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Metabolomics and Atherosclerosis

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Abstract

Metabolites reflect the dynamic processes underlying cellular homeostasis. Recent advances in analytical chemistry and molecular biology have set the stage for metabolite profiling to help us understand complex molecular processes and physiology. Metabolomics is the comparative analysis of metabolite flux and how it relates to biological phenotypes. As an intermediate phenotype, metabolite signatures capture a unique aspect of cellular dynamics that is not typically interrogated, providing a distinct perspective on cellular homeostasis. To date, there have been only a few metabolomics studies investigating cardiovascular diseases. In this review, we explore the principles of metabolomics and how it can provide further insight into the mechanisms of cardiovascular physiology and ultimately lead to improved diagnostic and therapeutic options for patients with cardiovascular disease.

Keywords

Metabolomics; Atherosclerosis; Coronary artery disease; Mass spectrometry; Nuclear magnetic resonance spectroscopy; Metabolite; Bioinformatics; Systems biology; Biomarker; Left ventricular dysfunction; Genomics; Proteomics

Introduction

Metabolomics examines metabolites and their flux in relation to specific biologic states. Because metabolites are the “currency” of cellular metabolism, in many ways, their flux reveals the homeostatic mechanisms that govern the responses to environmental stimuli. From a systems biology standpoint, relatively mature genomic and proteomic approaches have advanced our understanding of physiologic and pathologic conditions. These high-throughput approaches provide complementary insight into complex homeostatic networks [1•]. Metabolomics has similar potential, in that metabolites are the building blocks and substrates of all cellular processes. Additionally, as metabolites are not encoded by DNA,

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they provide another level of information that, if integrated and correlated with biological phenotypes, could provide another dimension of understanding biological systems. This, coupled with the relatively low amount of known metabolites (~6500 discrete small molecules compared with approximately 25,000 genes, 100,000 transcripts, and 1 million proteins) [2, 3], allows for high-resolution network analysis of cellular processes. Taking advantage of the unique characteristics of metabolites, metabolomics measures chemical signatures that are the culmination of genomic and proteomic utilization of metabolites, providing an integrated perspective of biological phenotypes.

However, two of the major barriers to metabolomics are related to the accurate resolution of large numbers of diverse molecules with wide dynamic ranges encountered in complex biological matrices and how to analyze and integrate these large data sets with other information. Technological advancements in mass spectrometry (MS) and nuclear magnetic resonance (NMR) [4] spectroscopy have improved the analytic resolution of metabolites to a point of application to metabolite–phenotype characterization and correlation [5, 6]. Additionally, novel bio-informatics techniques have improved the integration of metabolite data sets with other variables to uncover the complex relationships that define biological systems [7].

Modern Metabolomics: Approaches and Analytical Technology

Metabolomics usually employs one of two general approaches: targeted or nontargeted. The targeted approach requires *a priori* knowledge about the metabolites of interest and a general appreciation for the mechanisms underlying the physiologic process under investigation. This approach focuses on identification and quantification of discrete clusters of metabolites. Most targeted approaches utilize stable isotope dilutions for quantification of analytes [8, 9]. For example, Turer et al. [8] demonstrated distinct patterns of myocardial substrate use in patients with either coronary artery disease (CAD) or left ventricular dysfunction (LVD) during surgical ischemia-reperfusion [10]. This study utilized MS to profile distinct metabolites in patients undergoing cardiac surgery. The investigators demonstrated characteristic metabolic signatures associated with postoperative outcomes. One limitation to this approach is the reliance upon stable isotope–labeled standards to guide quantification of metabolite status and fluctuations associated with dynamic phenotypic states. Additionally, novel metabolites and relationships rarely can be identified because of the reliance upon candidate metabolites of interest.

In contrast, nontargeted metabolomic approaches are predicated on analysis of large amounts of diverse metabolites using powerful analytic techniques to query the vast amount of analytes with wide dynamic ranges. In general, the biological phenotype is determined, and then qualitative, relative differences in metabolites are assessed between the differing biological phenotypes. To date, no single analytical technology allows for the measurement of all metabolites simultaneously in complex biological matrices.

Both the targeted and nontargeted approaches to modern metabolomics involve three general steps: metabolite isolation, metabolite determination, and data analysis. The two predominant analytical tools employed towards metabolomic investigations are MS and NMR spectroscopy [11, 12].

NMR techniques allow for rapid, nondestructive analysis of metabolite profiles both from in vitro and in vivo sources [13]. Additionally, NMR methods facilitate the interrogation of large amounts of metabolites from a single sample. Not only does NMR provide detailed information on the molecular structure of metabolites, but the nondestructive nature makes possible the recovery of sample for further analyses. It is also a highly reproducible technique, which becomes especially important during the statistical analysis of the large

data sets. However, NMR spectroscopy does have limitations, including relatively poor sensitivity (nanomolar detection using high-field spectrometers), artifacts related to pH and ionic composition of the biological matrix, and the complex, overlapping spectra associated with the metabolite mixtures [4, 14]. The most common NMR experiment performed for metabolite detection is to obtain a one dimensional ^1H spectrum of the sample, which reports the chemical shift or chemical environment of all hydrogen atoms present and the spin–spin coupling between these nuclei, which is related to their connectivity. Thus, the spectrum contains the superposition of the ^1H NMR spectra of all metabolites in the biological sample, and because most of these hydrogens fall in the aliphatic region (between 0.5 and 4.25 parts per million), there is significant overlap. Spectral resolution can be enhanced through heteronuclear NMR, which monitors atoms other than hydrogen, and multidimensional experiments, such as J-resolved spectroscopy [11]. For example, ^{13}C spectra have greater chemical shift dispersion resulting in a less crowded spectrum and eliminate the need for suppressing the peaks from the aqueous solvent that do not contain carbon atoms. Relaxation-edited spectra, such as those employing the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, can improve the sensitivity of NMR to small molecules (amino acids) in the presence of larger metabolites (lipoproteins) with broad obscuring peaks by taking advantage of the different relaxation rates of molecules of varying size.

In contrast to NMR, MS techniques possess high sensitivity (picomolar range) and specificity and, in many instances, can be used as a stand-alone analytic technique for metabolite identification and quantification. Additionally, MS is most often combined with separation techniques like gas chromatography (GC) or liquid chromatography (LC), which improves the resolving power [15]. GC is based on differential partition between a carrier gas phase (containing the metabolites of interest) and the coating on a capillary. Thus, all analytes must be volatile or made volatile through chemical derivatization. For example, when using gas chromatography–mass spectroscopy (GC-MS) for measurement of fatty acids, reactive analytes must be stabilized using chemical derivatization of carboxyl, amine, and hydroxyl groups [16, 17]. This derivatization process adds complexity and introduces sample variability that can distort quantitative analysis. However, GC-MS was traditionally the preferred technique for analysis due to its sensitivity, high-resolution separation capability, and the existence of reference spectral libraries of species. Although its separation resolution is lower, LC does not require chemical derivatization of the metabolites. LC also has the advantage of providing the molecular ion peak, rendering this separation technique well suited for analysis of unknown metabolites. However, column chemistry can be difficult to optimize as polar compounds can be eluted with the void volume with commonly used reverse-phase ^{18}C columns, which renders no separation at all for these important metabolites [11]. Capillary electrophoresis (CE) is a third separation technique particularly well suited for polar or ionic metabolites, based on their charge–to-mass ratio. It offers complementary information with respect to GC-MS or LC-MS, and a combination of techniques provides broad metabolome coverage. The technical aspects of CE-MS are discussed thoroughly in a review by Ramautar et al. [18].

For both targeted and nontargeted metabolomics, data processing and statistical analysis are critical components of the process. Some elements of data processing include noise reduction, peak detection, chromatogram alignment, normalization, and data transformation [19, 20]. To increase the generalizability and improve the reproducibility of metabolomics studies, data standardization initiatives have been proposed, including those from the Metabolomics Society [21] (the Minimum Information About a METabolomics experiment [MIAMET]) [22], and the Metabolomics Standardization Initiative (MSI) [23]. Some bioinformatics approaches employed in metabolomics include principal component analysis, which identifies a small set of variables to explain the original data set, and discriminate

analysis, which fits a mathematical linear model to the data for classification [24]. Another method to integrate complex data sets is the grouping of metabolites through pathway analyses [25]. Metabolic pathway databases can then be used to reveal complex relationships and interactions by integrating molecular and phenotypic information [26].

Metabolomics and Cardiovascular Disease

Cardiovascular diseases, from CAD to cerebrovascular accidents (CVA), are the number one cause of death globally according to the World Health Organization. Atherosclerosis, the thickening of arteries, is the underlying pathologic process that affects not only the coronary arteries but also the cerebral, aortic, and peripheral vessels. Atherosclerosis involves the buildup of cholesterol particles, cellular byproducts, extracellular matrix deposition, and inflammatory cell infiltration within the vessel wall [27]. Chronic inflammation has recently been recognized as one of the key components of atherogenesis [28]. Additionally, many established risk factors for atherosclerosis are metabolic or are associated with significant metabolic derangements, including hypertension, smoking, diabetes, and dyslipidemia. However, the molecular mechanisms governing atherosclerosis are not yet fully known.

Traditional risk factors for CAD have been derived from epidemiologic studies [29, 30]. Although traditional risk factors capture much of the risk associated with CAD, there is still a need for further stratification to identify and treat patients at risk. Recent technological advances in the genomics, proteomics, and metabolomics fields have the potential to improve current diagnostic and risk assessment for clinical cardiovascular disease. Invasive coronary angiography is currently the gold standard for diagnosing patients with atherosclerotic disease, but metabolomic screening in conjunction with traditional risk assessments has the potential to improve noninvasive diagnostics and risk stratification of CAD. Although many investigators have used metabolomic techniques to study noncardiac diseases, cardiovascular metabolomics is still in its infancy.

One of the first studies to uncover the potential of unbiased metabolomic profiles in predicting patients with three-vessel CAD utilized a high-throughput approach based on NMR spectroscopy of clinical blood samples [31]. This study included patients with unstable CAD or prior myocardial infarction. The resulting metabolic signatures had greater than 90% predictive power for CAD patients on a major lipid region of the NMR spectra. A follow-up study was unable to reproduce these significant metabolomic signatures, citing significant confounding contribution from differences in gender and the use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors between the two study populations [32]. These conflicting reports emphasize the potential variability of metabolic profiling and the difficulty in reproducing metabolite profiles because of the complexity of quantitative metabolite assessment and inherent clinical variability of patient populations.

Metabolomics has also been used for profiling of low-molecular weight metabolites in the setting of myocardial ischemia. Iakoubova et al. [33] used high-sensitivity MS following LC separation to identify differences in plasma metabolites among 18 patients with exercise-induced ischemia compared with nonischemic patients. The ischemic group had significant changes in metabolites arising from the citric acid pathway, with decreased levels of γ -aminobutyric acid, citrulline, and argino-succinate. Another study used ^1H -NMR analysis of metabolites to predict the occurrence of exercise-induced ischemia in patients with suspected coronary artery disease [34]. In this study, NMR spectra were acquired from blood obtained immediately prior to a stress single-photon emission computed tomography (SPECT) in 31 patients with exertional angina. The metabolites identified from this analysis included lactate and glucose, as well as lipids and long-chain amino acids. Although both studies examined patients during exercise-induced ischemia, the variability in metabolic

profiles can partly be explained by protocol-specific differences. These studies suggest the role of citric acid cycle intermediates, modified lipids, and long-chain amino acids as key metabolic mediators of acute myocardial ischemia.

Another study utilized GC-MS to evaluate the major changes in plasma metabolites between nine patients with non-ST elevation myocardial infarction (NSTEMI), 10 patients with stable CAD, and 10 patients with no known CAD [35]. Citric acid, 4-hydroxyproline, aspartic acid, and fructose levels were decreased, whereas lactate, urea, glucose, and valine levels were increased in acute coronary syndrome patients compared with the control group. Citric acid, as previously described [33], was decreased in acute myocardial ischemia, reaffirming its role in aerobic metabolism and cardiovascular disease. Consistent with these results, high levels of hydroxyproline have been associated with the absence of CAD in an isolated population of elderly Okinawans, suggesting a component of metabolomic heritability [36].

The heritability of CAD and its associated risk factors is an established concept [37–39]. Recent studies suggest a differential risk pattern for CAD development and differential treatment responses related to genetic variability [40, 41]. CAD was associated with a genetic polymorphism of the kinesin-like protein KIF6. Patients carrying the KIF6 719Arg allele showed not only a higher risk of adverse coronary events but also derived a greater benefit from HMG-CoA reductase inhibitor treatment. Given the impact of cholesterol-reducing agents on metabolomic profiles and in parallel with the concept of a pharmacogenetic inheritance, metabolome heritability is another area of research that has just begun to be explored. Targeted metabolite heritability studies in plants [42] and mice [23] have already been completed. Additionally, another recent study performed quantitative profiling of 66 metabolites, including acyl carnitine species (byproducts of mitochondrial fatty acid, carbohydrate, and amino acid oxidation), amino acids, and free fatty acids, in plasma samples from eight families with premature CAD using tandem MS and GC-MS [43]. They found a high degree of heritability in all three groups. Several individual metabolites were heritable, including clusters composed of ketones, β -hydroxybutyrate, acetyl carnitines, and amino acids.

Recently, several reports have found added utility of metabolomics in the evaluation of the traditional risk factors, including diabetes and high cholesterol [44–46]. Lipidomics, a specific area of metabolomics that focuses on lipid metabolites, also has grown in conjunction with other “-omics” fields [47]. Lipid metabolites are the byproducts of diverse cellular processes including phospholipid homeostasis, cellular bioenergetics, and signal transduction pathways. Recent evidence suggests diabetes is also linked to enhanced cellular oxidative stress of lipid and protein components [48, 49]. Integrated genomic and metabolomics analysis in animal models of atherosclerosis, hyperlipidemia, and inflammation have revealed a wealth of information regarding the complex cellular relationships following atherogenic stimuli, such as a high fat diet [50, 51]. Combined profiling of substrate utilization, including lipid and carbohydrate metabolites, and gene expression revealed dysregulation of metabolic and inflammatory pathways prior to atherosclerotic disease appearance. These results support the hypothesis that modulation of energy metabolism and inflammatory processes precede athero-sclerotic lesions and are drivers in both the initiation and progression of CAD. Similar approaches, a marriage of molecular biology, analytical chemistry, and bioinformatics, will advance our understanding of complex diseases like atherosclerosis.

Conclusions

Metabolite profiles and their dynamic flux reflect the relationships between cellular processes and biochemical pathways underlying the full spectrum of physiologic states. The intrinsic characteristics of metabolites, including relatively low amounts, known cellular pathways, and independence from direct genetic modulation, make it a unique tool to complement existing methodologies in the basic sciences and clinical medicine. The greatest benefits of metabolomics will likely be realized through its integration with genetics, genomics, and proteomics to provide a comprehensive cellular topology of biological phenotypes. There already exist a number of diseases where insight into the underlying pathophysiology has been elucidated through metabolomics. However, only recently have metabolomic approaches been applied to cardiovascular diseases and, to date, only a few clinical studies exist. Metabolomics, coupled with traditional risk factor assessment, has the potential to greatly improve our understanding of cardiovascular disease. With this improved understanding, the realization of improved noninvasive diagnostics and targeted therapeutics will be one step closer to our patients' bedside.

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