Cell Metabolism
Review

Metabolomics and Metabolic Diseases: Where Do We Stand?

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Metabolomics, or the comprehensive profiling of small molecule metabolites in cells, tissues, or whole organisms, has undergone a rapid technological evolution in the past two decades. These advances have led to the application of metabolomics to defining predictive biomarkers for incident cardiometabolic diseases and, increasingly, as a blueprint for understanding those diseases' pathophysiologic mechanisms. Progress in this area and challenges for the future are reviewed here.

Introduction

The term "metabolomics" emerged at the dawn of the third millennium to describe attempts to measure all of the small molecule metabolites in a biological system, or at least a large number of metabolites at one time. In reality, metabolomics is nothing more than analytical chemistry, which of course has a much longer history. The advent of metabolomics has been fueled by major improvements in instrument technology, most notably in the sensitivity and mass range of mass spectrometers and associated gas and liquid chromatography techniques. Today, the most advanced systems deployed in a nontargeted mode (sometimes referred to as "shotgun" metabolomics) are able to detect up to 10,000 independent spectral features in a single biological specimen (Zamboni et al., 2015; Patti et al., 2012; Jin et al., 2016). However, in the best of such studies, only about one-third of the detected peaks can be linked to a specific chemical structure in an unambigious fashion, and only after many weeks of work. Nontargeted metabolomics is typically used to compare two biological conditions, e.g., drug-treated or genetically engineered cells compared to control cells. As such, nontargeted metabolomics is best suited as a discovery tool for identifying metabolites that change in response to manipulation of a biological system (relative concentration) rather than providing the exact concentration of a known metabolite (real concentration). Targeted metabolomics, which focuses on measurement of known metabolites in clusters with similar chemical structures (e.g., amino acids, acylcarnitines, organic acids, etc.) is a more quantitative tool, as it often involves the use of stable isotope-labeled metabolites (usually ^{2}H or ^{13}C labeled) as internal standards to allow the quantity of a targeted analyte to be deduced by the ratio of its peak area to that of the labeled standard added at a known concentration (referred to as isotope dilution mass spectrometry; Newgard et al., 2009; Ferrara et al., 2008; Bain et al., 2009). The evolution of targeted and nontargeted methods for static profiling of metabolites (referring to measurement of metabolite levels at one specific time point) has been complemented by advances in metabolic flux analysis, in which heavy atoms from stable isotope-labeled substrates are detected as they label downstream metabolic products in experiments involving multiple time points (Zamboni et al., 2015; Buescher et al., 2015; Fan et al., 2014; Alves et al., 2015). When used together, these assembled tools provide a deep and dynamic view of metabolic functions of cells, tissues/organs, and even whole animals and humans.

But the purpose of this article is not to review metabolomics technologies. Several reviews describing key technological advances in this domain have appeared recently and should be considered by interested readers (Zamboni et al., 2015; Patti et al., 2012; Bain et al., 2009; Buescher et al., 2015; Alves et al., 2015). Instead, this piece attempts to review the application of metabolomics to prominent metabolic diseases and conditions—particularly obesity, diabetes, and cardiovascular diseases—from the perspective of the physiologist, physician, or biologist, rather than from that of the analytical biochemist or technologist. In that context, the piece seeks to answer a specific question: In the past 15 years, what has metabolomics contributed to our understanding of complex cardiometabolic diseases, either in terms of our ability to detect and diagnose these conditions or via new insights into disease mechanisms?

Understanding Metabolic Diseases—Why Metabolomics?

Modern human society is encumbered with a pandemic of chronic diseases and conditions in which metabolic dysregulation plays a key role in pathogenesis and progression, including obesity, diabetes, and cardiovascular disease. Increasingly, dysregulated metabolism is also being recognized as a major contributor to diseases not traditionally considered as "metabolic" in origin, such as cancer, cognitive disorders, and respiratory pathologies. The successful sequencing of the human genome seemed to herald a new age of personalized medicine, in which genomic variation would be used to predict the impact of specific therapeutic interventions, leading to optimal management of disease in a given individual. However, many of our most prevalent chronic diseases are polygenic, including diabetes and cardiovascular diseases, and approaches such as genome-wide association studies (GWASs) have thus far explained only a small fraction of these diseases and made modest contributions to mechanism-based intervention strategies (O'Rahilly, 2009; Newgard and Attie, 2010). Moreover, better methods must be

developed to probe the interaction of genetics with environmental factors such as diet, the gut microbiome, and physical activity.

Comprehensive metabolite profiling, or metabolomics, defines the chemical phenotype of human subjects and animal models; as such, it has unique potential for defining biomarkers that predict disease incidence, severity, and progression, and for casting new light on underlying mechanistic abnormalities. Advantages of metabolomics relative to other ''-omics'' technologies include the following: (1) It has been estimated that humans contain about 6,500 discrete small molecule metabolites (Wishart et al., 2013), although new and more sensitive measurement technologies are gradually revealing a larger number of chemical species over time (Zamboni et al., 2015). Nevertheless, the number of metabolites is likely to remain smaller than the estimated 25,000 genes, 100,000 transcripts, and 1,000,000 proteins found in humans. (2) Metabolomics measures chemical phenotypes that are downstream of genomic, transcriptomic, and proteomic variability, thus providing a highly integrated profile of biological status. (3) Metabolomics also serves as a precise and noninvasive tool to discern mechanisms of action and possible toxicological effects of drug therapies, and to separate contributions of genetics, microbiome activity, and nutrition to overall metabolic phenotypes.

Metabolomics Reveals Associations of Metabolites with Cardiometabolic Diseases and Predicts Disease and Intervention Outcomes

Metabolomics Applied to Type 2 Diabetes

The association between cardiometabolic diseases and certain lipid metabolites commonly measured in clinical chemistry laboratories—including triglycerides, cholesterol, and total nonesterified fatty acids (NEFAs)—has long been recognized, leading some to conclude that cardiometabolic diseases are driven by perturbed lipid homeostasis. Application of metabolomics has led to a broader appreciation of metabolites that associate with these maladies and, in some cases, metabolites that predict intervention outcomes and future disease development.

As one example, targeted metabolomics has identified a signature of dysregulated metabolism of branched-chain amino acids (BCAAs) in subjects with various forms of cardiometabolic disease. The finding first emerged in a study of obese, insulin-resistant compared to lean, insulin-sensitive subjects (Newgard et al., 2009). Principal components analysis (PCA) performed on datasets comprised of plasma and urine amino acids, acylcarnitines, organic acids, and fatty acids identified a cluster of metabolites comprised of BCAAs, aromatic amino acids (Phe and Tyr), Glu/Gln, Met, and C3 and C5 acylcarnitines that strongly associated with insulin resistance as measured by the HOMA-IR score. In one sense, this finding was simply a rediscovery, given that association of BCAAs and aromatic amino acids with obesity and insulin resistance was reported 40 years earlier by Felig et al. (1969). However, the broader spectrum of analytes measured with metabolomics afforded two new insights: (1) the association of the BCAA-related metabolite cluster with insulin resistance was stronger than observed for several lipid-related clusters, including one principal component comprised of fatty acids and ketones and another comprised of medium-chain acylcarnitines derived from fatty acid oxidation (Newgard et al., 2009); (2) the clustering of glutamate and C3 and C5 acylcarnitines with BCAAs defined a signature comprising metabolites generated during BCAA catabolism, suggesting fundamental alteration of BCAA metabolism in insulin-resistant states. Glutamate is produced in the first step of BCAA catabolism, the transamination reaction catalyzed by branched-chain aminotransferase (BCAT), whereas C3 and C5 acylcarnitines are derived from three-carbon and five-carbon acyl CoA intermediates produced by mitochondrial metabolism of the carbon skeletons of BCAAs (Figure 1). In addition to the positive association between BCAAs, aromatic amino acids, and insulin resistance, glycine was found to have a strong negative association with insulin resistance when measured as HOMA score (Newgard et al., 2009) or by hyperinsulinemic-euglycemic clamp (Thalacker-Mercer et al., 2014).

The strong association of the BCAA-related metabolite cluster with insulin resistance was confirmed in multiple studies, including a cross-sectional study of subjects with metabolic syndrome and varying BMI (Huffman et al., 2009), a study in Chinese and Asian Indian subjects residing in Singapore in which BMI was controlled (average BMI of 24; Tai et al., 2010), a study on subjects at extremes of insulin sensitivity in the Insulin Resistance Atherosclerosis Study (IRAS) (Palmer et al., 2015), and studies of the effects of combined aerobic and resistance training in insulin-resistant subjects (Glynn et al., 2015). In all of these studies, the BCAA-related factor was more correlated with insulin resistance than any lipid-related factor. Also, IRAS cohort subjects that converted from prediabetes to diabetes during follow-up experienced an increase in BCAAs and a drop in glycine across this transition (Palmer et al., 2015). Importantly, these findings have been confirmed via application of metabolomics to large cross-sectional cohorts. Thus, in 7,098 young Finns (mean age 31 years), nuclear magnetic resonance (NMR) spectroscopy was used to profile 39 circulating metabolites and lipids, with findings of highly significant correlations between BCAAs, aromatic amino acids, ketones, and fatty acid composition and saturation with insulin resistance measured by HOMA-IR ($p < 0.0005$ for 20 metabolites; Würtz et al., 2012). Similarly, metabolomics profiling of 447 metabolites in 2,204 female subjects in the TwinsUK cohort found a strong association of BCAAs and their metabolites with type 2 diabetes and impaired fasting glucose levels (Menni et al., 2013). Several groups have also shown that BCAAs and related metabolites are associated with coronary artery disease (CAD), even when controlled for diabetes (Shah et al., 2010; Magnusson et al., 2013; Bhattacharya et al., 2014).

Targeted and nontargeted metabolomics studies have also revealed that BCAAs and their metabolites are prognostic for incident type 2 diabetes and obesity interventions outcomes. Thus, elevated plasma levels of Leu, Ile, Val, Phe, and Tyr were associated with up to a 5-fold risk for future development of type 2 diabetes in the Framingham Heart and Malmo Diet and Cancer study cohorts (Wang et al., 2011a). Analysis in the Framingham cohort compared 189 subjects that developed type 2 diabetes over the course of follow-up versus 189 matched controls that were diabetes free. Furthermore, targeted metabolomics was performed on baseline blood samples taken from 500 subjects in the weight loss maintenance (WLM) trial prior to a 6 month behavioral and dietary (DASH diet) intervention. At baseline,

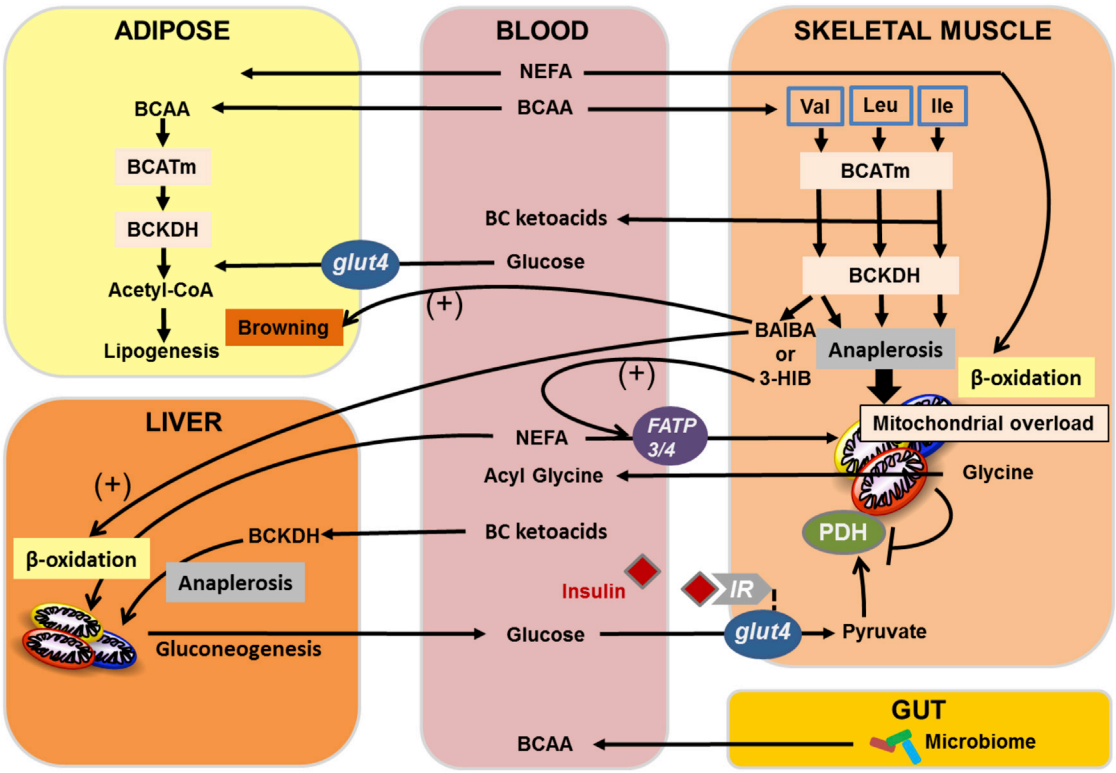

Figure 1. Emergent Mechanisms of Branched-Chain Amino Acid Metabolism in Cardiometabolic Disease Pathogenesis Unveiled with Metabolomics

Several mechanisms contribute to the accumulation of branched-chain amino acids (BCAAs) in plasma of obese, insulin-resistant humans, including increased de novo production of BCAAs by the gut microbiome and reduced utilization of BCAAs in liver and adipose tissue. BCAA utilization does not appear to be suppressed in skeletal muscle, and under obese conditions elevated BCAAs induce a decrease in skeletal muscle glycine levels, removing a potential escape valve for excess acyl CoAs. The combination of substrate pressure from elevated BCAAs and lipids in obesity contributes to accumulation of incompletely oxidized fatty acids in mitochondria ("mitochondrial overload") and reduced efficiency of glucose disposal. In addition, valine catabolism yields two new BCAA-derived factors that contribute to energy balance and metabolic homeostasis: β-aminoisobutyric acid (BAIBA), which stimulates thermogenesis and browning of white fat, and 3-hydroxyisobutryate (3-HIB), which stimulates transendothelial and muscle uptake of fatty acids. See text for details and discussion.

the factor score for a BCAA-related principal component very similar to that associated with insulin resistance strongly predicted improved insulin sensitivity, whereas lipid-related factors or the amount of weight lost had little or no predictive association (Shah et al., 2012b). Thereafter, NMR profiling of 1,680 subjects from the Cardiovascular Risk in Young Finns study identified a strong association of the three BCAAs, Tyr, and Phe with development of insulin resistance over a 6 year follow-up period (Würtz et al., 2013). This association was most pronounced in men. Finally, a recent study confirms both a strong association between BCAAs, Tyr, and Phe with insulin resistance in 429 Chinese subjects and a close association of these metabolites with future development of diabetes (Chen et al., 2016).

Another means of aligning metabolomics markers with disease is to study their pattern of response to efficacious disease interventions. As an example, obese subjects with type 2 diabetes undergoing gastric bypass (GBP) surgery have a much more dramatic decline in circulating BCAAs, C3 and C5 acylcarnitines, Phe, and Tyr than found in response to dietary intervention, despite equal weight loss (Laferrère et al., 2011). In another study, a similar large drop in BCAAs and related metabolites was observed in gastric bypass and gastric sleeve forms of surgical intervention (Magkos et al., 2013). These findings are significant because the surgical methods induce more dramatic improvements in glucose homeostasis than lifestyle interventions (Laferrère et al., 2011; Clifton, 2010). In aggregate, work from recent years has established that BCAAs and related metabolites are associated with insulin resistance, diabetes, and CAD; that they are predictive of diabetes development; that they are predictive of intervention outcomes; and that they are highly and uniquely responsive to therapeutic interventions.

Interestingly, these findings may not extend to all medical therapies. Thus, treatment of 30 insulin-sensitive and 30 insulin-resistant subjects with a single dose of the sulfonylurea drug, glibizide, which lowers blood glucose by stimulating insulin secretion, effectively lowered BCAA and aromatic amino acid levels in insulin-sensitive subjects, but not insulin-resistant subjects (Walford et al., 2013). In contrast, upon treatment of the same subjects with the insulin-sensitizing drug metformin (2 days of twice-daily injections), a lowering of glucose and insulin and an increase in the levels of BCAAs and aromatic amino acids occurred only in insulin-resistant subjects. Further studies of longer duration will be required to fully understand the impact of various diabetes therapies on metabolomics profiles.

More recently, application of nontargeted metabolomics has expanded the range of metabolites that predict risk of future

diabetes to include other amino acids, their metabolites, and lipids. For example, comprehensive lipidomic profiling of the same 189 diabetic and 189 nondiabetic subjects from the Framingham cohort previously subjected to LC-MS/MS-based metabolomics profiling revealed that higher diabetes risk is associated with triacylglyerols (TGs) that contain fatty acids of lower carbon number and double-bond content, whereas subjects with TGs containing fatty acids with higher carbon number and double-bond content had lower diabetes risk (Rhee et al., 2011). In addition, application of more comprehensive LC-MS/MS profiling to these subjects identified 2-aminoadipic acid (2-AAA) as a metabolite strongly correlated with incident type 2 diabetes, with subjects in the top quartile for this metabolite having a >4-fold increase in risk of disease (Wang et al., 2013). 2-AAA is thought to be derived from lysine catabolism, but surprisingly, 2-AAA levels were not associated with BCAAs or aromatic amino acids in this study, suggesting that this marker reports on an independent disease risk mechanism. Finally, application of a combined GC/MS and LC-MS/MS nontargeted metabolomics approach to plasma samples from 399 subjects from the Relationship of Insulin Sensitivity to Cardiovascular Risk (RISC) study confirmed that BCAAs were among the positively associated and glycine among the negatively-associated metabolites, with insulin resistance measured by hyperinsulinemic clamp (Gall et al., 2010). However, other metabolites, most notably α-hydroxybutyrate, linoleoyl-glycerophosphocholine, and oleic acid, were more strongly associated with the insulin-resistant state compared to BCAAs or glycine, and furthermore were selectively correlated with impaired glucose tolerance across multiple cohorts (Gall et al., 2010; Cobb et al., 2016).

Metabolomics Applied to Gestational Diabetes and Offspring Outcomes

All of the studies discussed above were performed in adult humans, but metabolomics has also recently been applied to pediatric populations to search for markers of early-stage insulin resistance, to predict future diabetes, and also to aid pregnant women in the search for biomarkers that assess the risk of conversion from gestational diabetes to type 2 diabetes and that predict outcomes in offspring. Findings in pediatric cohorts have been inconsistent (Frohnert and Rewers, 2016); two studies showed a positive correlation between BCAAs, BMI, and HOMA-IR (Newbern et al., 2014; Perng et al., 2014), and another showed an association with obesity (McCormack et al., 2013), but still others found no significant differences in BCAA levels in normal weight compared to obese adolescents (Wahl et al., 2012), or even a positive correlation between BCAAs/BCAA metabolites and insulin sensitivity (Si) (Michaliszyn et al., 2012). However, in a group of 17 pre- or early-pubertal children (age 8–13 years), baseline BCAA levels were found to be associated with HOMA-IR measured 18 months later, suggesting that, as in adults, BCAA levels may be predictive of future disease risk in adolescent subjects (McCormack et al., 2013). More work with larger cohorts of adolescent subjects is needed, and it remains possible that biomarkers other than BCAAs and related metabolites will have stronger associations with pediatric insulin resistance and type 2 diabetes.

Studies linking maternal metabolic status to offspring outcomes are just beginning to emerge. One study found positive associations of maternal triglycerides, leucine, isoleucine, ketone, and lactate levels—and negative association of glycine levels—with various measures of offspring body weight, although these were attenuated when corrected for glycemic status of the mothers (Scholtens et al., 2013). In another study, children of obese mothers were shown to have higher BCAA levels than those of lean mothers (Perng et al., 2014). Importantly, maternal metabolomics profiling provided an improved ability to predict newborn size outcomes beyond traditional risk factors, including maternal glucose (Scholtens et al., 2016). Future studies conducted over longer periods of follow-up should shed further light on the relationship between mothers' metabolic status and that of their offspring.

The use of metabolomics to predict the transition from gestational diabetes (GDM) to full-blown type 2 diabetes (which occurs in approximately 30% of GDM cases) is more immediately promising. A recent study used a plate assay technology to measure 163 metabolites and complemented this with direct amino acid analysis by LC-MS/MS and fatty acid analysis by GC/MS. These methods were used to compare 122 incident cases of type 2 diabetes with 122 noncases of incident type 2 diabetes matched by age, BMI, and race from a cohort of 1,035 pregnant women with GDM. Remarkably, metabolites which were significantly elevated in women with incident type 2 diabetes included all three BCAAs, Tyr, and 2-AAA, whereas glycine was negatively associated with diabetes risk (Allalou et al., 2016). Interestingly, multiple fatty acids were decreased in the subjects destined for incident diabetes as well. In contrast, metabolomic profiling of 96 women with GDM versus 96 women matched by age, BMI, and gravidity with normal glucose tolerance found six metabolites—anthranilic acid, alanine, glutamate, creatinine, allantoin, and serine—to be different between the groups, but found no differences in BCAAs, aromatic amino acids, or glycine (Bentley-Lewis et al., 2015). Similarly, few metabolites were found to be associated with fasting plasma glucose levels in 400 pregnant women of European descent from the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) cohort (Scholtens et al., 2013). However, many more metabolites were associated with glucose levels at the 1 hr time point of an oral glucose tolerance test in these subjects; these associations included positive associations with leucine, isoleucine, glutamate, phenylalanine, α-hydroxybutryate, and multiple acylcarnitines and fatty acids. Finally, a recent study comparing a cohort of GDM and normoglycemic mothers using a combined LC-MS and GC-MS approach revealed a striking (7-fold) increase in a furan fatty acid metabolite, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), in the GDM subjects (Prentice et al., 2014; Retnakaran et al., 2016). Furthermore, CMPF levels were even higher in a subset of GDM subjects that progressed to type 2 diabetes and were elevated in a separate analysis of subjects with type 2 diabetes compared to nondiabetic subjects (Prentice et al., 2014). Evidence for the involvement of CMPF in diabetes pathogenesis is discussed later in this article.

Metabolomics Applied to Prediction of Cardiovascular Events

Metabolomics has also been applied to predict incident cardiovascular events (Shah et al., 2012c). A principal component factor comprised of short- to medium-chain dicarboxylated acylcarnitines (DC-AC, including C4-DC, C5-DC, and C6-DC) predicted incident death/MI within 2 years in patients referred for

diagnostic catheterization in the CATHGEN study, a finding confirmed in a validation cohort (Shah et al., 2010). These findings were expanded via a cross-sectional survey of 2,023 subjects from the CATHGEN biorepository, demonstrating association of death/MI with a DC-AC factor ($p = 0.005$) essentially identical to the predictive signature from the case control study (Shah et al., 2012d). In addition, studies of 478 CATHGEN subjects who underwent coronary artery bypass grafting (CABG) revealed that the same short-chain DC-AC principal component was associated with adverse outcomes in univariate analysis ($p = 0.002$) and remained independently predictive of adverse outcomes in multivariable time-to-event analysis ($p < 0.001$) (Shah et al., 2012a). In a follow-up study, integrated -omics analysis, including metabolomics, GWAS, transcriptomics, and epigenetics in the CATHGEN cohort, found that the DC-AC diagnostic signature maps with high significance to several SNPs associated with genes involved in ER stress and the protein unfolding response (Kraus et al., 2015). Those SNPs in turn are independently associated with CVD events in time-to-event analysis. Transcriptomic and epigenetic profiling in the same subjects also identified associations between ER stress genes and the DC-AC cluster (Kraus et al., 2015).

Metabolomics has also been used to reveal a link between the diet, the gut microbiome, host metabolism, and biomarkers of incident CVD events (Wang et al., 2011b). Using a nontargeted LC-MS approach, 18 analytes were found to be associated with cardiovascular events, including three involved in choline metabolism—choline, betaine, and trimethylamine N-oxide (TMAO). Nutrients such as phosphatidylcholine, carnitine, and choline are metabolized by gut bacteria to generate trimethylamine (TMA). TMA is absorbed and carried by the portal circulation to the liver, where it is metabolized to TMAO by hepatic flavin monooxygenases. Several large studies have confirmed a link between TMAO and cardiovascular events, and cause and effect relationships between TMAO and cardiovascular diseases are emerging, as discussed below.

Metabolomics Applied to Fatty Liver and NASH

Overstorage of fat in the liver leads to the clinical syndrome of nonalcoholic fatty liver disease (NAFLD), which if left untreated develops an inflammatory component and evolves to nonalcoholic steatohepatitis (NASH). This in turn can progress to more advanced forms of liver disease, including cirrhosis and hepatocellular carcinoma (Lallukka and Yki-Järvinen, 2016). In the United States, approximately 30% of adults have NAFLD, and approximately 4% have NASH, but this varies across ethnic groups. The incidence of NAFLD is highest among Mexican Americans (estimated at 45%), intermediate in European-Americans, and lowest in African Americans (24%) (Sherif et al., 2016). Important insight into this imbalance in incidence across ethnic groups emerged with the identification of a variant allele (I148M) of the gene encoding patatin-like phospholipase A3 (PNPLA3), strongly associated with liver fat and highly prevalent in Hispanic individuals within a multiethnic cohort of subjects in the Dallas Heart Study (Romeo et al., 2008). The exact biological function of PNPLA3 remains unknown, but the recombinant protein has hydrolytic activity against various esterified lipids (Huang et al., 2011). The mutant form exhibits less of this activity and has enhanced propensity for association with lipid droplets (Smagris et al., 2015). Remarkably, recent evidence suggests that subjects with NAFLD induced by the PNPLA3 mutation are relatively protected against insulin resistance and type 2 diabetes, whereas NAFLD that develops independent of PNPLA3 mutations (“metabolic NAFLD”) is strongly predictive of future diabetes risk (Lallukka and Yki-Järvinen, 2016; Luukkonen et al., 2016). To investigate this further, comprehensive lipidomics analysis was performed on subjects with NAFLD with and without the PNPLA3 mutation (Luukkonen et al., 2016). The severity of steatosis was associated with insulin resistance as measured by HOMA-IR in subjects with the “normal” PNPLA3 allele, but no such relationship was found in subjects with the I148M PNPLA3 mutation. Remarkably, metabolic NAFLD with insulin resistance was associated with preferential storage of saturated fats and ceramides in liver, whereas the PNPLA3 NAFLD group had lower levels of these lipids and increased levels of polyunsaturated fats (Luukkonen et al., 2016). Obviously, there is huge opportunity embedded in understanding how lipid composition of excess liver fat influences systemic glucose homeostasis and insulin action (and vice versa), and this is likely to be an intensive area of investigation in the coming years.

New Metabolic Regulatory and Pathophysiologic Mechanisms Emerging from Metabolomics Studies

New Insights into Mechanisms of Insulin Resistance

Whereas the potential utility of metabolomics in the realm of disease prediction should be apparent from the foregoing summary, perhaps the most exciting windfall that has emerged from application of this technology is the blueprint that it provides for defining new physiologic and pathophysiologic mechanisms. As an early example, targeted metabolomics was used to show increases in a broad array of fatty acid-derived acylcarnitines and a concurrent decrease in TCA cycle intermediates in skeletal muscle of obese rodents (An et al., 2004; Koves et al., 2005, 2008). Genetic obesity or high-fat (HF) feeding results in upregulation of enzymes of fatty acid β-oxidation, but in sedentary animals this occurs absent a concurrent increase in enzymes of the TCA cycle and electron transport chain. This disconnect between the β-oxidation pathway and the terminal oxidative machinery results in incomplete fatty acid oxidation, leading to accumulation of mitochondrial-derived acyl-CoA and acylcarnitine metabolites. The potential pathophysiologic relevance of this finding was demonstrated in two studies in mice lacking expression of malonyl CoA decarboxylase or carnitine acetyltransferase (CrAT). In the former case, increases in levels of malonyl CoA, an allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT-1), resulted in reduced flux of fatty acids through the β-oxidative pathway and a decrease in muscle acylcarnitine levels in diet-induced obesity, accompanied by improved glucose tolerance (Koves et al., 2008). In the latter case, knockout of CrAT, which catalyzes conversion of short-chain acyl CoAs to membrane permeant acylcarnitines, resulted in accumulation of incompletely oxidized acyl CoA metabolites, exacerbating glucose intolerance and insulin resistance (Muoio et al., 2012).

Significant effort has also been applied to investigation of the strong association of BCAAs and related metabolites with metabolic diseases, and evidence for a role of BCAAs in disease pathogenesis has started to emerge. Thus, rats fed a HF diet supplemented with BCAAs develop insulin resistance despite

eating less food and gaining less weight than rats fed a HF diet alone (Newgard et al., 2009). In contrast, rats pair-fed on HF diet to match the caloric intake of the HF/BCAA group do not become insulin resistant. Targeted metabolomics revealed that the HF/BCAA group exhibited accumulation of a broad spectrum of acylcarnitines, to the same extent as observed in heavier HF-fed animals (Newgard et al., 2009). These findings are consistent with a model in which excess BCAAs contribute to impaired efficiency of fatty acid oxidation, resulting in accumulation of incompletely oxidized lipid species (Newgard, 2012).

Conversely, feeding obese rodents with a BCAA-restricted diet improves insulin sensitivity (White et al., 2016; Fontana et al., 2016), and possible mechanisms to explain this finding are emerging. In studies comparing Zucker obese rats fed a standard chow diet or an isonitrogenous diet in which BCAA content was lowered by 45%, feeding of the BCAA-restricted diet resulted in improved whole-animal insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp as well as enhanced muscle glucose uptake and glycogen synthesis (White et al., 2016). This improvement in glucose metabolism was accompanied by normalization of multiple short- and medium-chain acyl CoA species in skeletal muscle. In addition, muscle glycine levels are dramatically reduced in Zucker-obese compared to Zucker-lean rats, but are completely normalized in Zucker-obese rats by feeding of the BCAA-restricted diet, providing the first evidence of a direct connection between BCAA supply and glycine levels and helping to explain the consistent observation of high BCAA levels and low glycine levels in epidemiological studies. The restoration of the muscle glycine pool is accompanied by increased excretion of acetylglycine in the urine, suggesting that formation of acylglycine adducts may constitute a means by which BCAA restriction lowers muscle acyl CoA levels and restores muscle insulin sensitivity. Consistent with this, BCAA restriction lowers the respiratory exchange ratio in Zucker-obese rats, indicative of enhanced efficiency of fatty acid oxidation. These findings are aligned with a model wherein elevated circulating BCAAs contribute to the development of obesity-related insulin resistance by interfering with lipid oxidation in skeletal muscle (Figure 1). BCAA-dependent depletion of the skeletal muscle glycine pool may contribute to this effect by slowing acyl-glycine export to the urine (White et al., 2016). Interestingly, protein-restricted diets have recently been shown to increase circulating levels of FGF21, a hormone that increases lipid oxidation and improves glucose homeostasis (Laeger et al., 2014). However, induction of FGF21 does not seem to mediate the salutory effects of BCAA restriction on metabolic state, as FGF21 levels increase in response to generalized protein restriction, but not in response to specific restriction of BCAAs (Fontana et al., 2016).

Two new amino-acid-related mechanisms for regulation of metabolic homeostasis have recently emerged via application of metabolomics to rodent models of transgenic overexpression of the transcriptional coregulator PGC-1α. PGC-1α overexpression in skeletal muscle mimics the effects of exercise by inducing browning of white adipose tissue. Profiling of media from cultured muscle cells by LC-MS identified four metabolites that were significantly increased by forced overexpression of PGC-1α, including β-aminoisobutyric acid (BAIBA), which is derived from valine metabolism (Roberts et al., 2014). BAIBA increases expression of PPARα in white adipose and liver, induces thermogenic genes in adipose tissue, and activates fatty acid oxidation in hepatocytes. Administration of BAIBA to mice limits weight gain, enhances glucose tolerance, and induces browning of white fat. Exercise increases circulating BAIBA concentrations in rodents and humans, and it is proposed that BAIBA represents an endocrine factor that modifies liver and adipose metabolism in response to physical activity (Roberts et al., 2014). More recently, a different valine metabolite, 3-hydroxyisobutryate (3-HIB), was reported to be induced by PGC-1α overexpression and shown to function as an activator of transendothelial fatty acid transport (Jang et al., 2016). Like the BCAAs, 3-HIB levels were found to be elevated in db/db mice and in humans with type 2 diabetes, and administration of 3-HIB to rodents resulted in lipid accumulation in muscle and development of insulin resistance. Adding complexity, sedentary mice with muscle-specific overexpression of PGC-1α fed on a HF diet actually have impaired glucose tolerance relative to nontransgenic littermates (Choi et al., 2008), and mice of the two genotypes exhibit similar improvements in body weights and glucose control in response to a combined caloric restriction and exercise intervention (Wong et al., 2015). Targeted metabolomic profiling of muscles and plasma from these latter animals identified a principal component comprised of increased levels of ceramides, medium-chain acylcarnitines, Val, Met, His, and ornithine and decreased levels of the TCA cycle intermediates malate, fumarate, and succinate; these changes correlated with changes in energy balance, muscle insulin sensitivity, and glucose tolerance. BAIBA or 3-HIB were not measured in these studies.

How to reconcile these seemingly disparate findings? One unifying model requires that the regulatory significance of BAIBA or 3-HIB is different dependent on nutritional status (see Figure 1). Thus, in lean animals or humans, 3-HIB may act as a paracrine factor to increase fatty acid uptake by muscle during exercise, and BAIBA may function to mobilize and oxidize lipids as needed to support the increase in physical activity. By inducing valine catabolism, exercise increases the concentration of both mediators to engage these healthful effects. In contrast, whereas BAIBA levels are negatively correlated with fasting glucose, HOMA-IR, and circulating triglycerides and cholesterol in the Framingham cohort (Roberts et al., 2014), 3-HIB levels are positively correlated with blood glucose in a cohort of diabetic and normal subjects (Jang et al., 2016). Does this suggest that elevated BCAA in sedentary and metabolically unhealthy subjects is preferentially metabolized to yield 3-HIB rather than BAIBA, and that the consequence of this in a mileu of elevated lipids is to promote lipid overstorage and impair insulin action in muscle? Further studies will be required to investigate this issue and other issues related to these fascinating new mechanisms.

Serving as an important framework for these findings are recent discoveries relating to the mechanisms that cause BCAA and related metabolites to increase in the obese condition. BCAA catabolism is regulated at several early steps, including transport of BCAA into cells through the large neutral amino acid transporter LAT-1, transamination catalyzed by branched-chain aminotransferase (BCAT), and conversion of branched-chain keto acids to acyl CoAs by the branched-chain ketoacid dehydrogenase (BCKDH) complex. BCKDH is

a multisubunit enzyme complex of a design and subunit composition similar to that of the pyruvate dehydrogenase (PDH) complex. Like PDH, BCKDH activity is regulated by phosphorylation and dephosphorylation catalyzed by specific kinase (branched-chain ketoacid dehydrogenase kinase, BDK) and phosphatase (PPM1K) enzymes. Obesity has complex effects on regulation of BCAA catabolism that vary across key metabolic tissues and organs. In adipose tissue, obesity causes concerted suppression of all of the enzymes in the catabolic pathway at the transcriptional level (She et al., 2007; Herman et al., 2010; Hsiao et al., 2011). This mechanism does not seem to be active in liver, where instead hepatic BCAA catabolism is suppressed by increased inhibitory phosphorylation of BCKDH, secondary to increased expression of BDK and decreased expression of PPM1K (She et al., 2007). Remarkably, neither of these mechanisms appear to be operative in skeletal muscle (She et al., 2007), and BCKDH activity is actually increased in muscle of Zucker-obese compared to Zucker-lean rats (White et al., 2016). This differential regulation may contribute to mitochondrial substrate overload in skeletal muscle in obese animals by several mechanisms, including elevations in circulating BCAA due to reduced utilization by liver and adipose tissue, generation of anaplerotic substrates from BCAA catabolism in muscle that "fill up" the TCA cycle and make it more difficult for fatty acids to be completely oxidized (Newgard, 2012; White et al., 2016), and 3-HIB-stimulated transendothelial lipid transport (Jang et al., 2016). Interestingly, 2-AAA, a lysine-derived metabolite strongly associated with risk of type 2 diabetes discussed earlier (Wang et al., 2013), is also metabolized by an enzyme complex, known as α-ketoadipate dehydrogenase, that resembles PDH and BCKDH. Little is known about the regulation of this enzyme complex, but its similarity to PDH and BCKDH suggests that it may be downregulated in obesity, thereby contributing to the accumulation of its substrate, 2-AAA.

Another factor that may contribute to accumulation of BCAAs in obesity is the gut microbiome. BCAAs are "essential" amino acids, in the sense that humans are unable to synthesize them de novo, and it had been assumed that they are primarily obtained from the diet, particularly from meat and dairy products (which are rich in BCAAs). However, some gut bacteria are able to synthesize BCAAs from the simple organic acid precursors pyruvate and ketobutyrate. Transcriptomic analysis of microbiota from human twins discordant for obesity revealed induction of the entire de novo pathway for BCAA synthesis in the microbiota of obese compared to lean twins (Ridaura et al., 2013). Induction of this pathway correlated with increased production of BCAAs by the microbiota of obese subjects, and their transplantation into germ-free mice caused a significant increase in circulating levels of BCAAs and related metabolites in host animals. Remarkably, just 2 weeks after transplant, mice colonized with microbiota from obese subjects were glucose intolerant and exhibited a marked increase in levels of a broad spectrum of short-chain, medium-chain, and long-chain acylcarnitines in skeletal muscle compared to animals that received microbiota from lean twins (Ridaura et al., 2013). More recently, another group has extended these observations by performing metabolomics and microbiome profiling in 277 nondiabetic subjects, and inclusion of 75 subjects with type 2 diabetes for comparative analysis (Pedersen et al., 2016). A strong correlation between BCAA levels and insulin resistance was confirmed in this study, and the specific bacterial species *P. copri* and *B. vulgatus* were identified as those primarily responsible for driving BCAA biosynthesis in insulin-resistant subjects. Indeed, transplantation of *P. copri* into germ-free mice raised circulating levels of BCAA and caused insulin resistance and glucose intolerance. Taken together, these studies suggest that, in addition to regulation of energy balance and adiposity, the gut microbiome can have a substantive impact on metabolic homeostasis that contributes to disease pathogenesis. Further studies will be required to delineate the relative contributions of genetics, diet, gut microbiome, and selective suppression of BCAA catabolism in host tissues to the observed increases in BCAAs and related metabolites in obese and insulin-resistant subjects.

Metabolomics Applied to Pancreatic Islet Biology

Metabolomics has also been used to study pancreatic islet biology, including mechanisms leading to development of β cell dysfunction in diabetes. In one set of studies involving rat insulinoma (INS-1)-derived cell lines with different capacities for glucose-stimulated insulin secretion (GSIS), application of NMR-based mass isotopomer analysis was used to calculate relative flux rates of pyruvate through the oxidative enzyme PDH and the anaplerotic enzyme pyruvate carboxylase (PC). The studies showed that variable GSIS across the panel of INS-1-derived cell lines and in islets is tightly correlated to pyruvate anaplerosis and cycling activity rather than pyruvate oxidation (Lu et al., 2002; Alves et al., 2015). The significance of this finding is that anaplerotic metabolism of pyruvate through PC can produce excess TCA cycle intermediates that exit the mitochondria to engage with cytosolic enzymes. Indeed, surprisingly, flux through PC-catalyzed anaplerosis is more active in glucose-stimulated primary islet cells than is flux through the oxidative enzyme PDH (Schuit et al., 1997). Systematic study of several possible "pyruvate cycling" pathways implicated the cytosolic NADP-dependent isocitrate dehydrogenase (ICDc or IDH1) as a key extramitochondrial step, based on studies in which knockdown of the citrate-isocitrate carrier (responsible for export of citrate and isocitrate from the mitochondria) or IDH1 resulted in significant impairment of GSIS (Jensen et al., 2008; Joseph et al., 2006; Ronnebaum et al., 2006). The IDH1 reaction appears to play two key roles in mediating insulin release: first, by producing NADPH needed for the glutathione reductase reaction (Ferdaoussi et al., 2015; Ivarsson et al., 2005), and second, by generating α-ketoglutarate that can be transaminated to glutamate, a building block of glutathione (Figure 2). These pathways help maintain a robust pool of reduced glutathione (GSH) that in turn drives reduction of the desumoylation enzyme SENP1, thereby triggering insulin granule exocytosis via desumoylation of secretory granule proteins (Ferdaoussi et al., 2015). Intermediates in this pathway, including isocitrate, NADPH, and GSH (but not NADH or α-ketoglutarate), induce exocytosis in patch-clamped rodent and human β cells (Ferdaoussi et al., 2015; Ivarsson et al., 2005), an effect that is lost in mouse islets lacking expression of SENP1 or in human islets with shRNA-mediated suppression of SENP1 expression (Ferdaoussi et al., 2015). Importantly, isocitrate and NAPDH rescue exocytosis in glucose-unresponsive β cells from humans with type 2 diabetes (Ferdaoussi et al., 2015).

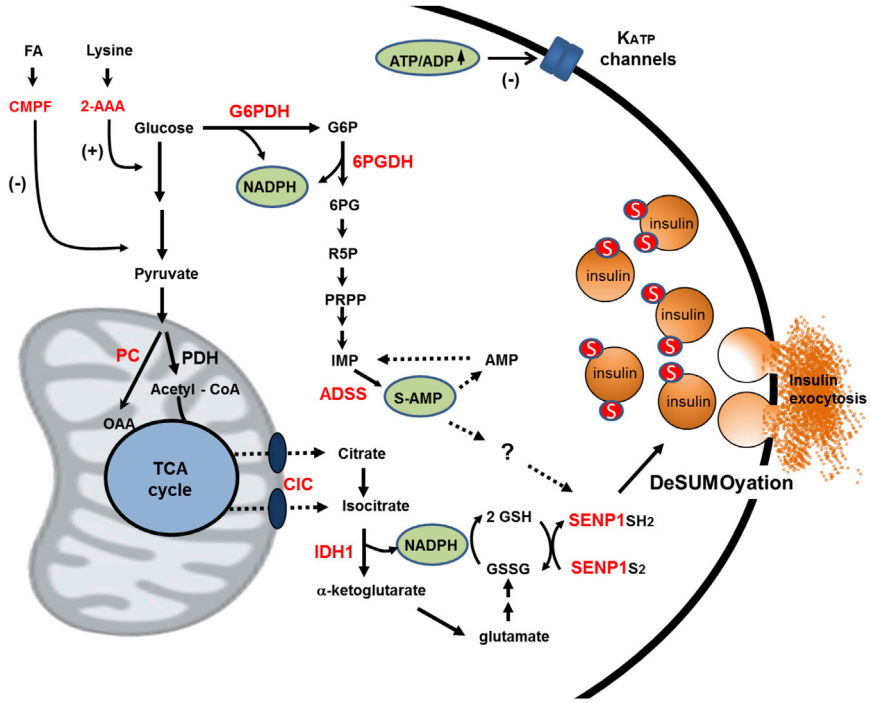

Figure 2. Emergent Mechanisms of Glucose-Stimulated Insulin Secretion from Metabolomics Studies

Application of metabolomics methods to the pancreatic islet has resulted in identification of mechanisms that may complement the classical K_{ATP}-channel-dependent pathway for glucose-stimulated insulin secretion (GSIS). This includes a pathway initiated by anaplerotic metabolism of glucose-derived pyruvate through pyruvate carboxylase (PC), egress of citrate, isocitrate, and α-ketoglutarate from the mitochondria to the cytosol via the citrate-isocitrate carrier (CIC), engagement of isocitrate with the cytosolic, NADP-dependent isoform of isocitrate dehydrogenase (IDH1), and reduction of glutathione to GSH by glutathione reductase. A second pathway involving metabolism of glucose through the pentose monophosphate shunt—including the first two NAPDH-producing steps, glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH)—results in a sharp increase in adenylosuccinate (S-AMP) produced from IMP via the adenylosuccinate synthase (ADSS) reaction. Importantly, intermediates generated by either the isocitrate/GSH (isocitrate, NADPH, GSH) or S-AMP pathways stimulate exocytosis in permeabilized human β cells and rescue loss of glucose regulation in β cells from humans with type 2 diabetes. Also for both pathways, the effects on exocytosis require expression of the insulin granule desumoylating enzyme SENP1. Metabolomics has, as is shown, identified two metabolites associated with risk of type 2 diabetes that modulate β-cell function: 2-aminoadipic acid (2-AAA), which enhances insulin secretion at basal glucose levels, and the fatty acid furan metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), which impairs glucose metabolism and insulin secretion. See text for details and discussion.

Metabolomics was also used to demonstrate glucose-induced changes in an array of purine and nucleotide pathway intermediates in islet cells, although the functional relevance of these findings was not initially defined (Huang and Joseph, 2014; Lorenz et al., 2013; Spégel et al., 2013; El-Azzouny et al., 2014). To investigate this further, a targeted, quantitative LC-MS/MS method was developed and used to demonstrate a robust decrease in inosine monophosphate (IMP) and an equally striking increase in adenylosuccinate (S-AMP) in glucose-stimulated 832/13 cells (Gooding et al., 2015). IMP is converted to S-AMP by adenylosuccinate synthase (ADSS), suggesting that this enzyme could play a regulatory role in β cell glucose sensing (Figure 2). Indeed, pharmacologic or molecular suppression of ADSS caused impairment of GSIS with an attendant decrease in S-AMP, effects that could be overcome by addition of adenine. Moreover, siRNA-mediated suppression of ADSL, a more proximal enzyme in the S-AMP biosynthetic pathway, impaired GSIS and lowered S-AMP levels independent of decreases in other adenine nucleotides or GTP. Infusion of S-AMP into patch-clamped β cells from nondiabetic humans revealed that it is equipotent to glucose for stimulation of exocytosis. Moreover, similarly to intermediates in the isocitrate/GSH pathway, S-AMP rescued exocytosis in glucose-unresponsive β cells from humans with type 2 diabetes. Finally, the stimulatory effect of S-AMP on exocytosis in patch-clamped human β cells is impaired by shRNA-mediated suppression of SENP1 (Gooding et al., 2015).

The new pyruvate/isocitrate/GHS and S-AMP pathways of GSIS are viewed as complementary to the classical initiating pathway that involves inhibition of ATP-sensitive K channels (K_{ATP} channels), membrane depolarization, and activation of voltage-gated Ca^{2+} channels (Henquin et al., 2003) (Figure 2). It remains to be determined if the newly described pathways are additive or synergistic with each other and with the K_{ATP} channel-dependent pathway in control of GSIS. The source of NADPH for reducing GSH also remains to be defined. A role for the pentose monophosphate shunt has been suggested by reports that inhibition of the first enzyme in the pathway—glucose-6-phosphate dehydrogenase (G6PDH)—by dehydroepiandrosterone (DHEA) (Spégel et al., 2013) or the second enzyme in the pathway—6-phosphogluconate dehydrogenase (6PGDH)—by siRNA or the chemical inhibitor 6-AN (Goehring et al., 2011) results in impaired GSIS. The effects of 6PGDH inhibition were ascribed to accumulation of early PPP intermediates and activation of p-ERK (Goehring et al., 2011), whereas DHEA treatment caused a decrease in GSH levels, although effects on NADPH were not reported (Spégel et al., 2013). These findings are not consistent with other work showing that shRNA-mediated suppression of IDH1 is sufficient to ablate the glucose-induced increment in GSH and the GSH:GSSG ratio in islet cells (Ferdaoussi et al., 2015). In addition, the finding that S-AMP has a direct effect of activating exocytosis in normal islets and is able to rescue dysfunctional exocytosis in human type 2 diabetes islets seems more aligned with the concept that S-AMP is the key regulator of GSIS produced in the pentose shunt (Gooding et al., 2015). Elegant metabolic flux methods have recently been reported for calculating the relative contributions of various pathways to total NADPH production (Fan et al., 2014). For example, absolute pentose pathway flux is measured by the classical method of the difference in $^{14}CO_2$ production

between [1-^{14}C] and [6-^{14}C] glucose. The contribution of the pentose shunt to NADPH synthesis is then measured by fractional enrichment (FE) of the NADPH and G6P pools in cells incubated with [3-D]glucose (Fan et al., 2014). Using these methods, a surprisingly large contribution of folate metabolism to total NAPDH production was recently reported in HEK293T cells (Fan et al., 2014). Application of similar methods to pancreatic islet cells should allow the fractional contributions of various NADPH-producing pathways to be quantified. Performance of such flux experiments in the presence and absence of strategic suppression of contributory enzymes such as G6PDH and IDH1 will help to define key operative pathways for control of GSIS.

Two metabolites that were discussed earlier in the context of their utility for prediction of diabetes, 2-AAA and CMPF, may contribute to diabetes pathogenesis by altering β cell function. Paradoxically, administration of 2-AAA to mice actually lowers rather than raises circulating glucose levels, which seems to be mediated by an effect of 2-AAA: to stimulate insulin secretion, even at low glucose levels (Wang et al., 2013). This raises the possibility that the link between elevated levels of 2-AAA and diabetes risk in humans could be the development of insulin resistance secondary to chronic hyperinsulinemia. This hypothesis requires further investigation. In contrast, CMPF seems to have a direct effect: to induce β cell dysfunction (Liu et al., 2016). Thus, treatment of diet-induced obese (DIO) or ob/ob mice with CMPF caused a decrease in glucose-stimulated insulin secretion and a worsening of glucose intolerance in these models. The decrease in β cell function in CMPF-treated mice was associated with a decrease in glucose metabolism and an increase in fatty acid oxidation accompanied by an increase in generation of reactive oxygen species. CMPF also caused an impairment in insulin granule maturation that led to a higher insulin content and an increase in the proinsulin:insulin ratio. The precise molecular target(s) of CMPF in mediating these effects remains to be defined. Interestingly, a clear increment in CMPF levels was observed in prediabetic subjects that progressed to overt diabetes, whereas those from this cohort that remained in the prediabetic state had stable CMPF levels (Liu et al., 2016). This finding implicates CMPF as a "tipping factor" that may help to explain why some individuals can remain in the prediabetic state for many years before suddenly progressing to type 2 diabetes.

Metabolomics Applied to Transgenic Mouse Models

As described earlier, new mechanisms linking BCAA metabolism and metabolic homeostasis were unveiled in studies of mice with muscle-specific overexpression of the transcriptional coregulator PGC-1α (Roberts et al., 2014; Jang et al., 2016; Choi et al., 2008; Wong et al., 2015). Metabolomics has also helped to identify metabolic pathways regulated by other transcription factors and coregulators in transgenic mouse models. Such studies can delineate the specific pathways affected, the tissues and organs in which regulation occurs, and in some cases, novel metabolites that mediate biological effects. For example, a series of studies have been conducted on transgenic mice with global knockout of the transcriptional coactivators SRC-1, SRC-2, and SRC-3. Metabolic profiling contributed to our understanding that SRC2 regulates glucose-6-phosphatase expression in liver (Chopra et al., 2008) and SRC1 integrates hepatic fatty acid and amino acid metabolism in liver and other tissues (Louet et al., 2010), whereas SRC3 knockout has focused metabolic effects in muscle and a particular signature to cause accumulation of very long-chain acylcarnitines (York et al., 2012). This latter finding led to the discovery that SRC3 controls expression of the mitochondrial carnitine-acylcarnitine translocase (CACT), which mediates entry of long-chain acylcarnitines into the mitochondrial matrix during fatty acid oxidation. Mutations in the gene encoding this protein underlie human CACT metabolic myopathy.

Another novel mechanism has emerged from studies in mice with genetic manipulation of GLUT-4 expression in adipose tissue. Mice with adipose-specific GLUT-4 knockout develop insulin resistance in liver and muscle (Abel et al., 2001), whereas transgenic overexpression of GLUT-4 causes improvement in glucose tolerance despite an increase in lipogenesis and circulating fatty acids (Shepherd et al., 1993). Surprisingly, the effect of GLUT-4 overexpression to improve glucose disposal depends on lipogenic activity in adipose tissue, as the effect on glucose homeostasis is eliminated in mice with dual knockout of GLUT-4 and the key lipogenic transcription factor ChREBP (Herman et al., 2012). This led to the hypothesis that specific lipids may mediate the glucoregulatory effects of GLUT4 manipulation in adipose tissue. To test this idea, comprehensive, nontargeted lipidomic analysis was performed on adipose tissue and plasma from GLUT-4-overexpressing mice and control mice (Yore et al., 2014). Among five lipids found to be enriched in the samples from GLUT-4 transgenic mice, four were identified as fatty acid-hydroxy fatty acid (FAHFA) esters, comprised of a typical long-chain fatty acid (palmitate, PA; oleate, OA; stearate, SA; or palmitoleate, PA) esterified to hydroxylated versions of one of the same four fatty acids. Among the 16 possible FAHFA dimeric species, six were increased in serum of GLUT-4 transgenic compared to wild-type mice, and the most clearly upregulated form, PA-hydroxy SA (PAHSA), was studied in more detail. Serum and adipose tissue PAHSA levels were positively correlated with insulin sensitivity in humans. A single oral bolus of PAHSA given to mice 30 min prior to a glucose tolerance test enhanced glucose clearance, and an effect of PAHSA, to enhance insulin-stimulated GLUT4 translocation, was demonstrated in cultured adipocytes. Remarkably, PAHSA also caused increases in circulating insulin and GLP-1 and suppressed inflammation, suggesting multiple sites of action (Yore et al., 2014).

FAHFA esters are not the first lipids to be implicated as therapeutic agents in metabolic disease. The monomeric fatty acid palmitoleate (cis-16:1n7) was reported to decrease hepatic fat and enhance skeletal muscle insulin sensitivity in mice (Cao et al., 2008). However, palmitoleate does not have the same strong associations with metabolic control in humans as those suggested for PAHSA. Omega-3 fatty acids (polyunsaturated fats found in fish oils) are also reported to be protective against metabolic diseases (Oh et al., 2010). Interestingly, both PAHSA and omega-3 fatty acids are ligands for the G protein-coupled receptor GPR120, possibly explaining their common effects of suppressing inflammation and promoting GLUT-4 translocation (Yore et al., 2014; Oh et al., 2010). This work has highlighted the power of untargeted metabolomics for discovery of new mechanisms, but several important issues remain to be

addressed, including the enzymatic pathways responsible for synthesis of FAHFA and their modes of regulation, the levels of FAHFA that might be present in different foods and supplements, and the effect of administration of PAHSA in human subjects with metabolic diseases.

Metabolomics Applied to Cardiovascular Disease Mechanisms

Recent studies have demonstrated surprising efficacy of "diabetes drugs" such as empagliflozin, an inhibitor of renal glucose reuptake by the sodium-glucose cotransporter SGLT2, and liraglutide, a GLP-1 analog, for reducing risk of cardiovascular events in diabetic subjects (Zinman et al., 2015; Marso et al., 2016). These findings highlight the strong metabolic interconnections between obesity, diabetes, and cardiovascular diseases and suggest that metabolic signatures that predict incident cardiovascular events, including the short-chain dicarboxylacylcarnitine (Shah et al., 2010, 2012c, 2012d) and TMAO (Wang et al., 2011b) signatures discussed earlier, may serve as biomarkers for engagement of diabetes drugs for mitigation of cardiovascular risk.

As discussed earlier, the gut microbiome may play an important role in the production of metabolites such as BCAAs that contribute to dysregulation of metabolic pathways and the eventual development of type 2 diabetes. More recently, strong evidence for a key role of the microbiome in regulating biological functions relevant to cardiovascular diseases has also been accumulating. TMAO, which is produced via sequential metabolism of choline and carnitine by gut microbes and host enzymes, is a strong predictive biomarker of atherosclerotic burden and cardiovascular events. Consistent with a cause and effect relationship, supplementation of mouse diets with TMAO or choline results in an increase in atherosclerotic plaque size in *apoE*$^{-/-}$ mice that is proportional to the increase in circulating TMAO levels. Moreover, the effect of dietary choline to enhance plaque formation in *apoE*$^{-/-}$ mice is abrogated by generalized ablation of the microbiome with broad-spectrum antibiotics (Wang et al., 2011b). More recently, TMAO has also been associated with risk of thrombotic events (MI or stroke) in a cohort of 4,007 clinically referred subjects (Zhu et al., 2016). Extending these findings, it has also been found that TMAO enhances platelet function in response to several agonists, including ADP, thrombin, and collagen, and also increases the rate of thrombus formation in the $FeCl_3$ mouse model of experimental thrombosis. Choline supplementation also enhances thrombosis in this model in a microbiome-dependent manner. Progress is occurring in identifying particular bacterial species that are strong producers of trimethylamines (TMAs), the precursors of TMAO formation, suggesting potential therapeutic strategies. Indeed, inhibition of bacterial TMA lyases with a choline analog, 3,3-dimethyl-1-butanol (DMB), was recently shown to reduce circulating TMAO and inhibit plaque formation in the *apoE*$^{-/-}$ mouse model (Wang et al., 2015). The effects of DMB on mitigating the thrombotic effects of TMAO remain to be demonstrated, and potential long-term side effects of the agent may emerge, but the approach has the potential to be one of the first broadly impactful examples of "drugging the microbiome." If this occurs, it should be remembered that the approach is based on an original discovery made with nontargeted metabolomics.

Integration of Metabolomics and Genetics for Understanding Cardiometabolic Diseases

Human genome-wide association studies have identified loci that map with cardiovascular risk and diabetes, but these explain only a small fraction of these diseases. This is likely due to the fact that both type 2 diabetes and cardiovascular diseases are driven by a mixture of genetic susceptibility, environmental factors, and (as has been illustrated herein) the gut microbiome, which conspire to perturb metabolic homeostasis and health. With the emergence of metabolite clusters that predict incident cardiometabolic disease and intervention outcomes, we are provided with intermediate phenotypes that present fresh opportunity to define underlying genetic architecture. This is particularly true given evidence that metabolic profiles are heritable. Thus, metabolomic analysis of eight multiplex families with familial early-onset CVD identified specific amino acids, fatty acids, and acylcarnitines with high heritability scores, even after adjustment for variables such as diabetes, hypertension, and BMI (Shah et al., 2009). Other studies have combined metabolomics profiling and genotyping in general population cohorts such as KORA and TwinsUK (Illig et al., 2010; Suhre et al., 2011; Shin et al., 2014). The most recent of these studies performed GWAS analysis and measured 333 known metabolites by a combined GC/MS and LC-MS/MS approach across 7,824 subjects in the two cohorts (Shin et al., 2014). Overall, 145 independent SNP/metabolite associations with genome-wide significance have emerged from these studies. New biological insights and disease detection strategies are implied by this work. For example, changes in carnitine levels were associated with SNP rs7094971 in the *SLC16A9* gene, also known as *MCT9*, which has homology to monocarboxylic acid transporters (Suhre et al., 2011). Expression of this gene in *Xenopus* oocytes demonstrated that it encodes a carnitine transporter. In addition, SNPs in several genes involved in aromatic amino acid metabolism, including TDO2 (tryptophan 2,3-dioxygenase), IDO1 (indoleamine 2,3-dioxygenase), TAT1 (T-type amino acid transporter1), and SLC7A5 (LAT1 amino acid transporter), were associated with multiple tryptophan metabolites, suggesting the potential for new blood-borne markers of altered tryptophan metabolism linked to mood disorders (Shin et al., 2014). Similarly, other recent studies have integrated GWAS or exome array analysis and metabolomics profiling in 2,076 Framingham Heart subjects and 1,528 validation cohort subjects from the Atherosclerosis Risk in Communities study to define novel coding variants that influence levels of metabolites such as histidine, phenylalanine, and ureidopropionate, as well as cholesterol ester and triglyceride metabolites (Rhee et al., 2013). Finally, as discussed earlier, a metabolite cluster (principal component) consisting of several short-chain dicarboxylated acylcarnitines that predict future cardiovascular events maps to genes associated with the protein unfolding response and ER stress pathways (Rhee et al., 2016). Further efforts to integrate metabolomics with genomics and other "-omics" datasets could yield new information about genetic pathways that control intermediate phenotypes contributing to polygenic metabolic diseases.

Summary and Conclusions

As the newest member of the stable of "-omics" methodologies for comprehensive molecular profiling, metabolomics has

undergone a rapid technological evolution and is now increasingly applied to human epidemiology and fundamental mechanistic research in almost equal measure. Consistent with its theoretical advantage in measuring the chemistry of biological systems and therefore being distal to genomic, transcriptomic, and proteomic variation, metabolomics has proven its utility for identifying new biomarkers of cardiometabolic diseases and, even more impressively, for detecting future disease events and intervention outcomes. New and unanticipated associations are emerging between cardiometabolic diseases and specific metabolites, leading in multiple cases to demonstration of their role in disease pathogenesis and to new insights into biological pathways that contribute to complex human diseases and disorders. Because a significant portion of metabolomics-based research to date has been descriptive, the full potential of metabolomics for defining new detection strategies and pathogenic mechanisms of cardiometabolic diseases remains to be realized. It is hoped that this perspective will encourage increased application of metabolomics to metabolic disease research and, more specifically, the use of disease-associated metabolomics signatures as a blueprint for defining novel disease mechanisms and targets.

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