomics in human nutrition.



MJG completed the literature research and prepared the first draft of the manuscript and all subsequent drafts after feedback from all other authors. None of the authors had any conflicts of interest.

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