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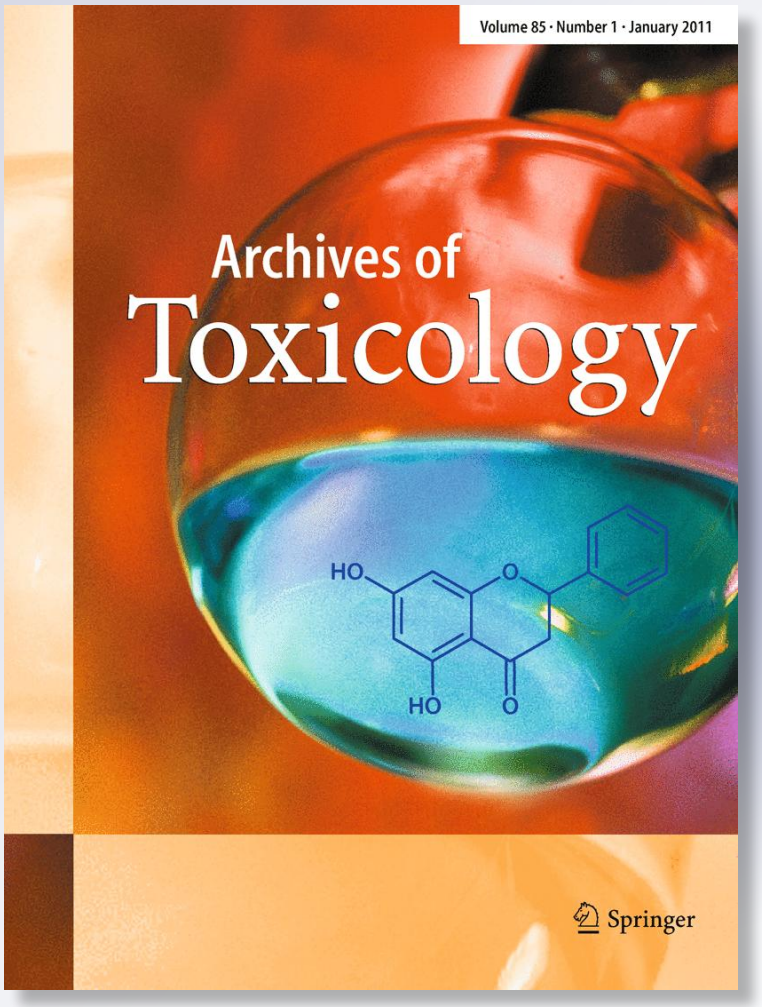

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REVIEW ARTICLE

The role of metabolites and metabolomics in clinically applicable biomarkers of disease

Mamas Mamas · Warwick B. Dunn · Ludwig Neyses · Royston Goodacre

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Abstract Metabolomics allows the simultaneous and relative quantification of thousands of different metabolites within a given sample using sensitive and specific methodologies such as gas or liquid chromatography coupled to mass spectrometry, typically in discovery phases of studies. Biomarkers are biological characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathological processes or pharmacologic responses to a therapeutic intervention. Biomarkers are widely used in clinical practice for the diagnosis, assessment of severity and response to therapy in a number of clinical disease states. In human studies, metabolomics has been applied to define biomarkers related to prognosis or diagnosis of a disease or drug toxicity/efficacy and in doing so hopes to provide greater pathophysiological understanding of disease or therapeutic toxicity/efficacy. This review discusses the application of metabolomics in the discovery and subsequent application of biomarkers in the diagnosis and management of inborn errors of metabolism, cardiovascular disease and cancer. We critically appraise how novel biomarkers discovered through metabolomic analysis may be utilized in future clinical practice by addressing the following three fundamental questions: (1) Can the clinician measure them? (2) Do they add new information? (3) Do they help the clinician to manage patients? Although a number of novel biomarkers have been discovered through metabolomic studies of human diseases in the last decade, none have currently made the transition to routine use in clinical practice. Metabolites identified from these early studies will need to form the basis of larger, prospective, externally validated studies in clinical cohorts for their future use as biomarkers. At this stage, the absolute quantification of these biomarkers will need to be assessed epidemiologically, as will the ultimate deployment in the clinic via routine biochemistry, dip stick or similar rapid at- or near-patient care technologies.

Keywords Metabolomics · Biomarkers · Systems biology

The authors Mamas Mamas and Warwick B. Dunn are joint first authors contributing equally to the manuscript.

M. Mamas · L. Neyses
Manchester Academic Health Science Centre,
University of Manchester, Oxford Road,
Manchester M13 9WL, UK

M. Mamas · L. Neyses
Manchester Heart Centre, Manchester Royal Infirmary,
Oxford Road, Manchester M13 9WL, UK

W. B. Dunn · R. Goodacre
Manchester Centre for Integrative Systems Biology,
Manchester Interdisciplinary Biocentre, University of Manchester,
131 Princess Street, Manchester M1 7DN, UK

W. B. Dunn · R. Goodacre (✉)
Department of Chemistry, Manchester Interdisciplinary Biocentre,
University of Manchester, 131 Princess Street,
Manchester M1 7DN, UK
e-mail: roy.goodacre@manchester.ac.uk

W. B. Dunn
Centre for Advanced Discovery and Experimental Therapeutics,
Manchester Biomedical Research Centre, York Place,
Oxford Road, Manchester M13 9WL, UK

Introduction to metabolomics

Discoveries in biochemistry during the late nineteenth and twentieth centuries have provided an array of valuable knowledge on metabolism. These include the metabolites,

proteins and co-factors involved and more importantly their interactions in metabolite biosynthesis or catabolism. Knowledge on the interaction between limited numbers of metabolites, proteins and co-factors in single metabolic pathways (e.g. glycolysis) has expanded to define metabolic networks composed of all areas of metabolism (for an example see http://www.genome.jp/kegg/pathway/map/map01100.html). Similar advances have been seen in understanding the interaction and function of proteins, transcripts and genes. These studies have developed from investigating a small number of biochemicals and their interaction (e.g. metabolites and proteins in a specific metabolic pathway) to studying the complex interactions of genes, transcripts, proteins and metabolites on a holistic level. The latter is described as systems biology (Bruggeman and Westerhoff 2007; Kell 2006; Westerhoff and Palsson 2004; Dunn et al. 2011). The development of holistic studies (genomics, transcriptomics, proteomics and metabolomics) have followed advances in technologies; for example, in high-throughput genome sequencing (Sanger et al. 1977) and methodologies for protein quantification (e.g. with iTRAQ; Ross et al. 2004). The role technological developments provides in scientific discoveries has been reviewed previously (Kell 2006), and we are often reminded of the quote by Sydney Brenner (*Nature*, 5 June 1980) that:

> Progress in science depends on new techniques, new discoveries and new ideas, probably in that order.

Metabolomics is one of the core disciplines of systems biology (Hollywood et al. 2006; Goodacre et al. 2004; Griffin 2006; Dunn et al. 2011). The discipline focuses on the study of low molecular weight organic and inorganic chemicals (metabolites) in biological systems. The quantitative complement of all metabolites is defined as the metabolome and sample-specific metabolomes can be studied, e.g. serum metabolome or liver tissue metabolome. Metabolomics is employed to study microbial (Mashego et al. 2007; van der Werf et al. 2007), plant (Hall 2006; Schauer and Fernie 2006), environmental (Bundy et al. 2009; Viant 2008) and mammalian (Nicholson 2006; Dunn et al. 2011) metabolomes. The readers are directed to a number of reviews that describe the role and experimental aspects of metabolomics (Dunn et al. 2011; Kell 2006; Go 2010; Broadhurst and Kell 2006; Dunn et al. 2005; Lindon and Nicholson 2008) as well as the related metabolic fingerprinting (Ellis and Goodacre 2006; Ellis et al. 2007).

An array of terms are applied in metabolomics. Metabonomics (Nicholson et al. 1999) and metabolomics (Fiehn 2002) are two terms that appeared from different scientific fields, mammals compared to plants and microbes, respectively. However, these two terms are employed interchangeably now (Nicholson and Lindon 2008). The holistic study of the complete metabolome is thus described as metabolomics or metabonomics. Metabolite/metabolic profiling or untargeted analysis can also be applied to the holistic study of a large subset of metabolites, though not all metabolites. In reality, the detection of all metabolites is not currently achievable and so many studies can be defined as metabolite/metabolic profiling.

Metabolomics offers a number of advantages (Dunn et al. 2011; Kell 2007). The metabolome is the final downstream product of transcription and translation and is thus closest to the phenotype. Dynamics of primary metabolism operate in timescales of seconds. These two characteristics allow the metabolome to be a sensitive and rapid measure of the system phenotype. For example, the authors have observed changes in the human serum metabolome 1 min after an ischaemic event in the heart (unpublished data). The number of metabolites is currently estimated to be lower than the number of genes and proteins, and therefore holistic studies should be easier. The current estimate of metabolites present in the human metabolome is 7800 (Wishart et al. 2009) compared to greater than 20,000 genes (http://www.ensembl.org/Homo_sapiens/Info/Index). However, our knowledge of the composition of the metabolome is incomplete and current databases do not include many lipids, drugs, drug metabolites, food nutrients and metabolites derived from interacting metabolomes such as mammalian-gut microflora metabolic cross-talk (Nicholson et al. 2004, Goodacre 2007). It is expected that the estimated size of the human metabolome will significantly increase. Metabolomics is a high-throughput strategy where hundreds of samples can be analysed per day or week, depending on the analytical instrument. Therefore, although instruments are expensive, costs per sample are relatively low and so large-scale experiments are achievable. Therefore, analysis of 100–1,000s of samples is readily achievable and so metabolomics can be applied as a discovery-phase tool in studies of the general population. Biological discoveries can be validated using small sample sizes in genomics, proteomics and transcriptomics where costs per sample are greater. However, the authors emphasize that in many human-focused studies that thousands of samples are required to define the biological variation in the human population adequately in, for example, biomarker validation studies.

Metabolomics is applied to define changes in the concentration of metabolites related to a genetic, biological or environmental perturbation. These changes are related to metabolism, regulation of metabolism by other biochemicals (e.g. allosterism, metabolite–protein interaction; Monod et al. 1965) or regulation of other processes by metabolites (e.g. riboswitches, metabolite–mRNA interaction; Henkin 2008). The objectives of metabolomic studies are dependent on the biological system and question being

asked. In human studies, metabolomics has predominantly been applied to define biomarkers related to prognosis or diagnosis of a disease or toxicity/efficacy of therapeutic interventions and to provide greater pathophysiological understanding of disease or drug toxicity/efficacy.

The first definitions of the metabolome and metabolomics were reported in 1998 (Oliver et al. 1998; Tweeddale et al. 1998). From this time, there have been huge advances in methodological and analytical technologies that have lead to the discovery of biomarkers and greater knowledge regarding disease pathophysiology. Technological advances from the 1960s with mass spectrometry (Horning 1968) and nuclear magnetic resonance (NMR) spectroscopy (Behar et al. 1983; Howells et al. 1992) allowed the first holistic studies of mammalian biofluids to be performed. In the last 15 years, technological advances have driven forward metabolomics to its current status. Today, metabolomics is a routinely applied tool with greater than 2,300 publications reported in PubMed (as of July 2010, and see Goodacre 2010). However, metabolomics is still the younger and smaller sibling of proteomics, transcriptomics and genomics.

Metabolomics operates with a workflow (Brown et al. 2005; Dunn et al. 2011) starting from a biological question and experiment, proceeding through sample collection and preparation, analytical experiment(s) to acquire data, data pre-processing and analysis followed by biological interpretation. This workflow is shown in Fig. 1.

Two generalized experimental strategies are applied, metabolic profiling (or metabolomics) and metabolite targeted analysis (Dunn et al. 2011). Metabolic profiling studies the metabolome in a holistic approach and from a point of limited a priori biological knowledge. The study is designed so to acquire data on a large subset of metabolites (100–1,000s) followed by interrogation of these data to define biological differences. The design of biological and analytical experiments is critical here as it is easy to

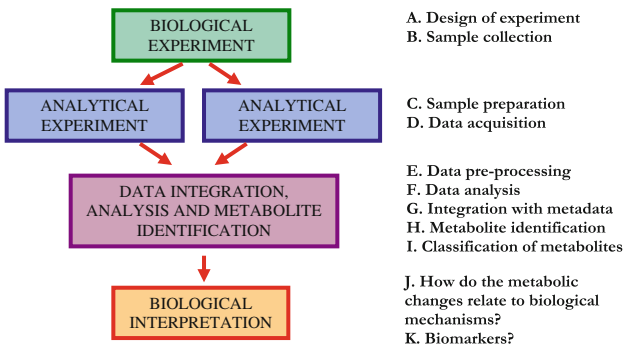

Fig. 1 The metabolomics experimental workflow involves the design of biological and analytical experiments, sample preparation, data acquisition, data pre-processing and analysis and data interpretation. This workflow leads to biological interpretation and reasoning

introduce confounding factors that are not recognized during data analysis and which can falsify biological conclusions (Broadhurst and Kell 2006). Powerful analytical technologies are applied (Begley et al. 2009; Zelena et al. 2009) to detect many hundreds or thousands of metabolites reproducibly in a single sample, without knowing which metabolites are of specific biological interest. Relative changes in metabolite responses or concentrations are reported, and at this stage the absolute concentrations of metabolites are not generally determined. Although the goal would be to detect all metabolites (defined as metabolomics), this is currently not technologically feasible. A combination of sample preparation and analytical platforms is recommended to acquire good coverage of detected metabolites (e.g. see the Husermet project where gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and NMR spectroscopy have all been applied; www.husermet.org). Data interrogation is performed with the application of univariate and multivariate analysis tools (Broadhurst and Kell 2006; Smilde et al. 2010; Madsen et al. 2010). These are discovery-phase studies, otherwise referred to as hypothesis-generating or inductive studies (Kell and Oliver 2004), sometimes by the non-cognoscenti as a 'fishing expedition' (Cantor 2011) and have the goal to discover new biology or biomarkers.

The second strategy is driven from known biology (potentially derived from a discovery study) where a limited number of metabolites (typically less than 20) are known to be biologically relevant before the biological experiment is designed (e.g. potential biomarkers), and *absolute* quantification of the metabolites is performed in a targeted approach. This strategy has been applied for decades and falls under the remit of traditional analytical chemistry and biochemical assays commonly found in clinical laboratories.

In the search for biomarkers, both of the above-mentioned strategies are important, have been discussed previously (Dunn et al. 2011) and is reviewed in Fig. 2. The discovery phase is less readily observed in general operation as these are highly specialized experiments. However, they provide putative biomarkers that were previously not reported or originate from a biological mechanism not known before. Once discovery studies have been performed, the putative biomarkers are validated in the general population where the biomarker will be applied to assess specificity and selectivity. This applies a targeted analytical method for absolute quantification. This targeted method can then be employed for future application in clinical biochemistry laboratories.

The sample size in these studies is dependent on the systems being studied, independent of whether a holistic or targeted approach is being applied. Animal studies in controlled laboratory environments with controlled genetic

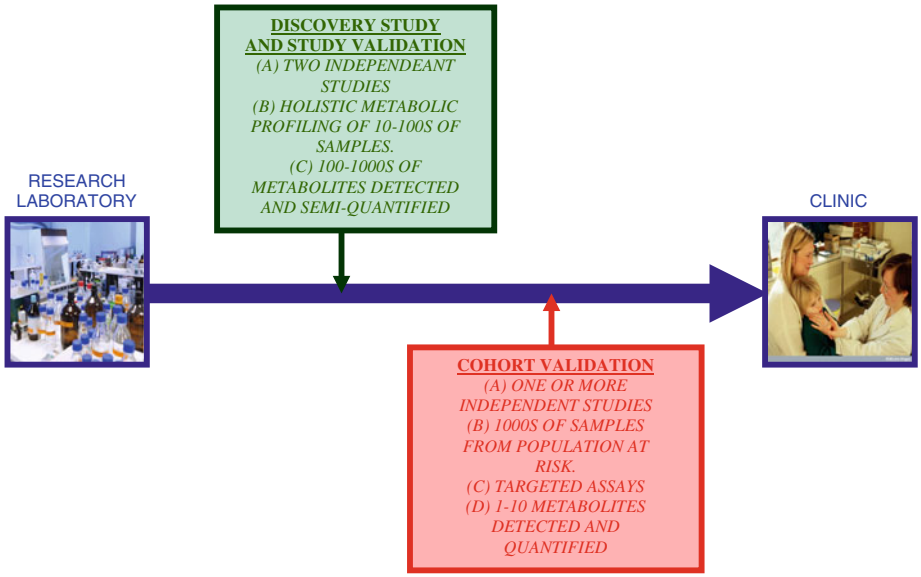

Fig. 2 The path from research laboratory to clinic for biomarker discovery and validation. The process involves detection of potential metabolic biomarkers in discovery studies for small sample populations with holistic detection of 100–1,000s of metabolites followed by small-scale and/or large-scale validation with targeted analytics of a limited number of metabolites in multiple independent studies

backgrounds can significantly reduce biological variability as detected in the metabolome. The number of samples can be low (5–20). However, in the general human population where genetic, environmental and lifestyle factors significantly differ, including physical activity, mental status as well as microflora (both in and on man), the biological variability will be high and many hundreds to thousands of samples are recommended. In metabolomic studies of the general population, the preferred strategy is to perform two independent small-scale holistic studies (defined as discovery and validation) where the number of samples is typically 10–100s and where many potential confounders (e.g. age, BMI, gender) are controlled so that the only 'random' variable between classes is the disease or drug treatment. These studies define potential biomarkers or disease pathophysiology. Sample sizes can be smaller if a time-series experiment is performed where multiple samples are acquired before and after a perturbation. Here, each individual acts as his or her own control (baseline), which with the appropriate statistical paired-test significantly reduces genetic, environment and lifestyle variability. These can be further validated if appropriate in a wider clinical cohort with a sample size of 100–1,000s. The metabolites of interest are generally known and so a targeted absolute quantification approach is applied for validation. This defines whether the biomarkers found in a somewhat controlled study are applicable in the general population where they will be applied.

Biomarkers

The development of biologically relevant tools, as shown above for metabolomics, has allowed the clinical scientist to assess biological parameters associated with the development, severity and treatment of a disease process or in the study of toxicity or efficacy of drugs. The measured biological characteristics applied are referred to as biomarkers.

The National Institute of Health (NIH) biomarkers definitions group has defined biomarkers as:

> Biological characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathological processes or pharmacologic responses to a therapeutic intervention
>
> (Biomarkers Definitions Working Group 2001).

Biomarkers are widely used in everyday clinical practice and have a number of important applications including their role as diagnostic tools for screening patients for the presence of disease, such as the use of elevated blood glucose for the diagnosis of diabetes (American Diabetes Association 2008; World Health Organization 1999), brain natriuretic peptide (BNP) for the diagnosis of heart failure (de Lemos et al. 2003) and prostate-specific antigen (PSA) for the diagnosis of prostate cancer (Brawer and Lange 1989). Biomarkers may also be used to determine the extent of disease, for example blood PSA concentration to indicate the extent of prostate cancer growth and whether it has spread to other organ systems (metastasis) (Brawer and Lange 1989). Many biomarkers are routinely used in clinical practice to provide a measure of disease severity and also provide important prognostic information relating to survival. For example, BNP in heart failure (Goto et al. 2003; Gardner et al. 2003), CA-125 in ovarian cancer (van Dalen et al. 2009), CEA in colorectal cancer (Kohler et al. 1980) and creatinine levels in renal failure. A number of articles have highlighted the role of biomarkers in a number of clinical fields (Kell 2007; Schnackenberg and Beger 2008; van

Ravenzwaay et al. 2007; Dunckley et al. 2005; Classen et al. 2008).

An important role of biomarkers in clinical medicine is the ability to assess the response to medical therapy of a clinical condition which is often difficult to assess on clinical grounds alone and more invasive imaging techniques are both time consuming, costly and may expose patients to unnecessary risks. As an example, measurement of the inflammatory marker c-reactive protein (CRP) as a measure of efficacy of antibiotic treatment of bacterial infections or CA-125 levels to assess the response of ovarian cancer to chemotherapy (Rocconi et al. 2009).

Biomarkers have also been used as substitutes for clinical endpoints both in clinical practice as well as the research setting particularly in novel drug development. As an example, human immunodeficiency virus (HIV) plasma viral load and CD4 cell counts are frequently used as surrogate endpoints for clinical outcomes (for example, death and occurrence of opportunistic infections) in the evaluation of antiviral agents in patients with HIV infection.

Most commonly, biomarkers used in clinical medicine are derived from the measurement of metabolites, proteins or DNA in blood samples since analysis of blood is widespread, is low cost and minimally invasive. Furthermore, in situations where biomarkers are needed to follow response to treatment over a short timescale i.e. measurement of CRP to determine response to antibiotic therapy, analysis of blood samples allows repeat biomarker measurement at multiple time points with ease. Furthermore, it is often not possible to obtain tissue samples in disease processes involving multiple organ systems due to the high risks associated with biopsy, particularly in cases where biomarkers are to be used for screening for disease where the diagnostic yield is often very low.

In certain clinical diseases such as cancer, measurement of biomarkers in tissue samples derived from the diseased organ systems is occasionally used. For example, the presence of biomarkers can be measured in tumours excised surgically to provide prognostic information, i.e. CEA levels/distribution in surgically excised colonic tumour samples (Hamada et al. 1985) or the assessment of oestrogen receptor status in breast cancer to provide information on prognosis and likely response to treatment (Spears and Bartlett 2009).

Metabolomics and biomarker discovery

Metabolomics has been applied to a number of clinical conditions for biomarker discovery (Madsen et al. 2010; Vinayavekhin et al. 2010). One of the first sets of studies involved diagnosis of genetic inborn errors of metabolism. Further advances have been observed in cancer and cardiovascular diseases, the two most common causes of morbidity and mortality in the western world.

Most biomarkers currently applied are single metabolites that provide suitable discrimination between populations. However, sets of metabolic biomarkers with which rules for diagnosis or prognosis can be determined may be appropriate in the future where a single metabolite does not provide adequate discrimination. This has been shown for pre-eclampsia, a pregnancy-related cardiovascular disease (Kenny et al. 2010).

Later, we review some of the metabolomic studies that have identified clinically relevant novel metabolic biomarkers, specifically in diagnosis of inborn errors of metabolism, in the field of cancer studies and in cardiovascular disease diagnosis.

Metabolomics and inborn errors of metabolism

One of the first applications of applying metabolites as disease biomarkers was observed for diagnosing inborn errors of metabolism (IEM) (Arn 2007). Currently, this has provided the most clinically applicable metabolic biomarkers in combination with glucose for diabetes diagnosis. Early pioneering work by Følling in the 1930s provided the discovery of phenylketonuria, as reviewed more recently (Centerwall and Centerwall 1961), and applications have grown to a level today where population screening for IEMs is performed in many countries around the world (Rashed 2001; Chace and Kalas 2005; Wilcken and Wiley 2008; Sahai and Marsden 2009). Inborn errors of metabolism are permanent with most (but not all) being inherited biochemical disorders. An inborn error of metabolism is often a defect in a single protein-encoding gene that leads to a lack of a functional enzyme, membrane transporter or other relevant protein. The biochemical result is a blockage in a particular metabolic pathway and/or in the transport/excretion of a metabolite. This blockage can result in a buildup of a metabolite before the blockage (which can be toxic) or a lack of a metabolite post-blockage. Once diagnosed, some may be treated by dietary restrictions to reduce the intake of a metabolite or through dietary supplementation of the depleted metabolite.

IEMs can be separated into distinct disorders including disorders of carbohydrate metabolism, amino acid metabolism, fatty acid oxidation and mitochondrial metabolism and purine and pyrimidine metabolism. Other IEMS including lysosomal storage conditions disorders (Fernandes et al. 2006) are also detectable.

One of the most common IEMs is phenylketonuria discovered by the Norwegian physician Ivar Asbjørn Følling in 1934 (Folling 1934; Centerwall and Centerwall 1961). The IEM is a result of a deficiency in the hepatic enzyme

phenylalanine hydroxylase (PAH) that converts the amino acid phenylalanine to tyrosine. The result is a buildup of phenylalanine and a reduction in tyrosine concentrations. The ratio of phenylalanine to tyrosine can be employed for diagnosis though more commonly the high concentration of phenylalanine is employed (traditionally this was by the Guthrie Inhibition Assay). Continued non-treatment can lead to mental retardation and brain damage and seizures. Treatment reduces the dietary intake of phenylalanine, which in itself can be difficult and so protein supplements (without phenylalanine content) are also recommended.

Typically, heel-prick blood samples collected on to paper and dried are applied for screening. This provides a robust and easy-to-apply sample collection method for population screening. Assays applied for IEM population screening are varied. The application of targeted biochemical assays for a single IEM (e.g. Guthrie Inhibition Assay (Partington and Sinnott 1964) for phenylketonuria) can be applied though mass spectrometry platforms that provide a tool for screening multiple IEMs. For example, tandem mass spectrometry is routinely applied for screening of amino acids, organic acids and carnitines for a range of IEMs followed by confirmation through targeted methods (Chace and Kalas 2005; Wilcken 2007). GC–MS is applied for assaying multiple organic acids for organic acid disorders in blood and urine (Kuhara 2007). Multiple screens on a single platform and sample provide cost-effectiveness and the ability to perform high-throughput assays.

Metabolomics and biomarker discovery in cancer studies

One of the most high-profile biomarker discovery studies of recent years in the field of cancer research was the unbiased metabolomics analysis performed using plasma, tissue and urine derived from patients with biopsy proven prostate cancer and biopsy negative controls (Sreekumar et al. 2009). Metabolomic analysis performed in prostatic tissue revealed significant metabolite differences between benign tissue samples, tissue samples in which the tumour was localized and those in which there were distant metastases. Notably, of the 628 metabolites identified in tissues sample, there were 60 metabolites (9.5% of the total detected metabolome) found in localized and/or metastatic tumours but not in benign prostate. Indeed, a subset of six metabolites (sarcosine, uracil, kynurenine, glycerol-3-phosphate, leucine and proline) were significantly elevated upon disease progression from benign to clinically localized prostate cancer and metastatic prostate cancer. One metabolite, sarcosine, was validated in a second cohort of tissue samples and identified as a potential candidate for future development in biomarker panels for early disease detection and aggressivity prediction in prostate cancer. For sarcosine to be a clinically applicable biomarker for prostate cancer screening it has to have similar discriminatory power if measured in readily available, easy to collect biofluids such as serum or urine. Although not detected in serum, sarcosine was found to be significantly higher in urine sediments and supernatants derived from biopsy positive prostate cancer patients when compared to biopsy negative controls with the overall receiver operator characteristic (ROC) curves suggesting a modest predictive value with AUCs of 0.71 and 0.67 for urine sediments and supernatants, respectively. Sarcosine may not only be a biomarker of prostate cancer, but a bio-actor that contributes to the disease process itself. Using molecular approaches and targeting metabolic pathways that influence sarcosine levels, the authors demonstrated a role for sarcosine in neoplastic progression modulating cell invasion and migration, which may in turn provide future novel avenues for therapeutic intervention.

Much of the initial enthusiasm regarding the success of metabolomics in identifying sarcosine as a screening biomarker in prostate cancer has however subsided (Shalken 2010). Recently, the first independent study to validate the role of urinary sarcosine measured in the urine supernatant (after centrifugation) by gas chromatography–mass spectroscopy (GC–MS) and normalized to creatinine (Jentzmik et al. 2010) suggested that urinary sarcosine cannot be used to diagnose prostate cancer and does not predict the histologic grade in the corresponding biopsy. These studies varied considerably in the clinical cohorts studied, substrate collection methodology as well as the biomarker assay used. Despite both studies using GC–MS, Sreekumar et al. (2009) determined log2 (sarcosine to alanine) ratios in urinary sediment, whereas Jentzmik et al. (2010) used urinary supernatant sarcosine-to-creatinine levels. This highlights the importance of standardization of assays used for biomarker validation studies as well as standardized substrate collection procedures (Schalken 2010). Further efforts to validate sarcosine as a biomarker are ongoing.

Serum metabolomics has been applied to other clinical populations with cancer with a view to developing novel early diagnostic biomarkers in renal cancer (Kind et al. 2007; Kim et al. 2009), colorectal cancer (Ritchie et al. 2010), pancreatic cancer (Urayama et al. 2010), leukaemia (MacIntyre et al. 2010), ovarian cancer (Guan et al. 2009; Odunsi et al. 2005) and oral cancer (Tiziani et al. 2009). Other studies have applied metabolomics analysis to body fluids other than blood in patients with cancer, with metabolomics analysis of urine from patients with breast cancer (Nam et al. 2009; Chen et al. 2009; Woo et al. 2009), ovarian cancer (Woo et al. 2009), cervical cancer (Woo et al. 2009) hepatocellular carcinoma (Wu et al. 2009) and bladder cancer (Issaq et al. 2008). Another biofluid subjected to metabolomics analysis is saliva, in one

such study (Sugimoto et al. 2010) derived saliva samples from 215 individuals (69 oral, 18 pancreatic and 30 breast cancer patients, 11 periodontal disease patients and 87 healthy controls) and identified 57 principal metabolites that accurately predicted the probability of being affected by each individual disease. The AUCs were 0.865 for oral cancer, 0.973 for breast cancer, 0.993 for pancreatic cancer and 0.969 for periodontal diseases. Although many of these studies are early exploratory metabolomic analyses for biomarker identification and to gain novel insight into pathophysiological processes that underlie key stages of cancer development, to date no biomarker that has been identified through metabolomics is in routine use in clinical practice. Metabolites identified from these early studies will need to form the basis of larger, prospective, externally validated studies in clinical cohorts for their future use as biomarkers.

Metabolomics and biomarker discovery in cardiovascular studies

Cardiovascular disease represents the most common use of biomarkers in clinical medicine both for diagnosis, risk stratification and prognosis. The majority of these biomarkers are currently not metabolites, for example troponins are proteins. Their use is recommended in both the European as well as North American guidelines for the management of a number of cardiovascular disorders including acute coronary syndromes (Anderson et al. 2007; Bassand et al. 2007) and heart failure (Dickstein et al. 2008; Hunt et al. 2005). These represent the commonest causes of cardiovascular morbidity and mortality worldwide.

Metabolomics has been applied to a number of cardiovascular conditions and we review the two most common which are encountered in clinical practice, heart failure and acute coronary syndromes. We were the first to apply metabolomic analysis to serum derived from 52 patients with chronic heart failure and 57 controls (Dunn et al. 2007) using GC–MS. Thirty-eight peaks showed a significant difference between case and control and two metabolites of particular interest were pseudouridine, a modified nucleotide present in tRNA and rRNA and a marker of cell turnover, as well as the tricarboxylic acid cycle intermediate 2-oxoglutarate. Indeed, in this small discovery study, pseudouridine and 2-oxoglutarate were at least as diagnostic of heart failure as the current gold standard biomarker brain natriuretic peptide (BNP) with areas under the ROC curve of 0.96 (pseudouridine), 0.93 (2-oxoglutarate) and 0.93 (BNP). More recently in a small validation cohort, we have shown that serum levels of 2-oxoglutarate correlate with clinical severity of heart failure as defined by New York Heart Association class (unpublished data). 2-oxoglutarate is a ligand of the GPR99 G-protein coupled receptor that may regulate the renin-angiotensin system (He et al. 2004), which is one of the major therapeutic targets in the treatment of heart failure. We have shown the presence of the GPR99 receptor expressed in rat neonatal cardiomyocytes and human foetal cardiomyocytes and that 2-oxoglutarate regulates the expression of VEGF receptor-1 and placental growth factor, regulators of angiogenesis (Nikolaidou et al. 2010) that may have a key role in the pathogenesis of heart failure.

Other groups have applied metabolomic analysis to the identification of novel biomarkers of myocardial ischaemia (Sabatine et al. 2005) in which LC–MS was applied to serum samples from patients undergoing exercise stress testing from 36 patients, 18 of whom demonstrated inducible ischaemia and 18 of whom did not (controls). Six members of the citric acid pathway were identified as among the 23 most changed metabolites in those patients with inducible myocardial ischaemia and these 6 metabolites, including citric acid, differentiated cases from controls with a high degree of accuracy. More recently, serum samples from patients with coronary angiography proven coronary artery disease and serum samples from age and sex match control patients was subjected to metabolomic analysis to determine whether the metabolome is able to discriminate the presence of coronary artery disease (CAD) and is able to predict the risk of cardiovascular events (Shah et al. 2010). Branched chain amino acid metabolites and urea cycle metabolites were associated with the presence of CAD, whilst dicarboxylacylcarnitines predicted death/myocardial infarction outcomes.

Others have studied changes in the metabolome further down the myocardial ischaemia cascade in patients undergoing planned myocardial infarction in the treatment of hypertrophic cardiomyopathy in which the pathologically hypertrophied cardiac septum is infarcted under controlled clinical conditions with alcohol injected down the septal artery that supplies the septum (Lewis et al. 2008). Using LC–MS changes in circulating levels of metabolites participating in pyrimidine metabolism, the tricarboxylic acid cycle and the pentose phosphate pathway were observed in patients undergoing planned myocardial infarction. A metabolic signature derived from patients with planned myocardial infarction consisting of aconitic acid, hypoxanthine, trimethylamine *N*-oxide, and threonine was able to differentiate patients with acute myocardial infarction from those undergoing diagnostic coronary angiography with high accuracy. Similarly, we have applied metabolomics analysis to patients following transient coronary artery occlusion during percutaneous coronary intervention (PCI) using LC–MS to study changes in the metabolome during transient myocardial ischaemia (Mamas et al. 2009) and shown that predominantly metabolic pathways involving lipids were perturbed with significant changes in diglycerides (DG),

lysophosphatidylcholines (LPC), phosphatidylcholines (PC) and free fatty acids (FFA).

As discussed previously, metabolomics can be applied in a targeted approach to biomarker discovery based on existing biological knowledge. For example, a targeted metabolomics approach towards post-translational modification products of arginine methylation has been performed in patients undergoing diagnostic cardiac catheterization in order to identify biomarkers for significant obstructive coronary artery disease as well as future major adverse cardiac events (MACE) (Wang et al. 2009). Wang et al. demonstrated that asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and arginine methylation index provided independent risk prediction for both significantly obstructive CAD and incident MACE in stable patients undergoing cardiac evaluation. Plasma ADMA levels have also been shown to be predictive of MACE in patients with acute decompensated heart failure (Dückelmann et al. 2008) and with chronic heart failure (Tang et al. 2008; Dückelmann et al. 2007). A small study has shown that treatment of patients with heart failure with ß-blockers is associated with a reduction in ADMA levels only in those who responded to ß-blocker treatment through an improvement in their cardiac function (Alfieri et al. 2008). However, this study does not determine whether this is a causal relationship or whether ADMA is merely a marker of heart failure severity whose serum levels alter following treatment with ß-blockers in the responder group. ADMA may have a role as a bio-actor with important biological actions that may contribute to key pathophysiological mechanisms underlying the disease process itself. For example, ADMA is an endogenous inhibitor of all three nitric oxide synthase (NOS) isoforms that impairs nitric oxide (NO) production, possibly by competing with arginine for the substrate-binding site of NOS (Boger et al. 2000). Clinical studies indicate that reduced NO release in the presence of elevated ADMA results in endothelial dysfunction (Boger et al. 1998) which is thought to play a key role in the development of cardiovascular disease.

Other cardiovascular-associated diseases have also been investigated to identify potential metabolic biomarkers. One example is pre-eclampsia, a pregnancy-related disease which effects 3–5% of pregnancies in the western world. Typical symptoms in the later stages of pregnancy (20 weeks and greater, typically greater than 28 weeks) are hypertension and proteinuria. Risk factors such as high maternal age, family history and pre-existing diseases are applied in the management of the disease. Currently, there are no curative therapies for pre-eclampsia and the mainstay of treatment is to prolong the pregnancy towards term, to treat the hypertension and provide supportive treatment as long as is possible without severely endangering mother or baby. The availability of a biomarker(s) to screen all women in early pregnancy would be hugely beneficial to enable appropriate management of the pregnancy before symptoms are observed later in pregnancy. Potential biomarkers have been identified in time-of-disease samples (Kenny et al. 2008), and recently putative biomarkers have been identified in a prospective cohort of women from samples collected at 15 weeks of the pregnancy (Kenny et al. 2010). Further large-scale validation is now being performed.

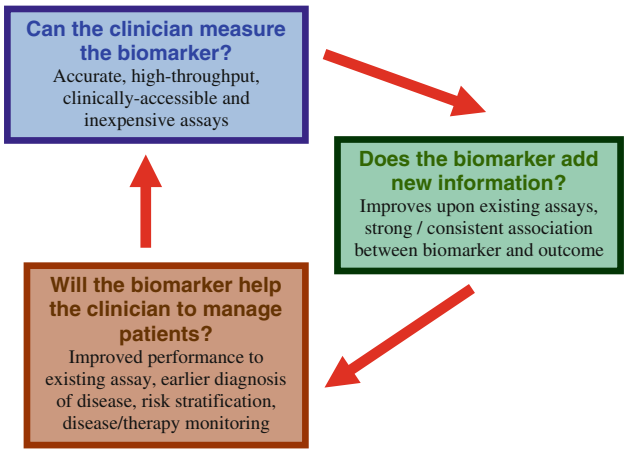

Fig. 3 Critical appraisal of biomarkers through the application of three criterions (modified from Morrow and de Lemos 2007)

Benchmarks for the assessment of novel biomarkers and metabolomics

High-throughput screening and application of metabolomics to the clinical disease state has led to the description of a number of metabolites that have the potential to be developed into robust biomarkers applied in the clinic. Recently, it has been suggested that novel biomarkers are critically appraised through addressing the following three fundamental questions (Morrow and de Lemos 2007):

1. Can the clinician measure it?
2. Does it add new information?
3. Does it help the clinician to manage patients?

Below we assess whether biomarkers discovered through metabolomic analysis are able to fulfil these three criteria. Figure 3 describes the appraisal requirements.

Can the clinician measure the biomarker?

For any biomarker discovered through metabolomic analysis to be of use in routine clinical practice, sample collection and an analytical methodology should be available that allows accurate quantification of the metabolite with the

capability for high-throughput, prompt turnaround time and low costs. As described previously most biomarkers used in clinical practice are serum (or plasma) based and most of the metabolomics studies that have led to potential biomarker discoveries have been performed on serum samples obtained from patients. Metabolites are currently routinely measured in clinical practice (glucose, cholesterol, creatinine, fatty acids etc.) hence much of the infrastructure that is required to measure metabolites that are found to be of interest following metabolomics analysis are present in hospital laboratories around the world enabling high throughput, prompt turnaround time and low costs associated with their analysis. Consequently, biomarkers discovered through metabolomics analysis could mostly be measured with existing technology that is commonplace in most hospital environments. A large cultural or technological change is therefore not required to allow metabolic biomarkers to be routinely assayed.

Does the biomarker add new information?

A key criterion with regards to the utility of a candidate biomarker is whether there are strong and consistent associations between the biomarker and the outcome or the disease of interest in multiple studies. Many of the metabolomics studies described above have been performed in small cohorts of clinical patients and have not been validated in larger study cohorts or where the biomarker is no longer predictive of the disease state. This may be related to potential confounders in the original study. For example published findings of ^{1}H-NMR spectral variation correlating with coronary artery disease severity (Brindle et al. 2002) were later found to be confounded by cholesterol-lowering (i.e. HMG-CoA reductase inhibition), therapy and gender (Kirschenlohr et al. 2006). Other confounders can be observed including significant differences in the clinical study cohorts studied, substrate collection procedures as well as the biomarker assay used as in the sarcosine studies in prostate cancer (Shalken 2010; Sreekumar et al. 2009; Jentzmik et al. 2010). External validation applying independent sample sets collected in different clinical locations is thus a critical step on the path towards clinical integration of any biomarkers discovered through metabolomics (Fig. 2). Such validation would include consistent findings from multiple studies that utilize prospectively collected samples among patients with well-characterized clinical outcomes in which the biomarker is independently associated. Most biomarkers recommended for routine clinical use in cardiovascular disease by professional society guidelines have demonstrated consistent risk relationships in 10 or more studies (The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction 2000). Biomarkers discovered through the application of this technology to the disease process may also provide novel insight into pathophysiology. For example, as described previously this is exemplified through the metabolomics based discovery of 2-oxoglutarate and the GPR99 receptor in the pathophysiology of heart failure (Nikolaidou et al. 2010) or sarcosine and the molecular mechanisms that underlie prostate cancer invasiveness (Sreekumar et al. 2009).

Will it help the clinician to manage patients?

Biomarkers have a variety of possible clinical applications that they may be used for such as: early detection of subclinical disease; diagnostic assessment of an acute or chronic clinical syndrome; risk stratification of patients with the clinical syndrome; selection of an appropriate therapeutic intervention; and monitoring the response to therapy. There are several biomarkers in clinical practice that serve this role and many have been outlined above so there is no reason to believe that biomarkers identified through metabolomics analysis would not be able to fulfil these roles, although none are in routine use in clinical practice to date. However, there is often a significant lead time lag between the first description of a potential biomarker metabolite identified through metabolomics analysis and the subsequent larger prospective validation studies that are require before its use can be considered in routine clinical practice. It should be noted that metabolic biomarkers are applied in drug development studies in Pharma, but most are never published in the scientific literature.

Conclusions

Metabolomics has been applied in two complementary strategies: (1) biomarker discovery studies through metabolic profiling that investigates the metabolome in a holistic approach from a point of limited biological knowledge and (2) through targeted analysis driven from known biology. Metabolomics has been successfully applied to clinical conditions including inborn errors of metabolism, cardiovascular disease and cancer studies resulting in the identification of novel biomarkers for the diagnosis, assessment of severity and provision of prognostic information in these conditions. Many of the metabolomics studies relating to biomarker discovery have only been performed in small clinical cohorts hence external validation will thus be a critical step on the path towards clinical integration of any biomarkers discovered through metabolomics (Fig. 2). Whilst there are examples of metabolites that are used in the clinic

to inform the clinician as to disease or efficacy of therapy, no biomarkers discovered through metabolomic analysis are used routinely in clinical medicine at this moment in time; although we note that there is often a significant lead time lag between the first description of a potential biomarker metabolite identified through metabolomic analysis and the subsequent larger prospective validation studies that are require before their routine use can be considered. Finally, for biomarkers discovered through metabolomic analysis to be utilized in everyday clinical practice they must fulfil criteria developed to assess biomarker utility, namely (1) Can the clinician measure them? (2) Do they add new information? and perhaps most importantly (3) Do they help the clinician to manage patients?

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