

Current challenges in Metabolomics for diabetes research: a vital functional genomic tool or just a ploy for gaining funding?

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Abstract:

Metabolomics aims to profile all the small molecule metabolites found within a cell, tissue, organ or organism and use this information to understand a biological manipulation such as a drug intervention or a gene knock out. While neither mass spectrometry or NMR spectroscopy, the two most commonly used analytical tools in metabolomics, can provide a complete coverage of the metabolome, compared with other functional genomic tools for profiling biological moieties the approach is cheap and high throughput. In diabetes and obesity research this has provided the opportunity to assess large human populations or investigate a range of different tissues in animal studies both rapidly and cheaply. However, the approach has a number of major challenges, particularly with the interpretation of the data obtained. For example, some key pathways are better represented by high concentration metabolites inside the cell and thus, the coverage of the metabolome may become biased towards these pathways (e.g. the TCA cycle, amino acid metabolism). There is also the challenge of statistically modeling datasets with large numbers of variables but relatively small sample sizes. This perspective discusses our own experience of some of the benefits and pitfalls with using metabolomics to understand diseases associated with type II diabetes.

Keywords: metabolomics, NMR spectroscopy, mass spectrometry, obesity, functional genomics

While it is difficult to date the start of any field this is particularly true of -omic technologies. The desire to profile a large number of entities involved in any tier of a biological system has been a common thread in biology. The field of metabolomics is no exception to this statement. While the terms metabolomics (23) and the related term metabonomics (22) were coined in the late 90s, it is difficult to distinguish some of the work conducted now under the umbrella of metabolomics from much earlier studies involving large scale profiling of metabolites by mass spectrometry (for example 16, 30) and NMR spectroscopy (for example 3, 5). Indeed many of the basic processes which occur in current metabolomic laboratories would not be that dissimilar to work carried out by the pioneers of metabolic research who gave their names to the various pathways we study. In this brief article I discuss some of the benefits modern metabolomic approaches provide to functional genomics, with particular reference to diabetes and the metabolic syndrome, and outline some of the challenges the field faces if it is to develop into a mature technology.

Citius, Altius, Fortius - Swifter, Higher, Stronger

While it would be possible to carry out metabolomics using classical biochemical assays based on enzymatic reactions the big conceptual leap forward in metabolomics is the desire to measure as many metabolites as possible and through this either categorize samples as part of a metabolic fingerprint (25) or footprint (1), or identify these metabolites as part of metabolic profiling to determine potential biomarkers of disease or drug response/toxicity (26). Currently, the majority of attention is focused on three

technologies – ^1H NMR spectroscopy, Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS).

High resolution ^1H NMR spectroscopy can in theory detect any organic molecule by virtue that they possess protons (hydrogen nuclei). When placed inside a high field magnet these nuclei possess different energies according to whether they oppose or are parallel to the applied magnet field. This energy difference also depends on the chemical environment the protons are found within providing a fingerprint for that molecule. This chemical environment can be interrogated by radiofrequency waves in a non-invasive manner. Of the commonly used metabolomic technologies it is the least sensitive of the approaches, although significant recent improvements have occurred in terms of increases in field strengths, cryoprobe technology, improvements in probe and spectrometer design and the development of microprobes. There is also the potential that hyperpolarisation (9) may one day produce a technology as sensitive if not more so than mass spectrometry. Even with current sensitivity though, NMR spectroscopy has a great deal to offer those involved in diabetes research. It is rapid, cheap on a per sample basis and highly robust. Type 2 diabetes, obesity and the Metabolic Syndrome are all disorders of the whole body and in order to interpret the biochemical changes that occur in one organ it is necessary to understand how it interacts with systemic metabolism. In this respect one can use surrogates for systemic metabolism examining blood plasma/serum or urinary profiles to monitor changes at the whole animal level (29; **Figure 1**) but to address the fundamental question as to what is occurring at an organ level, tissue extracts can be examined rapidly and cheaply by NMR spectroscopy. Using such an approach we

have used ^1H NMR spectroscopy to profile systemic metabolism in the PPAR- α knock out mouse by examining liver, skeletal muscles, heart tissue and adipose tissue, demonstrating that white adipose tissue is also affected by a failure to express PPAR- α , even though the overall expression level of PPAR- α in this tissue is negligible in wild type animals (2). Due to cost and time constraints it would be hard to envisage large number of such studies would be feasible using transcriptomics or proteomics, but metabolomics could be used as a tool for triage to identify the most relevant tissues to be characterized further at the transcriptional or proteomic level.

The GC-MS is the corner stone of many analytical chemistry laboratories and it too provides a highly robust tool for metabolic profiling (7). Mass spectrometry relies on identifying metabolites according to their mass after they have been ionized and accelerated across a distance. In GC-MS the metabolites are first vapourised into the gas phase and introduced into a GC column. This column is placed in an oven and the temperature raised across a time cycle, causing molecules to vaporize at different times, thus providing a chromatographic separation. Metabolites are then ionized and analysed by the mass spectrometer. As a metabolomic approach it has the immense benefit of a highly reproducible ionization method in the form of electron impact ionization which allows the user to identify metabolites by their mass spectra, making use of large databases such as NIST which contains over 163,000 compounds currently (<http://www.nist.gov/srd/nist1a.htm>). While metabolites do require derivatisation prior to analysis a number of robust methods exist for different classes of compounds (12). This approach is also probably the best way to distinguish fatty acids in the form of fatty acid

methyl esters. While under most circumstances these are cleaved from the backbone they are attached to, Watkins and co-workers (32) elegantly demonstrated a method involving the prior separation of different lipid classes using thin layer chromatography followed by GC-MS analysis as a cheap and reproducible alternative to LC-MS based lipidomics. Using this approach they investigated the changes in the lipidome of a mouse model of type 2 diabetes following treatment with rosiglitazone, a PPAR- γ agonist, including increased hepatic steatosis following chronic treatment. Indeed this method could have been performed using a GC-FID, placing the equipment within the price range of many physiology labs.

To examine intact lipid species and potentially definitively define the chemical groups that are changed in disease, rather than focusing on changes in the fatty acid profile of a particular class of lipids, LC-MS is the current method of choice. This approach separates metabolites according to their solubility in the liquid phase against their affinity for the solid phase contained within the column. The approach requires no derivatisation. Indeed, it is possible to bypass the chromatography stage and use direct infusion of lipid extracts into the mass spectrometer as part of an approach referred to as shotgun lipidomics. Han and colleagues (13) using this approach demonstrated a profound decrease in cardiolipin in the myocardium following streptozotocin-induced diabetes, as well as a remodeling of the overall cardiolipin profile with a 16-fold increase in 18:2-22:6-22:6-22:6 cardiolipin, preceding accumulation of triglyceride in the heart. Similar modulations of cardiolipin metabolism were observed in the ob/ob mouse. Alternatively, reverse phase chromatography can be used to separate different lipid species and reduce the potential of

ion suppression. Pietiläinen and co-workers (24) used LC-MS based lipidomics to examine whether acquired obesity was associated with a different serum lipid profile in young adult monozygotic twins who were either concordant or discordant (10-25 kg weight difference) for obesity. For the discordant twins blood serum had increased concentrations of proinflammatory and proatherogenic lysophosphatidylcholines and decreases in the antioxidant ether phospholipids, suggesting in these obese individuals the lipid profile was already such that it would promote atherogenesis, inflammation and insulin resistance. LC-MS has also been used to follow changes in the aqueous fraction of tissue extracts (2), blood plasma and urine (33) from rodent models of type 2 diabetes, but here the largest challenge is identifying the metabolites detected, and often such data has been largely used to classify samples as part of a metabolic fingerprinting study rather than carrying out biomarker identification.

The fact that all these technologies are relatively high throughput also means that large scale studies can be performed. This is particularly appropriate to type 2 diabetes, obesity and their related disorders which are on the whole polygenic disorders. This necessitates either the cross comparison of a number of monogenic animal models of the disease to produce an understanding of the extremes of the metabolic interactions associated with the diseases or the investigation of a disease process in a large population to investigate how a specific gene mutation or disease process interacts with the genetic diversity found in a normal population.

Dumas and colleagues (6) mapped metabolite resonances from ^1H NMR spectra of blood plasma from diabetic and control rats to quantitative trait loci (QTLs). Examining the

diversity in human populations directly, Makinen and co-workers (20) examined the use of ^1H NMR spectroscopy of blood serum to diagnose diabetic nephropathy in 182 sufferers of type I diabetes. This approach relied on in part modeling the different distributions of lipoprotein fractions and produced a test approximately as good as that used clinically. The group has since developed a Bayesian Markov chain Monte Carol approach to modeling the constituents of the saturated lipid profile in blood serum (31) and is exploring the use of this approach to model complications associated with type 1 diabetes. Similarly, Brindle and colleagues described a ^1H NMR spectroscopy based method for potentially diagnosing coronary artery disease through blood serum as a potential replacement to angiography (4).

There are also niche technologies that have been developed for specific problems. One such approach is high resolution magic angle spinning (HRMAS) NMR spectroscopy which is capable of producing high resolution spectra from intact tissues. This provides the opportunity to monitor changes in aqueous and lipophilic metabolites in the same sample. This is particularly appropriate for monitoring steatosis in liver samples, where the increase in lipid species is often accompanied by a dramatic loss in glycogen within the liver tissue (2, 10-11). Using such approach we have previously examined the lipid profile of fatty liver disease induced by orotic acid in situ directly within liver tissue (10). This approach nicely demonstrated why the Kyoto strain of rat was predisposed to fatty liver disease compared with the Wistar strain as liver tissue contained considerably more saturated fat.

'If you build a better mousetrap, you will catch better mice' - Mouse metabolomics as a tool for human pathogenic hypothesis generation and biomarker identification.

In our experience performing metabolomic studies in genetically modified rodent models poses significant advantages compared to human studies when investigating potential pathogenic mechanisms and identifying diagnostic/prognostic biomarkers. There are clear advantages derived from the more controlled genetic background, environmental and experimental conditions which allow narrowing the number of candidate metabolites /lipid species to become a meaningful therapeutic target or useful biomarker. For instance we have used LC/MS to identify reactive lipid species associated with lipid induced insulin resistance and diabetes in animals defective for PPAR γ . Using this approach we identified specific patterns of lipids accumulated in key metabolic organs such as liver, muscle, and adipose tissue that correlate with the severe metabolic phenotype of this mouse model (21). Subsequent studies in humans (24) confirmed lysophosphatidylcholines and ether phospholipids as bona fide lipid biomarkers correlated with insulin resistance and metabolic complications. This allows much smaller human studies to be conducted in order to confirm results from the more homogeneous population presented by mouse models. In this respect we think our research strategy of combined mouse and human profiling is valid and may complement other more traditional approaches. However, there is a word of caution. Strain background has a profound influence on the metabolome of mice, both in urinary profiles (8) and in tissues (17). This may beg the question as to what is a control mouse, and whether certain strains should be avoided for models of diabetes and obesity.

Lies, damn lies and statistics

One of the big conceptual changes from many classic metabolism studies and metabolomics is the idea that a change in metabolism may not be characterised by changes in individual metabolite concentrations but by a pattern of changes – the metabolic fingerprint or metabolic profile. This perhaps naturally arose from the measurement of many metabolite changes across the whole network of metabolism. Thus, almost as integral to metabolomics as the analytical devices to measure many metabolites are pattern recognition and data reduction tools from multivariate statistics (15, 18). These data analysis tools have provided correlations across metabolism demonstrating the high interconnectivity of many metabolic pathways, and providing many novel results from these types of analysis. However, there are still problems with the approach. With large scale multivariate data there is also the danger of over-fitting data, and where possible datasets should always be cross validated. A related issue is that datasets can be biased to the stratification of a particular population, and thus even in relatively large studies one should view with caution the results produced. Kirschenlohr and colleagues (19) demonstrated that the diagnosis of CAD by ^1H NMR spectroscopy of blood plasma was significantly complicated by related changes in the lipoprotein/saturated lipid region of the ^1H NMR spectra associated with gender and statin treatment that previous models had produced an artificially high classification of disease presence. Roussel and co-workers (27) similarly demonstrated that cardiovascular risk was poorly predicted by ^1H NMR spectroscopy and multivariate analysis in a population of type 2 diabetics. Thus, as in epidemiology studies it will be important to

follow up metabolomic biomarker discovery studies in humans with further studies in other populations to detect potential confounding factors.

If you only have a hammer everything looks like a nail.

One other area of bias to be aware of in metabolomic experiments is that the technologies employed may significantly bias the results. As mentioned NMR spectroscopy is relatively insensitive and so many of the changes detected are associated with high concentration metabolites. These same metabolites will probably be involved in part in osmoregulation given their high concentrations so a disease may look like a disorder in osmoregulation just because the metabolites that are detected have this role. Many of the metabolites we detect in our studies by GC-MS are involved in amino acid metabolism, the Krebs cycle, glycolysis and β -oxidation, and so it is perhaps not that surprising that the approach detects changes in these pathways! An alternative to the approach of global metabolite analysis is to provide a targeted approach where a number of pre-defined metabolites are targeted. This has the advantage that this type of approach can also be readily made quantitative by nature. Sabatine and co-workers (28) describe one such targeted LC-MS method where metabolites were selectively analyzed using a combination of different chromatographies, rather than the one size fits all approach used in most studies, and a triple quadrupole mass spectrometry based analysis to identify biomarkers of myocardial ischemia. Similarly Haaq and colleagues (14) have described the targeted analysis of 101 intermediary metabolites in an on-going patient study into nutrition and weight loss. It seems wise to carry out both hypothesis generating open profiling alongside hypothesis testing targeted analysis.

The things we forgot to remember – or standing on the shoulders of giants?

The temptation with any new field is to ignore the results of those that went before. There is a very great danger when producing new multivariate models of the network of metabolism that we may simply reinvent the wheel with some of these approaches. For example, we have commonly heard colleagues discussing how the pathway representation of metabolism in databases such as KEGG are wrong and inaccurately represent metabolism. While it is true that a simple flow diagram cannot represent the complexities of a metabolic network, in metabolomics we have a rich heritage of research into metabolism stretching back to the early days of biochemistry which should make the field the envy of other -omic approaches. This information can at least provide a first pass model of the interactions of metabolism in a cell, tissue, organ or organism and a context to the results from a metabolomic experiment. In common with other functional genomic studies it is also important to complete the cycle of hypothesis generation and hypothesis testing. As new genomic and metabolic pathways are deemed to be of importance in some disease, we must then return to the type of studies that will provide the important biochemical details of the metabolic states. When looking forward to what a new field will provide it will also be important for us not to forget what we already knew from classic metabolism and the approaches which have served the field well in the past.

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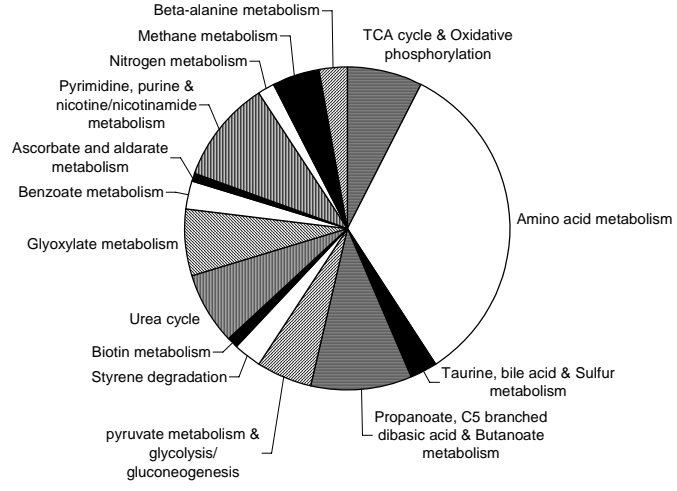
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Figure 1A: A summary of the metabolic perturbations detected in urine from the two animal models of type 2 diabetes and sufferers of the disease (taken from 29 with permission). The KEGG database was searched for each metabolite detected as perturbed and each KEGG pathway scored according to the number of metabolites listed within that pathway. For simplicity some related pathways have been combined to form a single group. **B:** A Partial Least Squares – Discriminate Analysis scores plot for male diabetic and control samples for human, rat and mouse. Clear species separation exists between human, mouse and rat samples although all three models demonstrate separation between control samples (black squares) and disease samples (white circles) along the same PC.

1a



1b

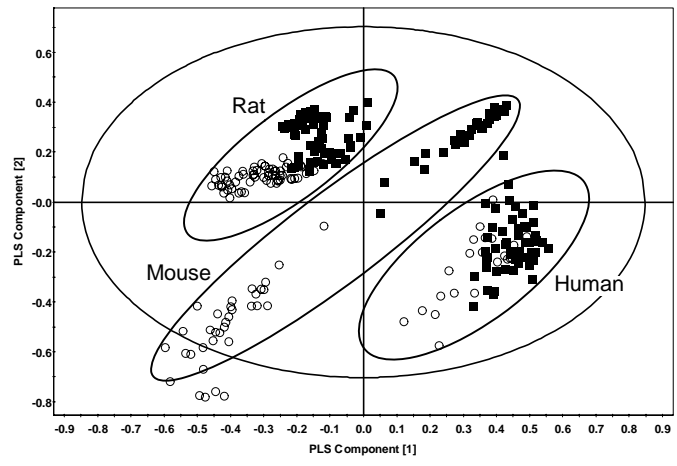


Fig 1