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Towards Metabolomic Signatures of Cardiovascular Disease

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Small biochemicals are the end result of all the regulatory complexity present in a cell, tissue, or organism, including transcriptional regulation, translational regulation, and post-translational modifications. Metabolic changes are thus the most proximal reporters of the body's response to a disease process or drug therapy. In 1971, Arthur Robinson and Linus Pauling conceived the core idea that information-rich data reflecting the functional status of a complex biological system resides in the quantitative and qualitative pattern of metabolites in body fluids.[1] In the same year, Horning and Horning[2] first used the term metabolic profiling to describe the output of a gas chromatogram from a patient sample. This new approach to the quantitative metabolic profiling of large numbers of small molecules in biofluids was ultimately termed "metabonomics" by Nicholson *et al*[3] and "metabolomics" by others.

Two core technologies are utilized to perform metabolic profiling: nuclear magnetic resonance (NMR) and tandem mass spectrometry (MS/MS), as previously reviewed in *Circulation: Cardiovascular Genetics*.[4] NMR requires relatively little sample preparation and is nondestructive, allowing for subsequent structural analyses. However, the method tends to have low sensitivity and can detect only highly abundant analytes. Tandem mass spectrometry (MS/MS), coupled with liquid chromatography (LC), on the other hand, has much higher sensitivity for small molecules and is also applicable to a wide range of biological fluids (including serum, plasma, and urine). Recent advances in MS technology now enable researchers to determine analyte masses with such high precision and accuracy that metabolites can be identified unambiguously even in complex fluids.

These technologies can be used to characterize biological samples either in a *targeted* manner, or in a *pattern discovery* manner. In the former, the investigator targets a predefined set of metabolites for analysis. In the latter, the investigator is faced with a complex pattern of peaks, many of which are anonymous—the molecular identities of the species giving rise to the peaks are generally not known. While the targeted approach is more limited, the analysis is more straightforward as the biochemicals giving rise to the signals have already been identified. The pattern discovery or fingerprint approach is inherently less biased, but the need for subsequent unambiguous identification of the peaks can be difficult.

The vision for human metabolic profiling also extends from seminal studies of inborn errors of metabolism in infants. Millington and colleagues pioneered the use of MS-based methods for monitoring fatty acid oxidation, as well as organic and selected amino acids.[5] Rapid identification of subjects with fatty acid oxidation disorders, organic acidemias and aminoacidopathies through metabolic profiling has led to dietary modification and amelioration of symptoms or disease onset. It is anticipated that a global metabolomic

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analysis of more common diseases such as atherosclerosis might identify new biomarkers or spotlight pathways for dietary or drug modulation.

As might be expected, however, the application of metabolomics to complex cardiovascular diseases has been more difficult than for Mendelian disorders. Important progress, however, is reported in this edition of *Circulation: Cardiovascular Genetics*. Shah and colleagues used a targeted MS/MS-based platform to profile approximately 70 metabolites in subjects from the CATHGEN biorepository who underwent cardiac catheterization to evaluate suspected ischemic heart disease. Particular strengths of this study include the relatively large number of patients studied, appropriate corrections for both clinical conditions and medications that may modulate metabolic profiles, as well as the use of derivation and replication groups. They demonstrate that peripheral blood metabolite profiles, one enriched for branched-chain amino acid (BCAA) metabolites and another comprising urea cycle metabolites, add to the discriminative capability for CAD compared with models containing only clinical variables. They specifically document improvement in the c-statistic with incorporation of metabolite factors, compared to clinical characteristics and established circulating biomarkers. Though the increment in the c-statistic is relatively modest, it underscores the capacity for metabolic profiling to provide new information on top of known risk factors. Furthermore, the group reports that a novel metabolite cluster composed of dicarboxylacylcarnitines predicts subsequent cardiovascular events (death and MI) in individuals with existing coronary artery disease (CAD).

The study from Shah and colleagues, which highlights BCAA from ~70 metabolites profiled (Factor 4 in the manuscript), is also noteworthy in the context of experimental and clinical data suggesting that certain amino acids may be markers of insulin resistance. [6–10] Furthermore, studies of branched chain amino acid supplementation in both animals[6] and humans,[11] including prior work by the authors of the present manuscript, indicate that circulating amino acids may directly promote insulin resistance, possibly via disruption of insulin signaling in skeletal muscle.[6, 9] Shah et. al. appropriately adjusted for diabetes in their clinical analyses, though their metabolomics platform may be identifying a more subtle metabolic syndrome. Their compelling new findings highlight potential cross-talk between insulin signaling and atherosclerosis that merits future investigation both in mechanistic studies in animal models as well as in clinical populations.

The authors also found an association between a group of urea cycle metabolites (Factor 9) and prevalent CAD, and note that this finding may reflect increased amino acid and ammonia catabolism. Alternatively, two of the four constituents of this factor (arginine and citrulline) are key substrates in the nitric oxide synthesis pathway, perhaps suggesting altered arginine bioavailability in modulating CAD. Indeed, another recent mass spectrometry-based study found that a low arginine to citrulline plus ornithine ratio in peripheral blood predicts CAD and adverse outcomes.[12]

The study from Shah and colleagues also highlights an unanticipated metabolite signature, enriched for small and medium chain dicarboxylacylcarnitines, that predicts future cardiovascular events. Carnitine and its acyl esters are essential compounds for the metabolism of fatty acids. The main function of carnitine is to assist in the transport and metabolism of fatty acids in the mitochondria, where they are oxidized as a major source of energy.[13] The acyl-CoA dehydrogenases, in turn, are a family of enzymes involved in the mitochondrial β-oxidation of fatty acids. Medium-chain acyl-CoA dehydrogenase acts on fatty acyl-CoA molecules from 4 to 12 carbons in length. Deficiency in this enzyme is the most common defect observed in the process of mitochondrial β-oxidation of fatty acids and one of the most common inherited disorders of metabolism. Affected individuals can suffer from severe hypoglycemia and catastrophic nervous system injury in the setting of fasting.

How the metabolomic findings in adults with CAD might relate in any way to Mendelian disorders remains unknown. Future studies must also address the source of the dicarboxylacylcarnitines, the atherosclerosis-relevant cell types in which they are acting, as well as the functional effects of their buildup.

The metabolomic studies were performed using a targeted-LC-MS/MS-based platform. At first glance, the number of metabolites assayed may seem modest in the context of the estimated universe of total human metabolites (~5000).[14] However, the analytes chosen are key intermediaries in the metabolism of proteins, carbohydrates, and fats. Moreover, use of the targeted approach that incorporates isotope labeled standards ensures the unambiguous identification of the analytes of interest, as metabolite assignments to mass spectral speaks can lead to spurious results. The use of standards also allows the investigators to report absolute as opposed to relative quantities of these metabolites. Their rigorous methodology will thus facilitate both animal studies to manipulate levels of these metabolites in physiologically relevant contexts, as well as future biomarker studies in other disease populations.

The relative contributions of diet and genetics to this newly described metabolic disarray also merits further investigation. Indeed, a prior study by this group has documented the heritability of metabolomic profiles in families with premature CAD. Ultimately, the integration of metabolomics findings with genetic data will provide an opportunity to study whether a biomarker also plays a role in disease pathogenesis. For a biomarker that has a causal role, the expected random distribution in a population of a polymorphism that determines high or low biomarker concentrations would be skewed in individuals depending on their disease status. Data from so-called “Mendelian randomization” studies are accumulating for several biomarkers.

The study by Shah and colleagues thus represents important progress in the integration of metabolomics towards our understanding of CVD. Though metabolite profiling technologies are still under development, they complement other functional genomic approaches, such as high throughput genome sequencing, RNA expression analysis, and proteomics. Together, they hold great promise to transform our ability to profile samples with the goal of illuminating biology and discovering valuable clinical biomarkers.

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