Metabolomics and ischaemic heart disease

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Abstract
Ischaemic heart disease accounts for nearly half of the global cardiovascular disease burden. Aetiologies relating to heart disease are complex, but dyslipidaemia, oxidative stress and inflammation are cardinal features. Despite preventative measures and advancements in treatment regimens with lipid-lowering agents, the high prevalence of heart disease and the residual risk of recurrent events continue to be a significant burden to the health sector and to the affected individuals and their families. The development of improved risk models for the early detection and prevention of cardiovascular events in addition to new therapeutic strategies to address this residual risk are required if we are to continue to make inroads into this most prevalent of diseases. Metabolomics and lipidomics are modern disciplines that characterize the metabolite and lipid complement respectively, of a given system. Their application to ischaemic heart disease has demonstrated utilities in population profiling, identification of multivariate biomarkers and in monitoring of therapeutic response, as well as in basic mechanistic studies. Although advances in magnetic resonance and mass spectrometry technologies have given rise to the fields of metabolomics and lipidomics, the plethora of data generated presents challenges requiring specific statistical and bioinformatics applications, together with appropriate study designs. Nonetheless, the predictive and re-classification capacity of individuals with various degrees of risk by the plasma lipidome has recently been demonstrated. In the present review, we summarize evidence derived exclusively by metabolomic and lipidomic studies in the context of ischaemic heart disease. We consider the potential role of plasma lipid profiling in assessing heart disease risk and therapeutic responses, and explore the potential mechanisms. Finally, we highlight where metabolomic studies together with complementary -omic disciplines may make further inroads into the understanding, detection and treatment of ischaemic heart disease.

Key words: atherosclerosis, biomarker, ischaemic heart disease, lipidomics, mass spectrometry, metabolite, metabolomics, risk

INTRODUCTION

IHD (ischaemic heart disease) is a major contributor to global mortality and morbidity, and accounts for approximately half of the global CVD (cardiovascular disease) burden. Of the 57 million global deaths in 2008, approximately 30% (17.3 million deaths) were attributable to CVD [1]. In the U.S.A., the leading cause of death is heart disease, translating into approximately 1 in 6 deaths in 2007 [2]. Similarly, in Australia, 34% (48,456) of all deaths were caused by CVD in 2008 and IHD accounted for 49% of these deaths [3]. Economic and healthcare costs in both the prevention and treatment of heart disease are substantial and constitute a major concern for public health policy. In 2004, the U.S.A. spent US $368 billion for direct and indirect costs due to CVD [4]. In addition, CVD costs the Australian health system approximately AUS $6 billion per year [5]. The World Health Organization estimates a global increase in IHD burden from 47 million DALY (disability adjusted life years; a measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or premature death) in 1990 to 82 million DALY by 2020 [6].

There is a growing urgency for the introduction of population screening for the early detection of atherosclerosis and the identification of ‘high-risk’ patients. The report from the SHAPE (Screening for Heart Attack Prevention and Education) Task Force [7] calls for non-invasive screening of all asymptomatic men 45–75 years of age and asymptomatic women 55–75 years of age to detect and treat those with subclinical atherosclerosis. The primary target for this screening strategy is to identify the ‘vulnerable patient’, that is those individuals who are at very high risk

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; apoB, apolipoprotein B; ARB, angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; CE, cholesterol ester; CI, confidence interval; CRP, C-reactive protein; CVD, cardiovascular disease; CVE, cardiovascular event; DALY, disability adjusted life years; ESI, electrospray ionization; GWAS, genome-wide association studies; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; 15-HETE, 15-hydroxyeicosatetraenoic acid; IHD, ischaemic heart disease; KAPS, Kuopio Atherosclerosis Prevention Study; LC, liquid chromatography; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; LIPID MAPS, Lipid Metabolites and Pathway Strategy; Lipo, lipoprotein lipid; LMW, low-molecular-mass; MS/MS, tandem MS; NFκB, nuclear factor κB; oxCE, oxidized CE; PC, phosphatidylcholine; lyso PC, lyso-phosphatidylcholine; oxLDL, oxidized LDL; oxPC, oxidized PC; PE, phosphatidylethanolamine; PLA2, phospholipase A2; Lp-PLA2, lipoprotein-associated PLA2; RADAR, Rosuvastatin and Atorvastatin in different Dosages And Reverse cholesterol transport; SM, sphingomyelin; SNP, single nucleotide polymorphisms; TMAO, trimethylamine-N-oxide.

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of a CVE (cardiovascular event), but are clinically asymptomatic. Although population-based screening is still controversial, newer screening strategies, often incorporating multiple biomarkers in multivariate models, have focused on re-classification, primarily of the intermediate risk group [8–10]. Such re-classification of individuals from intermediate-to-high risk can lead to more aggressive treatment with potentially improved outcomes. In addition to screening for risk assessment in the subclinical population, there is a growing need for better tools to evaluate those that present clinically with angina or other symptoms of CAD (coronary artery disease). Non-invasive screening to quantify coronary plaques could facilitate this diagnosis.

Statin (HMG CoA reductase inhibitors)-based lipid-lowering therapy has been the single most efficacious treatment of IHD in recent decades. However, this therapy only reduces approximately 30% of burden and, even with prolonged or higher doses of therapy, statins alone will not eliminate atherosclerosis [11]. Consequently, there is a growing need to understand the molecular basis of the residual risk such that new therapeutic strategies may be developed to reduce further the burden of IHD. In addition, accurate risk assessment will be essential to target new treatments and achieve maximal efficacy, while the ability to assess the response to treatment in terms of increased or decreased risk will be useful in the ongoing management of patients. Although the application of metabolomics in the clinical setting is still in its infancy, the high-throughput nature and advanced technologies currently available for metabolomics makes this a powerful approach to address these issues.

IHD is typically characterized by the narrowing of the vessel lumen and compensatory enlargement of coronary arteries due to an accumulation of an atherosclerotic plaque within the walls of the arterial intima [11]. The rate of progression of atherosclerosis, which underscores the manifestation of IHD, is, to a large extent, unpredictable and differs markedly among seemingly comparable individuals. Subclinical atherosclerotic lesions can progress to become advanced complex plaques via plaque necrosis, which results from a combination of macrophage apoptosis and dysfunctional phagocytic clearance of the apoptotic cells [12]. Complex plaques can become unstable (a ‘vulnerable’ plaque) and rupture. However, some plaques may undergo multiple rupture/stabilization cycles without necessarily leading to a CVE. Dyslipidaemia, inflammation and oxidative stress are key mediators of disease progression; however, the molecular basis of the local and/or systemic changes that precede plaque instability are still poorly defined. Despite our detailed knowledge of plaque pathology and progression, many individuals have no clinical symptoms and so are unaware of their risk. In 30–50% of these individuals, the first indicator of atherosclerosis is an acute heart attack, which is often fatal [13]. What is clear is that the risk of a CVE relates to both the severity (extent) of the coronary plaque and to the stability of the plaque.

The present review will examine the findings from recent metabolomic studies into IHD and how metabolomics has contributed to our understanding of dyslipidaemia, oxidative stress, and inflammation. The applicability of metabolomics to the monitoring of current therapies and the identification of new therapeutic targets, as well as the development of multivariate models to predict CVD risk, will also be discussed.

METABOLOMICS

The term ‘metabolome’ was first coined by Tweeddale et al. [14] and Oliver et al. [15] in 1998. ‘Metabolome’ was described as a set of metabolites present in cells or an organism. The term ‘metabolomics’ was first introduced by Fiehn [16], and was then further defined by the Metabolomics Society (http://www.metabolomicsociety.org/) 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”. Metabolomics is now a fast-growing field in modern integrated science. It represents a directional shift in metabolic research from approaches which concentrated on single pathways to those which attempt to gain a comprehensive understanding of complex metabolic networks [17]. As a result, metabolomics as with other ‘-omics’ is often a hypothesis-generating tool. Disease-related studies driven by genomics and proteomics technologies have largely focused on finding genetic markers and the subsequent expression or regulation of the proteins to understand disease mechanisms [18–20]. Metabolomics can provide another viewpoint of disease mechanisms in the form of metabolite levels and flux, which can subsequently be integrated into the existing knowledge gained via genomic, transcriptomic and proteomic approaches to provide a systems biology view of the disease process. Although this systems approach is still in its infancy, several examples are discussed later in the present review. Metabolomics is well suited to the study of chronic disease at a population level. The high-throughput nature of the technology [21] enables its application in epidemiological studies to identify biomarkers and define metabolic pathways relating to disease. A typical workflow of metabolomic studies is illustrated in Figure 1.

Technologies used in metabolomics

NMR (nuclear magnetic resonance) spectroscopy and MS (mass spectrometry) are the primary technologies driving current metabolomics. Developments in these technologies have improved the level of sensitivity by several orders of magnitude over the past decade. In addition, there has been an evolution of MS hybrid systems, combining the advantages of several mass analysers into a single MS instrument, as well as high-resolution instruments that provide accurate mass/charge measurements and thereby facilitate metabolite identification [22].

A number of strategies have been developed for metabolomic analyses; these can be loosely grouped into targeted and untargeted approaches. In the targeted approach, a set of pre-defined metabolites are analysed in the samples of interest. Although this approach can provide a high level of sensitivity, precision and accuracy by the use of stable isotope internal standards, it provides only limited coverage of the metabolome. The untargeted approach involves unbiased analysis of a large number of metabolites [23], both unknown and known. Although this can provide greater coverage of the metabolome, it is often associated
Metabolomics and ischaemic heart disease

Figure 1  Typical workflow of metabolomic studies
Samples are collected and, where necessary, metabolites extracted often with the addition of internal standards and/or derivatization. Metabolites are then quantified (LC or GC coupled with MS and/or NMR spectroscopy). Raw data are extracted, used for metabolite identification and further processed prior to statistical analysis. Bioinformatic tools are used to identify associations with disease states and outcomes, determine significant correlations, characterize metabolic signatures and to integrate results with existing biological knowledge.

with lower precision and accuracy. Such analysis is commonly carried out at initial stages of a study to identify novel metabolites which can then be identified, validated and integrated into the targeted profiling method to achieve the biological understanding of the phenotype [24]. For further details of metabolomic technology and its application to cardiovascular research, we refer the reader to reviews by Giovane et al. [25], Griffin et al. [17], Dunn et al. [26] and Rhee and Gerszten [27].

NMR spectroscopy
NMR can provide structural and quantitative information on complex mixtures of metabolites, often with little or no sample preparation. The technology is based on the measurement of the magnetic spin of nuclei (1H, 13C and 31P) contained in the metabolites of interest [17].

The application of NMR in metabolomics dates back to the 1970s and, despite the development of more sensitive MS technology, NMR maintains a number of unique advantages. NMR is non-destructive and highly reproducible [17]. Using current NMR technology, it is possible to detect and quantify up to 100 metabolites, depending on the biological samples [28]. NMR can also be used to generate detailed lipoprotein profiles in addition to metabolomic data. This approach was started more than 20 years ago [29] and quickly developed to include the quantification of lipoprotein subclasses [30]. NMR methodology based on two molecular windows, LIPO (lipoprotein lipid) and LMWM (‘low-molecular-mass’ metabolite) are commonly used to generate information on lipoprotein subclass distribution and LMWMs respectively [31]. The LIPO window consists of macromolecular signals arising from lipoprotein lipids, whereas, in the LMWM
Growing application in clinical screening and diagnostics. The development of MS technology over the past decade has largely contributed to the current advanced state of metabolomics and its growing application in clinical screening and diagnostics.

Sample application, with or without pre-fractionation, is an important consideration in MS-based metabolomics. Direct infusion permits an unbiased analysis of metabolites, but is susceptible to ion suppression and matrix effects. This has been largely overcome by newer instruments with improved sensitivity and mass accuracy such that direct infusion (shotgun) analysis of lipids (at low concentration) is routinely performed and is able to quantify hundreds of lipids within a short period of time [33,34]. However, although high mass accuracy can resolve isobaric species (which differ slightly in their mass), it does not resolve isomeric species (which have identical mass), which then require additional structural information or separation prior to MS analysis.

Chromatography (gas or liquid) coupled to MS can provide separation of metabolites prior to analysis, leading to improvements in metabolite coverage, as well as the accuracy and precision of quantification. GC–MS can provide a comprehensive analysis of a large variety of metabolites, but is limited by the nature of the chromatography, which requires the metabolites to be volatile. Thus, in most instances, derivatization is required prior to analysis. By contrast, LC (liquid chromatography)–MS does not usually require sample derivatization and has become the method of choice for global metabolite profiling due to its ability to routinely and simultaneously detect thousands of analytes in biological samples [35]. For further details of metabolomic technology, see reviews by Becker et al. [23] and Fiehn [36].

Lipidomics

Lipidomics is a subset of metabolomics that specializes in the characterization of the lipid complement in biological systems. The direct association between dyslipidaemia and altered lipid metabolism with metabolic diseases, including Type 2 diabetes and CVD, has prompted multiple studies in this field. Improvements in MS instrumentation have reduced analysis time and facilitated the high-throughput profiling of the lipidome in larger sample sets. A number of approaches have been used for the analysis of the lipidome: (i) 'shotgun lipidomics', which involves direct infusion of the sample followed by the application of multiple scan modes to obtain unbiased detection of lipids with high sample throughput [37,38]; (ii) 'targeted lipidomics', which employs LC in addition to multiple reaction monitoring and stable isotope internal standards to obtain accurate and precise measurements of known lipids of interest [39,40]; and (iii) 'untargeted lipidomics', which utilizes LC (with or without internal standards) and high mass accuracy to identify previously unknown lipid species. In our laboratory, we have developed a targeted lipidomics approach that can quantify over 300 lipid species in 14 min from 10 μl of human plasma (Figure 2) [41].

Bioinformatics

Advanced metabolomic technologies can generate a vast amount of data. The extraction, analysis, interpretation and integration of these data are growing challenges requiring specific statistical and bioinformatic approaches that are dependent on the experimental design and objectives of the study. However, some general principles apply and are outlined below.

Data extraction and preprocessing

Typically metabolomics data will be derived from NMR signal intensities or MS-derived chromatograms. For MS-derived data, particularly untargeted approaches, noise filtering, baseline correction, peak detection, de-isotoping and alignment are important steps toward integration of the signals to derive a numerical value for each metabolite. In NMR studies, similar issues relating to phase correction and baseline adjustment require preprocessing of the data to derive a dataset suitable for further statistical analysis. Multiple algorithms/software packages are available for these processes and have been reviewed recently [42,43].

Metabolite identification

Although in targeted approaches the metabolites are predefined, in untargeted analyses many/most metabolites will not be defined prior to analysis. Specific metabolites, often selected based on their biological relevance, will be characterized further. Typically, exact mass, retention time and fragmentation data, often combined with existing databases (see below), are utilized to provide structural information leading to identification. In NMR-based
metabolomics, identification can be based on comparison with reference compounds and libraries or on two-dimensional NMR spectroscopy to elucidate the connectivity between signals, thereby helping to identify the metabolite.

**Normalization and scaling**
Regardless of the source of the data, normalization is usually required to reduce the variation resulting from experimental sources while retaining the biological variation. Normalization is typically achieved by either statistical models to derive scaling factors based on the complete dataset or by utilization of internal or external standards.

**Data analysis (univariate)**
Once the dataset has been derived, analysis to identify metabolites or other covariates that encompass the bulk of the variance within the dataset can be performed. The selection of appropriate statistical methods will be dependent on the sample size and distribution or the ability to assess the normality of the distribution. Human or animal studies often have multiple covariates; regression analysis will allow adjustment for covariates in the determination of association with specific outcomes. In analyses involving multiple hypothesis testing (many variables), correcting for multiple comparisons is necessary. This is often made by the application of a Bonferroni correction factor. However, metabolomic data is typically highly correlated and so the stringent Bonferroni correction will probably lead to an under estimation of significance. The less conservative Benjamini and Hochberg procedure [44], which controls the false discovery rate is often more appropriate for metabolomics data.

**Data analysis (multivariate)**
Data reduction approaches such as PCA (principal component analysis) or supervised approaches, including PLS-DA (partial least squares discriminant analysis), can help to reduce complex data to a manageable form, identify associations within datasets and identify key metabolites contributing to the variance. Further analysis often targeted to the development of multivariate models to predict class assignment may also be performed. In these analyses more sophisticated approaches may be required. Such approaches include support vector machines, artificial neural networks, self-organizing maps and hierarchical cluster analysis and have been reviewed recently [45–48].

**Pathway analysis and integration with other ‐omics data**
Mapping of metabolomic data into pathways and the integration with genomic, transcriptomic and proteomic data has the potential to provide a unique and detailed view of metabolic processes, as well as the complex interplay with regulatory and signalling pathways. However, the limited coverage of the metabolome and the existing tools and databases limits such studies. Nonetheless, a number of websites containing databases and bioinformatic tools for metabolite characterization and identification, as well as statistical and pathway analysis, have been developed. The Human Metabolome Database (http://www.hmdb.ca/) [49] contains over 7900 metabolite entries, whereas the LIPI D MAPS (Lipid Metabolites And Pathway Strategy) structure database (http://www.lipidmaps.org/) [50] contains over 30000 unique lipid structures. The LIPI D MAPS structure database also provides a number of bioinformatic tools, including the ‘LIPI D MAPS MS Prediction Tool’, to predict a mass spectrum for a given metabolite, and the ‘LIPI D MAPS Pathway Editor’ to create, manage and edit metabolic pathways. Other resources include; the METLIN (Metabolite and Tandem MS) database (http://metlin.scripps.edu/) [51] and MCCD (Madison Metabolomics Consortium Database; http://mmcd.nmrfam.wisc.edu/) [52]. These databases often provide links to other resources such as KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/), thereby facilitating the integration of metabolomic information with other biological data.

**METABOLOMIC STUDIES DEFINING THE PATHOGENESIS OF IHD**

Inflammation, oxidative stress and dysfunctional lipid metabolism are key mediators in the pathogenesis of IHD. Dyslipidaemia and dysfunctional lipid metabolism are also thought to be key mediators of the disease process. Metabolites are the end-product of the ‐omics pathway (genomics ‐ transcriptomics ‐ proteomics ‐ metabolomics) and therefore closest to the patient phenotype, although the feedback loops from metabolites to regulatory pathways are both complex and extensive. As such, metabolomic analysis can provide a unique viewpoint of the dynamic and complex metabolic changes that occur in response to a pathophysiological perturbation. The complementary nature of metabolomics with other ‐omic approaches (genomics, transcriptomics and proteomics) also provides the potential for integration to produce a systems approach to understanding the disease process. In contrast with the other ‐omic approaches, metabolomics has only recently been utilized to study the pathogenesis of IHD (Table 1).

**Inflammation and oxidative stress**
In aerobic cells, oxidants are normal by‐products of cellular metabolism [53]. However, the production rate of these oxidants is often elevated in diseased states, resulting in a disparity in the levels of oxidants and antioxidants, leading to a state of oxidative stress [53]. Oxidative stress has been implicated in the underlying pathology of IHD. Myeloperoxidase, a major producer of ROS (reactive oxygen species), is secreted by activated polymorphonuclear leukocytes and macrophages at the sites of inflammation [54] and has also been found in human vascular lesions [55]. Elevated levels of myeloperoxidase in blood and leukocytes have been shown to be a strong independent predictor of CAD in angiographically defined patients [56,57]. In addition, an elevation of the lipid peroxidation biomarker, F2‐isoprostane, was associated with increased BMI (body mass index) in Framingham Heart Study subjects [58,59], suggesting a systemic oxidative stress in patients with obesity‐related heart disease.

On the basis of immunological studies [60–62], oxidative stress has been shown to result in the modification of the lipid and protein components of LDL (low‐density lipoprotein). This modification translates to atherogenic and dysfunctional forms
Table 1  Major findings from metabolomic studies of IHD

<table>
<thead>
<tr>
<th>Area of investigation</th>
<th>Samples analysed</th>
<th>Metabolite composition/findings</th>
<th>Relevant reference(s)</th>
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<tbody>
<tr>
<td>Oxidation</td>
<td>Plasma</td>
<td>Nine species of oxysterols found in healthy volunteers</td>
<td>[63–65]</td>
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<td>7β-Hydroxycholesterol found in KAPS subjects with a fast progression of carotid atherosclerosis</td>
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<td>A total of 16 species of oxPC and lyso-PC found in plasma of patients with alcoholic liver disease</td>
<td>[57,76]</td>
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<td>oxLDL</td>
<td></td>
<td>A total of eight major oxPC species derived from 1-palmitoyl-2-arachidonyl PC and 1-palmitoyl-2-linoleoyl-PC, which together constitute oxPCCD36, that are recognized by macrophage CD36 scavengers</td>
<td>[71,76,81,82,85]</td>
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<td>Elevated levels of 29 species of saturated and mono-unsaturated lyso-PC, short-chain oxPC and long-chain oxPC that make up the substrates/products of Lp-PLA2</td>
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<td>oxPCCD36, which are recognized by macrophage CD36 scavengers, were enriched in rabbit atherosclerotic lesions</td>
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<td>Elevated levels of 26 out of 29 species of Lp-PLA2-specific saturated and mono-unsaturated lyso-PC, short-chain oxPC and long-chain oxPC found in human atherosclerotic lesions</td>
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<td>Seven groups of oxidized CE found in human atheromata</td>
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<td>67-fold and 82-fold increase of lyso-PC-chlorohydrin (C16:0) and (C18:0) respectively in human atherosclerotic tissues compared with normal tissues</td>
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<td></td>
<td></td>
<td>Increased level of oxylipin 15-HETE in human atheromata</td>
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<td>Inflammation</td>
<td>Plasma</td>
<td>Increase in palmitic acid (C16:0; 8-fold), stearic acid (C18:0; 3-fold), and 1-monolinoleoylglycerol (C18:2-glycerol; 3-fold) respectively in patients with stable atherosclerosis compared with healthy subjects</td>
<td>[152]</td>
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<td></td>
<td>Increase in linoleic acid (C18:2), oleic acid (C18:1), palmitic acid (C16:0) in human atherosclerotic plaques</td>
<td>[93,94]</td>
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<tr>
<td></td>
<td>Plaque</td>
<td>Enrichment of taurine</td>
<td>[97]</td>
</tr>
<tr>
<td>Symptomatic/</td>
<td>Plasma</td>
<td>Comparison of patients with unstable CAD compared with stable CAD showed: an increase in total ceramide, dihexosylceramide, trihexosylceramide, odd-chain PC, PE, free cholesterol and diacylglycerol; and a decrease in total monohexosylceramide, phosphatidylglycerol, phosphatidylinositol, alkyl-PC, PC plasmalogen, alkyl-PE, PE plasmalogen, lyso-PC, lyso-PE, CE and triacylglycerol</td>
<td>[41]</td>
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<tr>
<td>asymptomatic IHD</td>
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<td>Comparison of patients with unstable CAD compared with stable CAD showed: an increase in total ceramide, dihexosylceramide, trihexosylceramide, odd-chain PC, PE, free cholesterol and diacylglycerol; and a decrease in total monohexosylceramide, phosphatidylglycerol, phosphatidylinositol, alkyl-PC, PC plasmalogen, alkyl-PE, PE plasmalogen, lyso-PC, lyso-PE, CE and triacylglycerol</td>
<td>[41]</td>
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<td>Plaque</td>
<td>A total of 19 lipid species signature in symptomatic/asymptomatic lesions and 12 lipid species signature in stable/unstable plaque areas</td>
<td>[103]</td>
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<td></td>
<td>Enrichment of CE, lyso-PC, PC and certain SM in carotid endarterectomies compared with control radial arteries</td>
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<td>Other metabolic</td>
<td>Plasma</td>
<td>Significant discordant of six metabolites in the citric acid pathway in subjects of inducible ischaemia compared with controls</td>
<td>[24,106–108]</td>
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<td>processes</td>
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<td>Significant difference in 54 metabolites, including 3-hydroxybutyrate and amino acids, in patients with stable carotid atherosclerosis compared with healthy individuals</td>
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<td>Significant suppression of myocardial fuel (especially acetylcarnitine and 3-hydroxybutyrylcarnitine) in patients with impaired ventricular function at baseline and after ischaemia/reperfusion</td>
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<td>Three metabolites of PC (choline, TMAO and betaine) were shown to predict cardiovascular risk in an independent large clinical cohort</td>
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</table>

of LDL, which are subsequently taken up by macrophages via scavenger receptors, such as CD36 [57], thus leading to foam cell formation and progression to atherosclerotic plaque, as discussed above. Some of the early metabolomic studies involved the identification of oxidation products from human plasma. Nine species of oxysterols (cholesterol oxidation products) were detected and identified in human plasma from 31 subjects using GC–MS [63]. Using the same approach, 7β-hydroxycholesterol, a predominant cholesterol oxidation product in membranes and lipoproteins, as well as seven other cholesterol oxidation products, were detected in serum from 20 subjects from KAPS (Kuopio Atherosclerosis Prevention Study) with progressive carotid atherosclerosis,
supporting further the association of lipid oxidation with atherogenesis [64]. More recently, 16 species of PC (phosphatidylcholine) and lyso-PC (lyso-phosphatidylcholine) oxidation products were identified in human plasma from five male subjects with alcoholic liver disease (also associated with increased oxidative stress) using quadrupole time-of-flight MS [65].

To elucidate further the mechanism of lipid accumulation and foam cell formation, Podrez et al. [57,66,67] conducted several metabolomic studies to identify the structural details of ligands for the macrophage scavenger receptor CD36. CD36 is a glycosylated membrane protein, expressed on the surface of cells, such as microvascular endothelial cells and macrophages [68,69], where it is involved in the uptake of oxidized lipoproteins (oxLDL (oxidized LDL) and oxHDL [oxidized HDL (high-density lipoprotein)]) [69,70]. Employing cell-binding assays, as well as multiple chromatography and MS platforms, Podrez et al. [57] demonstrated that a novel class of oxidized phospholipids with sn-2 acyl group, possessing terminal \( \gamma \)-hydroxy (or oxo) -\( \alpha \)-\( \beta \)-unsaturated carbonyl groups (oxPC\(_{\text{CD36}}\)) serve as high-affinity ligands for CD36. To validate these results, each of the oxidized lipids was synthetically produced, their structures were confirmed using NMR and MS, and their binding activity determined. Further in vitro analyses of oxPC\(_{\text{CD36}}\) showed that they were generated in oxidized lipoprotein via multiple distinct pathways, and promoted CD36-dependent macrophage binding and foam cell formation when incorporated into cholesterol-laden particles [71], as well as activation of platelets at pathophysiological levels [67]. In addition, their levels were markedly elevated in plasma of humans with low HDL levels [67]. oxPC\(_{\text{CD36}}\) were also found to be enriched in atherosclerotic lesions in rabbits [71]. Therefore oxPC\(_{\text{CD36}}\) appear to function in atherogenesis by mediating the recognition and uptake of oxidized forms of LDL via CD36 on macrophages.

Lp-PLA\(_2\) [lipoprotein-associated PLAs (phospholipase A\(_2\)) is of interest in atherosclerosis studies because of its increased expression in vulnerable atherosclerotic lesions [72,73] and the hypothesized pro-inflammatory nature of its enzymatic products, such as lyso-PC and oxidized NEFAs (non-esterified fatty acids) [74,75]. Davis et al. [76] identified 29 different species of oxPC (oxidized PC) and lyso-PC from human oxLDL that made up the substrates and/or products for Lp-PLA\(_2\) by employing a direct infusion (shotgun) approach on an ESI–MS/MS (electrospray ionization tandem MS) platform. These species were differentiated into three classes: (i) short-chain oxPC, which were substrates for Lp-PLA\(_2\); (ii) long-chain oxPC, which were also substrates for Lp-PLA\(_2\), but were less efficiently hydrolysed; and (iii) saturated and mono-unsaturated lyso-PC, which were predominant PC products of Lp-PLA\(_2\). The concentrations of the three classes of lipids were shown to increase during LDL oxidation. To assess the physiological relevance of these findings, they identified 26 out of the 29 different species in human carotid artery plaque samples (\( n = 5 \)), although the quantities differed between plaque samples [76].

Previous studies have shown elevated levels of lyso-PC in both sera of patients with atherosclerosis [77] and in human atherosclerotic lesions [78]. Lyso-PC are oxidation products that can come from a variety of sources, including LDL oxidation and the enzymatic action of Lp-PLA\(_2\), as already mentioned above, as well as the oxidation of membrane PC plasmalogens. Plasmalogens are a subclass of glycerophospholipids with a unique acid-labile vinyl-ether linkage at the sn-1 position of the glycerol backbone [54]. The vinyl-ether moiety renders plasmalogens more susceptible to oxidative stress compared with the fatty acyl analogues [79,80] and triacylglycerols (triglycerides) [81]. Thus plasmalogens have been proposed to serve as an endogenous antioxidant. Secondary attack on lyso-PC by HOCl (hypochlorous acid) can lead to the oxidative production of lyso-PC–chlorohydrin [80]. By employing a direct infusion approach, Messner et al. [82] identified lyso-PC–chlorohydrin (C\(_{16:0}\) and C\(_{18:0}\)), and demonstrated that their concentrations were increased by 67- and 82-fold respectively in human atherosclerotic tissue (\( n = 2 \)) compared with normal tissue (\( n = 2 \)). These findings further support the concept of increased levels of oxidative stress within atherosclerotic lesions leading to elevated levels of oxidative by-products.

CEs (cholesterol esters) are neutral lipids that make up a major component of LDL particles [83,84]. Recent metabolomic studies have identified oxCEs (oxidized CEs) in atherosclerotic lesions [81,85]. By employing ESI–MS/MS, seven groups of abundant oxCE species, including novel oxidation products, were identified from human atheromata (\( n = 6 \)) [81]. One of the three most abundant oxCE species was identified as the CE (C\(_{20:4}\)-derived 15-HETE (15-hydroxyeicosatetraenoic acid) CE [81]. Consistent with this finding, Gertow et al. [85] identified 15-HETE as the most abundant arachidonic-acid-derived oxidative product of 15-lipoxygenase. They also determined that 15-lipoxygenase mRNA was more highly expressed in atherosclerotic lesions from symptomatic subjects (\( n = 102 \)) compared with asymptomatic subjects (\( n = 30 \)). However, in contrast with the findings by Gertow et al. [85], Hutchins et al. [81] did not propose the implication of 15-lipoxygenase in atherosclerosis based on their findings of regio- and stereo-specificity studies. Instead, a non-enzymatic mechanism involving free radicals was suggested to dominate during atherosclerosis development. Further studies are warranted to clarify the aforementioned mechanisms which may not be mutually exclusive.

These studies exemplify how metabolomics can define the relationship of oxidation and inflammation with IHD through the comprehensive and systematic characterization of oxidized lipids and the subsequent identification and quantification of these lipids in biological samples.

**Plaque composition**

Different characteristics of plaques such as plaque volume and echogenicity have been studied to assess the progression and regression of atherosclerotic plaques, as well as to better define plaque stability [86–89]. Although these characteristics have been demonstrated to be positive predictors of stroke and acute coronary syndromes [90–92], the focus in research has shifted from the qualitative approaches, investigating the morphology of plaque and the degree of carotid stenosis [11], to more quantitative approaches, looking at the different composition of plaque at different stages of atherogenesis. Metabolomics lends itself to such studies.
Atherosclerotic plaque represents an end-product of the metabolic abnormalities associated with IHD and, as such, it provides a metabolic readout of the altered metabolism. There have been a number of studies aimed to characterize the plaque metabolome. By employing ESI–MS/MS, targeted quantitative analysis of atherosclerotic plaque has identified three predominant species out of ten NEFAs present in plaques: linoleic acid (C18:2), oleic acid (C18:1) and palmitic acid (C16:0) [93]. Consistent with this finding, Mas et al. [94] utilized MS-imaging techniques to characterize both the location and composition of NEFAs and they found significantly elevated levels of C18:2, C18:1 and C16:0 in the neointima of atheromatous plaques of Type 2 diabetic patients compared with non-diabetics (total n = 40) [94]. The local enrichment of plaque by NEFAs, especially C18:2, was accompanied by increased expression of Lp-PLA2 and MCP-1 (monocyte chemoattractant protein-1) [94], which are implicated in inflammation and tissue injury [95,96]. In addition, C18:2 triggered the activation of NF-κB (nuclear factor κB), as well as the expression of Lp-PLA2 and NF-κB, suggesting a bidirectional relationship between inflammation and NEFAs [94]. Taken together, these findings support the link between local inflammation, which is accompanied by altered metabolite composition, and atherosclerotic plaque progression.

The association of inflammation and oxidative stress in plaque progression was suggested further in a study by Mayer et al. [97], where they investigated the pathophysiological role of microparticles. Microparticles arise from blebbing of cell membranes due to cell activation. The origin of the plaque-derived microparticles was determined to be primarily leucocytes and smooth muscle cells by proteomic analysis [97]. This finding suggests that microparticles may partially underscore the contribution of oxidative micro-environments in the development of atherogenesis. 1H-NMR analysis of the metabolite composition of plaque microparticles indicated taurine as the predominant metabolite [97]. Taurine is the most abundant free organic acid in human neutrophils and is involved in the negative feedback of the monocyte/neutrophil oxidative burst via its scavenging of myeloperoxidase-derived HOCl [98]. The administration of taurine to high-cholesterol-fed rabbits was shown to result in decreased oxidative stress and cholesterol accumulation in the aortas [99]. Therefore the enrichment of cytoprotective taurine in plaque may suggest an elevation of free radical scavenging activity as a defence mechanism in response to increased oxidative stress.

The majority of myocardial infarcts result from the rupture of the atherosclerotic fibrous cap [100,101]. Much is known about the cellular and pathophysiological causes of this rupture, such as the production of collagenases triggered by the inflammatory mediator IFN (interferon)-γ [11], and the different levels of shear stress in the carotid artery that differentiate stable/unstable areas of a maximum stenosis [102]. However, at the molecular level, the systemic nature of plaque that precedes plaque instability is not fully understood. One study, in particular, aimed to differentiate the lipid compositions of atherosclerotic plaques from carotid endarterectomy samples of symptomatic and asymptomatic patients, and stable and unstable areas of the same symptomatic atherosclerotic lesions [103]. Employing a shotgun lipidomic approach on a nanoflow ESI–MS/MS system, 150 lipid species from nine different classes were identified and quantified from the atherosclerotic plaque samples. Compared with control radial arteries (n = 3), plaque samples (n = 3) were characterized by the enrichment of CE, PC, lyso-PC and certain SM (sphingomyelin) species. The difference in the lipid composition between the unstable and stable areas of plaques (n = 8) was statistically significant for several species, including CE (C18:0), CE (C20:3), SM(C18:1/C15:0), and PC (C16:4) [103]. System-wide analysis revealed plaque-specific lipid signatures containing 19 lipid species for the asymptomatic–symptomatic lesions (n = 6 per group), and 12 lipid species in the stable–unstable plaque areas [103].

The study by Stegemann et al. [103] represents a milestone in our understanding of plaque biology by detailing the dynamic nature of plaque composition. Future studies in this area may ultimately give us an insight into the lipid signatures defining plaque vulnerability/stability, as well as the metabolites involved in the local inflammatory cascade of atherogenesis. However, although metabolomic analysis of plaques provides information of local plaque characteristics, the relationship to the patient’s systemic inflammatory state is unclear. Similarly, the analysis of circulating metabolites does not necessarily define the nature or even the burden of plaque. Further studies combining plaque and circulating metabolites are required to better define this relationship. The current clinical approach to reducing CVE has been focused on plaque stabilization using lipid-lowering statin-based drugs [11]. This will be discussed further in the Response to therapy section below.

### Lipid metabolism

The studies described above demonstrate that oxidation of lipids within plaque is a major metabolic process and that these same lipids can also be isolated from plasma, suggesting that they can leave the plaque tissue. However, the primary contributing source of plaque lipids is from circulation in the form of lipoproteins that cross the arterial wall to the intima. As such, the analysis of plasma lipids may provide important insight into metabolic processes that can contribute to plaque progression.

In a targeted lipidomics study utilizing LC–ESI–MS/MS, we profiled 305 known lipid species in plasma from 220 individuals representing stable angina (n = 60), unstable coronary syndrome (n = 80) and matched healthy controls (n = 80) [41]. Although there was an overlap of lipid species between the two analyses, we identified multiple lipid species that were associated with either stable disease (relative to healthy controls) or unstable disease (relative to stable disease) only. In addition to CE and triacylglycerols, we identified ceramide, phosphatidylinositol and PE (phosphatidylethanolamine) species positively associated with stable CAD, whereas lyso-PC and ether-linked and PC plasma-laden species were negatively associated with stable disease. In contrast, we observed phosphatidylinositol to be negatively associated with unstable CAD along with the ether-linked and PE plasmalogen species (Figure 3).

These and other findings [41] point to the involvement of a number of lipid metabolic pathways in inflammation and oxidative stress that may be contributing to disease onset and progression. Interestingly, although lyso-PC, generated by the
action of Lp-PLA₂, has been observed to be elevated in plaque [76,103,104], we observed this lipid to be negatively associated with both stable and unstable CAD in the circulation. One possible explanation for this apparent contradiction may be the presence of CAD and left ventricular dysfunction. They performed metabolic profiling using LC–ESI–MS/MS to measure 63 metabolites in paired (peripheral artery and coronary sinus) plasma samples. They observed significant suppression of myocardial fuel uptake in those patients with impaired ventricular function at baseline and after ischaemia/reperfusion. These studies highlight the importance of myocardium energy metabolism and its relationship with cardiovascular disease. These studies have not only identified potential biomarkers to predict cardiovascular risk after adjustments for traditional cardiac risk factors and medication usage in an independent large clinical cohort (n = 1876). Further studies in mouse models demonstrated that supplementation with choline or TMAO promoted atherosclerosis and that gut flora played a critical role in TMAO production. These studies have not only identified potential biomarkers to assess cardiovascular risk, but have identified new metabolic relationships with CVD, thereby providing avenues for the development of future therapeutic interventions.

The approaches described above are providing valuable insight into the metabolic processes associated with, and contributing to, IHD. These studies highlight the complexities of atherogenesis and go some way toward explaining the residual risk following correction of cholesterol levels. As such, they open new
avenues of investigation for the development of new therapeutic strategies to further reduce the burden of IHD.

BIOMARKERS FOR IHD

Early detection and treatment of those individuals at high risk of a future CVE (the vulnerable patient) will be an important factor in the effort to reduce the burden of CVD. However, current screening is limited by the predictive power of available tests, the high cost of these tests or a combination of both. Non-invasive screening can currently be performed by the assessment of traditional risk factors (e.g. smoking status, BMI and blood cholesterol), or by direct measurement of arterial structural changes associated with atherosclerosis, such as carotid intima medial thickness measured by ultrasound, coronary artery calcification score determined by computed tomography, ankle/brachial blood pressure ratio and magnetic resonance imaging techniques.

In addition to screening for risk assessment in the subclinical population, there is a growing need for better tools to evaluate those that present clinically with angina or other symptoms of CAD. Following clinical evaluation (including electrocardiography and troponin measurements) to identify those with unstable disease, the differential diagnosis for the remainder of patients can be difficult, often requiring invasive and/or expensive imaging techniques. Accurate risk assessment in this already ‘high-risk’ group could also be useful in the design of treatment strategies. In both the stable and unstable patients, the ability to assess the response of treatment in terms of increased or decreased risk would be useful in their ongoing management.

New biomarkers, resulting from current and future metabolomic studies may help address some of these needs. According to the NIH (National Institutes of Health; http://www.nih.gov/), a biomarker is defined as “a biologic specimen that may be a marker of exposure to some substance, of its metabolism, or of the integration of exposure and metabolism, and may also reflect host characteristics”. However, the deluge of candidate biomarkers in the research and clinical communities has led to an effort to further define a set of assessment criteria for novel cardiovascular biomarkers by Morrow and de Lemos [109]. The assessment was structured around three main criteria: (i) the availability of reliable analytical method to measure the biomarker, (ii) the ability of the biomarker to add new information, and (iii) the biomarker should have a range of potential clinical applications that may improve patient care [109].

The technology associated with metabolomics lends itself to high-throughput applications for the measurement of multiple analytes and, as such, developments in this area have been toward establishing metabolic profiles that can predict disease status or progression. Typically, individual biomarkers offer only limited prognostic ability, whereas a combination of markers or a biomarker profile, usually combined into a multivariate algorithm, may provide improved discrimination of disease states. Such profiles can then be assessed by the same criteria described above and can be combined with traditional risk factors which may include: demographic risk factors (ethnicity, geography, age and sex); lifestyle contributions (smoking, physical inactivity and poor diet); physiological risk factors (BMI and Type 2 diabetes); biochemical risk factors {level of serum LDL, HDL and and concentration of Lp(a) [lipoprotein(a)]}.

The technology for metabolic profiling is increasingly being applied in pathology laboratories with many hospital-based laboratories taking up this technology for a range of diagnostic and monitoring assays. Newborn screening programmes for inborn errors of metabolism typically measuring 20 or more metabolites using MS were first established in the 1990s. Since that time, many of the technical issues have been resolved and development in this area is continuing [110]. Translation of this approach into chronic diseases in the adult population will require additional developments in many areas, including biomarker identification, assay development and validation, as well as data analysis, interpretation and reporting.

Lipoprotein and metabolite profiling by NMR spectroscopy

The early application of NMR to the assessment of cardiovascular risk met with limited success; Kirschonlohr et al. [111] examined the power of NMR spectra of serum to predict angiographically defined CAD. They found that the analysis was mainly dependent on the lipid regions of the spectra, but that age, sex and drug treatment were confounders. Although prediction was 80.3% accurate for those not treated with statins, this fell to only 61.3% for those on statin treatment. They concluded that detection of CAD by NMR was weak compared with angiography [111]. Rousselet et al. [112] performed a nested case-control study of 190 diabetic patients in the placebo arm of a larger clinical trial DIABHYCAR (type 2 DIabeTes, HYpertension, Cardiovascular events and Ramipril) [113], a randomized clinical trial of high-risk Type 2 diabetic patients who were followed for 4 years to ascertain fatal or non-fatal acute myocardial infarction. They analysed the spectra for lipid subclass by deconvolution of the lipid domain or by whole-spectra analysis. However, models based on the lipid domains only were 62% accurate in the training set and only 53% accurate in the validation set and, although models based on the whole spectra (207 elements) could achieve 87% accuracy in the training group, this was decreased to only 53% in the validation group. Thus this approach performed even worse for the prediction of future events that it had in the previous classification study [111].

These early studies highlight the challenges in the development and validation of new biomarkers for IHD. However, several recent studies have given reason for optimism that metabolomics may yet provide useful biomarkers for IHD. Bernini et al. [114] applied NMR-based metabolomics to the analysis of plasma samples from 864 healthy volunteers to investigate the relationship between cardiovascular risk and plasma metabolites. They compared the metabolomic profiles of healthy individuals with increased cardiovascular risk parameters (low HDL, high LDL, high total cholesterol, high triglycerides and high Framingham score) against individuals with low risk using univariate and multivariate statistics. They found that subjects with high or low risk could also be discriminated with NMR metabolomics using complex fingerprints that contained not only the traditional risk factors (cholesterol, triglycerides, LDL and
Although additional validation and prospective studies are also needed to demonstrate the independent nature of these lipid biomarkers compared with traditional risk measures of CVD, the Framingham risk score. These studies demonstrate the independent nature of these lipid biomarkers that are able to add to traditional risk factors for IHD, particularly those with a high residual risk, such as those following myocardial infarction or in those who express co-morbidities (such as the metabolic syndrome, insulin resistance and Type 2 diabetes, and hypertension). The residual risk, together with potential, albeit infrequent, statin-related myopathy, remains a significant concern.

More recently Wurtz et al. [115] have performed a larger epidemiological study of 4309 healthy young adults and related the NMR-derived lipoprotein and metabolite profiles to carotid intima medial thickness. Their analysis focused on both the LIPO subclass distribution and LMW metabolites respectively [31]. In this cross-sectional study, intima medial thickness was associated with previously characterized lipoprotein disturbances such as elevated VLDL (very-LDL) and LDL and decreased HDL. However, they also observed association with LMW metabolites, including amino acids, as well as fatty acid oxidation metabolites. A subsequent analysis used the NMR metabolomic measures to predict high intima medial thickness at the 6-year follow-up [116]. They reported improved prediction of intima medial thickness when HDL-C (HDL-cholesterol) was replaced with NMR-determined LDL-C (LDL-cholesterol), medium HDL, docosahexaenoic acid and tyrosine, together with risk factors from the Framingham risk score. These studies demonstrate the independent nature of these LMW metabolites and their potential to add to the conventional risk measures of CVD.

The plasma lipidome and cardiovascular risk

An alternate approach to combining lipoprotein measures (either traditional or NMR based) with LMW metabolites is to dissect out the individual lipid species present in the lipoproteins to provide additional information, and potentially mechanistic insight, into cardiovascular risk. Our targeted lipidomics study on plasma from stable and unstable CAD patients, together with healthy controls (see the Lipid metabolism section) identified multiple lipid species that were associated with either stable disease (relative to healthy control) or unstable disease (relative to stable disease) [41]. We subsequently developed computational models using correlation minimization, followed by the ReliefF feature selection in tandem with L2-regularized logistic regression-based classification within a three-fold cross validation framework (1000 repeats). Models created using a combination of only 15–20 lipid species and traditional risk factors performed significantly better {C-statistic, 0.875 [95% CI (confidence interval), 0.874–0.877]} compared with models containing only traditional risk factors [C-statistic, 0.796 (95% CI, 0.795–0.798)] [41].

As with the NMR studies, this targeted lipidomics approach demonstrated the independent nature of these lipid biomarkers and their ability to add to traditional risk factors for IHD, although additional validation and prospective studies are also required in this area.

RESPONSE TO THERAPY

Statins are commonly used for the primary and secondary treatment of coronary heart disease and for reducing CVD risk. In particular, there is strong evidence in support of statin use in individuals who have suffered a myocardial infarction in reducing risk of recurrent coronary events and improving survival rates post-infarction [117–122]. The risk reduction in cardiovascular morbidity and mortality by statins is chiefly associated with its LDL-C-lowering effect. Despite this reduction, and even on maximal statin therapy, high-risk individuals exhibit significant residual risk of future CVEs. That is, CVEs still occur among statin-treated individuals, although at a significantly lower rate. The risk is amplified further in patients following myocardial infarction or in those who express co-morbidities (such as the metabolic syndrome, insulin resistance and Type 2 diabetes, and hypertension). The residual risk, together with potential, albeit infrequent, statin-related myopathy, remain a significant concern.

Different statins have also been shown to lower LDL-C to various degrees with rosuvastatin and atorvastatin achieving, on average, greater reductions in plasma LDL-C levels [28,123,124], and, in the case of rosuvastatin, an increase in LDL-C concentration and reduction in plaque formation [125,126]. On the other hand, the response of individuals to statin treatment has also been reported to vary with respect to the degree of LDL-C-lowering, even in a relatively homogenous group with good drug compliance [127,128]. Inter-individual variation notwithstanding, the mechanistic rationale that underpins the differential responses to statin treatment is not fully understood.

Combination therapy (ranging from the use of ACEIs, ARBs and β-blockers, fibrates, ezetimibe, niacin and fish oil) may confer an additional level of protection. Indeed, the complementary and beneficial effects of other lipid-lowering agents may outweigh the complexities associated with developing and maintaining such an approach [129]. As we move towards a personalized medicine approach to the treatment of IHD, combination therapies tailored to the individual are likely to play an increasing role. In this setting a metabolomics approach may provide the power necessary to tease out the complex interplay of individual responses to combination therapies.

Differential effects of statins on plasma lipid metabolites (RADAR substudy)

Using a targeted lipidomics approach, Bergheau et al. [130] showed for the first time in a subgroup of 80 well-matched subjects derived from the RADAR (Rosuvastatin and Atorvastatin in different Dosages And Reverse cholesterol transport) study [131] that rosuvastatin and atorvastatin exert differential effects on molecular lipid metabolites, and that these effects were, on average, dose- and time-dependent. Both agents lowered plasma levels of SM, with atorvastatin exerting a greater reduction. Interestingly, rosuvastatin increased, whereas atorvastatin decreased, plasma levels of PC and its molecular species at the same time points and at equivalent doses. The mechanistic processes that underlie the discordant changes in plasma PC by rosuvastatin and atorvastatin are not clear. The pronounced lowering effect of SM by
atorvastatin compared with rosuvastatin is also noteworthy given the higher potency of rosuvastatin.

In the RADAR study, rosuvastatin lowered the LDL-C/HDL-C ratio, the primary end point of the study, to a greater degree compared with atorvastatin, although this did not reach statistical significance in the subgroup analysis. That the reduction in the LDL-C/HDL-C ratio by either statin could be predicted by alterations in certain plasma lipid metabolites and that the lipid signature was specific for statin type, is of interest and provides a potential rationale for the differential effects of statins on lipids [130]. In addition, they showed that both statins lowered the plasma SM/(SM + PC) ratio, a marker of atherogenesis, again, with rosuvastatin achieving a greater reduction at any dose and time point compared with atorvastatin. The precise mechanisms that account for the aforementioned observations are not clear. However, PC and SM are important constituents of the plasma membrane and of plasma lipoproteins, and alterations in the relative content of PC and SM may alter membrane integrity and lipoprotein metabolism, both of which have been implicated in atherosclerosis. The improvement in the SM/PC ratio by statins may suggest reduced susceptibility of SM to hydrolysis by sphingomyelinase within the vessel wall [132,133]. Inhibition of sphingomyelin synthesis has been shown to reduce plasma levels of cholesterol and triacylglycerols and increase HDL-C in apoE (apolipoprotein E)-knockout mice. Importantly, these lipid changes were accompanied by an increase in the regression of atherosclerotic plaques [134,135]. Elevated sphingomyelinase and PLAr2 activities may cause oxidative and lipolytic remodeling of LDL particles, increasing the likelihood of LDL entrapment within the arterial wall causing further inflammation and promoting atherosclerosis; this effect is partly mediated by the increased release of LDL-derived arachidonic acid by PLAr2 [135]. Furthermore, SM inhibits LCAT- and LPL (lipoprotein lipase)-mediated triacylglycerol hydrolysis thereby affecting cholesterol esterification and potentially clearance of apoB (apolipoprotein B)-containing lipoproteins respectively [136,137]. Whether lowering of plasma phospholipids and sphingolipids by statins translates to improved clearance of apoB-containing lipoproteins requires further study utilizing a tracer lipidomics approach.

**Differential lipidomic signatures in response to statin therapy**

The notion that metabolic signatures could predict individual outcome to statin treatment could potentially translate into a useful tool with clinical applications. Using a targeted lipidomics approach, Kaddurah-Daouk et al. [138] showed in a subgroup of 48 subjects sampled from the CAP (Cholesterol and Pharmacogenetics) study [127] that certain baseline lipid metabolites were predictive of statin treatment responses based on the change in LDL-C as a primary end point. The lipidomic signature comprised chiefly metabolites derived from CE and PC and, to a lesser extent, PE. Of these metabolites, three were plasmalogens. In addition, it was demonstrated that subjects who responded well to simvastatin treatment based on their reduction in LDL-C (‘good responders’) exhibited discordant changes in a greater number of lipid metabolites compared with poor responders. Although all subjects achieved reductions in plasma levels of triacylglycerol, as well as total cholesterol and LDL-C, only the good responders achieved significant reductions in the fatty acid moieties in triacylglycerol, CE, PC and PE. By contrast, fatty acids derived from diacylglycerol were significantly decreased only in the poor responder group.

Interestingly, both groups showed increased $C_{20:4,n-6}$ fatty acid levels (arachidonic acid) in lyso-PC, as well as most other lipid classes. In light of the anti-inflammatory effects of statins, together with the reported reduction in CRP (C-reactive protein) levels in this study, the increased plasma levels of arachidonic acid in response to simvastatin appears counter-intuitive. However, lowered utilization of arachidonic acid as a substrate for the synthesis of inflammatory lipids, such as eicosanoids and prostaglandins, may directly reflect the improved inflammatory profile with statin therapy. In addition, simvastatin has previously been shown to increase $\Delta^5$- and $\Delta^7$-desaturases via the sterol-regulatory-binding protein, leading to increased arachidonic acid formation [139]. Using precursor/product ratios as estimates of metabolite production, Kaddurah-Daouk et al. [138] showed increases in the ratio of $C_{20:4,n-6}/C_{20:3,n-6}$, a measure of $\Delta^5$-desaturase activity. However, this increase was only observed in the good responder group, thus the increased $\Delta^7$-desaturase activity only partially explains the increased arachidonic acid levels that occurred in both groups. Interestingly, alterations to the lipidomic signature specifically by simvastatin have been associated with increased muscle expression of the arachidonic acid 5-lipoxygenase-activating protein [140], albeit this was at the highest simvastatin dose (80 mg/day); whether these processes occur at a lower dose warrants further investigation.

**CRP: beyond LDL-C-lowering**

The reduction in plasma LDL-C by statins may not fully account for changes in other biomarkers, for instance CRP, which may relate more to alterations in inflammation. That baseline PE plasmalogen and PC plasmalogen levels were directly and inversely associated respectively, with a reduction in CRP levels in response to simvastatin supports the notion that statins exert pleiotropic effects beyond LDL-C-lowering [138]. Moreover, there was no overlap in the correlation matrix between changes in lipid metabolites and LDL-C-lowering and with changes in CRP, suggesting that the changes in CRP were independent of LDL-C-lowering. The precise mechanisms that account for these correlations in a statin-treated group is not clear, but may relate, in part, to improvements in the anti-inflammatory and antioxidant profiles, as well as improved vascular and endothelial function that usually accompanies statin therapy. Whether these improvements are mediated by plasmalogens warrants further experimental studies.

**INTEGRATION OF GENOMIC AND METABOLOMIC STUDIES**

GWAS (genome-wide association studies) using cardiovascular outcomes have identified biologically relevant genes and gene sets that are providing new insight into cardiovascular risk and disease
mechanisms. Many follow-up studies to unravel these pathways are currently underway and a number of recent reviews have been published to cover this field [141–143]. Such studies have also been used to identify loci associated with plasma total cholesterol, LDL, HDL and triglycerides [144–147].

An extension of these studies then is to combine GWAS with metabolomic profiling to enable many ‘metabolic phenotypes’ to be mapped in a single dataset. The pre-existing or co-determined relationships between the metabolic profile and disease phenotypes will provide a framework for the subsequent analysis to define metabolic pathways and/or regulatory pathways. Hicks et al. [148] have applied ‘targeted lipidomics’ to examine the association between 318,237 SNPs (single nucleotide polymorphisms) and levels of circulating SM, dihydrosphingomyelin, ceramide and glucosylceramide (33 species) in 4400 subjects from five diverse European populations. The strongest associations were observed in or near seven genes functionally involved in ceramide biosynthesis and trafficking with an additional 70 variants across 23 candidate genes involved in sphingolipid-metabolizing pathways also achieving significance. This was the first application of metabolomics to GWAS and has demonstrated that circulating concentrations of a number of sphingolipids are under strong genetic control. A follow-up study from the same group extended these analyses to include 24 SMs, nine ceramides, 57 PCs, 20 lyso-PCs, 27 PE-based plasmalogens, as well as their proportions in each major class [149]. They identified four novel loci significantly associated with phospholipids and two with sphingolipids explaining up to 3.1% of the variance. Analysis with respect to within-class molar proportions identified three additional loci for phospholipids thought to be involved in fatty acid elongation/saturation processes or specific fatty acid turnover mechanisms. Given the established or proposed roles of ceramides, other sphingolipids and phospholipids in multiple disease states, including IHD, these loci can now be tested for their roles in disease pathogenesis. The combination of studies involving metabolomic and other –omics, including GWAS, will be an important strategy to identify the mechanistic links between lipid species and other metabolites with disease status.

CONCLUSIONS

Metabolomics presents a new frontier in metabolic medicine by empowering us to comprehensively and systematically study and characterize LMW metabolites in biological systems. Metabolomic studies provide insight into metabolic changes in these biological systems in response to perturbation and thus are by their nature hypothesis-generating. Larger-scale human studies, either population-based or clinical trials, can form the basis for a priori hypothesis testing in an experimental setting. Subsequent studies could be extended to appropriate animal models to identify relevant mechanistic pathways and further validate the population findings. Although a reductionist approach has historically been employed in the investigation of disease, a global systems biology approach is significantly more powerful and yields more information. By the same token, the wealth of information generated by metabolomic studies represents another level of complexity that requires careful and considered applications of statistical and bioinformatics methodologies to better analyse and interpret the data. Nonetheless, adopting a systems biology approach will (i) facilitate our understanding of mechanistic pathways that underscore IHD, (ii) allow for biomarker discovery and better CVD risk stratification and estimation, and (iii) allow for more detailed monitoring and determination of optimal efficacy of current and novel therapeutic agents to achieve a significant reduction in disease burden.

CVD risk stratification and estimation remains a challenge, with current measures based primarily on plasma lipids and lipoproteins, as well as other anthropometric measures. We [41] and others [150] have demonstrated, using a lipidomics approach, that molecular lipids can form a sound basis for the development of models which can detect, stratify and predict risk above and beyond traditional plasma lipids and other risk factors. Furthermore, when the lipidomic model is combined with traditional risk factors, the combined model performs significantly better. However, metabolomics is by its very essence a ‘snapshot’ of the phenotype and, although it provides valuable information pertaining to atherosclerotic disease, it is at best an association study linking lipid metabolites to atherosclerosis. To better elucidate causal factors and to enhance our understanding of metabolism and pathways in CVD to identify potential therapeutic targets, kinetic or ‘fluxomics’ studies are required. Such studies are complicated and require specialized expertise, but may provide the necessary experimental evidence to complement large-scale population studies, test existing and novel therapeutic agents and identify new therapeutic targets.

Statins are the current recommendation for cholesterol-lowering. However, there still remains a significant residual risk of recurrent CVE in statin-treated patients, so statin therapy alone will not eliminate CVD. Statins affect both lipids and lipoproteins, and alterations to the lipid composition of lipoprotein particles may affect its metabolism. The precise mechanisms that relate statins to lowered cardiovascular risk in high-risk individuals, beyond cholesterol-lowering, have not been fully elucidated. More importantly, identifying potential therapeutic targets to significantly lower the residual risk of recurrent CVEs in statin-treated patients has now become imperative. Metabolomics provides the necessary technology to facilitate advancements in identifying such therapies. Presently, it is difficult to fully discriminate between the direct effects of statins on the lipidome or whether the alterations to lipid metabolites are secondary or a consequence of the global reduction in cholesterol. In addition, changes to the lipidome by statins and other agents may be subtle, requiring the use of a sensitive and powerful platform to discern any significant differences. Metabolomics empowers us to study more comprehensively the effects of statins and other therapies on lipoprotein-specific changes despite no significant changes in whole plasma [151]. Given the pleiotropic effects of statins and other therapies and their complex interaction with other therapeutic agents on metabolites, in particular lipids, utilizing a metabolomics platform, together with appropriate experimental designs, may provide a more comprehensive readout and facilitate the identification of multiple players and pathways that may extend beyond cholesterol-lowering.
Metabolomics technology has the potential to play a leading role in the development of personalized medicine. Emerging metabolomic studies have demonstrated individual variation in the response of lipid metabolites to the same statin and, conversely, different statins have been shown to exert differential effects on lipid metabolites in a relatively homogenous population. However, further studies are required to demonstrate whether metabolite or lipid profiling can be exploited to tailor therapy. Ultimately, the goal of metabolomics is to better understand the pathophysiology of heart disease by utilizing a holistic systems biology approach encompassing environmental and genetic influences, leading to early detection and improved treatment strategies.

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Metabolomics and ischaemic heart disease


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303


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