Highlights of the 2012 Research Workshop : Using Nutrigenomics and Metabolomics in Clinical Nutrition Research

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Abstract

The American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.) Research Workshop, "Using Nutrigenomics and Metabolomics in Clinical Nutrition Research," was held on January 21, 2012, in Orlando, Florida. The conference brought together experts in human nutrition who use nutrigenomic and metabolomic methods to better understand metabolic individuality and nutrition effects on health. We are beginning to understand how genetic variation and epigenetic events alter requirements for and responses to foods in our diet (the field of nutrigenetics/nutrigenomics and epigenetics). At the same time, methods for profiling almost all of the products of metabolism in plasma, urine, and tissues (metabolomics) are being refined. The relationships between diet and nutrigenomic-metabolomic profiles, as well as between these profiles and health, are being elucidated, and this will dramatically alter clinical practice in nutrition. (*JPEN J Parenter Enteral Nutr*. XXXX;xx:xx-xx)

Keywords

Nutrigenetics; metabolomics; intervention

Clinical Relevancy Statement

Nutrigenomics and metabolomics provide methodology that allows clinicians to view a broader footprint of what is going on in metabolism than they can get using current clinical chemistry panels. Instead of having to assess sugar metabolism from a glucose and insulin measurement, by using metabolomic methods almost all of the metabolites of the pathways of energy metabolism can be assessed at a cost that is reasonable. This could greatly refine the practice of clinical nutrition. When a nutrition clinical trial is conducted, nutrigenomic methods can help investigators to understand why a subgroup of study subjects responded to treatment, while others did not. This could reduce the "noise" that often clouds such clinical studies.

Overview (Steven H. Zeisel)

The toolsets available that allow for detailed analysis of genetic variation and metabolic footprints can revolutionize the practice of clinical nutrition. Whereas now our approach is to design nutrition interventions based on how the average person will respond, we soon will be able to individualize these interventions based on a deeper understanding of individual differences in metabolism. The methods available in genetics and metabolomics are ready to use now, but the catalog of how these measurements can be used to predict responders and nonresponders to nutrition interventions is still very small and needs many more entries before the approaches will be clinically useful. Building this catalog is one of the most important tasks for nutrition scientists in the next five years.

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Nutrigenetics and Metabolomics Can Help Define Responders and Nonresponders in Clinical Nutrition Studies (Steven H. Zeisel)

One of the characteristics of nutrition research often is that there are significant variations in response to a nutrition intervention, and these result in large standard errors. This variance makes it harder to prove that the nutrition intervention had biological significant effects. One of the assumptions made by nutrition scientists is that people are metabolically similar; however, it is becoming apparent that this may not be the case and there is significant metabolic individuality. This, in part, underlies the recent interest in individualized nutrition. For the clinical nutrition researcher, it is important to realize that combining metabolic responders with nonresponders to an intervention is an important source of variance in the study data and may explain why studies sometimes result in divergent conclusions. If we could find biomarkers that identify the responders from the nonresponders, we could appropriately group study subjects, not combine them, and greatly reduce variance in data, thereby enhancing identification of biologically significant effects using smaller numbers of study subjects. It would be best to separate responders from nonresponders before a study is initiated; however, if the appropriate samples are collected during the study, it is possible to retrospectively analyze the data using appropriate grouping of subjects and thereby resuscitate an apparently failed clinical study.

Sources of Metabolic Variation

Metabolism can be modified by many factors, including diet, other environmental exposures, and drugs and body composition. Possibly the most important modifiers of metabolism are genetic and epigenetic.[2,3] Whatever the source of variation in metabolism, this variation should be detected by metabolomic profiling, which identifies a footprint of metabolism that is composed of the many small molecules generated by metabolic pathways. Thus, a clinical nutrition researcher should collect data on diet, exposures, and body composition, as well as collect samples of DNA for genetic analyses and of biological fluids for metabolomic profiling (usually plasma and/or urine are used, but any fluid can be analyzed as appropriate to the study problem). With the help of a bioinformatician, one can usually differentiate responders from nonresponders in a clinical study.

Metabolomic Profiling

In the old days, nutrition scientists measured a small set of targeted metabolites and extrapolated from these data to predict what was changing in metabolism. We often looked at what we knew best to find only what we expected to find—akin to looking under the streetlamp for lost keys. Modern technology makes it possible to measure hundreds of small molecules in a single sample (less than half a milliliter) of plasma or urine.[4] This untargeted metabolite profiling permits scientists to use many more data points in developing their estimate of changes in metabolism, and it permits them to look at pathways they never suspected would be changed by an intervention. There are 2 main platforms available for performing metabolic profiling, and most laboratories use one or the other. Nuclear magnetic resonance methods have the advantage that little sample processing is required, but sensitivity can be a problem. Molecules present in low concentrations are often not seen. Mass spectrometry methods require some form of separation of the metabolites using chromatography before entry into the mass spectrometer and are quite sensitive. Usually a metabolomic profiling platform will split the sample and pass it through a gas chromatography mass spectrometer and a liquid chromatography mass spectrometer because these 2 separation methods complement each other for isolating the widest variety of small molecules. Once the data are generated, rather complicated analyses need to be performed to interpret the data, and this is best done by a team that consists of a bioinformatics expert and a metabolism expert.

Nutrigenetic Profiling

The field of nutrigenetics/nutrigenomics is growing rapidly. For now, it is sensible to focus on 2 aspects that can be practically applied today in clinical nutrition research. Single-nucleotide polymorphisms (SNPs; stated simply, these are spelling errors in the genetic code) are very common, with more than 1 million identified.[5] It is estimated that every person has about 50,000 SNPs. Some portion of these alters the expression or function of genes at critical steps in metabolism, and these changes result in metabolic inefficiencies that underlie metabolic variation between people. Current commercial technology allows us to measure a million or more of these SNPs in a study subject, but this may not be wise unless you are studying tens of thousands of subjects.[1] This is because of biostatistical corrections that must be made when you make multiple comparisons. For clinically sized studies (tens to hundreds of subjects), it is better to preselect a small number of SNPs to be studied based on suspected underlying mechanisms.

It is now possible to measure epigenetic marks on genes.[6] These marks influence whether genes are expressed. When DNA cytosines are methylated, they attract capping proteins that block off the binding site for transcription factors, and this blocks gene expression. This inhibitory signal can be reinforced by epigenetic marks on the proteins around which DNA is coiled (histones) as well as by microRNAs that bind to the gene and prevent it from being transcribed. Soon we will understand enough about epigenetics to study how these marks contribute to metabolic variation in human clinical nutrition studies. The major problem impeding progress is that these marks differ tissue by tissue. Although SNPs are the same in all DNA, epigenetic marks on genes in peripheral white blood cells may not reflect the marks present in liver or brain or heart. It is a rare study that can obtain samples from such target tissues, and most have to extrapolate from blood sample data.

A second problem for epigenetics is that these marks may change during development, so a single measure in time may not fully explain metabolic variation.

An Example of the Utility of Nutrigenomics and Metabolomics in Clinical Nutrition Research

When the dietary requirement for choline in humans was studied, it was found that most men and postmenopausal women had to eat a diet containing choline or they developed liver and/or muscle damage that resolved when they were fed again with a choline-containing diet.[7] However, less than half of premenopausal women developed liver or muscle damage when deprived of choline.[7] This finding led to studies that determined that the pathway in liver for endogenous synthesis of phosphatidylcholine (a source of choline) is induced by estrogen; premenopausal women have extra capacity to make their own phosphatidylcholine and thus need to eat less choline in the diet.[8] Why, then, did almost half of premenopausal women still need to eat choline? They had SNPs in genes of choline and folate metabolism that made these metabolic pathways inefficient.[9,10] The most common SNP that made women require choline in the diet was an SNP in the gene responsible for endogenous synthesis of phosphatidylcholine (*PEMT*); women with this SNP could not turn this gene on with estrogen.[11] Thus, like men who have little estrogen, these women had to eat choline to prevent liver damage. More than 70% of women in North Carolina had 1 minor allele for this SNP, and approximately 20% had 2 minor alleles; the increase in relative risk for choline deficiency imparted by 2 copies of the minor allele was 24-fold.[9]

Thinking about the above experiment, it would have been quite a puzzle if the data from young women were combined and analyzed because there were really 2 groups of women, responders and nonresponders, to the diet restriction. When combined, there would be large standard deviations of the means, and many subjects would need to be studied to determine if there was a statistical difference between pre- and postmenopausal women and men in dietary requirements for choline. However, the ability to separate responders (those with the SNPs that increased the requirement for dietary choline) from nonresponders (those women who did not need to eat choline) markedly reduced data variance and made it easy to detect significant differences in a study of 80 subjects.

As noted above, these SNPs in genes of 1-carbon metabolism created metabolic inefficiencies that should be present even when a person is not challenged by being fed a low-choline diet. These inefficiencies should be detected by metabolomic profiling. In the above study on human choline requirements, plasma samples at baseline (before any diet manipulation) were subjected to metabolomic profiling, and it was possible to predict with high confidence which subjects would develop liver damage when deprived of choline.[12] Even when on a diet adequate in choline, these individuals had a group of metabolite changes caused by the SNPs in genes. In addition, metabolic profiling can be useful when studying the response to an intervention because it provides a picture of metabolism with a greater scope than is usually obtained by measuring a few targeted metabolites. In the choline study, after subjects were fed a low-choline diet, there were expected changes in choline metabolites in plasma (they dropped), but there were unexpected changes in other metabolites—for example, changes in acylcarnitines suggested that mitochondrial function was disturbed during choline deficiency.

Summary

In summary, nutrigenetic and metabolomic methodologies have great utility for scientists studying human nutrition. Every clinical study should plan to collect appropriate samples so that these methods can be employed to refine data analysis. This approach may reduce the "fuzziness" associated with human nutrition studies and may identify new mechanistic pathways because we are looking beyond the light shed by the streetlamp we were trained under.

Epigenetics—The Role of Early Diet in Shaping Our Gene Expression Potential (Robert A. Waterland)

Epigenetics is the study of mitotically heritable alterations in gene expression potential that are not caused by DNA sequence alterations.[13] By stably regulating gene expression potential in differentiated tissues, epigenetic mechanisms such as DNA methylation play a critical role in mammalian development. In the past decade, it has increasingly been recognized that dysregulation of epigenetic mechanisms may play an important role in human disease.[14,15] Indeed, nearly any disease with a genetic basis could also have an epigenetic basis. The inherent tissue specificity of epigenetic gene regulation, however, presents a major obstacle to an improved understanding of the epigenetic basis of human disease.[16] To determine if genetic variation is associated with a specific disease, any easily obtainable DNA sample—such as from peripheral blood—is sufficient since essentially all cells in the body contain the same DNA. If, on the other hand, one wishes to determine if epigenetic variation is associated with, say, Alzheimer disease or type 2 diabetes, epigenetic marks in peripheral blood DNA may be completely irrelevant.

Mouse studies of metastable epialleles (MEs) suggest the potential to bypass in some cases this obstacle of tissue specificity. At murine MEs, DNA methylation is established stochastically (ie, randomly), even among genetically identical individuals,[17] and interindividual epigenetic variation is influenced by maternal nutrition before and during pregnancy.[18-21] Moreover, interindividual epigenetic variation at MEs occurs systemically, affecting all tissues.[19,20] The best characterized

murine ME is the *agouti viable yellow* (A^{vy}) locus, which affects coat color and body weight regulation. Whereas 2 genetically identical A^{vy} heterozygous mice appear indistinguishable at birth, differences in DNA methylation at A^{vy} can subsequently cause one to become yellow, hyperphagic, and obese, whereas the other grows up with a normal brown coat and lean body type. One could take a few drops of blood from each of several newborn A^{vy} heterozygous mice, however, and by measuring DNA methylation at A^{vy} predict with absolute certainty which will become obese and which will be lean as adults. Hence, although the obesity of yellow A^{vy} mice is caused by dysregulated *agouti* expression in the hypothalamus,[22] the epigenetic lesion causing this misexpression is detectable in peripheral blood! The implications for human epigenetic epidemiology are obvious. Interindividual epigenetic variation at human MEs, detectable in peripheral blood DNA, may enable inferences about epigenetic dysregulation in internal organs and cell types of relevance to disease.

We performed a 2-tissue epigenomic screen to identify MEs in humans.[23] Using a DNA methylation microarray approach, we performed 4 interindividual comparisons, in each case comparing the same 2 healthy white adults using both peripheral blood and hair follicle DNA (representing the mesodermal and ectodermal lineages of the early embryo, respectively). Gene regions that exhibited concordant interindividual variation in both tissues were candidate MEs. Candidates were validated by testing for interindividual variation in DNA methylation in 3 tissues representing all 3 germ layers of the early embryo (liver, kidney, and brain) in postmortem samples from Vietnamese accident victims. Moreover, several of the loci demonstrated substantial interindividual epigenetic variation among monozygotic twins, providing further evidence that this epigenetic variation occurs stochastically.

Seasonal variation in maternal dietary intake and nutrition status in rural Gambia, West Africa,[24] provided a "natural experiment" by which to test whether the establishment of epigenotype at these loci is affected by maternal nutrition. In collaboration with Andrew Prentice and colleagues at the London School of Hygiene and Tropical Medicine, we obtained peripheral blood DNA samples from children (average age 9 years) who were conceived during either the rainy or the dry season in West Kiang, The Gambia (n = 25 per season). On the basis of the mouse studies, we anticipated that individuals conceived during the nutritionally challenged rainy season—when villagers are running low on staple foods from the previous crop—would have lower DNA methylation at the MEs. We found just the opposite. At all 5 putative MEs tested, DNA methylation was significantly higher in children conceived during the rainy season.[23] (This seemingly contradictory result may be due to the high availability of folate-rich leafy vegetables during the rainy season.[25])

In summary, our results show that epigenetic metastability does occur in humans. At select human genomic loci, establishment of systemic interindividual epigenetic variation occurs stochastically and is influenced by maternal nutrition before and during pregnancy. We anticipate that the identification of more such loci will highlight excellent candidate genes at which to study associations among early nutrition, epigenetic regulation, and human disease.

Nutrition Genomics: The Case for Heart Disease (José M. Ordovás)

The progress of genomics, fostered by the Human Genome Project, has been spectacular. In just 1 decade, we have gone from having a rough draft of the human genome to being close to achieving the "$1000 genome." The ability to conduct genome-wide association studies (GWAS) using denser gene arrays has made it feasible to conduct comprehensive genomic analysis in hundreds of thousands of individuals and the identification of hundreds of new loci associated with most common genetic disorders. Nevertheless, the translation of these findings into practical applications is still lacking. Moreover, most of the genetic variability attributed to cardiovascular disease (CVD)–related risk factors remains unaccounted, suggesting that additional genetic variants and genetic mechanisms need to be identified. Some have proposed that the "missing heritability" will be found buried in the epigenome or by conducting whole-genome sequencing and identifying a myriad of new rare mutations associated with the phenotypes of interest.[26] Another plausible explanation for this apparent "missing heritability" may be the presence of gene-by-environment interactions.[27] Among the most relevant to health may be those resulting from gene-diet interactions that, as highlighted earlier (in the Overview by Steve Zeisel), once properly identified and characterized, could dramatically alter the translation of nutrition research into clinical practice.

One of the traditional contributions of nutrition research to public health has consisted of the definition of optimal dietary recommendations aimed to prevent disease and promote optimal health. For this purpose, and based on the best scientific evidence available at each time in history, several dietary guidelines have been implemented to improve the health of the general population and of those at high risk for specific diseases. However, past and current dietary guidelines have been based for the most part on observational epidemiological evidence and have not considered the dramatic differences in the individual's response to changes in nutrient intake (see "Nutrigenetics and Metabolomics Can Help Define Responders and Nonresponders in Clinical Nutrition Studies," by Steven Zeisel). These differences in response may greatly affect the efficacy of these recommendations at the individual level.

The mechanisms responsible for the interindividual differences in response to intervention, and particularly dietary response, are far from being fully understood.[28] Nevertheless, although the presence of a genetic component has been proposed for several decades, only recently have researchers begun to examine these nutrient-gene interactions at the molecular level.

The Application of Nutrigenomics: Understanding Interindividual Differences in CVD Risk Factors in Response to Diet

The different response of traditional CVD risk factors to diet, depending on the particular characteristics of an individual, is not a new observation but has already been widely observed and documented for decades. Having admitted that each individual may respond differently to the same diet, it becomes crucial to identify the factors defining such differential response.

The traditional approach to the identification of genetic factors implicated in differential dietary response has been based on the candidate gene approach. More recently, with the ability to agnostically interrogate the entire genome, we can begin to identify unsuspected genes and biological pathways. However, a major barrier to the progress of nutrigenomics toward the personalization of recommendations for CVD prevention has been the lack of consistency of the reported interactions across populations and studies.[29] Nevertheless, some cases can be used as proof of concept for this approach. The one summarized below demonstrates a significant interaction between a functional polymorphism in the apolipoprotein A2 locus (*APOA2*), dietary saturated fat (SFA), and obesity risk.

Apolipoprotein A-II (APOA2) plays an ambiguous role in lipid metabolism, obesity, and atherosclerosis. Initially, we studied the association between a functional APOA2 promoter polymorphism (-265T>C) and plasma lipids (fasting and postprandial), anthropometric variables, and food intake in 514 men and 564 women who participated in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study.[30] We obtained fasting and postprandial (after consuming a high-fat meal) measures, including lipoprotein particle concentrations by proton nuclear magnetic resonance (NMR) spectroscopy and estimated dietary intake by use of a validated questionnaire.

We found recessive effects for this SNP that were homogeneous by sex. Thus, -265C/C subjects had statistically higher body mass index (BMI) than did carriers of the T allele, and their odds ratio for obesity was 1.70 ($P = .039$). This could be explained in part by the fact that C/C individuals had a total energy intake that was statistically higher (mean [SE] 9371 [497] vs 8456 [413] kJ/d, $P = .005$) than in T allele carriers. This association remained statistically significant even after adjustment for BMI. Moreover, we found no associations with fasting lipids and only some associations with high-density lipoprotein (HDL) subfraction distribution in the postprandial state. Therefore, this initial analysis demonstrated that the -265T>C SNP was consistently associated with food consumption and obesity, suggesting a new role for APOA2 in regulating dietary intake.

Next, we investigated the potential interaction among the APOA2 -265T>C SNP, food intake, and BMI. For this purpose, we carried out cross-sectional, follow-up (20 years), and case-control analyses in 3 independent populations.[31] We analyzed gene-diet interactions between the APOA2 -265T>C SNP and SFA intake on BMI and obesity in 3462 individuals from 3 populations in the United States: the Framingham Offspring Study (1454 whites), the GOLDN Study (1078 whites), and Boston–Puerto Rican Centers on Population Health and Health Disparities Study (930 Hispanics of Caribbean origin).

The prevalence of the C/C genotype in study participants ranged from 10.5%–16.2%. Most interesting, we identified statistically significant interactions between the APOA2 -265T>C SNP and SFA regarding BMI in all 3 populations. Thus, the magnitude of the difference in BMI between the individuals with the C/C and T/T + T/C genotypes differed by SFA. A mean increase in BMI of 6.2% ($P = .01$) was observed between genotypes with high (≥22 g/d) but not with low SFA intake in all studies. Likewise, the C/C genotype was significantly associated with higher obesity prevalence in all populations only in the high-SFA stratum. Meta-analysis estimations of obesity for individuals with the C/C genotype compared with the T/T + T/C genotype were an odds ratio of 1.84 ($P < .001$) in the high-SFA stratum, but no association was detected in the low-SFA stratum (odds ratio, 0.81; $P = .18$). Therefore, we identified a gene-diet interaction influencing BMI and obesity that was strongly replicated in 3 independent populations.

To increase the level of evidence, we extended our findings to European and Asian populations.[32] For this purpose, we did a cross-sectional study in 4602 subjects from 2 independent populations: a high-CVD risk Mediterranean population (n = 907 men and women; aged 67 ± 6 years) and a multiethnic Asian population (n = 2506 Chinese, n = 605 Malays, and n = 494 Asian Indians; aged 39 ± 12 years) participating in a Singapore National Health Survey. In this case, the frequency of C/C subjects differed among populations (1%–15%). Most important, we confirmed the recessive effect of the APOA2 SNP and replicated the APOA2-SFA interaction on body weight. In Mediterranean individuals, the C/C genotype was associated with a 6.8% greater BMI in those consuming a high ($P = .018$) but not a low ($P = .316$) SFA diet. Likewise, the C/C genotype was significantly associated with higher obesity prevalence in Chinese and Asian Indians only, with a high-SFA intake ($P = .036$). We also found a significant APOA2-SFA interaction in determining insulin resistance in Chinese and Asian Indians ($P = .026$). Therefore, the influence of the APOA2 -265T>C SNP on body weight–related measures was also modulated by SFA in these Mediterranean and Asian populations.

Furthermore, we investigated some of the behavioral and hormonal mechanisms underlying our previous findings.[33] For this purpose, we evaluated relationships between APOA2 and obesity risk with particular focus on patterns of eating and ghrelin. The design was cross-sectional, and we investigated overweight and obese subjects (n = 1225) in 5 weight loss clinics in southeastern Spain. Our data show that C/C subjects were more likely to exhibit behaviors that impede

weight loss and less likely to exhibit protective behaviors. Plasma ghrelin for C/C subjects consuming low SFA was lower compared with (1) C/C subjects consuming high SFA, (2) T/T + T/C carriers consuming low SFA, and (3) T/T + T/C carriers consuming high SFA (all $P < .05$). In summary, the APOA2 -265 T/C SNP was associated with eating behaviors and dietary modulation of plasma ghrelin, providing some mechanistic basis for our previous findings.

An example of the use of the more agnostic knowledge generated by GWAS in combination with the large sample size available from consortia is presented next. The focus of this work was the interaction between genetic variants associated with glucose homeostasis and type 2 diabetes and zinc intake. Zinc is an essential micronutrient that is important for β-cell function and glucose homeostasis. Therefore, we tested the hypothesis that zinc intake could influence the glucose-raising effect of specific variants.[34] For this purpose, we conducted a 14-cohort meta-analysis to assess the interaction of 20 genetic variants known to be related to glycemic traits and zinc metabolism with dietary zinc intake (food sources) and a 5-cohort meta-analysis to assess the interaction with total zinc intake (food sources and supplements) on fasting glucose levels among individuals of European ancestry without diabetes.

Our analyses revealed a significant association of total zinc intake with lower fasting glucose levels (β-coefficient ± SE per 1 mg/d of zinc intake: –0.0012 ± 0.0003 mmol/L, summary P value = .0003), whereas the association of dietary zinc intake was not significant. Moreover, we identified a nominally significant interaction between total zinc intake and the SLC30A8 rs11558471 variant on fasting glucose levels (β-coefficient ± SE per A allele for 1 mg/d of greater total zinc intake: –0.0017 ± 0.0006 mmol/L, summary interaction P value = .005). This suggests a stronger inverse association between total zinc intake and fasting glucose in individuals carrying the glucose-raising A allele compared with individuals who do not carry it. Therefore, our results suggest that higher total zinc intake may attenuate the glucose-raising effect of the rs11558471 SLC30A8 (zinc transporter) variant. Our findings also support evidence for the association of higher total zinc intake with lower fasting glucose levels.

These examples provide some proof of concept about the potential use of gene-diet interactions to identify individuals with differential response to dietary factors. However, in practice, we need to provide a more complete and clinically relevant picture. Most important, we still need to demonstrate whether personal risk information will trigger changes toward a healthier diet considering that most people tend to connect healthy eating with eating less and with less pleasurable food.[35] Therefore, we need evidence-based data to ensure that the knowledge generated by nutrigenetic science is properly implemented and scrutinized. Furthermore, as nutrition becomes increasingly integrated with preventive medicine, it is essential that dieticians and medical practitioners as well as geneticists are properly educated in the field of nutrigenetics/nutrigenomics. Therefore, it is essential to prove the initial hypothesis that individual approaches can improve public health better than global recommendations, and this benefit is available to the entire population and not only restricted to those with higher socioeconomic status and education.[36]

Comprehensive Metabolic Profiling Links Muscle Insulin Resistance to Carnitine Imbalance (Deborah M. Muoio)

In metabolic diseases such obesity and diabetes, skeletal muscle fails to respond appropriately to the master counter-regulatory hormone, insulin, resulting in impaired glucose disposal after a meal. The onset of this "insulin-resistant" condition is intimately associated with generalized increases in adiposity as well as ectopic lipid deposition within the muscle and other nonadipose tissues.[37] A major quest in this field has been to identify specific lipid molecules that universally discriminate insulin responsive vs resistant states. To this end, our laboratory has employed a targeted metabolomics approach to survey several 2-state models of insulin sensitivity. The methods used focused on quantifying approximately 150 intermediary metabolites measured in serum, urine, and tissue extracts.[38] Results of these analyses identified a subclass of lipid-derived metabolites, known as the acylcarnitines, that correlated negatively with glucose tolerance.[39,40] Most of the even chain acylcarnitines are formed as metabolic by-products of incomplete fatty acid β-oxidation and are derived from their respective acyl-CoA intermediates by a family of carnitine acyltransferases that reside principally in mitochondria. Our interpretation of the muscle acylcarnitine profiles was informed by experiments in which substrate flux, mitochondrial function, and metabolic capacity were assessed by several complementary methods. In aggregate, the findings established a strong connection between mitochondrial bioenergetics and insulin action while raising new questions regarding the roles of incomplete β-oxidation and acylcarnitines as potential biomarkers and/or mediators of metabolic disease.

The observation that tissue acylcarnitines accumulate in several instances of insulin resistance tempts speculation that these metabolites might act as "lipotoxic" culprits. However, this suggestion is at odds with evidence that production of carnitine conjugates helps to avert mitochondrial dysfunction, owing at least in part to regeneration of free CoA.[41,42] Presuming that acylcarnitine production and efflux actually benefit mitochondrial function, we considered the possibility that prolonged exposure to lipid stress disrupts this defense mechanism by compromising carnitine availability. Consistent with this prediction, we uncovered a recurrent signature of carnitine diminution in multiple rodent models of overnutri-

tion, metabolic disease, and aging.[42,43] The decline in free carnitine in obese and/or aged rodents was associated with increased whole body fat oxidation, muscle accumulation of long-chain acylcarnitines, a corresponding fall in short-chain acylcarnitine species, and impaired substrate switching from fatty acid to pyruvate when assessed in isolated mitochondria. Subsequent studies showed that obesity-related derangements in mitochondrial fuel selection were reversed by dietary supplementation with L-carnitine, in parallel with improved glucose tolerance and insulin responsiveness.[42-44] Interestingly, the antidiabetic effects of L-carnitine were accompanied by a shift in whole-body fuel preference toward glucose oxidation[8]—a surprising result given the prominent role of this nutrient in permitting mitochondrial import and β-oxidation of long-chain acyl-CoAs.

The initial step in fat oxidation is executed by carnitine palmitoyltransferase 1 (CPT1), which catalyzes the reversible transesterification of long-chain acyl-CoA with carnitine. The long-chain acylcarnitine product of CPT1 traverses the inner mitochondrial membrane via carnitine/acylcarnitine translocase (CACT) and is then delivered to CPT2, which regenerates acyl-CoA on the matrix side of the membrane where β-oxidation occurs. Importantly, however, in addition to its requisite role in fatty acid oxidation, carnitine also facilitates mitochondrial efflux of excess carbon fuels. Thus, in the event that rates of substrate catabolism exceed energy demand, accumulating acyl-CoA intermediates are converted back to their membrane-permeant acylcarnitine counterparts, which readily exit the organelle and tissue. Fitting with the latter function, carnitine-supplemented rodents had robust increases in tissue efflux and urinary excretion of acetylcarnitine.[42,43] This specific metabolite derives from acetyl-CoA via the action of carnitine acetyltransferase (CrAT), a mitochondrial matrix enzyme that converts short-chain CoA species to their corresponding acylcarnitine esters. Acetyl-CoA holds a prominent position in intermediary metabolism as the universal end product of fatty acid, glucose, and amino acid oxidation. As its major metabolic fate, acetyl-CoA typically enters the tricarboxylic acid (TCA) cycle, where it drives production of reducing equivalents that in turn fuel adenosine triphosphate (ATP) synthesis by the electron transport chain. During conditions of lipid surplus, a rise in the mitochondrial pool of acetyl-CoA results in feedback inhibition of pyruvate dehydrogenase (PDH), the enzyme complex that connects glycolysis to glucose oxidation.[45] We therefore surmised that increased flux through the CrAT reaction might serve to mitigate lipid-induced suppression of PDH. Direct experimental evidence that CrAT activity can indeed affect glucose homeostasis came from studies wherein the metabolic consequences of overexpressing the enzyme were examined in primary human skeletal myocytes. As predicted, the resulting enhancement of acetylcarnitine production and efflux increased cellular PDH activity, glucose oxidation, and glucose uptake,[43] thus mimicking the therapeutic actions of L-carnitine supplementation. Our current working model suggests that carnitine buffers intramitochondrial imbalances between acyl-CoA load and TCA cycle flux, thereby affording protection against nutrient-induced mitochondrial stress.

In summary, targeted metabolomics analyses have pointed to a heretofore unappreciated role for carnitine and CrAT in regulating skeletal muscle glucose disposal, and this interpretation was subsequently corroborated by direct experimental evidence from animal and cell-based models. Ongoing investigations are now testing the antidiabetic potential of L-carnitine therapy in human subjects with impaired glucose tolerance. Taken together, this work provides an example of how metabolomics approaches can be used to identify potential sites of metabolic dysfunction while also serving as a guide for more traditional, hypothesis-driven nutrition research.

Metabolomic Profiling in Patients With Diabetes (Wei Jia)

Metabolomics measures metabolic phenotypes that are the net result of genomic, transcriptomic, and proteomic variability, therefore providing the most integrated profile of biological status. The pathological development and the drug intervention of diabetes mellitus (DM) involve altered expression of downstream low molecular weight metabolites, including lipids and amino acids, and carbohydrates such as glucose. Currently, a small number of markers used for clinical assessment of type 1 and 2 DM treatment may be insufficient to reflect global variations in pathophysiology.

Metabolomic biomarker discovery is a young research area that carries great hopes for both medicine and the nutrition sciences, particularly for the early detection of well-characterized metabolic disorders such as DM. The principal concept of metabolomic biomarker research is to identify key metabolites (other than glucose) differing in a control and a diabetic group, with diagnostic or prognostic abilities.

In this workshop, we would like to discuss the clinical application of metabolomics by means of 2 metabolomic studies of diabetes conducted by our group. Recently, we performed a metabolomics study on a new phenotype of DM, fulminant type 1 diabetes mellitus (T1DM). Fulminant T1DM (FT1DM), newly discovered as a subtype of T1DM (first reported in 2000), is defined as the acute destruction of pancreatic β-cells as well as α-cells, leading to extremely rapid progression of hyperglycemia and ketoacidosis.[46] The prevalence of FT1DM was estimated to be 8.9% in all patients with T1DM and 0.2% in all patients with newly diagnosed diabetes.[47] Although there is no report on the prevalence of fulminant T1DM in China, the number may be comparable given a similar genetic background and lifestyles between the 2 East Asian populations.

The rapid progression of hyperglycemia and ketoacidosis of fulminant T1DM leads to almost total destruction of β-cells within a few days (typically less than 1 week) and thus a high death rate if appropriate therapies are not in place. To date, the pathogenesis of this disease has not been established. In addition, there is no early detection method for such a rapidly progressing disorder.

Subjects

Four groups of age-matched human subjects, healthy controls (n=20), patients with T1DM (n = 6 + 20, with and without ketoacidosis), patients with type 2 DM (T2DM; n = 20), and patients with FT1DM (n = 6) were recruited by the Shanghai 6th People's Hospital affiliated with Shanghai Jiao Tong University. Only male subjects were selected in the current study. The diagnostic criteria for FT1DM were based on published literature[48] and included all of the following: (1) occurrence of diabetic ketosis or ketoacidosis within 7 days after the onset of hyperglycemic symptoms (elevation of urinary and/or serum ketone bodies at first visit), (2) plasma glucose ≥16.0 mM and glycated hemoglobin level (HbA_{1c}) <8.5% at first visit, and (3) fasting serum C-peptide level <0.3 ng/mL (<0.10 nmol/L) and <0.5 ng/mL (<0.17 nmol/L) after intravenous (IV) glucagon (or after meal) load at onset. Venous blood samples were taken from individuals after overnight fasting for at least 10 hours, and the serum samples were obtained in the normal manner. Aliquots of serum samples were stored at –80°C until metabolic analysis was performed.

Metabolomics Profiling and Data Analysis

Serum samples were prepared, chemically derivatized, and measured with a LECO's gas chromatography time-of-flight mass spectrometry (GC-TOFMS; LECO Corporation, St Joseph, MI), following our published protocols.[49] GC/MS data files were pretreated as previously described.[50] The mean-centered and autoscaled data were then introduced into the SIMCA-P 11.5 Software (Umetrics, Umeå, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) was used to obtain an overview of metabolic variations among the different groups. Orthogonal projections to latent structures discriminant analysis (OPLS-DA), a supervised pattern recognition approach, was used to construct a predictive model to identify key metabolites that differentially expressed each disease phenotype.

Results

FT1DM patients exhibited a distinct metabolic profile in the scores plots compared with healthy controls and patients with T1DM and T2DM, indicating a robust identification through serum metabolite expression levels. A panel of serum metabolites that are differentially expressed in FT1DM was identified as potential diagnostic markers for FT1DM. The differential metabolites between FT1DM and classic T1DM (with and without ketoacidosis) mostly overlapped with those derived from comparison between FT1DM and healthy controls, which include significantly altered ketone bodies and free fatty acids. These metabolites reflect the perturbed metabolism of ketone bodies and fatty acids under the condition of insulin deficiency. Only those differential metabolites that are distinct in FT1DM were chosen as potential biomarkers.

Conclusion

The study demonstrated that metabolomics profiling can contribute to the development of a panel of biomarkers for more sophisticated classification of the diabetic diseases, which may ultimately serve as an early diagnostic approach for FT1DM.

In the second example, a metabolomic study was performed to determine metabolic variations associated with T2DM and the drug treatments on 74 patients who were newly diagnosed with T2DM and received a 48-week treatment of a single drug: repaglinide, metformin, or rosiglitazone.[49] Fasting overnight and 2-hour postprandial blood serum of patients was collected at 24 and 48 weeks to monitor the biochemical indices (FPG, 2hPG, HbA_{1c}, etc). GC/MS coupled with multivariate statistical analysis was used to identify the alteration of global serum metabolites associated with T2DM as compared with healthy controls and responses to drug treatment. Significantly altered serum metabolites in patients with diabetes include increased valine, maltose, glutamate, urate, butanoate, and long-chain fatty acids (C16:0, C18:1, C18:0, octadecanoate, and arachidonate) and decreased glucuronolactone, lysine, and lactate. All 3 treatments were able to downregulate the high level of glutamate to a lower level in serum of patients with T2DM, but rosiglitazone treatment was able to reverse more abnormal levels of metabolites, such as valine, lysine, glucuronolactone, C16:0, C18:1, urate, and octadecanoate, suggesting that it is more efficient to alter the metabolism of patients with T2DM than the other 2 drugs.

Linking the Gut Microbial Community to Disease States in Humans (Anthony Fodor)

There are on the order of 10 times more microbial cells in the human body than human cells.[51] Within the genomes of those microbial cells, there are on the order of 100 times more genes than genes encoded by the human genome. The metabolomic capacity of the microbial community is thought to substantially exceed that of the liver.[52] Determining how the microbial community affects human phenotypes will require the execution of metagenome association studies in which next-generation sequencing is used to link the state of the microbial community to human health and disease phenotypes. To understand the extent of normal variation in the microbial community, the Human Microbiome Project (HMP) is performing a survey of 18 tissue types in over 200 healthy individuals.[53] An initial analysis of this healthy cohort is encouraging in suggesting that the complexity of the microbial community is not infinite and therefore that metagenome association studies may be tractable. For ~5000 samples within the

HMP cohort, 454 sequencing technology targeting the V3–V5 region of the 16S rRNA gene was used to generate more than 30,000,000 sequences.[54,55] If we cluster these sequences into operational taxonomic units (or OTUs, defined as groups of sequences with an average percent identity of 97%) with a clustering strategy[56] that requires that an OTU consist of sequences that are seen often enough that they can form consensus sequences, we find that a very modest number of V3–V5 OTUs (695) can account for nearly all (~98%) of the V3–V5 sequences in the HMP cohort.[57] Moreover, when we compare the consensus sequences that represent these 695 OTUs with an existing database of full-length sequences, nearly all of them have a very high-quality match (>97% identity).[57] Taken together, these data suggest that, at least viewing the microbial community through the lens of taxa defined by 97% average sequence identity, the complexity of the human-associated microbiome is not infinite but rather consists of a small number of taxa that have been seen in multiple cohorts. In a metagenome association study, each taxa will be considered in a null hypothesis that the taxa is not associated with phenotypes of interest. If there were vast numbers of taxa, we would need vast sample sizes to evaluate large numbers of null hypotheses. The relatively modest number of taxa discovered within the more than 5000 V3–V5 samples within the HMP suggests that, by contrast, modest sample sizes will be required to see significant effects. Moreover, since nearly all of the V3–V5 OTUs have been previously observed in other studies, we can have some confidence that the results of a metagenome association study performed on one cohort may be informative for other cohorts.

Although there appear to be only a modest number of taxa in the nonrare biosphere, there is still tremendous individual variation within these taxa.[54] Nearly all V3–V5 OTUs within the HMP cohort show 2 to 3 orders of magnitude in variation in different people.[58] That is, in samples from some people, a particular OTU may represent nearly all of the sequences in the sample, whereas in other people, that OTU may represent <0.01% of the sequences or be completely absent.[58] This variation is seen in all tissue types with both V1–V3 and V3–V5 primers.[58] These data tell us that although the number of "parts" that make up the microbiome is limited, these "parts" exist at very different levels of abundance in different people. This is consistent with the observation that each person has a distinct microbiome that is stable over time.[59,60] This individual variation provides a great challenge to metagenomic association studies: it is an open question as to whether the high degree of individual variation will confound attempts to link individual or sets of taxa to health and disease phenotypes across multiple subjects.

One intriguing idea that has recently been introduced in an attempt to reduce the complexity of individual variation in the gut microbial community is the concept of enterotypes.[61] Arumugam et al[61] argue that despite individual variation, people can be clustered into 2 or 3 distinct types based on their gut community. The enterotype concept is attractive because it offers a substantial simplification of metagenome association studies; if subjects can be easily classified into enterotypes, hypothesis testing can proceed based on which enterotype classification subjects were assigned rather than on other more complex and more variable characteristics of the microbial community. In the HMP cohort, there was some support for the enterotypes concept when classifying gut taxa at the genus level but not at the more detailed OTU level,[58] suggesting that individual variation at the subgenus levels could potentially confound enterotypes. The appropriate level of taxonomic resolution for metagenome association studies remains an open question,[62] and it will be interesting to see if future metagenomic association studies have more success when working at more or less refined levels of the taxonomic tree.

Although the technologies that enable metagenomic association studies are still undergoing rapid change, metagenomic association studies with modest samples sizes are finding some success in establishing statistically significant associations. For example, a recent study showed several taxa that were associated with the tendency of subjects to develop fatty liver on a low-choline diet.[59] When this taxanomic information was combined with host SNP information for a gene involved in choline synthesis, a simple model could be established that was nearly perfectly correlated with the degree of fatty liver observed in response to a low-choline diet.[59] Although this sort of successful metagenomic-associated study is encouraging, it remains to be seen how reproducible such observations will be across multiple cohorts. Next-generation sequencing technology is continuing to rapidly evolve, and this will make obtaining metagenomic profiles both faster and less expensive, reducing the barriers to achieving substantial sample sizes on multiple future cohorts. In particular, the increasing read-length of the Illumina platform is making it possible to achieve great sequencing depth on 16S rRNA gene-based community profiling for only a few dollars a sample.[63] As sequencing costs continue to decline and bioinformatics pipelines become more refined, we are moving toward a future where a personalized view of each individual's microbiome may become part of the individual genetic background that drives personalized medicine.

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