Metabolomics as a tool for cardiac research

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Abstract | Metabolomics represents a paradigm shift in metabolic research, away from approaches that focus on a limited number of enzymatic reactions or single pathways, to approaches that attempt to capture the complexity of metabolic networks. Additionally, the high-throughput nature of metabolomics makes it ideal to perform biomarker screens for diseases or follow drug efficacy. In this Review, we explore the role of metabolomics in gaining mechanistic insight into cardiac disease processes, and in the search for novel biomarkers. High-resolution NMR spectroscopy and mass spectrometry are both highly discriminatory for a range of pathological processes affecting the heart, including cardiac ischemia, myocardial infarction, and heart failure. We also discuss the position of metabolomics in the range of functional-genomic approaches, being complementary to proteomic and transcriptomic studies, and having subdivisions such as lipidomics (the study of intact lipid species). In addition to techniques that monitor changes in the total sizes of pools of metabolites in the heart and biofluids, the role of stable-isotope methods for monitoring fluxes through pathways is examined. The use of these novel functional-genomic tools to study metabolism provides a unique insight into cardiac disease progression.

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Introduction

The philosophy underlying functional genomics is that to understand a given genetic, physiological, or pathological intervention, the point of initial perturbation should not be viewed in isolation, as advocated by so-called reductionist approaches. Instead, the complete network of changes within an organism must be understood by measuring as many entities as possible at any level of organization (Figure 1). For example, 'transcriptomics' and 'proteomics' aim to measure the mRNA and protein complement of a system, respectively. Likewise, 'metabolomics' measures the small molecules—metabolites—of an organism. The term 'metabolome' was first coined by Oliver and co-workers[1] and Tweeddale and colleagues,[2] whereas Nicholson and co-workers coined the term 'metabonomics' around the same time.[3] Metabolomics is the more-commonly used term in the literature, but because both names have rather similar definitions, one must consider both words in order to have a true reflection of the literature. For the sake of consistency, the term metabolomics is used throughout this Review.

The concept behind metabolomics is the application of tools from analytical chemistry to profile the maximum number of metabolites found within an organism, tissue, cell, or biofluid as is feasible. The first major caveat to this approach is that no analytical tool can measure all the metabolites found within even the simplest organism. This problem partly relates to the complexity of measuring chemical entities that exist over a wide range of polarities (making metabolite extraction and chromatography challenging), molecular masses, and concentrations (from millimolar to femtamolar). The Human Metabolome Project has a central aim to identify, quantify, catalog, and store data on all metabolites that can potentially be found in human tissues and biofluids at concentrations >1 micromolar. Over 8,500 metabolites have already been identified and recorded in the Human Metabolome Database,[4,5] demonstrating the huge challenge faced by researchers in the field of metabolomics. Despite these analytical issues, metabolomics is used widely as a functional-genomic tool, for biomarker discovery, and in systems biology; the use of metabolomics in general biology has been reviewed previously.[6–10]

In this Review, we discuss how metabolomics has been used to study cardiac metabolism and cardiovascular disease. We briefly describe the key technologies used to conduct metabolomic research and then discuss their application to cardiology, including functional-genomic studies in animal models of disease, fundamental mechanistic studies in biochemistry, and the search for novel biomarkers in patient groups.

Analytical tools in metabolomics

A wide range of analytical tools have been used for metabolomics (Table 1), including infrared spectroscopy, fluorescence spectroscopy, and Coulombic arrays, but by far the two most-commonly employed approaches utilize NMR spectroscopy and mass spectrometry (MS; Figure 2). A brief introduction to these two techniques is provided, but they are reviewed in more detail elsewhere.[10] Despite the limitations of these technologies, a combination of MS-based and NMR-spectroscopy-based metabolomics can identify, detect, and quantify hundreds[11,12]

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(and potentially in the future, thousands) of metabolites at a high throughput (assay times of 20–40 min) and with some degree of automation.

Key points

- Metabolomics (also called metabonomics) is the general measurement of the metabolites found within a cell, tissue, biofluid, or organism
- Although no single analytical tool can measure all the metabolites within an organism, NMR spectroscopy and mass spectrometry can profile wide ranges of metabolites
- Metabolomics is hypothesis-generating rather than hypothesis-based
- Metabolomics has been used to determine the roles of specific genes in the development of cardiac diseases and to define metabolic phenotypes associated with given genetic modifications
- Metabolomics has been used in the search for biomarkers for cardiac disorders in humans; however, a novel, validated biomarker for cardiac disease is still being sought

NMR spectroscopy

NMR spectroscopy relies on the fact that certain nuclei possess the property of magnetic spin and, when placed inside a magnetic field, can adopt different energy levels that can be observed using radiofrequency waves. The most-commonly used nuclei for biological metabolites are ^{1}H, ^{13}C, and ^{31}P. The chemical environment of the nucleus has an effect on the exact frequency at which it resonates (referred to as chemical shift), as does coupling with adjacent nuclei (known as spin coupling). The combination of these two effects ensures that NMR spectroscopy is a highly discriminatory tool for structure elucidation, even for mixtures of molecules such as tissue extracts or biofluids. How the physical properties associated with the NMR effect give rise to its many uses in biology has been reviewed previously.[13]

In metabolomics, ^{1}H-NMR spectroscopy is used predominantly because it is quick in terms of acquisition (within minutes); it has the highest sensitivity of all but one nucleus for the NMR effect; the NMR spectrometer does not get dirty because the sample is physically isolated from the instrument, and thus the spectrometer maintains a constant performance; the resultant spectra are not greatly affected by salty biofluids (including urine); and because protons are found in all organic molecules. However, ^{1}H-NMR spectroscopy has relatively low sensitivity (particularly compared with MS), operating in the high micromolar to millimolar range and has a small chemical-shift range, which produces crowded spectra when acquired as a 1D spectrum, meaning that discrimination of resonances from the various compounds in complex mixtures can be difficult.

Despite these limitations, NMR spectroscopy already has an excellent track record in determining metabolic changes in the heart, both in animal models and as a clinical tool. NMR spectroscopy with ^{31}P has proved to be a versatile tool for assessing energetics both in perfused hearts and *in vivo*, allowing quantification of the levels of ATP, phosphocreatine, and inorganic phosphate, the intracellular pH, and the activity of creatine kinase.[14–16] These parameters have been quantified in studies of myocardial ischemia,[15,17] heart failure,[18,19] reperfusion injury,[20–22] and the cardiac effects of diabetes mellitus.[23,24]

In addition, ^{13}C-NMR spectroscopy has been used to monitor the uptake of given metabolites and to probe changes in substrate metabolism within the intact heart.[25,26] Using this method, substrates such as glucose, acetate, lactate, and free fatty acids are labeled with the stable ^{13}C isotope, which can be detected using NMR spectroscopy or MS. Although ^{13}C-NMR spectroscopy is less sensitive than ^{1}H-NMR spectroscopy, it has the major benefit over MS that NMR spectroscopy allows the radioactive label to be localized within a given metabolite. This information can be used to probe particular pathways, such as the activity across the malate–aspartate shuttle.[27,28] This approach has witnessed a resurgence with the introduction of hyperpolarized-^{13}C-NMR spectroscopy.[29,30] In this technique, the sample containing the labeled substrate is first frozen and pressurized, and polarization can be produced greatly in excess of the normal NMR experiment, even rivaling the sensitivity of MS.

Given the wide use of ^{31}P-NMR spectroscopy and some of the unique benefits of ^{13}C-NMR spectroscopy, the use of ^{1}H-NMR spectroscopy to follow *in vivo* metabolism in cardiac studies has been surprisingly rare. However, ^{1}H-NMR spectroscopy has been used to monitor lactate production *in vivo* in the ischemic heart,[31] and to follow lipid infiltration of the heart after ischemic reperfusion injury.[32] In many ways, these early studies of heart

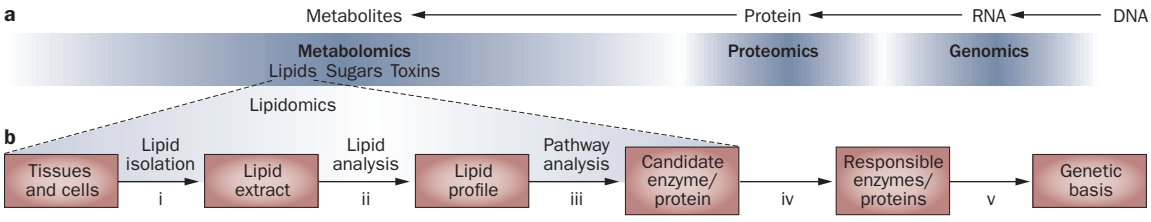


Figure 1 | Metabolomics in the spectrum of functional-genomic approaches, with emphasis on lipidomics. **a** | Genes encode proteins that interact with environmental factors, producing the metabolite complement of a cell, tissue, or biofluid. **b** | Lipidomics is a subdivision of metabolomics and describes the complete lipid complement of a cell, tissue, or biofluid. Lipids are extracted from the source material using organic solvents (step i), and analyzed using techniques such as liquid chromatography and mass spectrometry (step ii). The lipid profile of a tissue or biofluid can then be used to identify patients with a particular disease, or perturbed molecular pathways in animal models (step iii). This complements the information obtained by other approaches such as genomics and proteomics (steps iv and v). Modified with permission obtained from Nature Publishing Group © Wenk, M. R. *Nat. Drug Disc.* **4**, 594–610 (2005).

Table 1 | A comparison of the key approaches used in metabolomics, with particular reference to cardiac research

Technique	Coverage of the metabolome	Cost	Throughput*	Advantages‡	Disadvantages‡
High-resolution NMR spectroscopy	20–30 in heart tissue 30–40 in blood plasma 40–100 in urine	Low per sample	~10 mins	Very consistent results because sample does not come into contact with detector Good with biofluids with a high salt content, such as urine Good libraries of spectra Easy to process	Poor sensitivity Co-resonances for 1D-NMR spectroscopy 2D-NMR spectroscopy is time consuming
In vivo NMR spectroscopy	~10 by ^{1}H ~5–6 by ^{31}P ~5–10 by ^{13}C (dependent on substrate)	High	~30 mins with preparation time	Can be used to observe metabolism *in vivo*, so is truly representative of the working heart Imaging can map metabolite distributions —very useful for fat distribution	Very poor sensitivity, but which can be addressed with hyperpolarization or higher field strengths
High-resolution magic-angle-spinning NMR	20–30 in heart tissue	Low	~15 mins	Can be used to monitor the cellular environment (e.g., compartmentation) in intact tissue Tissue can be chilled to reduce *post-mortem* effects	Tissue cannot be perfused, so viability of tissue is limited
Direct-infusion MS	Hundreds of aqueous or lipid metabolites	Low	3–4 mins	Has been used to profile both aqueous and lipophilic metabolites in various studies Minimal carry-over, as no chromatography involved Good reproducibility Simple to optimize	Ion suppression can be a substantial problem Identification can require chromatography, e.g., for isobaric species Metabolite identification is a major challenge and requires MS^{n} acquisitions Semiquantitative at best
GC–MS	20–30 fatty acids 100–150 aqueous metabolites Higher for GC/GC–MS	Low–medium	20–30 mins for fatty acids 30–45 mins for aqueous metabolites	Chromatography is robust and reproducible Metabolite identification is aided by adoption of common ionization parameters in electron impact and wide availability of the NIST library and others Can be quantitative	Fatty-acid analysis requires the removal of the fatty acid from the head group, which complicates identification Metabolites need derivatization and not all metabolites are suitable for derivatization
LC–MS	Hundreds to thousands?	Medium	Typically 15–30 mins	Chromatography reduces the effect of ion suppression and can separate isobaric species Suitable for measuring intact lipids, dipeptides, tripeptides, and other macromolecules	Chromatography can drift during a sample run, which makes data processing difficult Metabolite identification is a major challenge
Triple–quadrupole (targeted) MS	Typically 30–40; up to 100–150 across assays	Medium to high, depending on number of assays	15 mins per chromatography run ~60 mins for comprehensive screens	Highly sensitive Highly quantitative Targeted, so metabolite identification not an issue Results readily transferable because concentrations can be measured	Targeted, so discovery of novel biomarkers unlikely Time-consuming to set up quantitative assays

*All the techniques can take a substantial amount of time to process, or they can be highly automated, depending on the lab where the work is undertaken. Furthermore, the sample volumes required vary widely depending on what is being measured and the exact piece of equipment used (for example, microprobes versus potential probes). ‡These comments are meant as general guidelines, and the reader is alerted to the fact that the field uses a diverse range of approaches. Abbreviations: GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NIST, National Institute of Standards and Technology.

metabolism with ^{1}H-NMR spectroscopy can be viewed as the forerunners to the development of metabolomics for cardiac metabolism.

Mass spectrometry

Gas chromatography–mass spectrometry

In contrast to NMR spectroscopy, definitive MS studies that could be claimed to be the forerunners to metabolomic research are difficult to pinpoint. MS is one of the few approaches that can profile low-concentration metabolites and xenobiotics in biological samples. Arguably, the development of MS to profile a range of chemically diverse metabolites for medical diagnosis began with the use of gas chromatography (GC) combined with MS to profile organic acids in both rat and human heart tissue.[33,34] GC–MS was also used to track ^{13}C-labeled metabolites, by virtue of the fact that these metabolites are heavier than those containing the ^{12}C isotope.[35] GC has the advantage of being a robust form of chromatography compared with liquid chromatography (LC), with only modest shifts in retention time during a typical analytical run (particularly since the development of modern, prepacked GC columns), and because the analytes must be volatile and are, therefore, easily introduced into the mass spectrometer. Metabolites fragment in a fairly consistent manner under typical GC–MS conditions, which allows libraries of spectra to be created.

However, many metabolites are not volatile and require some prior derivatization. Fatty acids are analyzed after conversion into methyl esters, whereas amino acids,

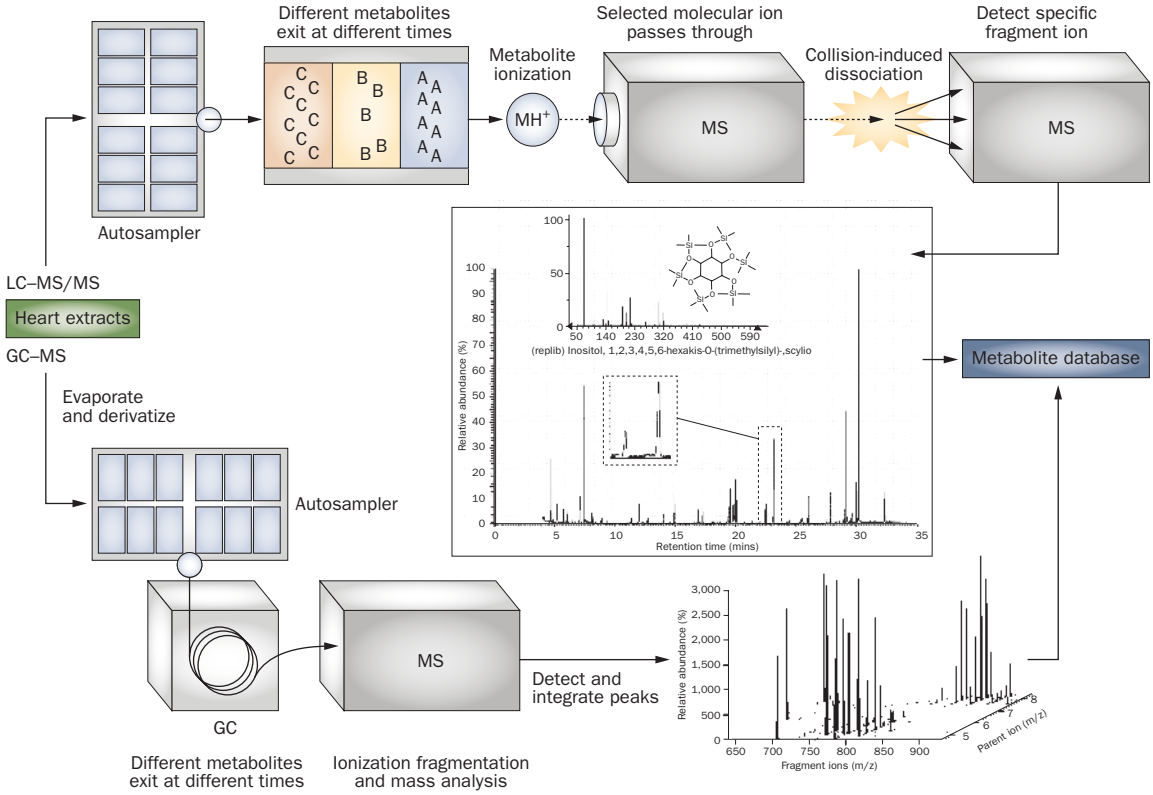


Figure 2 | Metabolomic analysis by chromatography with MS. To simplify the complexity of the metabolite complement of a cell, tissue, or biofluid before analysis with MS, chromatography is used to separate individual metabolites. Separation is achieved by injecting the sample extract onto a GC or LC column. For GC, chemical conversion of metabolites to less polar forms (derivatization) is often required. Metabolites are separated according to their affinity for the solvent (LC) or gas (GC) and the material of the column, and so pass through the column at different rates ('retention times'). Further discrimination is provided by the mass spectrometer. Individual metabolites are identified by their masses, fragmentation patterns, and retention times. Abbreviations: GC, gas chromatography, LC, liquid chromatography; MS, mass spectrometry; m/z, mass-to-charge ratio. Modified with permission obtained from Nature Publishing Group © Last, R. L. *et al. Nat. Mol. Cell Biol.* **8**, 167–174 (2007).

sugars, and oxoacids can be made volatile through addition of one or more trimethylsilyl groups.[36] The sensitivity of MS means that, even with a long chromatography run, substantial overlap between a number of co-eluting metabolites exists, which makes quantification of each individual metabolite difficult. This inconvenience can be addressed by 2D-GC or the use of time-of-flight (TOF) instruments, which can sample more points across peaks in the chromatographic run than traditional methods.[37] Furthermore, the very act of derivatization can destroy metabolic information. For example, to measure total fatty acids, individual fatty acids must be cleaved from the backbone to which they are attached during transesterification (Figure 3a). Determination, therefore, of whether a given fatty acid came from, for example, a triglyceride or a phospholipid becomes difficult.

Liquid chromatography–mass spectrometry

To investigate complex macromolecules such as lipids or peptides, an alternative approach to GC–MS is needed to ensure that metabolites are ionized without destruction. In electrospray ionization, macromolecules are dissolved in a liquid that is then turned into a fine aerosol of charged particles by its introduction into the mass spectrometer.[38] This process allows the coupling of MS with LC. The low amount of energy required for ionization does not tend to cause a large amount of fragmentation of the metabolites, which means that the molecular ion can be detected in many cases. A wide range of biomolecules can be readily ionized by this approach, which has led to the common use of LC–MS in proteomics and lipidomics —the analysis of intact lipids (Figure 3b).[39,40]

The two major limitations that have slowed the development of LC–MS as a tool for metabolomics are metabolite identification and the reproducibility of the chromatograph. Metabolite identification in LC–MS can be difficult if a large number of isomers present are present (Figure 4a), or if biomolecules are ionized when attached to other molecules and form 'adducts', which complicates structure elucidation. Also, LC is more variable than GC, which can make comparisons of metabolites across an analytical run difficult.

To address these problems, some researchers have resorted to removal of the chromatography step altogether and instead use direct infusion. To improve discrimination of metabolites, either high-resolution instruments, such as Fourier transform mass spectrometers, or triple–quadrupole instruments are employed to detect

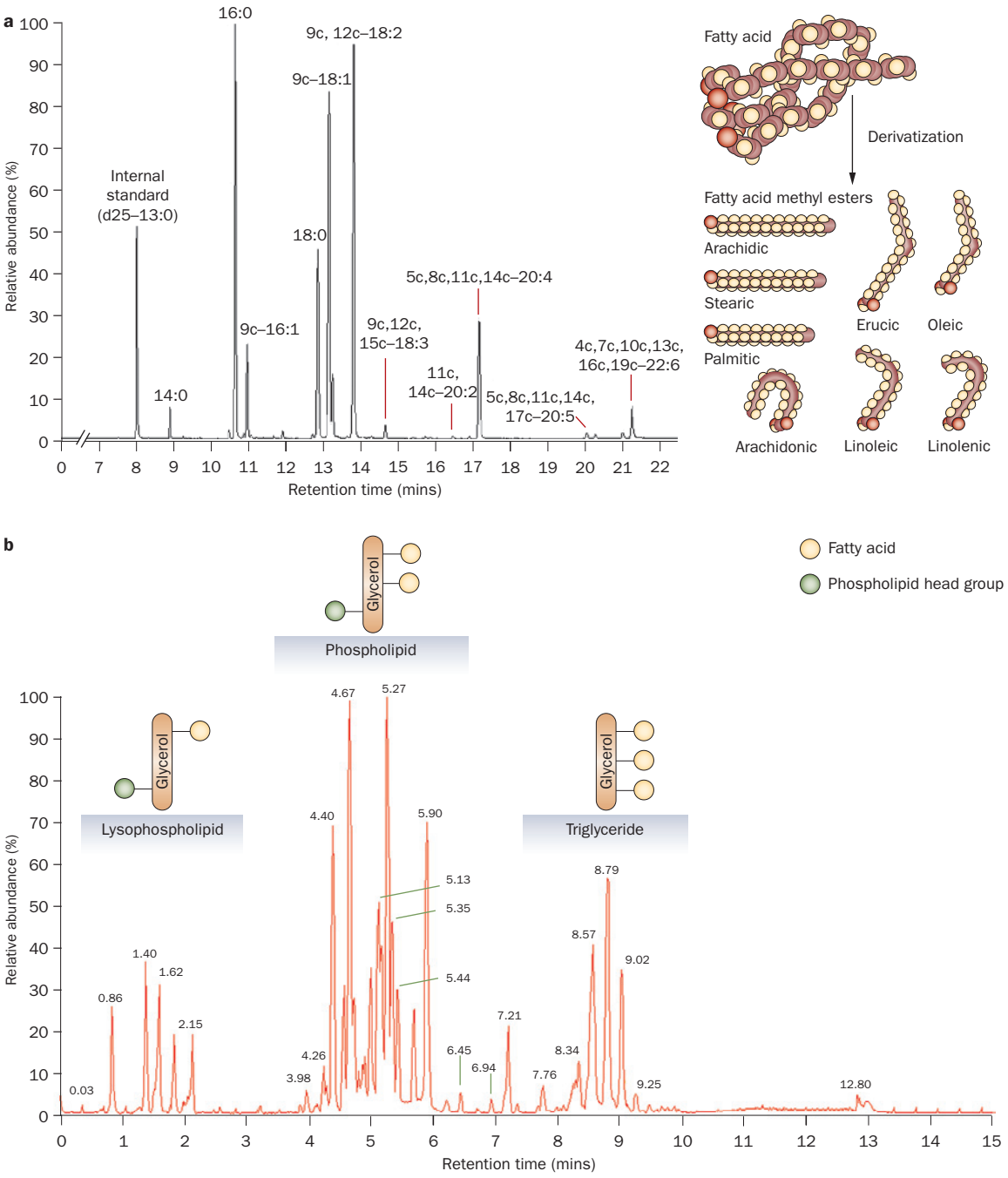


Figure 3 | Techniques for measuring the components of the lipidome of the heart. **a** | The total fatty-acid complement of an extract of heart tissue can be measured by gas chromatography. Fatty acids are converted into fatty acid methyl esters as part of a transesterification derivatization (right). Each peak in the chromatogram (left) represents an individual fatty acid methyl ester, which is analyzed by either mass spectrometry or with a flame ionization detector. The labels indicate the length and degree of unsaturation of each fatty acid; for example, 9c–18:1 indicates a fatty acid that is 18 carbons in length, with one double bond positioned at the ninth carbon from the carboxylate group. **b** | A range of intact lipids can be detected by liquid chromatography–mass spectrometry. Chromatography separates intact lipids according to polarity, and high-resolution mass spectrometry can be used to identify individual lipid species.

metabolites by their distinctive fragmentation patterns. A triple–quadrupole-based approach for lipid analysis is often referred to as 'shotgun lipidomics'.[41,42] However, direct infusion of metabolites can be subject to ion suppression, whereby the ionization capability of one analyte is reduced because the charge is associated with another analyte.

Open and closed profiling

The approaches outlined above are often described as open profiling or untargeted analyses because a wide range of lipid species are analyzed. By contrast, targeted or closed approaches are used in MS to target a limited set of metabolites, often structurally related, in a quantitative

manner (Figure 4b). Closed analyses are usually more sensitive than open approaches, but are unlikely to identify novel biomarkers because they are aimed at predefined metabolites. Instead, such analyses are important for biomarker validation after a putative biomarker has been identified using an open-profiling approach.

Metabolomics in cardiology

Analysis of gene modifications

In cardiology, metabolomics has been used alongside proteomics to provide an holistic investigation of genetic modifications, such as the effect of genetically modifying protein kinase Cε in the heart.[43] A profound set of changes in metabolism was demonstrated, including in the malate–aspartate shuttle, glucose metabolism, creatine kinase, pyruvate kinase, and lactate dehydrogenase.

Proteomics and metabolomics were similarly combined in investigations to profile tissues from the mdx mouse —a murine model of muscular dystrophy that lacks the protein dystrophin as a result of a spontaneous mutation in its X-chromosome-linked gene.[44–46] Metabolic profiles of the heart, skeletal muscle, diaphragm, and two regions of the brain were generated using ^{1}H-NMR spectroscopy and a principal-components analysis to process the data. This analysis demonstrated that each of the three isoforms of dystrophin were characterized by very different metabolic changes depending on the tissue type in which they were found, with heart and skeletal muscle being characterized by perturbations in creatine and taurine metabolism. This approach was complementary to previous, hypothesis-driven research, which also showed that one of the major perturbations in muscle tissue, including the heart, was an increase in taurine as measured by ^{1}H-NMR spectroscopy; taurine has previously been correlated with the capacity to repair muscle damage in the mdx mouse.[47,48]

Furthermore, these metabolic changes in taurine metabolism were also used to monitor treatment of dystrophic muscle tissue by genetic upregulation of utrophin in mice.[49] A GC–MS-based analysis of *ex vivo*, working, perfused mdx mouse hearts showed a decrease in fatty-acid oxidation and a concurrent increase in carbohydrate oxidation.[50] However, despite the switch to carbohydrate metabolism—which is usually associated with a decrease in oxygen consumption—this change in metabolic substrate was accompanied by an increase in oxygen consumption in the dystrophic mouse heart. This finding suggests that the heart of the dystrophic mouse possesses a mitochondrial deficit that impairs normal function. These changes also occurred alongside remodeling of the nitric oxide–cyclic GMP pathway, demonstrating that a single gene modification can affect many parts of the metabolic network.[50,51]

In a nontargeted approach, metabolomics was used in conjunction with an *N*-ethyl-*N*-nitrosourea mutagenesis screen to identify that the mitochondrial trifunctional enzyme subunit β was involved in cardiac arrhythmogenesis and fibrosis.[52] Metabolically, the mouse generated in the *N*-ethyl-*N*-nitrosourea screen was characterized by increased levels of circulating long-chain acylcarnitines compared with the wild-type strain, as measured by LC–MS. Hepatic steatosis was detected in addition to cardiac fibrosis, which suggests that lipotoxicity might be the cause of the arrhythmogenesis.

A mouse model with a transgene encoding cardiac-specific vascular endothelial growth factor B surprisingly had concentric cardiac hypertrophy without a change in cardiac function.[53] The cardiomyocytes were enlarged and showed less damage after ischemia compared with the control strain, whereas the cardiac tissue from the transgenic mouse had increased levels of ceramides and decreased triglyceride levels, as measured by LC–MS. The transgenic mice died following lysis of mitochondria, possibly as a result of lipotoxicity in the heart.

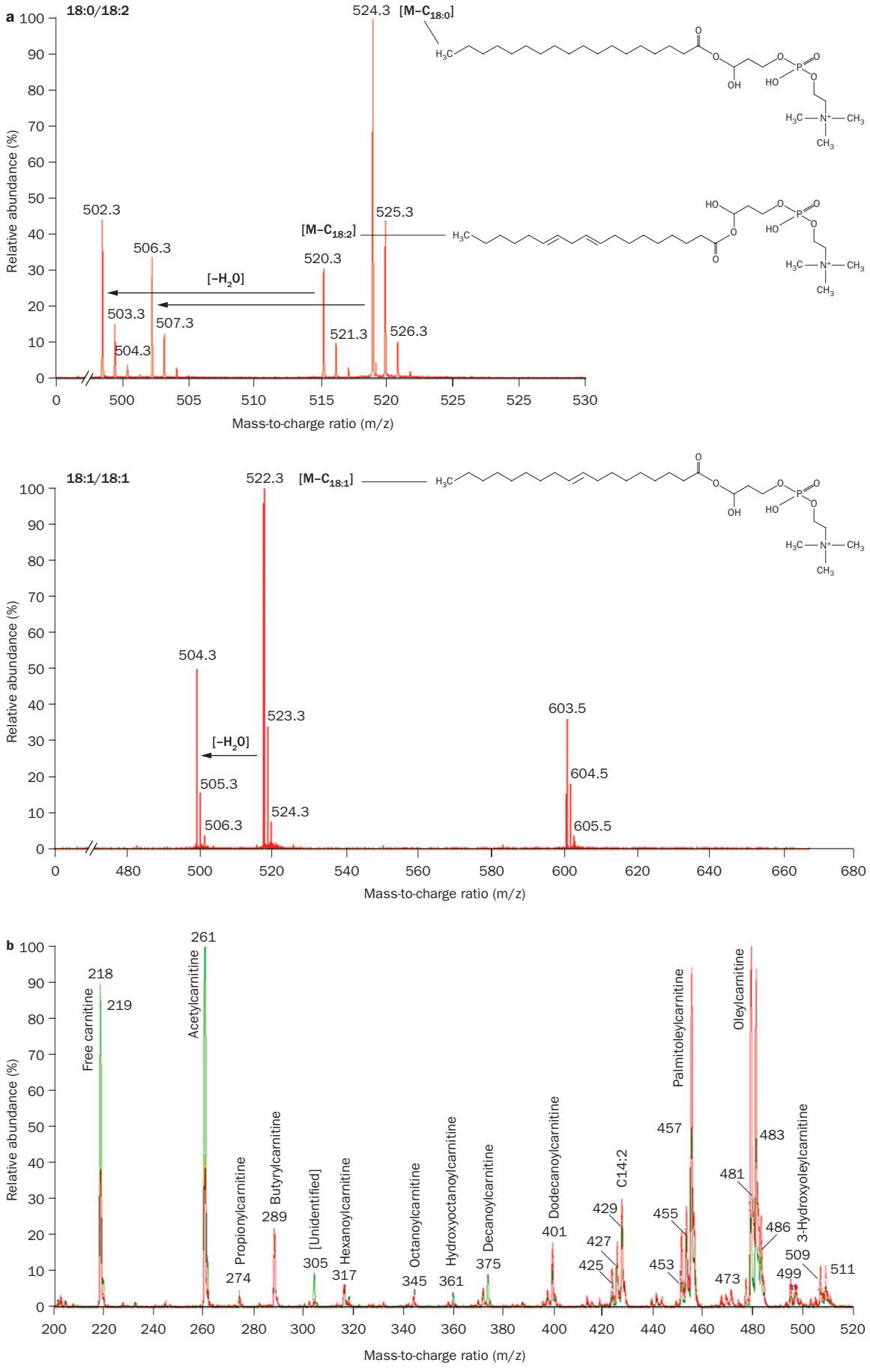
The effect of angiotensin II-induced mitochondrial dysfunction and subsequent cardiac hypertrophy in the rat has been investigated by GCxGC–TOF MS.[54] A wide range of metabolic changes were identified in a transgenic model of angiotensin stimulation (where rats had both human renin and angiotensinogen genes), including an increase in hypoxanthine consistent with purine degradation compared with the wild-type strain, as measured by GC–MS.

Genetic modifications can also be traced through changes in blood plasma; for example, a number of metabolic changes were identified in blood from patients with dilated cardiomyopathy.[55] This approach has the added advantage that the changes are readily applicable to diagnosis in the clinic, if they are robust when extended to large cohorts. Although metabolomics is becoming increasingly popular in functional-genomic studies of the heart, many have been proof-of-concept studies or, alternatively, metabolomics has generated more questions than answers about the function of a gene. If the approach is really to benefit cardiac-metabolism research, improved tools are needed to model changes in pool sizes of metabolites.

Substrate selection

The peroxisome proliferator-activated receptor α knockout (*Pparα*$^{-/-}$) mouse has become a versatile model for probing substrate selection in the heart because of the central role of PPARα in regulating fatty-acid metabolism in humans.[56] The *Pparα*$^{-/-}$ mouse was used as a model of cardiac decompensation in the diseased heart, because substrate preference is known to switch from fatty-acid metabolism to increased carbohydrate utilization in a number of disease states.[57] As expected, the perfused *Pparα*$^{-/-}$ heart had a reduced rate of fatty-acid oxidation and an increased rate of carbohydrate metabolism compared with wild-type control hearts, measured using a combination of stable isotopes and GC–MS. Additionally, the genetically modified heart had a reduced capacity to increase lactate production with increased workload, which suggests a restricted ability to upregulate glycolysis.

Metabolomics was used to build a systems-biology atlas of response to the gene deletion across the *Pparα*$^{-/-}$ mouse, by investigating the interaction between changes in the heart, liver, skeletal muscle, and adipose tissue across the whole organism.[11,58] A combination of ^{1}H-NMR spectroscopy and GC–MS demonstrated that a failure



◀ **Figure 4** | Further characterization of the lipidome by tandem MS. **a** | One of the main challenges of liquid chromatography–MS is lipid identification because of the large numbers of isomers present. MS/MS can be performed to address this problem. Two isobaric (same mass) phosphocholines can undergo time-aligned parallel fragmentation, a pseudo MS^3 technique, to demonstrate that they are phosphocholine (18:0/18:2) and phosphocholine (18:1/18:1). **b** | In addition to open-profiling methods, which examine a wide range of metabolites, targeted analyses by tandem MS can be performed on particular classes of metabolites. A mass spectrum of carnitine derivatives, acquired using a triple–quadrupole method targeted to this class of compounds, can be used to assess flux of fatty acids across the inner mitochondrial membrane. Abbreviation: MS, mass spectrometry.

to express *Pparα* led to a decrease in the pool sizes of a number of endogenous metabolites within the heart, including glucose, glycogen, alanine, lactate, ketone bodies, and fatty-acid-oxidation products, which supported the conclusion that the heart of the *Pparα*$^{-/-}$ mouse has a reduced metabolic capacity.[57]

Given that agonists for the PPARα are potent treatments for type 2 diabetes and obesity in humans, the action of this class of drugs has been investigated in Swiss–Webster mice using LC–MS-based lipidomics.[59] The heart was the second most-affected organ (after the liver), and was characterized by an increase in docasahexenoic acid and phosphatidylserine (which suggests activation of phosphatidylserine synthase-2), and a decrease in cardiac sphingomyelin (which indicates activation of sphingomyelin phosphodiesterase, with a preference for polyunsaturated sphingomyelin). Detailed measurements of the full complexity of some classes of lipids in the heart have been made possible by improvements in LC–MS and bioinformatics. Consequently, the roles of individual lipid species in contributing to diseases in the heart, such as diabetic cardiomyopathy or steatosis, are still inconclusively determined and detailed lipidomic studies are needed, although the accumulation of ceramides, diacylglycerides, and plasmalogens has been implicated in pathogenesis. Wide-scale use of LC–MS to profile lipid changes in cardiac tissue will enable the formulation of a mechanistic understanding of how dyslipidemia affects the 'lipidome' and, in turn, how this produces the complex phenotype of disease.

Substrate selectivity during cardiac hypertrophy has been examined using a combination of ^{13}C-NMR-spectroscopy-based isotopomer analysis of glutamate and real-time PCR to investigate fatty-acid oxidation.[60] In a pressure-overload model of hypertrophy in the rat, fatty-acid oxidation was found to be impaired in conjunction with downregulation of Pparα and its target genes. Similar decreases in palmitate oxidation were established in the same animal model compared with controls using a combination of dynamic modeling of ^{13}C labeling *in situ* and high-resolution ^{13}C-NMR spectroscopy *in vitro* to distinguish between exogenous and endogenous fatty-acid oxidation, as well as metabolism of lactate, pyruvate, and glycogen.[61] The conclusion was that, under pressure-induced hypertrophy, the heart had reduced capacities for triglyceride storage and mobilization, which contributed to the reduced palmitate oxidation in pathological hypertrophy. In addition, some of these changes might, in turn, arise from a change in activity of the anaplerotic pathway, with increased NADP-dependent malic enzyme expression in conjunction with stimulated oxidation of glycolytic products in the hypertrophic heart.[62]

Toxicity monitoring

Metabolomics has also been used in toxicity monitoring. The effects of 3,4-methylenedioxymethamphetamine (commonly known as ecstasy) on cardiac metabolism in rats were monitored using *in vivo* ^{1}H-NMR spectroscopy.[63] The drug produced a number of metabolic changes in the heart, including an increase in the pool size of carnitine and a decrease in choline compared with wild-type rats. Doxorubicin cardiotoxicity has been monitored using a similar approach.[64]

Metabolomics in cardiovascular disease

The identification of novel biomarkers of cardiovascular disease is a widespread focus for research. Given the metabolic basis of many cardiovascular pathologies, metabolic changes could reasonably be used to monitor relative risk, understand pathophysiological mechanisms, and track treatment. MS and NMR spectroscopy have been used to profile a wide range of metabolites in blood plasma and urine, some of which seem to be highly correlated with cardiovascular disease under certain conditions, particularly when multivariate patterns are used to profile risk or disease status. Although no novel, validated biomarker has yet been found using metabolomics, research in this field is ongoing.

Atherosclerosis

Mice

Investigators in human studies must balance the desire to profile a large number of individuals in order to allow for dietary effects and genetic variation across the population, with the challenges involved in the analysis of multiple samples. An alternative approach is to use animal models. Laboratory strains have far less genetic variation than between humans, and their diet and environment can be closely controlled. Animal studies, therefore, require smaller replicate numbers than human trials, and have the added benefit that tissues can be readily obtained. However, most mouse strains are naturally resistant to the development of atherosclerosis, so extreme models of the disease are often required in mice.

Liver lipids of the apolipoprotein E3 (*Apoe3*) Leiden transgenic mouse were profiled using a combination of transcriptomics and metabolomics with LC–MS.[65] The combination of approaches allowed the construction of a topology network of responses to the induced atherosclerosis. In a follow-up study, a dietary intervention was added.[66] As the cholesterol content of the diet was increased, the liver switched from a low inflammatory state to a proinflammatory and proatherogenic state, with a tight correlation between increased lipid metabolism and the development of atherosclerosis. In a similarly

multidisciplinary approach, NMR-spectroscopy-based metabolomics was combined with proteomics using 2D-gel electrophoresis to examine atherosclerosis in aortas from Apoe-deficient mice.[67] Atherosclerosis was associated with an increase in oxidative stress, reflected in a change in the oxidation state of peroxiredoxin 6 and the expression of NADPH-dependent malic enzyme involved in replenishing the glutathione pool, and a decrease in the energy status of the vessels.

Changes in lipid metabolism were determined using ^{1}H-NMR spectroscopy of blood plasma from hyperlipidemic hamsters in order to monitor the proatherogenic properties of various diets, with the aortic cholesteryl-ester level considered to be an early marker for atherosclerosis.[68] VLDL cholesterol, total cholesterol, and *N*-acetylglycoproteins were positively correlated with the aortic cholesteryl-ester level, whereas the region of the NMR spectrum at 3.75 parts-per-million, albumin-lysyl residues, and trimethylamine-*N*-oxide were negatively correlated. The interactions between diet and genotype were modeled using a multivariate approach in the *Ldlr* knockout mouse, which revealed a profound change in the oxidation of choline-containing metabolites.[69] Both the genetic modification and feeding with a diet high in fat, cholesterol, and cholate reduced the excretion of trimethylglycine (also known as betaine) and dimethylglycine in the urine of these animals. Furthermore, these metabolic changes were found in another mouse model of atherosclerosis—the *Apoe* null ($Apoe^{-/-}$) mouse.[69]

Metabolomics has also been applied to novel models of atherosclerosis in mice. The $Atm^{+/-}/Apoe^{-/-}$ mouse was generated to investigate the interaction between the reduced capacity for DNA repair associated with heterozygosity for the ataxia-telangiectasia mutated gene (*Atm*), and the known proathrogenic phenotype of the $Apoe^{-/-}$ mouse.[70] The $Atm^{+/-}/Apoe^{-/-}$ mice displayed increased atherosclerosis and effects of metabolic syndrome when compared with the $Apoe^{-/-}$ mouse. These features were accompanied by increased DNA damage in macrophages, and decreased mitochondrial function, which was measured directly by PCR for mitochondrial DNA damage and by generation of reactive oxygen species from isolated mitochondria, and could also be inferred by the metabolic changes in blood plasma and liver tissue detected by NMR spectroscopy and MS. The incidence of atherosclerosis has been linked with the oxidation of choline to trimethylamine-*N*-oxide in both humans and mice; however, whether trimethylamine-*N*-oxide has a direct effect on atherogenesis, or whether this metabolite is a proxy for meat consumption—more likely given the profound influence of diet on the concentration of trimethylamine-*N*-oxide—in the cohort examined is unknown.[71]

Humans

Atherosclerosis, because of its high global prevalence, is an important target for metabolomic studies, with a particular focus on biomarker discovery (reviewed previously[72–74]). Angiography is the gold standard for diagnosing coronary artery disease (CAD), the most-common form of atherosclerosis; however, this procedure is invasive, carries a small, but finite, risk to the patient, and requires a clinician to be present. A great deal of interest was generated, therefore, when reports were published that ^{1}H-NMR spectroscopy could be used not only to diagnose CAD in patients, but also to assess its severity.[75]

After this original study, aspects of the metabolic profile of blood serum (measured by ^{1}H-NMR spectroscopy) were linked with hypertension.[76] The main metabolic changes associated with both CAD and hypertension were in the regions of the NMR spectra containing resonances from lipids in lipoproteins, particularly those associated with LDL cholesterol and VLDL cholesterol, as well as the choline-containing region. On closer inspection, however, a number of issues with how the analysis was performed were noted. Severity was divided into CAD affecting one, two, or three vessels, but in many respects, the real determinant of whether an individual is predisposed to CAD-induced sudden cardiac death is the stability of the plaques within the arteries. Furthermore, the original study consisted of ~150 individuals, and the investigators stated that the diagnostic power was sufficient and unlikely to be a result of chance.[75] Of concern, a high degree of stratification was used to build the multivariate models. For example, in the comparison between disease presence (triple-vessel CAD) and no CAD, the control group consisted of seven men and 23 women, whereas the diseased group comprised 34 men and two women. Given the known association between sex and CAD, this stratification is problematic. Furthermore, both studies used a supervised pattern-recognition approach, termed orthogonal signal correction partial least-squares discriminant analysis. Although some cross-validation had been performed, samples should have been introduced that were completely naive to the model, rather than following the orthogonal signal correction filter. As a result, the test samples were already influenced by the original pattern-recognition model, which makes assessment of the validity of the model difficult.

In a larger study, the same analytical and statistical procedure was employed, although a rigorous test-and-train routine was used to predict the status of each sample.[77] Initially, sex was predicted according to the spectrum profile of each serum sample (Figure 5a). This prediction was 100% accurate, with classification largely driven by the relative proportions of VLDL, LDL, and HDL cholesterol—with many of the chemical shifts responsible for this classification also having been implicated in the original models for CAD and high blood pressure.[75,76] Similar models for statin treatment were not so robust on an individual basis, although clear differences could be detected between groups. Predictions for CAD and non-CAD groups were only 80.3% correct for male patients not treated with statins, and 61.3% correct for male patients receiving statins, compared with randomly correct predictions of 50% (Figure 5b). Even more disturbingly, diagnostic tests achieved a confidence limit of >99% for only 36% and 6% of predictions for the untreated and treated groups, respectively. The conclusion was that the ability to detect CAD using ^{1}H-NMR spectroscopy with >99% confidence was very weak compared with angiography and, therefore, unsuitable for clinical use.

In the Non-Insulin-Dependent Diabetes, Hypertension, Microalbuminuria or Proteinuria, Cardiovascular Events, and Ramipril (DIABHYCAR) randomized, clinical trial,[78] researchers investigated whether ^{1}H-NMR spectroscopy could be used to predict CAD in patients with type 2 diabetes. A nested case–control study of 190 patients was used (150 men, 40 women), with disease defined as those who had a myocardial infarction or suffered sudden cardiac death over the 4 years of the study; the controls were individuals with no evidence of cardiovascular disease. The investigators used a train-and-test routine containing 70% and 30% of the study participants, respectively. For whole-spectra analysis, the accuracy of prediction was 87% of the training group, but only 53% in the independent validation group.[78]

In a large study of cardiovascular disease, ^{1}H-NMR spectroscopy was used to profile the relative distribution of lipoprotein fractions in the blood plasma of 27,673 women.[79] During the 11 years of follow-up, 1,015 women had incident cardiovascular disease. Prediction of the risk of cardiovascular disease using NMR spectroscopy was comparable, but not superior, to predictions obtained from standard clinical chemistry measures of lipoproteins and lipids.

Although poor for predicting disease risk for an individual, ^{1}H-NMR spectroscopy might be useful in terms of stratifying patient groups—for example, to discriminate between patients with kidney disease and those with metabolic syndrome in addition to type 1 diabetes.[80] In a study of 4,309 individuals with type 1 diabetes, ^{1}H-NMR spectroscopy of serum was used to stratify patients according to levels of VLDL, LDL, and HDL cholesterol, which in turn correlated with the atherosclerotic burden as measured by the intima–media thickness of carotid arteries.[81] Spectral profiles can even be correlated with gene variation. In a cohort of Finnish individuals, correlations between metabolic and genetic variations were associated with a number of genes involved in inflammation, metabolism, and adiposity.[82]

Ceramides and diglycerides in blood plasma from patients with coronary heart disease were correlated using LC–MS with the inflammatory markers IL-6, tumor necrosis factor, and high-sensitivity C-reactive protein, as well as with insulin resistance.[83] A strong correlation was found between circulating ceramide levels and IL-6, even when allowing for correlations with other risk factors of inflammation, indicating a potential role for ceramides in IL-6-mediated inflammation.

Metabolomics can also be used to add power to genome-wide association studies. In a study targeting 363 metabolites (including amino acids, carnitines, and phospholipids) in 284 men, a number of correlations were identified between single nucleotide polymorphisms and the resultant metabolic phenotype, including two of medical relevance to atherosclerosis.[84] One polymorphism of fatty acid desaturase 1 was highly correlated with a number of glycerophospholipids and, therefore, might have a role in regulating the metabolism of LDL and VLDL cholesterol. These paired associations gained statistical significance when compared with either the individual metabolites or gene polymorphisms, which suggests that this approach might reduce variation in a study. The combination of GC–MS and ^{1}H-NMR spectroscopy has also been used to identify metabolic changes associated with atherosclerosis, albeit in a small number of individuals.[85]

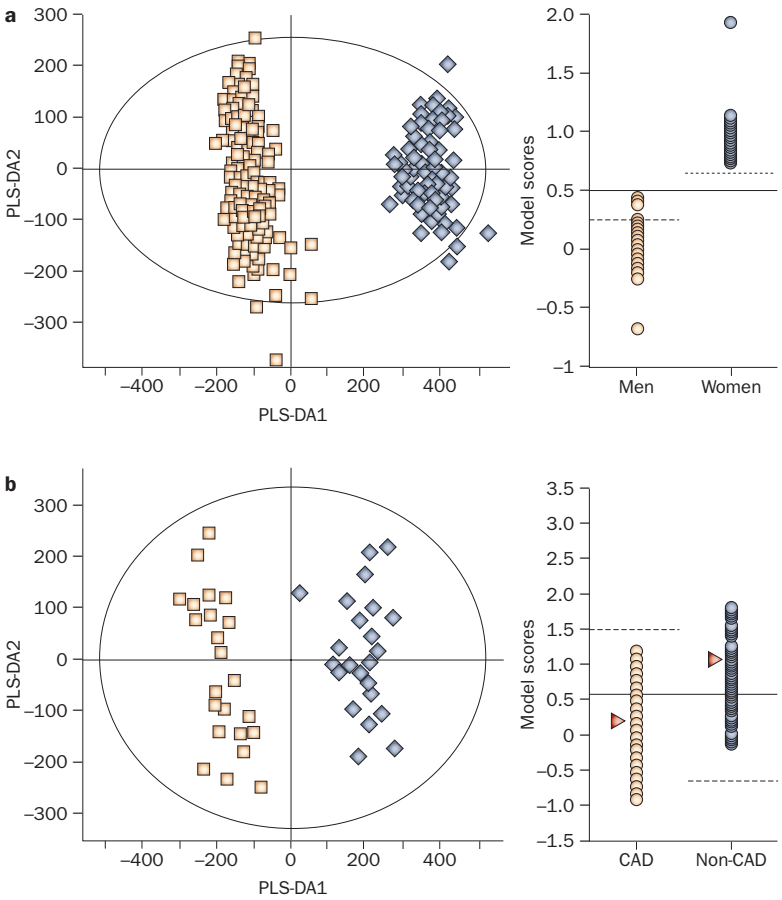


Figure 5 | Using metabolomics to model. **a** | Prediction of patient sex according to the spectrum profile of each serum sample was 100% accurate. This model was used as a positive control in the study by Kirschenlohr *et al.*[77] to demonstrate how a robust PLS-DA model behaves in terms of predictability. The prediction scores for sex are shown (right). This model approaches perfect classification, where men would score 0 and women would score 1. **b** | Orthogonal signal correction PLS-DA of ^{1}H-NMR spectra of blood serum from men with or without CAD. Good separation according to disease presence is apparent (left), but when individual samples are predicted, poor separation is produced (right). In a perfect model, individuals with CAD would score 0 and those without CAD would score 1. Abbreviations: CAD, coronary artery disease; PLS-DA, partial least-squares discriminant analysis.

All the studies discussed above involved blood plasma or serum, but it might also be possible to find markers of cardiovascular disease in urine. The urinary profiles of 4,630 individuals from the UK, the USA, China, and Japan were examined by ^{1}H-NMR spectroscopy and a number of common metabolites (including formate, hippurate, and alanine), many associated with diet, were found to be highly correlated with blood pressure.[86]

Myocardial ischemia and infarction

A targeted analysis of intermediates of the tricarboxylic acid (TCA) cycle, nucleotides, and amino acids using triple–quadrupole LC–MS enabled investigators to identify patients with inducible myocardial ischemia

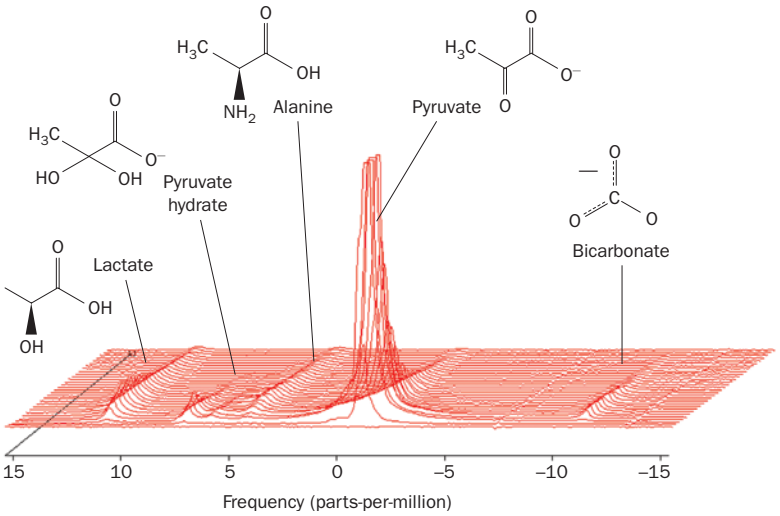


Figure 6 | *In vivo* spectra from the heart of a male Wistar rat. A time course of spectra acquired each second over a 60 s period after injection of hyperpolarized [1-^{13}C]pyruvate into the rat is shown. The signal is localized to the heart with a surface coil positioned over the chest. Hyperpolarization increases the magnetic resonance signal-to-noise ratio by more than 10,000-fold. The arrival and subsequent decay of the injected [1-^{13}C]pyruvate signal can be detected, along with the generation of lactate, alanine, and bicarbonate. The equilibrium product pyruvate hydrate is also observed. The signal decay is attributable to several factors including loss of polarization and metabolic conversion. Courtesy of Dr Damian Tyler.

during exercise stress.[87] The ischemia was characterized by increased lactate levels, and changes in TCA-cycle intermediates and the breakdown products of AMP. The underlying biochemistry was already known, but the LC–MS approach provided quantitative data and enabled scoring of relative degrees of ischemia. A targeted approach was also applied to a small, but highly controlled, population of 36 patients undergoing alcohol septal ablation for hypertrophic obstructive cardiomyopathy, to identify markers associated with medically induced myocardial infarction.[88] The metabolic profiles of these patients were compared with profiles from individuals undergoing elective angiography (negative control group) and with spontaneous myocardial infarction (positive control group). Importantly, blood was sampled directly from the coronary sinus so that metabolites released from cardiac tissue were not diluted by systemic metabolism. Both types of myocardial infarction produced similar metabolic changes in the sampled blood plasma.

Myocardial infarction induces remodeling of the heart with cardiomyocyte loss that can subsequently develop into cardiac failure. LC–MS-based metabolomics was used to investigate the potential beneficial effects of inhibitors of soluble epoxide hydrolase, thought to increase the concentration of epoxyeicosatrienoic acids, which have vasodilatory properties and inhibit nuclear factor κB-mediated gene transcription.[89] These inhibitors reduced the inflammatory state of the heart after myocardial infarction, as measured by changes in the profile of eicosanoids, and might provide novel therapeutic strategies.

Blood serum was examined for markers of cardiac failure using GC-TOF MS in 52 patients with systolic heart failure (ejection fraction <40%) compared with 57 control individuals.[90] As was found in the studies of myocardial infarction, nucleotide metabolism (particularly of pseudouridine and 2,4,6-trihydroxypyrimidine) was important in identifying individuals with heart failure. Substrate metabolism in isovolumic, Langendorff-perfused hearts from rats (6–24 months) was measured using ^{13}C-NMR isotopomer analysis of glutamate-labeling in order to investigate age-related changes to substrate selectivity in heart failure.[91] An age-related increase in palmitate oxidation and a subsequent decrease in lactate oxidation were detected. The metabolic changes that accompany heart failure have also been investigated using ^{31}P-NMR spectroscopy *in vivo*.[92] Left ventricular hypertrophy was distinguished from congestive heart failure using ^{31}P-magnetization-transfer to monitor the flux through creatine kinase.

A problem with the observation of ^{13}C labeling by NMR spectroscopy is the limited time resolution of the process; it takes several minutes to acquire enough signal for ^{13}C resonances to be reliably detected. This concern has been overcome with hyperpolarized-^{13}C-NMR spectroscopy (Figure 6). The increased polarization afforded by this approach was used to show that, immediately after an ischemic insult, the perfused heart relies largely on the production of lactate and alanine, with essentially no flux through pyruvate dehydrogenase during the early recovery phase.[93]

Challenges remain with this novel approach. In particular, many substrates cannot be polarized to a sufficient level to be used *in vivo*. Furthermore, the labeling is not at steady state and so is more challenging to model mathematically (and all labeling studies *in vivo* suffer from the potential for systemic metabolism to deplete the intended substrate and replace it with other metabolites that might be taken up by the heart). Finally, the use of hyperpolarized-^{13}C-NMR spectroscopy has been focused primarily on labeling pyruvate in the heart; whether the technique can reveal more about the regulation of pyruvate dehydrogenase is unclear, given that the enzyme complex has already been well-characterized both classically and using ^{13}C-NMR isotopomer analysis in the heart.[94–96] If this technique is to develop further, novel substrates are needed to probe less well-characterized pathways.

Diabetic cardiomyopathy

Diabetes increases the risk of mortality from cardiovascular disease[97,98] and, although atherosclerosis has a major role in the causes of death, a subpopulation of patients with type 2 diabetes develop myocardial dysfunction that is independent of vascular disease. How heart failure develops in this subgroup, especially in the absence of ischemic disease, is still unknown. In this respect, a subset of patients with diabetes and abnormal cardiac performance or structure is diagnosed with diabetic cardiomyopathy in the absence of CAD, hypertension, or valvular disease.

To investigate diabetic cardiomyopathy and the effects of dyslipidemia *in vivo*, magnetic resonance spectroscopy can be readily used to detect the accumulation of fat, which contains a high proportion of CH_2 groups that contribute to a prominent peak at ~1.3 parts-per-million in NMR spectra of the heart. The lipid content

of the heart increases before overt type 2 diabetes and subsequent development of diabetic cardiomyopathy.[99] Furthermore, *in vivo* magnetic resonance spectroscopy showed that an increase in myocardial triglycerides was correlated with impaired contractility.[100]

Given that diabetes can be viewed as a disorder of lipid metabolism as much as of glucose homeostasis, lipidomics should provide a unique insight into impairments in the diabetic heart. The use of solid-phase extraction GC–MS to follow the action of rosiglitazone in a mouse model of diabetes demonstrated that cutting-edge LC–MS equipment is not necessarily needed to perform lipidomics.[101] The drug substantially altered metabolism of free fatty acids and cardiolipin in the heart. Shotgun lipidomics was used to profile lipid species altered by diabetes in the heart, and showed a large increase in carnitine derivatives, including 3-hydroxycarnitine.[102] The production of these acylcarnitines was, in turn, linked to the expression and activity of calcium-independent phospholipase A_2. A follow-up study linked mitochondrial dysfunction to depletion of cardiolipin, a critical component of the inner membrane of mitochondria.[103]

In addition to the measurement of pool sizes of metabolites in tissues and biofluids, an important technique for investigating the diabetic heart is the measurement of substrate metabolism using stable isotopes. Substrates can be labeled at different positions to assess the relative metabolism of two or three substrates under various conditions, which is particularly important in the heart where a range of substrates, including glucose, ketone bodies, fatty acids, and lactate, are metabolized. Using this approach, lactate oxidation was shown to be impaired in the diabetic heart, in addition to the well-known reduction in glucose oxidation, presumably resulting from inhibition of pyruvate dehydrogenase by increased fatty-acid oxidation.[104]

The diabetic heart has increased fat reserves within its tissue, but ^{13}C-NMR spectroscopy has shown surprisingly that the turnover of this pool is faster than in the non-diabetic heart. The triglyceride pool was roughly twice as large, and the rate of turnover of triglycerides was fourfold higher, in the hearts of rats with streptozotocin-induced diabetes compared with wild-type animals.[105]

Conclusions

Metabolomics is already influencing research in the field of cardiac metabolism, in part because of the increasing importance of NMR spectroscopy since the 1980s for *in vivo* monitoring of cardiac metabolism. Perhaps the biggest difference between classical approaches to metabolism and metabolomics is the move from hypothesis-directed towards hypothesis-generating research. Although both NMR spectroscopy and MS have been used widely to phenotype novel models of disease or genetic modifications in animals, before the rise of metabolomics and similar approaches, a specific question was commonly investigated. By contrast, the goal of metabolomics is to profile as many metabolites as possible in order to understand how the overall metabolism of an organism is changed and to place a given gene modification or disease process in a general context. As improvements in MS allow the creation of increasingly comprehensive catalogs of metabolites, and the advent of hyperpolarized-^{13}C-NMR spectroscopy improves the sensitivity of *in vivo* NMR spectroscopy, the field of metabolomics is set to grow exponentially.

Much interest has been shown in the use of metabolomics to identify biomarkers of cardiovascular disease. Although, to date, no novel biomarkers that can be used in isolation have been validated, the use of pattern-recognition techniques has identified profiles of metabolic changes that can be used to follow various disease processes in both animal models and man. In addition, metabolomic approaches have powerfully elucidated biological mechanisms of pathology, particularly for lipid metabolism, where advances in LC–MS have allowed intact lipids to be studied in more detail than previously possible with other analytical techniques.

Review criteria

Full-text manuscripts were selected for inclusion in this Review from the authors' previous reading lists as well as searches on PubMed. For the latter, the search terms used were: "cardiac metabolism", "NMR spectroscopy", "magnetic resonance spectroscopy", "mass spectrometry", "metabolomics", and "metabonomics" across all published papers.

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Author contributions

J. L. Griffin, H. Atherton, and J. Shockcor researched the data for the article. J. L. Griffin and L. Atzori discussed the content of the Review, and J. L. Griffin wrote the manuscript. All the authors were involved with reviewing and editing the manuscript before submission.