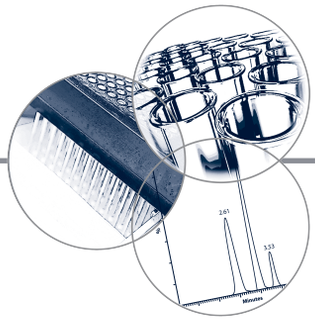

Integration of metabolomics in heart disease and diabetes research: current achievements and future outlook

Metabolomics is an emerging and powerful discipline that provides an accurate and dynamic picture of the phenotype of mammalian systems through the study of endogenous and exogenous metabolites in cells, tissues, culture supernatants as well as biofluids. In the last 5 years an increase in the number of metabolomic investigations of cardiovascular diseases and diabetes has been observed. In this article the experimental strategies applied and recent examples of their application in disease and drug efficacy/toxicity biomarker detection and the employment for the discovery of new molecular pathophysiological processes related to disease onset and progression, as well as their usefulness in drug efficacy/toxicity, will be reviewed. An outlook of the requirements for future successes will also be discussed.

Introduction to metabolomics

Low-molecular-weight chemical compounds (metabolites) present in biological systems play an important role in many mammalian biological processes [1]. Their biosynthesis and catabolism, through a complex metabolic network, provides energy (e.g., ATP), components for higher molecular weight biochemicals (proteins, transcripts, DNA) and biological structures (e.g., cell membranes and organelles). Regulation of biological processes is also observed through binding or covalent modification by metabolites of proteins (post-translation modifications, allosterism) and mRNA (riboswitches).

History

The scientific field of metabolomics emerged at the turn of the 21st century with the first reported definitions of the metabolome (the complete complement of metabolites in a biological system) [2,3] and was closely followed by the first holistic studies of the metabolome [4,5]. The closely associated metabonomics also developed in a similar timeframe [6]. Although significant analytical and computational advances were observed in the last century, which have led to the emergence of metabolomics, the first holistic profiles of mammalian biofluids were reported in the 1960s and 1970s [7,8], long before the term metabolomics was reported. Pioneers of biochemistry were characterizing metabolic profiles in the early 20th century; for example, the characterization of glycolysis in 'yeast juice' by Buchner. Whilst there are many definitions of metabolomics, a good place to start is The Metabolomics Society who define metabolomics as the comprehensive characterization of the small-molecule metabolites in biological systems, which can provide an overview of the metabolic status and global biochemical events associated with a cellular or biological system [201].

Experimental strategies

Three experimental strategies are applied in metabolomics studies [1]. The first is metabolic profiling or untargeted analysis which performs a holistic study of large numbers of chemically diverse metabolites, typically 100–1000s of metabolites, with limited sample preparation and with the application of powerful analytical technologies (typically chromatography–MS or NMR). This strategy is applied in discovery-based studies where the objective is to detect, semi-quantify (based on peak areas or heights but with no comparison to calibration curves) and identify as many metabolites as is achievable. No knowledge of which metabolites are present in the sample and are of biological interest is available prior to data acquisition. Data pre-processing and analysis post-data acquisition defines changes in the metabolic profile related to the biological question being asked. The second strategy is the targeted analysis of a small number of metabolites, typically less than 20 metabolites, performing more complex sample preparation and fractionation and applying

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analytical platforms that provide high sensitivity and selectivity (typically GC–MS or LC–MS with triple quadrupole [QQQ] mass spectrometers). Absolute quantification of metabolites is performed through appropriate use of internal standards and calibration curves. A third strategy, defined as semi-targeted analysis, has developed more recently. This is again applied in discovery studies, although the metabolites to be detected are known before data acquisition and LC coupled to QQQ mass spectrometers are applied to provide greater sensitivity and selectivity to the analytical methods compared with metabolic profiling. The metabolites studied, typically tens to hundreds of metabolites, are chosen based on previous biological knowledge and authentic chemical standard availability. Quantification is performed to a less strict level, typically applying a single calibration curve for a small number or chemical class of metabolites. Each of these strategies has advantages and limitations [1]. For discovery studies, semi-targeted analysis provides methods of high sensitivity, selectivity and precision with linear calibration achieved in the nanomoles–millimoles/liter range. Multiple methods can be applied to increase the range of metabolites studied, with the disadvantage of lower throughput. This strategy allows a rapid advance from data to biological knowledge but is limited by the choice of metabolites to study (normally based on somewhat limited biological knowledge and the lack of availability of authentic chemical standards). Metabolic profiling detects a greater number of metabolites in a single analytical run and, therefore, provides a greater probability of observing unexpected biological changes but metabolite identification and conversion to biological knowledge is currently a major issue in these studies [1]. Metabolite coverage is also a problem and all intermediates within a pathway are often not seen due to dynamic range and detection limits and detected metabolites highlighted on metabolic maps appear to be randomised – like a blind person throwing darts at a Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolite network [9]. By contrast, targeted studies are limited by the number of metabolites investigated but provide the highest levels of analytical sensitivity, selectivity, accuracy and precision.

Metabolomics is routinely applied as a discovery or hypothesis-generation strategy where biological experiments are designed to acquire robust and valid data from which biological hypotheses can be created through inductive reasoning [10]. From these discoveries further validation studies can be performed to test the hypotheses [1]. All discovery-based metabolomics studies operate with a workflow; from biological study, through the analytical experiment (sample preparation and data acquisition) to data preprocessing, analysis and hypothesis generation (schematic examples of the generalized workflow are shown in [1,11]). Experimental design of both the biological and analytical experiments is hugely important to ensure biological hypotheses are valid and robust [12]. This is particularly important in human-based studies where large variations in the genotype and environment (which cannot be easily controlled) impact on the composition of the metabolome studied. The types of metabolomics studies performed have been discussed in detail recently with emphasis on appropriate numbers of samples studied, which range from tens to thousands of samples [1]. Following the collection of samples, data are acquired on powerful analytical platforms with NMR spectroscopy and chromatography–MS systems being the most frequently, but not exclusively (for example see [13]), applied platforms [1,14]. Protocols have been developed and are available for the analysis of different sample types [15–17]. The stability of MS compared with NMR has to be considered in large-scale studies. MS platforms provide a lower degree of response stability compared with NMR because of sample interaction with the instrument and subsequent time-related contamination and drift in the measured response. Typically less than 150 samples can be analyzed before a detrimental drop in stability is observed [18,19]. Therefore, appropriate separation of large-scale MS-based studies in to small-scale analytical experiments where robust data can be acquired and integrated post-data acquisition should be performed. This requires the use of quality control (QC) samples for quality assurance, signal correction of low level instability and data integration [20–22]. Following acquisition, pre-processing and analysis of data is performed to define biological differences between classes. A range of different univariate and multivariate techniques are applied during data analysis procedures [1,12,23].

Types of metabolomes

In mammalian systems the quantitative collection of metabolites, defined as the metabolome, are composed of thousands of metabolites and fully test the capabilities of analytical platforms. No single analytical platform can be applied to detect all metabolites in a single metabolome analysis and the integration of data from multiple analytical

platforms is therefore recommended. The Human Metabolome Database (HMDB) [24] is the most comprehensive information source describing the human metabolome and describes 7800 metabolites. However, a range of endogenous (in particular lipid metabolism) and exogenous (drug metabolites, gut microflora-derived metabolites, phytochemicals and nutrients) metabolites are currently absent. Mammals are complex systems composed of complex interactions between cells, tissues and biofluids. Each of these sample types and the same sample type from different areas of the body are composed of distinct metabolomes. For example, the metabolomes of urine and serum/plasma are significantly different, a result of different biological processes involved in their construction. Urine and blood provide representative pictures of organism function through the integration of signals from multiple cell types and organs. Other biofluids can be acquired, although these are ethically more difficult to collect in large numbers, for example CSF, amniotic fluid and brain dialysate. However, to acquire specific metabolic signals related to the biological processes of specific cells or tissues requires the direct study of these cells and tissues, either following tissue biopsy and metabolic inhibition from mammals or following culturing in the laboratory.

In human studies, the investigation of cell, tissue or biofluid metabolomes provide a number of advantages [1]. The metabolome is the final downstream product of gene expression and provides a sensitive measure of the phenotype of the system, controlled by both genetic and environmental interactions. The genome defines what may happen; the metabolome defines what has happened or is happening. Changes in response to genetic and environmental perturbations are observed rapidly (seconds to minutes) in the metabolome. Metabolomics is a high-throughput strategy as it provides the analysis of tens to thousands of samples per week and is, therefore, applicable to the study of hundreds to thousands of samples in discovery-based studies. This is particularly important in the study of humans in the general population where genetic and environmental factors are highly variable and, therefore, large sample sizes are required to define this complex variability. Some of the studies discussed below were only achievable because of this level of throughput. High-throughput studies also provides the opportunity to perform longitudinal studies, which allows subtle changes over time to be observed, which with single time point sampling could be missed.

Objectives of metabolomics studies in mammals

The study of mammalian systems generally has one or both of the following objectives. The first objective is to discover prognostic biomarkers that provide information regarding survival and clinical outcomes associated with the disease process or diagnostic biomarkers that are able to are able to diagnose:

- The risks of developing disease;
- The presence of clinical disease before the development of overt clinical signs or without the need for invasive investigations;
- The progression of disease;
- Measurement of drug toxicity or efficacy in targeting disease.

The second objective is to define molecular pathophysiological mechanisms related to disease onset and progression or drug toxicity and efficacy. The review below will describe some specific applications in detail to highlight the differences between these objectives. Both objectives can be fulfilled in a single study as biomarkers can be indicative of biological processes at the molecular level. For example, a study by Dunn and colleagues discovered biomarkers of heart failure [25] and further biological studies were performed to validate these markers and to define the biological actions of one of these biomarkers, 2-oxoglutarate, in cardiac cells [26]. Figure 1 highlights the different types of metabolomics applications performed in drug discovery/development and diseases studies.

The review presented here will discuss the role of discovery-phase metabolomics, and its integration with other omic studies, in the investigation of cardiovascular diseases (CVDs), with an emphasis on heart disease and diabetes mellitus (DM). CVDs are diseases affecting the heart or blood vessels and are currently the most common causes of morbidity and mortality in the western world. The WHO reported that 16.7 million deaths in 2003 (29.2% of total global deaths) were caused by some form of CVD. A common cause of CVD is DM, the commonest metabolic disease in the world which is characterized by glucose dysregulation and abnormalities in insulin secretion and/or impaired insulin action at the level of the insulin receptor or further downstream. According to the WHO, DM has a prevalence of over 171 million worldwide and this is expected to double over the next 20 years [27]. CVD and DM are

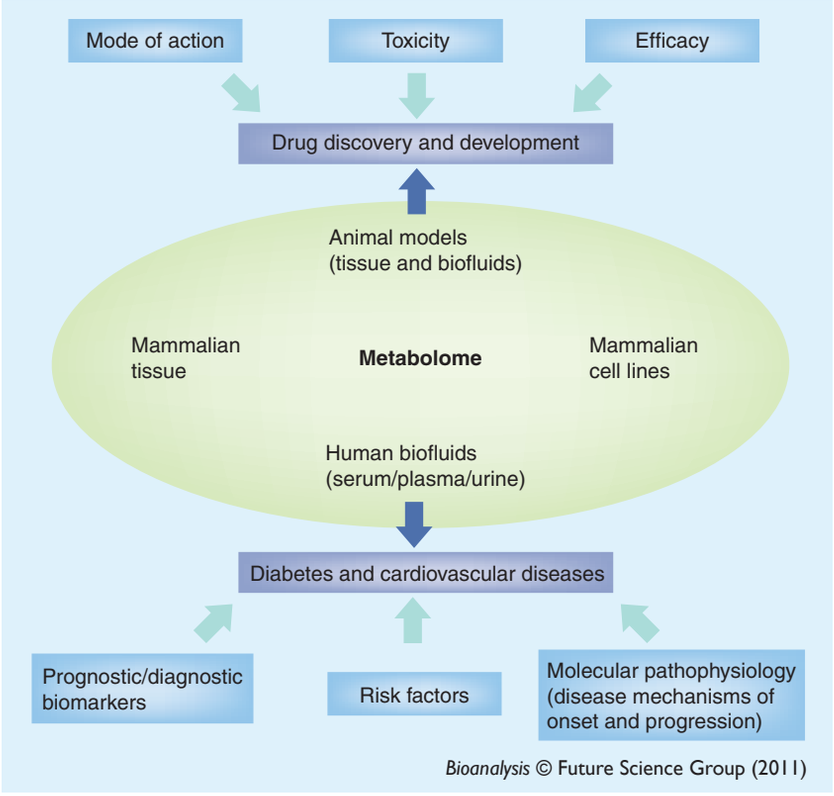

Figure 1. The application of metabolomics in disease studies and drug discovery/development. The investigation of mammalian cell, tissue and biofluid metabolomes provides molecular knowledge and biomarkers related to disease risk, onset and progression and increasing knowledge on drug mode of action, toxicity and efficacy.

inter-twined, with CVD being the commonest cause of mortality amongst patients with DM [28] and the presence of one disease routinely (but not exclusively) predicts the development of the other. Diabetes is over-represented in many cardiovascular disorders, for example the prevalence of DM in the general population is 5% whereas its prevalence in some CVD such as heart failure is as high as 40% [29]. Appropriate citations will illustrate metabolomics role in the discovery of biomarkers and novel molecular pathophysiological processes related to disease onset and progression and drug efficacy and toxicity. Table 1 summarizes the types of studies performed with appropriate citations.

Cardiovascular diseases: heart disease & diabetes

Cardiovascular diseases are the commonest cause of death worldwide. The most frequent cause is coronary heart disease. It is estimated in 2030 that 23.6 million deaths will result from CVDs and the majority of these will be observed in low- and middle-income countries [202].

More than 220 million people in the world are currently diagnosed with diabetes and this is expected to double by 2030 [203]. Individuals with DM are 2–6 times more likely to develop CVD than those individuals without DM, and the commonest cause of death amongst patients with DM is CVD. It has been estimated that 40–50% of deaths associated with DM are secondary to CVD composed mainly of heart disease and strokes [30,31]. DM, often described as diabetes, can be classified in to three categories; Type 1 diabetes (T1D), Type 2 diabetes (T2D) and gestational diabetes. All categories show an elevated blood glucose level, although the causes are different. T1D involves the failure of the body to produce insulin, a result of the autoimmune attack and loss of β-cells in the pancreas, the source of insulin in the body. T2D is a result of the inability of cells to use insulin effectively most probably through defective insulin receptors. Impaired glucose tolerance (IGT; or insulin resistance) is an early indicator of the later onset of T2D, though not at a level that results in elevated glucose levels applied for diagnosis of T2D. Gestational diabetes is only observed during pregnancy, has a similar pathology to and can precede T2D. DM can affect multiple organ systems outside of the heart through its effects on macrovascular and microvascular function as well as direct effects on cellular structures. Diabetic retinopathy is observed through damage to the blood vessels in the eye. Approximately 2% of people who have been diagnosed with diabetes for 15 years are blind and 10% develop severe visual impairment. Diabetes is a major cause of kidney failure, with 10–20% of individuals with DM dying of kidney failure. DM may damage the blood vessels that supply the limbs to cause peripheral vascular disease (PVD). It is reported that over 4 million patients with DM in the US have PVD and this is a leading cause of disability amongst individuals with DM. PVD may result in a spectrum of clinical manifestations including claudication (pain when walking) through to limb amputation [32].

The social and economic impact of CVDs including diabetes is immense. Research has focused on defining early prognostic biomarkers of CVDs, understanding molecular pathophysiological processes related to CVD development, developing novel therapeutic agents to prevent the development/progression of the disease state and promoting prevention through lifestyle/risk factor modification (e.g., dietary changes, weight loss, increased exercise or quitting smoking).

Metabolomics has been used to focus research on these components and a number of publications have reviewed the application of metabolomics in cardiovascular research [33–39].

Biomarkers of heart disease & diabetes

The NIH biomarkers definition group has defined biomarkers as biological characteristics that measure and evaluate biological, pathological or pharmacological responses to disease or therapeutics [40]. In the clinical environment biomarkers are applied for diagnostic and prognostic purposes in disease states and have been used to monitor both drug toxicity or therapeutic efficacy. We have recently reviewed biomarker discovery through application of metabolomics and their application in clinical disease [41].

Metabolomic studies have discovered a number of potential biomarkers related to heart disease and diabetes. The applicability of these biomarkers is limited until multiple studies have been performed to validate and characterize the specificity of the biomarker(s) in the population where the biomarker(s) will be applied. These validation studies are relatively infrequent at present, and if undertaken are only performed in small single-center cohorts rather than in a large multicenter setting. Due to these limitations, the potential for false discoveries within the metabolomics field should be considered and lessons should be learnt from the significant problems of false discoveries observed in proteomics and genomics.

The applicability of the biomarker in the population where it will be applied is extremely important and for the study of CVDs in the human population this requires the study of thousands of subjects to ensure the biomarker(s) are valid and robust when taking in to account the large variation in the genotype and environment of humans. Smaller studies are less robust as ensuring a homogenous sample of the population has been sampled is difficult and a significant reason for failures of these studies is that the biomarker is valid in this population but not in a larger population, which is more metabolically homogenous. If smaller matched case-control studies are performed it is advisable, as a first step, to validate the initial observations in a second independent population.

Diabetes

A study by Wang and colleagues to define potential prognostic biomarkers of T2D has shown how robust and valid studies can be performed [42].

Table 1. Different types of metabolomics studies performed in the investigation of diabetes and cardiovascular diseases and appropriate references.

Generalized research area	Specific research area	Ref.
Disease prevalence and relationships between CVD and DM		[27–31,202,203]
Reviews of the role of metabolomics in research	Cardiovascular disease	[33–39]
	Diabetes	[82–85]
DM: biomarkers	Prognostic biomarkers or risk factors	[42,48]
	Prediabetes changes	[45,49,50]
	Impaired glucose tolerance	[51,52]
CVD: biomarkers	Exercise-induced cardiac ischemia	[53,54]
	Silent myocardial ischemia	[55]
	Early cardiac ischemia	[59]
	Coronary heart disease	[56,57]
	Heart failure	[26]
	Planned myocardial infarction	[58]
	Atherosclerosis	[60]
	Abdominal aortic aneurysm	[61]
	Adverse cardiac events	[62]
DM: molecular disease mechanisms	The role of PPARs applying mouse model	[86]
	Other diabetic animal models	[87–89]
	Insulin treatment in T1D	[90]
	Beta cell lines	[91]
	Insulin resistance/sensitivity	[92–96]
	Glucose tolerance test metabolic changes	[46,97,98]
	Branched-chain amino acid changes in diabetes	[42,43,45–47,92,96]
CVD: molecular disease mechanisms	Atherosclerosis (apoE mouse model)	[63–65]
	Atherosclerosis (humans)	[72]
	Coronary artery disease	[80,81]
	Dilated cardiomyopathy	[73]
	Cardiac hypertrophy	[75]
	Effect of diet and gut microflora on cardiovascular disease and insulin resistance	[68–71]
	Heart disease (mouse models)	[76–79]
	Other animal models	[66,67]
	Heart failure	[74]
Drug development and toxicity: CVD	Statins	[100]
	Soluble epoxide hydrolase inhibitors	[101]
	Doxorubicin	[102]
	Traditional Chinese medicine	[103]
	Rofecoxib drug toxicity	[105,106]
Drug development and toxicity: DM	Triethylenetetramine	[107,108]
	Nutraceutical extracted from rosemary seeds	[109]
	Chinese medicine berberine	[110]
	Metformin	[111]
	Leptin therapy compared with insulin therapy	[112]
	Rosiglitazon	[113–115]

CVD: Cardiovascular disease; DM: Diabetes mellitus; PPAR: Peroxisome proliferator-activated receptor; T1D: Type 1 diabetes.

The study involved a large prospective cohort of 2422 individuals from the Framlingham Offspring study assessed for normal glycemia and from which serum samples were collected and subjects were followed-up for 12 years. Of the original cohort, 201 developed diabetes. Semi-targeted analysis of 189 T2D cases and 189 matched controls were performed for 61 polar metabolites using a LC–MS/MS QQQ system. Five branched-chain and aromatic amino acids (isoleucine, leucine, valine, tyrosine and phenylalanine) showed significant associations with the later diagnosis of T2D, all amino acids were elevated in those subjects who developed T2D. A second study was subsequently performed with an independent, prospective cohort (Malmö Diet and Cancer study), which validated these findings. The results show that a combination of three amino acids (isoleucine, phenylalanine and tyrosine) could predict future diabetes with a more than fivefold higher risk for individuals who were in the top quartile of the amino acid scores. This study was based on prediction in high-risk groups where the metabolic separation between cases and controls would be expected to be the greatest, as is generally the case in matched case-control studies. The validation of these results in a general population composed of high- and low-risk groups can be more difficult and the authors applied the system to assess 400 randomly chosen subjects from the Framlingham Offspring study and showed a reduced relative risk associated with the elevated amino acid concentrations (twofold risk for individuals in highest quartile), although they still remained independent predictors for the development of T2D. Such studies may not only identify potential biomarkers of the disease process, but may identify bioactor roles for these biomarkers, namely that these 'biomarkers' may actually contribute to the pathophysiological mechanism that commences with the development of the disease process itself. As an example, branched-chain amino acids may directly promote insulin resistance in humans [43] through the activation of insulin receptor substrate-1 signaling pathways [44] and so may directly contribute to the development of diabetes. It is of further interest that independent studies by other researchers have also shown that branched-chain amino acids have a role to play in the onset of diabetes or during hyperglycemia [45–47].

A related study profiled the lipid components of serum in the samples acquired from the Framlingham Offspring study since dyslipidemia is often observed in patients with diabetes, due to the close cross-regulation between carbohydrate and lipid metabolism [48]. Serum samples from 189 subjects who developed diabetes and 189 matched controls were subject to metabolomic profiling. Lipids with lower carbon number and double bond content were associated with increased risk of developing diabetes. Lipid acyl chain content could be applied for risk assessment and for pharmacological interventions. For example, it was shown that those lipids associated with greater risk of developing diabetes decreased in concentration after insulin treatment.

Other studies have investigated early metabolic changes associated with the later progression to diabetes. Orešič and colleagues have applied UPLC–MS and comprehensive GCxGC–MS to study cord blood and serum of children in the Finnish birth cohort study T1D prediction and prevention study (DIPP) to show that dysregulation in lipid and amino acid metabolism precedes islet autoimmunity in T1D [45]. Metabolic changes were observed in subjects studied longitudionally who later developed T1D and included increases in the concentrations of glutamate, leucine, isoleucine and lysophosphotidylcholines (which are proinflammatory lipid mediators) and decreases in succinate, citrate, triglycerides and phospholipids including ether phosphocholines, phosphocholines and sphingomyelins. This work highlights the power of metabolomics in challenging old ideas, the research effectively shows that there is a possibility that increases in the concentrations of toxic metabolites may damage β-cells (and others) before the antibody response observed later in the progression to T1D. Bougneres and Valleron have also shown metabolic changes, which precede T1D development, although with no strong biological reasoning as to how these changes are linked to autoimmunity and β-cell destruction [49]. A separate paper has discussed the potential role of infant feeding in an increased risk of developing T1D later in life [50] and highlights how early environmental factors can influence the onset of specific diseases. These studies not only identify potential biomarkers for predicting the development of DM many years prior to when overt abnormalities in serum glucose levels are present, but as importantly they define novel roles for both amino acids and lipids in the pathophysiology of DM development, which precede the well-described changes in glucose metabolism. These branched-chain amino acids and lipid metabolites and pathways

may represent novel targets for the development of future therapies that target the very earliest pathophysiological processes that lead to the eventual development of DM.

Impaired glucose tolerance

Other studies have investigated metabolic changes associated with IGT, one of the earliest clinical manifestations of insulin resistance and a precursor of diabetes. Zhao and colleagues have studied the plasma and urine of IGT and control subjects and shown that the metabolic profiles are different. Changes in fatty acid, tryptophan, uric acid, bile acid, lysophosphatidylcholine and TCA-metabolism, were observed along with lower concentrations of metabolites derived from gut microflora (hippuric acid, methylxanthine, methyluric acid and 3-hydroxyhippuric acid) [51]. Lucio and colleagues have applied Fourier Transform MS to define that a decline in insulin sensitivity was associated with changes in arachidonic acid, essential fatty acid and bile acid metabolism and the biosynthesis of unsaturated fatty acids and steroid hormones [52]. These studies suggest that the diabetic biochemical phenotype is present many years before clinically overt diabetes is present, which is currently defined on the basis of elevations in fasting or post-prandial glucose levels [204] and may eventually lead to newer diagnostic classifications based on such early metabolite signatures.

Heart disease

The discovery of putative biomarkers associated with the presence, progression or severity of numerous cardiac-related diseases have been reported. An early study in 2005 provided a good example of how metabolomics can be applied in a longitudinal manner and defined novel biomarkers of significant flow-limiting coronary artery disease (CAD) associated with the development of significant cardiac ischemia following exercise testing [53]. Blood samples were collected before and after an exercise stress test for 36 patients (18 cases in which the stress test demonstrated stress inducible ischemia and, therefore, the presence of flow limiting obstructive CAD and 18 control cases in which the stress test was negative). The collection of samples before and after stress testing allowed genetic and environmental variation to be minimized as each subject acted as their own control. Semi-targeted analysis was performed and showed a strong link between the presence of inducible cardiac ishemia following stress testing and changes in metabolism with six of the top 23 most changed metabolites being present in the TCA cycle. The citric acid cycle plays a central role in metabolism within the myocardium, with several catabolic pathways converging on the cycle including substrates derived from both glycolysis and β-oxidation of fatty acids to form NADH intermediates that enter the oxidative phosphorylation pathway to produce ATP. It is unclear whether elevation of these TCA cycles intermediates points to metabolic stress within the ischemic myocardium or whether they are derived from other tissues such as skeletal muscle. The strengths of such studies lies in the ability to derive biological information relating to pathophysiology; however, as with many of these biomarkers studies, these findings have not been validated in a larger validation cohort. Similarly, Barba and colleagues have applied serum-based ^{1}H NMR to predict exercice-induced ischemia in patients with suspected CAD. In this small study of 31 patients (22 subjects with and nine subjects without reversible myocardial perfusion), 84% were correctly predicted based on a cross-validated PLS–DA model [54], although again from this study it is unclear whether metabolite changes seen where derived from ischemic myocardium or other tissues such as skeletal muscle. Lin and colleagues have shown that changes in four classes of phosholipids have been reported and related to silent myocardial ischemia in a study of 14 diagnosed silent myocardial ischemia subjects and 25 matched controls [55].

An earlier study published in 2002 applied ^{1}H NMR to diagnose the presence and severity of coronary heart disease with a specificity greater than 90% reported for distinguishing subjects with severe atheromatous lesions in their coronary arteries, subjects with stenosis of all three major coronary vessels and subjects with angiographically normal coronary arteries [56]. Coronary heart disease is the most common disease affecting the heart and is defined as the failure of the coronary blood supply to provide sufficient nutrients to the cardiac muscle and related tissues. The most common disease type is CAD, a result of the development of atherosclerotic plaques leading to reduced blood flow. However, further studies have not been able to replicate these findings, and discussed potential confounding factors and their impact on the biomarker signature of lipids (gender and statin drug treatment) [57], which highlights the importance of appropriate experimental design in metabolomics experiments to minimize the risk of false discovery.

A study by Dunn and colleagues applied GC–TOF-MS to discover biomarkers of heart failure in the serum of 52 cases and 57 matched controls. The study highlighted changes in a range of metabolites related to heart failure. Of these, 2-oxoglutarate (2-OG) and pseudouridine showed the greatest differences between cases and controls, with 2-OG being an intermediate of the TCA cycle and pseudouridine being a marker of cell turnover [25]. These initial findings have been used to define hypotheses that have been tested and validated [26], showing that discovery-based metabolomics can in fact be used to discover biomarkers and provide greater molecular pathophysiological understanding.

Studies to define early myocardial injury have also been performed. Lewis and colleagues have applied metabolomic analysis to 36 patients with hypertrophic obstructive cardiomyopathy undergoing alcohol septal ablation in which alcohol is injected down a septal artery to cause planned myocardial infarction (PMI) of the hypertrophic left ventricular septum, thereby, reducing the outflow tract gradient in these patients and improving symptoms [58]. Cardiomyopathies are a collection of diseases affecting the myocardium (heart muscle). Hypertrophic cardiomyopathy is an inherited autosomal condition in which cardiac hypertrophy (thickening), associated with sudden cardiac death, is caused by mutations in one of a number of sarcomere proteins. Blood samples were collected before and after PMI. Metabolic changes were observed 10 min after PMI and included perturbations in pyrimidine metabolism, the tricarboxylic acid cycle and its upstream contributors, and the pentose phosphate pathway. The results were validated in an independent set of subjects. A metabolic signature composed of aconitic acid, hypoxanthine, trimethylamine *N*-oxide and threonine separated patients presenting with acute myocardial infarction secondary to occlusive coronary vascular disease. Mamas and colleagues have applied a similar strategy, using transient coronary artery occlusion during percutaneous coronary intervention and serum metabolomics with UPLC–MS to define changes related to early cardiac ischemia [59]. Here increases in lysophosphotidylcholines, arachidonic acid, docosahexaenoic acid and bilirubin and a decrease in tryptophan was observed between pre- and post-transient coronary artery occlusion samples, which may highlight the importance of lipid changes during the early stages of myocardial infarction especially given that many of the lipid species identified are thought to play a critical role in the development of cardiac arrhythmias that underlie the development of suddent cardiac death, an important cause of mortality especially in early stages of myocardial infarction.

Fewer reported studies have been observed to discover biomarkers of atherosclerosis, an inflammatory condition that occurs within blood vessels in which deposition of lipids within the vessel wall activates inflammatory processes that result in the formation of a plaque that reduces the luminal area of the blood vessel, thereby resulting in a decreased blood flow through it. This process may occur in any part of the vascular tree, and the clinical manifestations will depend on which organ system is affected. Atherosclerotic disease in coronary arteries may manifest as anginal symptoms when the blood flow within the diseased vessel is insufficient to meet the metabolic requirements of the heart whilst rupture of these atheromatous plaques with the development of occlusive thrombus within the vessel may result in myocardial infarction or stroke. A Chinese study of 16 stable patients with clinically evident atherosclerosis and 28 healthy control subjects applying GC–MS has described changes in fatty acid metabolism (palmitate, stearate, and 1-monolinoleoylglycerol). These validated previous findings showing that palmitate targets apoptosis and inflammation pathways in atherosclerosis [60].

As an example of non-heart-based CVD, a separate study by Ciborowski and colleagues have searched for potential biomarkers of abdominal aortic aneurysm (AAA) by the culturing of aortic tissue and analysis of the conditioned medium (metabolic footprint) after culture [61]. The culture medium mimics blood, and differential secretion of metabolites related to tissue type or pathology provides the discovery of tissue-specific biomarkers. These markers can then be searched for in blood. The method discovers tissue specific biomarkers in a controlled environment before their validation in the general population rather than searching for these in a complex media (blood or urine) where the probability of success can be expected to be lower. AAA is a permanent and localized aortic dilation (>3 cm) where a rupture can occur leading to hemorrhage. The rupture can be a direct result of the dilation or a result of the formation and growth of a intraluminal thrombus. In this study, tissue from intraluminal thrombus (both luminal and abluminal), aneurysm aortic wall and healthy aortic wall were studied. Changes in hippurate, 5-oxoproline, acylcarnitines,

lysophosphotidylcholines, leukotrienes, fatty acid amides and vitamin E were observed when comparing aneurysm aortic wall and healthy aortic wall. Changes in fatty acids, fatty acid amides, octylamine, glycerophosphotidylcholines, vitamin B6, leukotrienes, lysophosphotidylcholines, acylcarnitines, glutamylhydroxyproline and docosahexaenoic acid were observed when comparing intraluminal thrombus and aneurysm aortic wall.

The earlier examples show how metabolomics is applied as a discovery tool. The high-throughput capabilities can also be applied in targeted analysis where the metabolites or biomarkers of biological interest are known. For example, previous discovery studies (which did not apply metabolomics strategies) implicated arginine methylation in the increased risk of adverse cardiac events. A targeted metabolite analysis of asymmetrical dimethylarginine (ADMA), symmetrical dimethylarginine (SDMA), *N*-monomethylarginine, methyl-lysine, arginine and its major catabolites (citrulline and ornithine) was performed in 1011 subjects to validate the hypothesis [62]. ADMA and SDMA showed a positive risk predictor (in stable patients undergoing cardiac evaluation) of obstructive CAD and major adverse cardiac events, which include myocardial infarction, stroke and death. The power of this study comes in the ability to validate initial hypotheses in a large at-risk cohort of subjects.

Molecular pathophysiological mechanisms associated with heart disease & diabetes

The role of metabolomics in biomarker discovery has been reviewed earlier. A greater number of metabolomic studies have the objective to increase knowledge related to molecular pathophysiological mechanisms of disease or drug efficacy/toxicity so to aid in defining drug targets and therapuetics.

■ Atherosclerosis

A number of animal and human studies have investigated atherosclerosis. The apolipoprotein E-deficient (apoE) mouse is one of the most commonly used murine models of atherosclerosis. ^{1}H NMR analysis of urine from this model has shown that xanthine and ascorbate were elevated in normal mice compared with apoE mice and were possible markers of atherosclerotic plaque formation [63]. Mayr and colleagues have integrated data from metabolomic and proteomic analysis of atherosclerotic vessels from apoE-deficient mice to study atherogenesis [64]. Proteomic analysis showed redox imbalance, oxidative stress, immunoglobulin deposition and impaired energy metabolism preceding lesion formation in apoE$^{-/-}$ mice. ^{1}H NMR metabolomics showed decreases in alanine and the adenosine nucleotide pool which validated the impaired energy metabolism discovery in proteomics.

DNA damage is linked to atherosclerosis development and the effect of DNA damage has been studied in apoE$^{-/-}$ mice that were haploinsufficient for the protein kinase *ATM* gene, which coordinates DNA repair. Profiling of mouse tissue showed changes related to mitochrondrial defects, including reduced oxidative phosphorylation. DNA damage and non-repair resulted in ketosis, hyperlipidemia and increased fat storage, which resulted in atherosclerosis, and the view that mitochrondria dysfunction may represent a pharmacological target in CVD [65]. A study in rats has shown changes in phenylalanine, tryptophan, bile acids and amino acid metabolism and the study also described increases in lysophosphotidylcholines after atherosclerotic plaque development [66]. Other rodent models have investigated time-dependent development of early atherosclerosis and have shown changes in amino acid and fatty acid oxidation metabolism during this plaque development, again implicating mitochrondrial dysfunction in atherosclerosis [67]. Whilst these studies may provide initial insights into pathophysiological changes in metabolism that may contribute to the development of atherosclerosis, it is important to appreciate that the development of atherosclerosis is a multifactorial process and only a vanishingly small proportion of cases seen in the clinical arena have abnormalities or mutations in apoE and so it remains unclear whether these pathways are of relevance in the human condition. These models are also limited in that the patterns of atherosclerosis seen and the organ systems affected are different to that observed in the clinical setting. Nevertheless such metabolomic studies allow elucidation of pathways that may be validated and further investigated in targeted analysis in human subjects with clinically/subclinically evident atherosclerosis.

■ Influence of diet & gut microflora on heart disease

There is clear evidence of the relationship between diet and the risk of atherosclerosis development, less is known on how gut microflora may modulate this risk through their metabolic pathways

since intestinal microbial communities can influence the efficiency of harvesting energy from diet, and consequently influence susceptibility to obesity [68], and may also have an active role in the susceptibility to insulin resistance [69]. Wang and colleagues have discovered a link between gut flora metabolism of phosphotidylcholine and an increased risk of CVD. Choline, trimethylamine-*N*-oxide (TMAO) and betaine were all shown to predict risk for CVD in a large clinical cohort. Dietary supplementation with TMAO and betaine promoted atherosclerosis in a murine model whereas dietary-enhanced choline atherosclerosis was inhibited in germ-free mice and in atherosclerosis-prone mice whose intestinal microflora were suppressed [70]. Interestingly, mice supplemented with TMAO or betaine in their diets all showed enhanced macrophage levels of two macrophage scavenger receptors implicated in atherosclerosis, CD36 and SR-A1 and that the intestinal microflora had a key role in the endogenous formation of cholesterol-laden macrophage foam cells, one of the earliest cellular hallmarks of the atherosclerotic process. The discovery of such a relationship between gut flora-dependent metabolism of dietary phosphatidylcholine and the development of CVD provides exciting opportunities for the development of novel therapies that may target gut microflora as a means of modifying cardiovascular risk.

Cheng and colleagues have shown a distinct diet–genotype interaction in rats between low-density lipoprotein receptors (LDLRs) and high fat/cholesterol diets. Dysfunction of LDLRs result in hypercholesterolemia, elevated blood cholesterol, atherosclerosis and coronary heart disease. ^{1}H NMR analysis of plasma and urine of LDLR knockout mice showed changes in TCA and fatty acid metabolism and a change in the choline oxidation pathway, observed through a decrease in betaine and dimethylglycine in urine [71]. These changes are observed in different apoE mice strains on the same diet and so could be seen as a first step towards the development/validation of markers of atherosclerosis in larger human cohorts. Fewer studies of atherosclerosis have been observed in humans. Teul and colleagues studied nine subjects with stable carotid atherosclerosis and 10 healthy controls and showed changes in amino acid metabolism [72]. This study also highlighted the presence of insulin resistance (but not diabetes) in carotid atherosclerosis subjects showing the potential link between insulin resistance and atherosclerosis.

Other heart diseases

Dilated cardiomyopathy (DCM) is one of the most common non-ischemic cardiomyopathies seen in clinical practice and is characterized by left ventricular dilation, which is often accompanied by a deterioration in left ventricular function. It has a number of different etiologies including infective, metabolic, drug and familial forms although often an underlying etiology cannot be identified. DCM has been studied in 39 patients and 31 matched controls and showed increases in TCA metabolites and fatty acid β-oxidation products and decreases in steroid metabolites, glutamine, threonine and histidine. Decreased glutamine and increased concentrations of 3-methylhistidine and prolylhydroxyproline levels implicated increased myofibrillar and collagen degradation/deposition in DCM patients [73] that have been confirmed in post mortem studies. Interestingly, these authors found that pseudouridine and 2-OG where elevated in their DCM patients, which was similar to the observation of Dunn and colleagues [25] who found that pseudouridine and 2-OG were highly predictive of the development of heart failure in a cohort of patients with chronic heart failure. 2-OG is a citric acid cycle intermediate. It is a co-substrate in hypoxia signaling and collagen biosynthesis. It is abundant mostly in the kidney and is the ligand of GPR99, a G-protein coupled receptor and we have shown that GPR99 is expressed in cardiomyocytes and that exposure of neonatal rat cardiomyocytes to 2-OG increase mRNA levels of VEGF receptor-1 and PGF that are involved in angiogenesis and growth [74].

Mervaala and colleagues have described distinct metabolic changes related to angiotensin II-induced cardiac hypertrophy. Decreased levels of fatty acids are potentially associated with changes in β-fatty acid oxidation, whereas an increase in hypoxanthine suggested an increase in purine degradation. These metabolic changes are potentially related to changes in substrate use and mitochrondrial dysfunction [75].

Mutant mouse models have been used to study cardiac fibrosis through a mutation in the mitochondrial trifunctional protein β-subunit [76]. Metabolomic-guided screening coupled to *N*-ethyl-*N*-nitrosourea-mediated mutagenesis identified mice that exhibited elevated levels of long-chain acylcarnitines and the mice displayed symptons of cardiac fibrosis. De Souza and colleagues have studied how congestive heart failure can lead to atrial structural remodeling and increased susceptibility to atrial fibrillation [77].

Metabolomic and proteomic profiling of left-atrial cardiomyocytes and cardiac tissue from sham and ventricular-tachypaced congestive heart failure dogs was performed. High-resolution ^{1}H NMR showed increased levels of glucose and alanine, accumulation of ADP in relation to ATP and decreases in the concentration of α-ketoisovalerate. These metabolic changes indicated a reduction in energy utilization and a change from glycolysis to α-keto acid metabolism.

Abnormalities in protein kinase C (PKC) signaling have been implicated in heart failure, myocardial hypertrophy, and ischemic preconditioning. Using PKC-δ knockout mice, Mayr and coworkers have performed combined proteomic and ^{1}H NMR metabolomics studies to define changes in metabolism that may contribute to the phenotype associated with PKC-mediated CVD disease development. Research has shown that PKC-δ loss changes cardiac metabolism, specifically a large decrease in the ratio of glycolytic end products compared with end products of lipid metabolism. Combined with proteomic data, the results suggest that a loss of PKC-δ creates a shift from glucose to lipid metabolism for energy production [78]. A separate study has investigated PKC-ε-mediated cardioprotection in relation to regulating glucose metabolism in murine hearts and how PKC-ε activity influences cardiac glucose and energy metabolism [79]. A study of CAD and left ventricular dysfunction has also shown differences in myocardial fuel uptake and its association with disease and ischemia/reperfusion during cardiac surgery [80]. Shah and colleagues have integrated genetic and metabolomic data from 117 individuals in the GENECARD study to show that several classes of metabolites are highly associated with high heritabilities of metabolites in premature CAD including amino acids, free fatty acids and acylcarnitines which are all associated with fuel uptake and energy production. The data suggest a genetic basis for these changes in profiles [81].

Insulin resistance & diabetes

The study of diabetes, a metabolic disease, has been widespread and review articles discussing the role of metabolomics and its advantages and limitations have been recently published [82–85].

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes and play essential roles in the regulation of cellular differentiation, development and metabolism and have several different subtypes (PPAR-α, -β, -γ and -δ) that differ both in the tissues that they are expressed and their function. PPARs are targeted by a number of drug therapies used in clinical practice, such as fibrates (PPAR-α) and thiazolidinediones used in the treatment of diabetes (PPAR-γ). Animal models have been applied to study diabetes and the metabolic syndrome. Atherton and colleagues applied ^{1}H NMR spectroscopy and MS to study PPAR-α null mice. PPAR-α partly regulates genes encoding mitochondrial, microsomal and peroxisomal β-fatty oxidation in the liver. Heart, skeletal muscle, liver and adipose tissue of PPAR-α null mice were analyzed at 1 month of age. Perturbations in glycolysis, TCA cycle and glucogenesis were observed along with a metabolic perturbation in adipose tissue, even though PPAR-α is only weakly expressed in this tissue [86]. These findings may identify how pathophysiological changes in metabolic pathways within adipose tissue, which may contribute to changes in adipose tissue function, and may modulate secretion of adiponectins and leptins that have been shown to predict future diabetes development.

A systems biology study integrating metabolomic and transcriptomic data has been performed to define metabolic changes asociated with diabetes in symptomatic mice. Connor and colleagues have integrated liver adipose and muscle transcriptomic data and ^{1}H NMR urine metabolomic data for diabetic db/db mice and control db/+ mice [87]. A total of 24 distinct metabolic pathways were perturbed in diabetic mice including mitochrondrial dysfunction and changes in lipid metabolism, glucogenesis, oxidative stress and protein/amino acid metabolism. Again similar to the data derived from large clinical cohorts, branched-chain amino acids (BCAA) were observed to change, potentially indicating that elevated BCAA are key in the pathophysiology of diabetes development. Although studying symptomatic mice, the results show the importance of a systems-level approach to clinical research, through the integration of multiple datasets. This will undoubtedly become more important in the years to come. A separate study has assessed changes in prediabetic mice (ddY strain mice [ddY-H]), which naturally develop diabetes during aging. The most striking change observed was an alteration in lysine biosynthesis and degradation metabolism [88].

A number of studies have discovered perturbed metabolic pathways associated with insulin resistance and diabetes. In streptozotocin-induced

T1D rats, Zhang and colleagues have shown increases in urinary concentrations of glucose, alanine, lactate, ethanol, acetate and fumarate in T1D diabetic rats compared with controls. Correlation analysis showed specific changes in glucose, TCA and gut-microflora metabolism along with choline metabolism [89]. Lanza and coworkers applied ^{1}H NMR and LC–MS/MS to study metabolic differences between T1D during insulin treatment (I+) and during acute insulin depreviation of non-diabetic controls (I-). I+ and I- were clearly separated in multivariate space and several pathways were implicated in the separation including protein synthesis and breakdown, gluconeogenesis, ketogenesis, amino acid oxidation, mitochondrial bioenergetics and oxidative stress [90]. Spegel and colleagues have studied glucose-responsive and glucose-unresponsive β-cell lines. Dysregulated secretion in the glucose-unresponsive cell line indicated a decoupling of glycolysis and TCA metabolism, which was recoupled when mitochrondria fuel which bypassed glycolysis was introduced [91]. Tai *et al.* have shown in a relatively large study of 263 non-obese Asian-Indian and Chinese men that changes in amino acids and related metabolites are associated with insulin resistance including increased levels of alanine, proline, valine, leucine/isoleucine, phenylalanine, tyrosine, glutamate/glutamine and ornithine, and a cluster of branched-chain and related amino acids [92]. These indicate a change in protein metabolism in insulin resistant men.

Skeletal muscle plays a central role in regulating insulin resistance through a number of mechanisms including the regulation of glucose transport by GLUT-4, a glucose transporter protein found in skeletal muscle, which is insulin regulated, and targeted disruption of GLUT-4 in skeletal muscle causes insulin resistance [93]. Changes in skeletal muscle metabolism has also been studied in relation to insulin resistance. Koonen and colleagues have studied changes in young and middle-aged wild-type and CD36 mice fed either a standard or high-fat diet for 12 weeks [94]. Aged mice fed on a normal diet showed an increase in triglycerides without insulin resistance. However, aged mice fed on a high-fat diet showed an increased susceptibility to insulin resistance, although this could be reversed in CDC36 mice where skeletal muscle fatty acid transport and excessive lipid accumulation are reduced. Koves and colleagues have shown that there is a strong association between skeletal insulin resistance and lipid-induced mitochrondrial stress [95]. The study showed increased fatty acid β-oxidation, inhibited switching to carbohydrate substrates during the fasted-to-fed state and a reduction in organic acid intermediates of the TCA cycle. Lipid-induced insulin resistance was inhibited by a reduction of fatty acid uptake into the mitochrondria. Kuhl and coworkers have studied exercise-induced improvements in insulin sensitivity in T2D subjects [96]. The specific aims were to identify individuals with improved insulin sensitivity as a consequence of increased physical activity. Insulin sensitivity was shown to be improved in subjects after the exercise regimen. A total of 44 metabolites defined differences between trained and non-trained diabetics and included inositol-1-phosphate, an inositol phosphate that is part of a group of mono- to polyphosphorylated inositols that play crucial roles in diverse cellular functions, such as cell growth, apoptosis, cell migration, endocytosis and cell differentiation.

The oral glucose tolerance test (GTT) is applied to assess for the presence of abnormalities in glucose regulation and forms part of the diagnostic criteria for DM and impaired glucose tolerance. Shamam and colleagues performed two independent GTT human studies following overnight fasting of nondiabetic individuals. Bile acids, urea cycle intermediates, and purine degradation products all showed relative changes between pre- and post-GTT samples. This showed the effect of insulin on four processes; proteolysis, lipolysis, ketogenesis and glycolysis. In the GTT results of subjects who later developed diabetes, changes in glycerol (indicative of lipolysis) or leucine/isoleucine (proteolysis) compared with controls were the strongest indicators of progression to diabetes and the authors suggested that individuals can be unaffected by either lipolysis or proteolysis during the development of insulin resistance [46]. Zhao and colleagues have studied the metabolic changes in plasma of healthy subjects following a GTT. The results showed a decrease in saturated and monounsaturated free fatty acids (with the fastest rates of change observed for palmitic and oleic acids), an increase in polyunsaturated free fatty acids and a decrease in C_{10}, C_{12} and $C_{14:1}$ acylcarnitines, which all imply a switch from β-oxidation to glycolysis and fat storage. There were also increases in bile acid concentrations and lysophosphotidylcholine concentrations [97]. Lin and colleagues have applied the GTT to control rats and rats fed high-fructose diets, a model of T2D. Changes in amino acids, the biosynthesis of purines, polyunsaturated fatty acids and

phospholipids were observed and allowed differentiation between control and rats fed high-fructose diets. These data suggested that oxidative stress, including lipid peroxidation, the elevated amino acids and the perturbation of fatty acids, were caused by the high-fructose diet in liver and skeletal muscle tissues in rats [98].

Development of therapeutics to treat cardiovascular diseases

Cardiovascular drugs comprised the largest pharmaceutical drug category in 2006 worldwide with a market value of US$100.8 billion representing 17.8% of the pharmaceutical market. The treatment of CVD is divided into two categories: preventive treatment and the treatment of existing conditions. The development of therapeutics to treat CVD forms a major business opportunity for the pharmaceutical industry in view of the number of potential users, which is increasing hand in hand with the global obesity epidemic, and thus the financial benefits achievable. It is expected that metabolomics, and in particular lipidomics, will play an important role in the study of toxicity and efficacy of drugs in CVDs in the future [99].

■ Heart disease

One of the most common drugs applied to reduce the risk of CVD are statins, used in primary prevention to inhibit the development of CVD in high-risk individuals as well as secondary prevention to inhibit recurrence once CVD, such as myocardial infarction, stroke and so on, have occurred. Increased serum low density lipoprotein cholesterol (LDL-C) levels are associated with increased risk of developing CVD. Statins are used to lower cholesterol by inhibiting the enzyme HMG-CoA reductase in the liver. Kaddurah-Daouk and colleagues have studied the lipidome response to simvastatin in the Cholesterol and Pharmacogenetics study. Subjects from the upper and lower tails of the LDL-C response distribution were studied. Correlations between cholesterol ester, phospholipid metabolites and LDL-C response to statin treatment were observed along with correlations between C-reactive protein and plasmalogens, indicating their involvement in inflammation processes [100].

Li and colleagues have studied the beneficial effects of soluble epoxide hydrolase inhibitors on cardiac hypertrophy and remodeling in a myocardial infarction murine model [101]. These catalyze the conversion of epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids. EETs have vasodilatory properties and inhibit the activation of nuclear factor-κB-mediated gene transcription, whose downstream effects include stimulation of TNF-α and interleukin production that have important roles in the development of inflammation and apoptosis. Results showed a decrease in the ratio of EETs/dihydroxyeicosatrienoic acids demonstrating an inflammatory status, which, when treated with epoxide hydrolase inhibitors, showed a change from an inflammatory state towards its resolution through changes to a baseline concentration in a range of lipid mediators.

Doxorubicin (DXR) is a potent antineoplastic agent, with widespread clinical use, although one of the main limitations associated with the use of this therapy is cardiotoxicity, specifically oxidative stress and changes in cardiac energy production and usage. Andreadou and colleagues have identified biomarkers of DXR toxicity in rat tissue 72 h after dosage in Wistar rats after a 20 mg/kg single dosage [102]. Metabolite markers of cardiac DXR toxicity identified included acetate, succinate and branched-chain amino acids and these markers returned to a metabolic status similar to pre-dosage of DXR after treatment with oleuropein; a phenolic antioxidant, present in the olive tree and reported to confer protection against DXR cardiotoxicity. Chinese traditional medicines are also being evaluated in relation to their potential treatment effects. Lv and colleagues have studied the intervention effects of Compound Danshen Tablets, a herbal treatment, on myocardial ischemia in rats. An OPLS model showed differences between sham and MI rats and the reversal of these changes (particularly for four metabolites in purine metabolism; hypoxanthine, xanthine, inosine and allantoin) to sham levels for MI rats treated with compound Danshen tablets [103].

Toxicity of drugs prescribed for other ailments can also result in cardiotoxicity. Force recently reviewed the cardiotoxicity of kinase inhibitors applied in the treatment of cancers [104]. Non-steroidal anti-inflammatory drugs are amongst the most commonly used class of drugs in clinical practice. The primary effect of non-steroidal anti-inflammatory drugs is to inhibit the enzyme cycloxygenase (COX), thereby inhibiting conversion of arachidonic acid to prostaglandins, prostacyclin and thromboxanes, which are key mediators of inflammation. Two related isoforms of cycloxygenase have been identified, COX-1 and -2. Selective COX-2 inhibitors, such as rofecoxib and celecoxib, were developed to have

anti-inflammatory effects without the associated adverse sequelae associated with COX-1 inhibition, such as gastrointestinal bleeding. However, COX-2 inhibitors have more recently been shown to significantly increase the risks of developing CVDs and some COX-2 inhibitors, such as rofecoxib, have been withdrawn from the market by the manufacturer. A metabolic profiling study has shown in a murine model that oral administration of rofecoxib for 3 months results in a 120-fold elevation of 20-hydroxyeicosatetraenoic acid (20-HETE), which correlates with a shorter bleed time of the tail (i.e., enhanced clotting ability and the detrimental effect this can have). This discovery led to evidence demonstrating that rofecoxib inhibits 20-HETE degradation, does not promote its biosynthesis and increases rat platelet aggregation, which may have detrimental effects associated with CVDs. This may partly provide mechanistic insight in to the findings of major clinical studies such as the VIGOR study comparing rofecoxib to naproxen in patients experiencing rheumatoid arthritis [105], in which rofecoxib was associated with significantly more thrombotic events, notably myocardial infarctions. More recently data from a placebo-controlled trial for rofecoxib in the prevention of colon cancer recurrence also suggested an excess of thrombotic events after long-term (>18 months) consumption of rofecoxib [106].

Diabetes

Interesting research by Cooper and colleagues have indicated that diabetic complications are, in part, a consequence of increased copper concentrations in tissues, which through catalysis of the Fenton-like or Haber-Weiss reactions increase the production of reactive oxygen species (ROS) and their subsequent accumulation in tissues. Copper-selective chelation (applying triethylenetetramine, otherwise known as Trientine hydrochloride, which is already a drug prescribed for Wilson's disease) is suspected to decrease the unbound copper concentration and leads to a decrease of ROS concentrations. Trientine hydrochloride has been shown to improve function and antioxidant defences in cardiovascular tissues of a rat model of diabetes [107] and to retard the onset and slow the progression of diabetes in human amylin/islet amyloid polypeptide transgenic mice [108].

Other potential or currently prescribed diabetic treatments have also been studied applying metabolomic techniques including a nutraceutical extracted from rosemary seeds [109], a chinese medicine berberine, which reduced the high levels of free fatty acids [110], metformin [111] and leptin therapy, which implies that this has significant short and long-term effects/improvements compared with insulin monotherapy [112]. Zhang and colleagues have studied the effect of rosiglitazone treatment, currently a drug prescribed for diabetes, on diabetic nephropathy in streptozotocin-induced diabetic mice [113]. The metabolomic study showed that of 56 metabolites, which changed between diabetic and nondiabetic mice, 21 returned to baseline levels after administration and the authors imply that the drug reduced ROS accumulation in these tissues.

Van Doorn and colleagues have discovered putative early responding biomarkers of rosiglitazone treatment in human subjects with T2D and healthy controls. No metabolic changes were observed for treated healthy controls but changes in urinary hippurate and aromatic amino acids and increases in plasma BCAAs, alanine, glutamate and glutamine in the T2D group were observed [114]. Bao and colleagues have shown that rosiglitazone treatment is able to reverse abnormal concentrations of valine, lysine, glucuronolactone, C16:0, C18:0 and C18:1 fatty acids and urate to nondiabetic levels [115].

Future perspective

This review of the role metabolomics has played to date in the study of CVDs and diabetes highlights the potential of metabolomics: to define molecular pathophysiological processes implicated in pre-symptomatic and symptomatic stages of diseases; to define metabolic changes and their link to risk association; to discover potential prognostic or diagnostic biomarkers of disease; and to study the efficacy and toxicity of therapeutics.

The impact of many of these studies in routine clinical practice is limited and currently there has been little progress towards development of new treatments (although some of the therapeutics are already in use) or application as clinically relevant biomarkers. There is an essential requirement to progress developments in the laboratory to benefit patients in hospitals, defined as translational medicine or bench-to-bedside research. However, the integration of results from the studies discussed highlight some commonality in the areas of metabolism being perturbed. For example, in diabetes perturbations in branched-chain amino acids, glycolysis, TCA metabolism and mitochondrial metabolism has been implied in multiple studies.

However, further validation of the discoveries are required in the future to provide increased confidence of the validity. Only from continued application of metabolomics and an increasing number of success stories validated in independent sample sets from independent biological studies will metabolomics be taken onboard to be applied routinely in these areas of research. The integration of data from multiple functional levels (as shown above in a limited number of studies) and the integration of *in silico* modeling techniques with experimental data in a systems biology strategy will undoubtedly improve the successes of understanding molecular processes in the complex system that is the mammal.

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Executive summary

- Metabolomics is a powerful and emerging strategy to investigate the phenotype of mammalian systems.
- Many metabolomics applications are discovery based to define molecular processes associated with disease onset or progression or drug efficacy/toxicity.
- Metabolomics has been applied to discover biomarkers of disease risk. The currently most robust study applying two independent at-risk populations has described three amino acids as predictors of later onset of Type 2 diabetes.
- Metabolomics has been applied to study the metabolic changes which precede hyperglycemia in Type 1 and Type 2 diabetes and which are associated with a range of cardiovascular diseases.
- Metabolomics has been applied to discover biomarkers of drug efficacy and toxicity and apply these to investigate the efficacy of potential or currently prescribed therapeutics.

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