METABOLOMICS
a 'systems' contribution to pharmaceutical discovery and drug development

The potential of metabolomics, a systems approach to biochemical pathway analysis, to yield scientific and commercial benefits to healthcare and biotechnology enterprises is increasingly recognised. This article discusses current applications and future developments and places an emphasis on incorporation of metabolomics within all aspects of the value chain of drug development.

By Dr George G. Harrigan

Escalating research and development costs continue to challenge the pharmaceutical industry. While pharmaceutical ventures remain productive[1], rising expenditures have precipitated an increasing number of mergers and business acquisitions by the larger pharmaceutical companies. The number of such agreements in the pharmaceutical industry increased from 24 in 2003 to 45 in 2004[2]. External factors, including a demanding regulatory environment, have been implicated in the onerous time and cost burdens associated with drug development. Factors intrinsic to the industry include escalating development costs associated with target validation and high-throughput screening and with later-stage clinical trials where trial populations have increased 135% over the past 10 years[1]. This clinical population increase does not appear to have significantly reduced candidate drug attrition. There is thus a need to develop innovative strategies and technologies that identify drug candidates (or targets) unlikely to succumb to attrition at preclinical and clinical trials. Outcome and mechanistic biomarkers which can accelerate candidate progression are also needed. No one scientific discipline can fully address such challenges but an increasing interest in metabolomics, a systems approach to understanding phenotypic changes, is clearly evident. The proximity of the metabolome to phenotype may offer increased insights into functional changes associated with pharmacological or nutritional intervention.

This article briefly describes metabolomics and discusses current applications and potential developments. It also proposes that hypothesis-driven approaches, as opposed to untargeted profiling, may prove more relevant to prerogatives within the pharmaceutical industry. Metabolomics also has the potential to impact several therapeutic areas; the test case studies in this article highlight cancer, neuro-degeneration, cardiovascular dysfunction and dyslipidemia.

What is metabolomics?

Concentrations, fluxes and transport mechanisms of metabolites are known to be highly sensitive to disease and to drug intervention. Changes in recorded levels of, for example, glucose, choles-

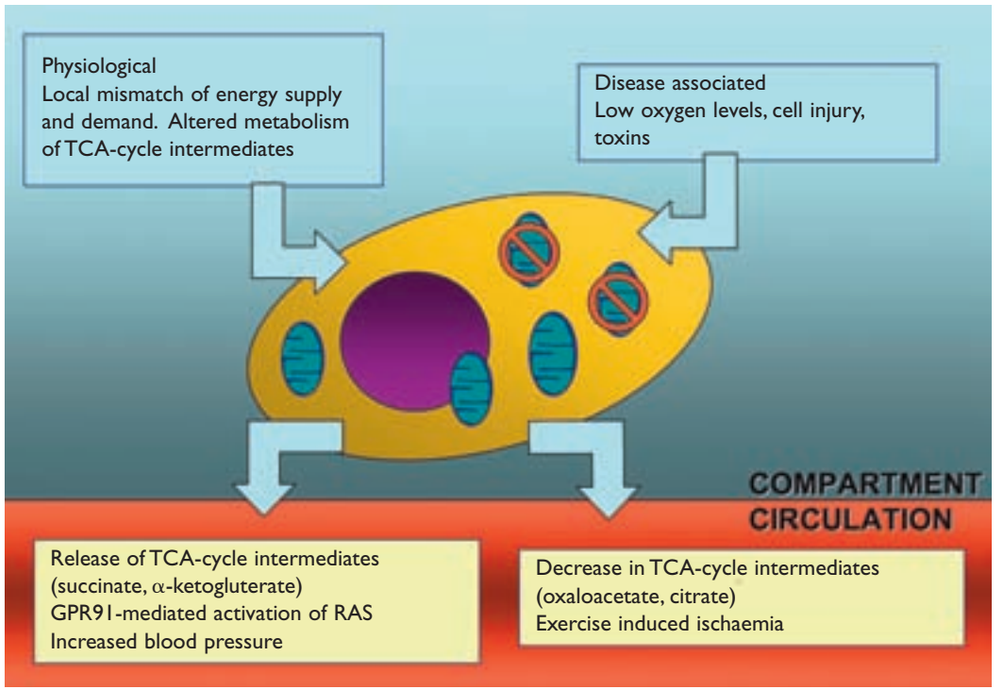

Figure 1
Mitochondrial metabolites are implicated in many intracellular and extracellular signalling functions. Distinct perturbations in TCA cycle metabolism have been observed during exercise inducible ischaemia in human patients and may also be associated with hypertension. This figure is based closely on that presented by Herbert SC in Nature, 429, 143-145 (2004)

terol and triglycerides not only have clinical implications but these metabolites can be used as outcome biomarkers to support drug candidate progression. Adopting the argument that fluctuations in small molecule metabolites closely reflect the functional responses of an organism to perturbation, metabolomics seeks to probe the mechanisms that contribute to changes in metabolic levels and fluxes and understand the implications of such changes on disease, drug response and nutritional intervention. A principal goal is to increase the range of available metabolite biomarkers, further understand their cellular and systemic distribution and facilitate their application in accelerating drug candidate progression.

Metabolism and metabolomics share as their root the ancient Greek word, *metabol*, which means change, and obviously both terms are equally applicable to cell, tissue or whole organism. Metabolomics research is characterised, at least in part, by two different (but not necessarily mutually exclusive) conceptual approaches broadly defined as 'targeted' and 'non-targeted'[3-5]. Non-targeted approaches provide a hypothesis-free global overview of high abundance metabolites most affected by experimental perturbation or disease. Targeted approaches which highlight identified and pre-selected metabolic pathways may prove more relevant to evaluating the impact of a drug candidate on metabolic regulation. Technology platforms for targeted approaches are based on discrete optimised analytical strategies for different classes of metabolites or pathways. This approach represents both an accommodation with the wide differences in physiochemical structure, stability and differential abundance of metabolome components and with the recognition that metabolome analyses may operate best in a mechanistic, hypothesis-driven framework. It clearly facilitates greater evaluation of low abundance, biologically remarkable metabolites such as eicosanoids, other signalling lipids, hormones and neurotransmitters. A key challenge for vendors providing instrumentation to support metabolomics is in establishing an optimal balance between the accuracy and range of metabolite measurements.

Targeted metabolomics approaches emphasising biological expertise in experimental strategy additionally allow adoption of flux-based methodology where specifically designed tracer-labelled substrates can be incorporated into test biological systems and their distribution and metabolic fate recorded. As will be discussed later, metabolic flux analyses provide an operational 'moving picture'[6] rather than a compositional 'snapshot' of a biological system. At present this approach is somewhat under-represented in metabolomics but it may be pointed out that the data acquisition technologies used here are essentially that used in compositional

studies. The data acquisition technologies utilised in metabolomics, primarily nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) have been extensively reviewed and will not be covered here. One point to be made, however, is that many instrument vendors are actively involved in designing platforms specifically for the metabolomics community.

Clinical applications

An intriguing impetus for advancing metabolomics was suggested by a report that implicated circulating TCA cycle intermediates in hypertension[7]. Elevated levels of succinate and α-ketoglutarate (putative GPCR ligands) were suggested as linking mitochondrial metabolism with blood pressure (see **Figure 1**). The signalling role of mitochondrial metabolic products is well recognised[8] and this research area clearly offers opportunities for targeted metabolomics. Applying such an approach the Gerszten laboratory demonstrated that circulating levels of functionally related metabolites of the TCA cycle differed in the plasma of patients susceptible to exercise-induced ischaemia relative to that of healthy individuals[9]. Utilising an HPLC-ESI-MS/MS-based analytical platform that allowed evaluation of 173 known analytes, Gerszten demonstrated that i) circulating oxaloacetate and citrate decreased in ischaemia-susceptible subjects but not controls and that this was also true of γ-aminobutyric acid, an end-product of α-ketoglutarate metabolism and citrulline and arginosuccinate, members of the urea cycle which feeds into the TCA cycle. Lactic acid, an end product of glycolysis, greatly increased in both ischaemia-susceptible and healthy subjects. Gerszten emphasised that "an important rationale for unequivocally identifying analytes or surveying analytes is to gain insight into the functionally relevant cellular mechanism contributing to disease pathways."

By considering metabolites, not as single discrete entities, but as co-ordinate members of a network, it is also implicit that metabolome data acquisition could provide sufficient functional differentiation to classify human disease states. An analogous approach to that discussed above was recently

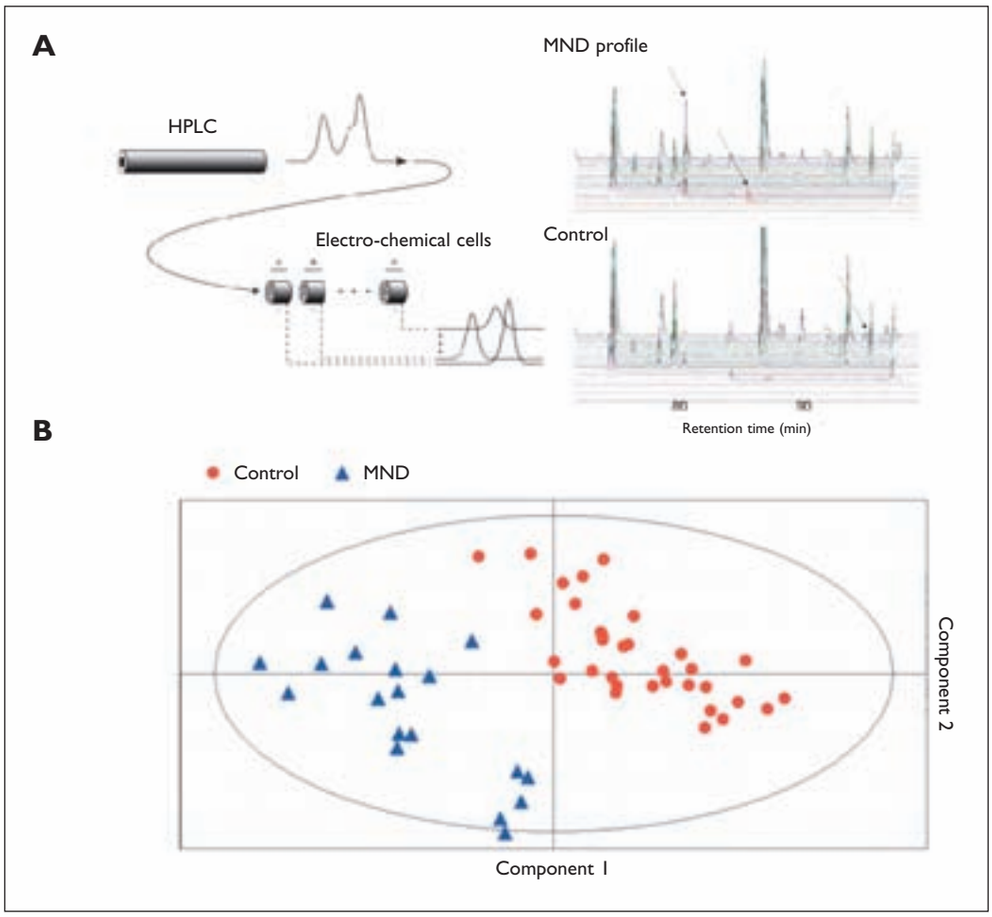

Figure 2
A study of the electrochemically active plasma metabolome revealed distinct signatures between patients with motor neuron disease and a healthy cohort. A) presents the application of electrochemical detection methodology in metabolome data acquisition and B) shows how partial least squares-discriminant analysis distinguished MND patients from a healthy cohort based on the acquired metabolome data

employed to identify metabolomics signatures in motor neuron disease (MND). Pointing out that no single biochemical differentiator has been unambiguously associated with MNDs such as amyotrophic lateral sclerosis (ALS), Rozen et al (2005)[10] exploited a HLPC-electrochemical detection system that allowed quantitation of 317 plasma metabolites. The data derived from analyses of blood collected from 28 MND patients and 30 healthy controls did indeed reveal a metabolic signature of disease (see **Figure 2**). Elucidation of the structures of signal molecules in ALS and other MNDs may now yield insights into aberrant biochemical pathways and provide targets for drug design. Electrochemical detection methods are particularly powerful for neuroactive metabolites derived from, for example, tyrosine metabolism but given increasing evidence that diverse metabolic pathways impinge upon neuro-degeneration[11], targeted analyses may require co-adoption of other technologies more responsive to electrochemically inert metabolites.

Metabolic flux-based approaches remain somewhat under-represented in the metabolomics research community despite their clear importance[6,12,13]. Such analyses involve administration of a tracer-labelled metabolite to a test biological system. Recording the incorporation of the tracer atoms into other metabolites facilitates simultaneous measurement of flux through multiple pathways. As an illustration of the widespread applicability (and safety) of tracer-based metabolic flux analysis in humans one can consider a recent report by SIDMAP (www.sidmap.com) on *de novo* lipogenesis in low-birth-weight infants[12]. Such infants have high energy requirements and are dependent on high fat intake to maintain adequate postnatal growth. Researchers at SIDMAP hypothesised that *de novo* lipogenesis plays an important physiological role in adapting to nutrients derived from breast milk or parenteral feeding. Administration of the stable isotope tracer [2-^{13}C] acetate to low-birth-weight infants revealed that synthesis of palmitate, stearate and cholesterol was indeed active in those infants maintaining standard postnatal growth. SIDMAP was able to make a major metabolomic conclusion that *de novo* lipogenesis permits newborn infants to meet the fat energy needs of peripheral tissues for growth and storage and to maintain plasma fatty acid composition in adaptation to different dietary fat intake.

The above examples illustrate contrasting but effective metabolomics contributions to a range of human health concerns ranging from cardiovascular dysfunction, neuro-degeneration and issues at early infancy.

Applications in early drug discovery

There is considerable emphasis on metabolomic applications in later stages of drug discovery including clinical trials, yet the most expensive part of pharmaceutical development resides within early discovery which encompasses disciplines such as target validation and high-throughput screening (HTS). Strategies to reduce attrition may be most cost-effectively implemented at the earliest stages of drug discovery[14]. In many Pharma enterprises, biomarker operating plans place an emphasis on substrate/product ratios as mechanistic markers of target enzyme function; it is critical that a drug candidate be shown to act on its target throughout all stages of the value chain and that modulation of that target does indeed impact disease progression. An example of how targeted metabolomics approaches could facilitate the progression of early leads throughout the value chain can be conceptualised through discussion of eicosanoid-acting chemistry. Many HTS campaigns identify inhibitors of eicosanoid production using high-throughput, but indirect, fluorescence assays; leads identified from such screens can be subsequently validated in MS-based 'orthogonal' assays that directly measure enzyme substrate and product. These assays can easily be translated from isolated enzyme reactions to cell, tissue and biofluid analysis and, more to the point, in the process of translation can be refined to increase metabolite coverage. Additionally, since changes in eicosanoid metabolism influence, and are greatly influenced by, lipid supply lipidomic profiling represents an appropriate adjunct to studies on eicosanoid metabolism. This concept was first successfully put into practice by Wheelock et al (2005)[15] in a study on soluble epoxide hydrolase, a lipid modifying enzyme implicated in hypertension.

Two case studies exemplifying the type of targeted metabolomics approach that, in addition to the concept described above, could be incorporated into early discovery are now presented.

Metabolic flux analysis of drug candidate action in cell systems

Early discovery studies on candidate drug action or target validation typically utilise cell-based systems and such systems are eminently suitable for metabolic flux evaluations. This is exemplified in a SIDMAP study of different pathways associated with *de novo* nucleic acid synthesis in cancer cells[13]. Cancer cells exhibit a high rate of metabolism with glucose as a primary substrate. Tracer-based metabolic profiling using [1,2-$^{13}C_2$]-D-glucose has revealed that alternative routes of *de novo*

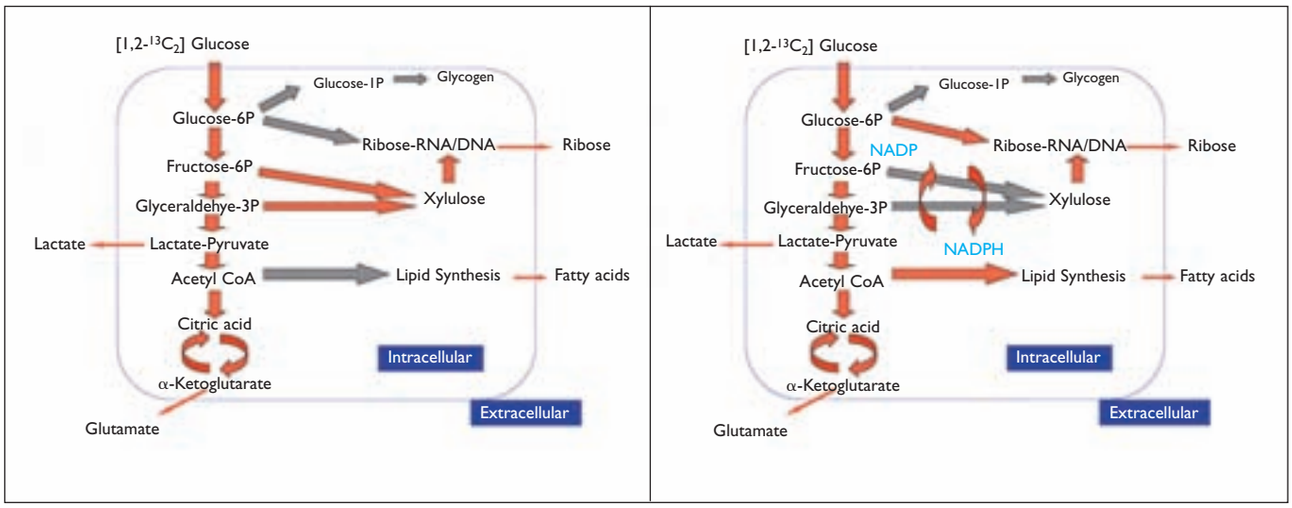

Figure 3: Metabolic flux analysis using [1,2-$^{13}C_2$]-D-glucose as a tracer substrate reveals that cancer cell types (eg apoptosis resistance versus apoptosis sensitive) use differential and preferred routes for *de novo* nucleic acid synthesis. Apoptosis resistant cell lines have high rates of fatty acid synthesis and desaturation providing an adequate reservoir of the oxidised form of $NADP^+$ that is used as the sole hydrogen acceptor for oxidative pentose synthesis. Reduced rates of fatty acid synthesis do not provide such an acceptor and the non-oxidative pentose cycle, which does not require it, predominates. It has also been demonstrated that BCR-ABL^+ leukaemia cells prefer use of the oxidative branch of the pentose cycle and that resistance to Gleevec in this cell line results through increased non-oxidative capacity

nucleic acid synthesis from glucose carbon are differentially used in certain cancer types. In apoptosis sensitive cells lines, such as thiamine transport deficient human fibroblasts or pancreatic adenocarcinomas, the pentose cycle predominantly operates by the non-oxidative branch whereas apoptosis resistant cell lines, such as inflammatory breast cancer cell lines predominantly use the oxidative branch. Highlighting the connectivity of metabolic pathways, the preferential use of a given pentose phosphate pathway route can be correlated with relative rates of fatty acid synthesis and desaturation (see **Figure 3**).

A notable application of this concept is a SIDMAP investigation of the mechanism of action of imatinib mesylate (Gleevec) on BCR-ABL^+ leukaemia cells. The gene product from the breakpoint cluster region-Abelson (BCR-ABL) chromosomal fusion induces increased glucose uptake, hexokinase activity and oxidative pentose phosphate pathway metabolism. Gleevec inhibits this process effectively reducing *de novo* nucleic acid synthesis. SIDMAP has now shown that cells develop Gleevec resistance by increasing functional capacity in non-oxidative pentose phosphate pathway metabolism, a pathway not appreciably inhibited by Gleevec. In the same manner that stable isotope tracer studies can be readily performed in cell culture, peripheral blood cells can also be profiled *in vivo* in the presence of Gleevec treatment of human subjects. Thus the effect of Gleevec therapy and early indications of resistance can potentially be predicted from SIDMAP demonstrations of increased non-oxidative pentose metabolism. It is significant that the SIDMAP technology can translate from contributions in early discovery to clinical applications.

The SIDMAP approach has also been successful in discriminating the effects of thiazolidinedione analogs on fatty acid metabolism in cultured liver cells[16] and clearly applications to demonstrating the effects of drug candidates on metabolic regulation can be widely expanded.

Metabolomic analysis of animal model systems

The development of drugs for regulation of diseases such as obesity, diabetes and cardiovascular dysfunction clearly requires considerable efficacy and toxicological testing in animal models. Efficacy assessments also require that regulation of metabolism in animal models is reasonably well understood. A metabolomics study utilising an analytical platform developed by Lipomics Technologies, Inc (www.lipomics.com) that allows quantitative measurement of more than 500 lipid metabolites in blood and tissue samples was recently employed to probe metabolic regulation in the low density lipoprotein receptor null (LDLrKO) mouse, a model for atherosclerotic progression[17]. This

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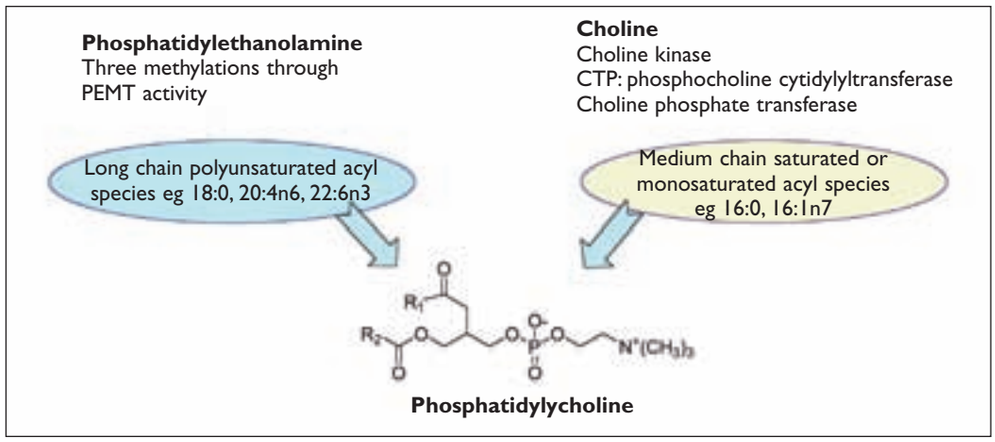

Figure 4: The cytidine diphosphate (CDP)-choline pathway and the hepatic phosphatidylethanolamine methylation (PEMT) pathway produce different profiles of phosphatidylcholine species based on the structure of the acyl chains. The CDP-choline pathway preferentially utilises saturated or monounsaturated medium-length acyl chains, and the PEMT pathway preferentially utilises polyunsaturated long-chain acyl chains. This preference can be quantitatively assessed through lipidomic profiling to provide estimations on the relative activity of these two pathways. CTP Cytidine triphosphate

lipidomic platform has profound implications for approaches to assessing disease and drug intervention. Thus, while cholesterol is an important predictor of disease, the information generated through lipidomic profiling can facilitate dissection of different and distinct pathway contributions regulating cholesterol synthesis, adsorption and transport. Lipidomic profiling in the LDLrKO model allowed estimates of the relative activities of two pathways which contribute to serum cholesterol ester (CE) levels; the lecithin:cholesterol acyltransferase (LCAT) pathway which preferentially uses polyunsaturated acyl species and the acyl-coenzyme A:cholesterol acyltransferase (ACAT) pathway which typically uses saturated or monounsaturated acyl species. Lipidomic profiling revealed that a 'Western' diet-induced increase in serum CE in the LDLrKO mouse sustained over 12 weeks was derived from progressively differential and opposing activities in the ACAT (increased) and LCAT (decreased) pathways. Significantly, ACAT and LCAT have competing effects on lipoprotein profiles and atherosclerotic potential with ACAT being implicated as the more atherogenic. In the same study, an understanding of substrate specificities associated with two phosphatidylycholine (PC) synthesis pathways allowed a means to correlate elevated PC levels in the LDLrKO mouse with increased CDP-choline pathway activity (see **Figure 4**). Lipidomic dissection is therefore a powerful tool in assessing not only new anti-atherosclerotic drug candidates but compounds acting on pathways known to modulate lipid metabolism.

Correlating metabolome data sets with proteome and transcriptome data sets

An increasing area of interest is correlation of metabolomic data sets with proteomic and transcriptomic data sets[18]. While the goal of such studies is presumably to provide a deeper understanding of biological systems it may be worth pointing out that if the metabolome were truly closer to phenotype then the use of complementary data could be perceived as merely ancillary or even potentially confounding. In an integrated transcriptomic and NMR-based metabolomic study of fatty liver in the rat, an inverse correlation between stearoyl Co-A desaturase mRNA levels and levels of unsaturated fatty acids described as 'surprising' by the authors was revealed[19]. This observation is somewhat less surprising when placed in the context of a metabolomic demonstration that gene expression patterns in the adipose tissue of obese mice resulted in assessments of rates of fatty acid synthesis and lipogenesis that are inconsistent with direct measurements of metabolic flux[6].

Emerging metabolomics technologies

In the US a metabolomics roadmap sponsored by the NIH has now been initiated and distribution of $70 million over five years is projected. Selected examples of funded projects are now briefly described. Development of new methodology for determining the concentration of protein-unbound free fatty acids in intracellular and extracellular milieu entitled 'Fluorescent Probes for Hydrophobic Metabolites' represents a particular-

ly intriguing programme. Although free fatty acid levels are clearly critical in many diseases, monitoring of unbound free fatty acid profiles remains technically challenging and improved detection methodologies can only help facilitate drug candidate progression in a range of therapeutic areas. The use of fluorescently labelled metabolite probes is also a theme in a proposal 'Glycolipid Metabolism in Single Cells' to dissect glycolipid metabolism in single neurons. This proposal will focus on the development of different fluorescently labelled substrates within two different glycolipid metabolite pathways and offers particular promise in pharmacological evaluations of neuroactive agents. Another cellular neuro-metabolomic proposal 'Technologies for Cellular Neurometabolomics' addresses the use of a suite of technology developments including "unique sampling protocols, micro-fluidically-based sample conditioning unit with integrated electrophoretic separations, followed by native fluorescence and mass spectrometric detection and capture of appropriate metabolites into nanoliter volume capillaries for nanoliter volume NMR spectroscopic characterisation."

Also intriguing in terms of exploiting metabolomics in the early phases of drug candidate progression is a project 'Biological Oscilloscopes Spatio-Temporal Metabolomics' to develop real-time *in vivo* spatiotemporal quantification of metabolic fluctuations. By combining, among other aspects, quantitative real-time fluorescent microscopy and fluorescent nano-sensor proteins that report on specific metabolites and "it is anticipated that many of the signal pathway perturbations and alterations in metabolic profiles will provide a large database of correlative changes, recapitulation and diagnostic potential of disease states and novel biological discovery of signalling networks."

In summary, the NIH is currently supporting well conceived and technically feasible innovations that have the potential to drive metabolomics as a biologically informed science and provide metabolomic data that can be integrated into early drug candidate evaluation and also guide subsequent biomarker-enabled *in vivo* studies.

Consortia involved in metabolomics

The first organised multi-site collaboration between academic and industrial metabolomics researchers was that initiated by Professor Jeremy Nicholson, Imperial College, UK. COMET (Consortium of Metabonomics in Toxicology) comprised Imperial College and six pharmaceutical partners and emphasised the role of NMR-based urinary metabolite profiling in assessing animal model toxicity associated with new drug candidates. Many of the metabolites observed to be associated with observations of chemically-induced toxicity were shown to be derived from gut microflora. Conversely, in the original gut-microflora population of a test system could confound metabolic profile responses. An intriguing development from COMET's work has been a refocus on the role of gut-microflora in modulating host homeostasis and health.

A more recent academic-industrial partnership is HUMSERMET funded by the UK Biotechnology and Biological Sciences and Research Council (BBSRC) and the Medical Research Council (MRC). Led by Professor Doug Kell, University of Manchester, in partnership with Astra Zeneca and GlaxoSmithKline, this consortium seeks to develop novel methods for the acquisition and analysis of the human serum metabolome. Consortium goals, as documented in its website, www.metabolomics.co.uk, include optimising data acquisition technology (primarily GC-MS and LC-MS), assessing the 'normal' range of the human metabolome, identifying prognostic and diagnostic biomarkers for disease and determining the efficacy of drugs and other treatments. Diseases currently under investigation include Alzheimer's disease and ovarian cancer.

While one emphasis of HUMSERMET is on the application of metabolomic technologies in hypothesis generation, it may be speculated that this initiative will still need to demonstrate that it can test hypotheses. Are changes in serum cholesterol levels due to modulation of the ACAT-2 pathway? Can the acquired metabolome data generate lipid desaturation indices and make statements about important lipid-modifying enzymes, such as stearoyl-CoA desaturase? Does the metabolomic platform indicate if the drug target is being directly inhibited? It may be relevant here to point out the increasing acceptance of the omega-3-index (O3I) as a risk measure of coronary heart disease[20] and contrast it with the lack of impact of multivariate approaches which fail to identify metabolites or provide mechanistic linkage.

The US National Institute of General Medical Sciences has, since 2003, sponsored a LIPIDMAPS consortium of 16 academic research institutes and two private enterprises to fully map metabolic pathways in the macrophage cell. In many respects the LIPIDMAPS project has become possible through the refinement of mass spectrometers and six new mass spectrometry facilities have been

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established to support this effort. The goals of LIPIDMAPS are listed as "i) separate and detect all of the lipids in a specific cell and discover and characterise any novel lipids that may be present; ii) quantitate each of the lipid metabolites present and quantitate the changes in their levels and location during cellular function; and iii) define the biochemical pathways for each lipid and develop lipid maps which define the interaction networks." LIPIDMAPS will focus on mice macrophage lines with specific gene mutations in order to associate single gene changes with changes in synthesis, processing, and transport of lipid metabolites. Because macrophages play a significant role in the formation of atherosclerotic lesions understanding and defining changes in macrophage biology in terms of changes in lipid metabolism will prove invaluable to drug discovery. To quote one of the leading LIPIDMAP members, Professor Murphy of the University of Colorado Health Sciences Center, "aspirin, ibuprofen, naproxen, COX-2 inhibitors, statins – these are all drugs that work to alter lipids. It is hard to watch your evening news or pick up a magazine without seeing advertising that relates directly to lipids."

Concluding remarks

The current business model of the pharmaceutical industry is characterised by increasing research and development costs yet, according to the FDA, approximately 90% of drug candidates tested fail during clinical development. Many of these failures are simply due to a lack of efficacy and clearly candidates are progressing through the value chain of drug development without a full understanding of their impact on the metabolic regulation of disease. This low success rate coupled with continual increases in the cost of pharmaceutical research has had a critical impact on return on investment. It is increasingly apparent that the long-term viability of a candidate drug must be established at the earliest stages possible. Investment in innovative strategies to ensure that only truly efficacious candidates enter clinical trials is required. Integrating targeted hypothesis-driven metabolomic approaches with preclinical and clinical drug development may facilitate a redesign of scientific paradigms within the current business model of the pharmaceutical industry. The technical hardware and expertise currently exists and, indeed, instrument and informatics vendors continue to enhance the value of their product to metabolomics researchers. Further acceptance of metabolomics will require that researchers exploit that technology to promote further understanding of the metabolic regulation of biochemical pathways implicated in disease and translate such findings to the development of therapeutic interventions that truly benefit humankind. DDW

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