Ernst Schering Foundation Symposium Proceedings, Vol. 4, pp. 79–98
DOI 10.1007/2789_2008_090

Published Online: 06 June 2008

Biomarker Discovery for Drug Development and Translational Medicine Using Metabonomics

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Abstract. There exists at present an urgent desire for better biomarkers, especially in the context of pharmaceutical drug development and in the detection and management of disease. Many researchers in the area of biomarker discovery and development have turned to the "-omics" sciences as a way of addressing these needs. Metabolic profiling, or metabonomics, defines the metabolic phenotype and offers a source of novel biomarkers that have better potential to translate effectively. This review will discuss the broad philosophy and motivations behind metabonomics, and illustrate the case with applications relevant to pharmaceutical development and patient management. Particular focus will be paid to the potential of metabonomics to contribute to biomarker discovery in toxicology and cancer research.

1 The Potential of the Metabolome to Fill the Biomarker Gap

The high candidate drug attrition rate, particularly late in the development process, is extremely costly and is often due to the poor translation/prediction of drug metabolism, efficacy or toxicity from preclinical models to humans. These problems are exacerbated and intertwined with the lack of mechanistic understanding relating to many disease processes and indeed even in terms of pharmacological action itself. Given that most disease is heterogenenous in phenotype, it is also widely recognized that there has been a dearth of biomarkers able to stratify patients into those groups most likely to benefit from a particular treatment, and that this has led to costly failures of development programmes (Frank and Hargreaves 2003). Such biomarkers are in fact a fundamental part of chemopreventative strategies, which require by default a means by which to select subpopulations at particularly high risk of disease or disease progression. Hence the need for biomarkers that can personalise medicine for the individual and that translate effectively between models and humans is paramount.

The establishment of platforms that can characterise a biological sample in an untargeted and highly parallel manner have revolutionised modern biological research. Operating in a primarily hypothesis-generating rather than hypothesis-testing mode allows for efficient screening of candidate biomarkers without making prior (and not always correct) assumptions about what relationships may be detected. Where it is possible to operate in a near comprehensive manner, such as in the definition of the genome or the associated transcriptional profile (transcriptome), one can be confident in obtaining a truly global perspective of a system at the chosen biomolecular level. By its nature "-omics" science is clearly technology driven, and its growth has only been possible by major and continuous advances in analytical science and bioinformatics. While this paradigm for research is somewhat challenging to the traditional reductionist approach to biology, it has begun to be routinely used by many investigators.

Analogous to the concept of the genome or proteome, the metabolome can be defined as the complete description of metabolite levels in a biological system (Tweeddale et al. 1999). Seen from a geno-

centric perspective, it offers a holistic description of the metabolic phenotype for functional genomics studies (Raamsdonk et al. 2001). From a more integrated viewpoint, knowledge of the metabolome, in addition to gene expression and regulation, is a vital component of systems biology. In reality, the metabolome is also subject to major exogenous inputs in the form of exposure to pharmaceutical, environmental and dietary compounds, and in most multicellular organisms is manipulated by means beyond the host genome or proteome via commensural microbes (Nicholson et al. 2004, 2005). Thus the metabolome is a diffuse concept and the researchers in this area (metabonomics/metabolomics) tend to work with data that can be more readily recognised as metabolic profiles: descriptions of small molecule composition that are not necessarily comprehensive but are largely unbiased in scope and amenable to quantitative interpretation. Analytical techniques used to measure metabolites, such as NMR spectroscopy and mass spectrometry, can be used to generate such profiles in a targeted or untargeted manner, efficiently defining detectable portions of the metabolome. While obviously desirable, metabolic profiles need not be fully resolved and annotated, i.e. all metabolites defining the profile are identified a priori). Analytical profiles can be analysed directly by statistical pattern recognition to identify factors that correlate to exposure to known toxicants or to the presence or likelihood of disease, and hence target metabolite characterisation. This distinction between targeted and untargeted metabolic profiling has some parallels with the difference between bottom-up versus top-down strategies for systems biology.

Among the ideas that surround metabolic profiling, the concept of metabonomics (Nicholson et al. 1999) in particular embraces a top-down approach, and traditionally has exploited the analysis of biofluids such as urine or plasma, lending it towards efforts to understand integrative physiological and systemic change (Fig. 1). Also, as part of metabonomic studies, intact tissue is often analysed by magic-angle spinning (MAS) NMR spectroscopy, which provides observations that have some relevance to *in vivo* NMR spectroscopy (MRS/MRI). The combination of biofluid analysis and untargeted metabolic analysis makes metabonomics an ideal platform for translational biomarker research. Noninvasive or minimally invasive biomarkers derived from body fluids or that can be detected by imaging are inherently more practical to take from

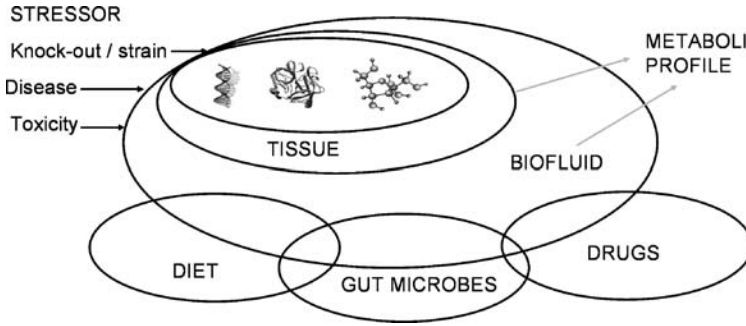

Fig. 1. Metabonomics works at the interface between an organism and its environment

the bench to the bedside. In addition, a metabolite is a defined chemical entity that is the same across all cell types, species and individuals, unlike gene products which change in sequence, splicing and are modified post-translation. This makes the analytical protocols used for metabolite detection fundamentally more likely to translate from models to humans and vice-versa.

Metabonomic profiles have been shown in principle to reflect the presence of pathological events in a number of disease models, including those for diabetes and metabolic syndrome (Dumas et al. 2007), infection (Wang et al. 2006), cancer (Al-Saffar et al. 2006; Bundy et al. 2006; Glunde et al. 2006; Teichert et al. 2008) and neurological disease (Tsang et al. 2006). Importantly, there are several key examples of metabonomics studies demonstrating the ability to detect the presence of, or potential for, disease in humans, namely atherosclerosis (Brindle et al. 2002; Makinen et al. 2007), cancer (Odunsi et al. 2005; Beger et al. 2006), schizophrenia (Holmes et al. 2006) and congenital defects in metabolism in infants, including those of unknown aetiology (Wevers et al. 1999). While the role of metabolism in the aetiology of disease processes such as metabolic syndrome is obvious, there are metabolic phenotypes for other pathologies such as neurological disease where the link is less clear, highlighting the potential of metabonomics to provide novel biological insight into already well-studied areas. Much of this new biology arises from the fact that metabolic profiles will not only be

determined by altered regulation of metabolic pathways and enzymatic activity, but also reflect environmental exposures and the functional integrity of cells, and tissues. Nongenomic factors already form the basis of most systemic biomarkers currently used in pathological reporting, particularly in toxicology.

2 Metabonomics in Toxicology

In toxicology, there are several ways in which new biomarkers can make an impact:

- By detecting otherwise silent pathologies;
- By being more translatable/relevant to humans;
- By predicting the individual susceptibility to an adverse event;
- By being less invasive and allowing response dynamics to be defined using less compound and fewer animals;
- By predicting traditional outcomes (i.e. acting as surrogate endpoints) and thus allowing risk and safety margins to be evaluated using less compound and fewer animals earlier in development;
- By revealing the mechanism leading to toxicity or the potential for toxicity and thus aid risk assessment.

There is a wealth of data in preclinical models demonstrating how the relationships between metabolic profiles and the severity, timing, site and mechanism of chemical toxicity could be exploited for all of these purposes (Robertson 2005; Keun 2006; Keun and Athersuch 2007).

Metabolic profiling can add significant value to the samples routinely generated by preclinical studies in drug discovery and development. In the context of such studies, endogenous metabolites are largely seen as interferences to the study of drug metabolites or other biomarkers of exposure. However, a substantial body of work has demonstrated that specific urinary metabolite changes could be associated with liver toxicity (Nicholson et al. 2002). Using model compounds, a number of particular biomarkers have been reported in metabonomic studies, including taurine for general liver dysfunction (Sanins et al. 1990); bile aciduria

for billiary toxins (Robertson et al. 2000); N-methyl nicotinamide for peroxisome proliferation (Connor et al. 2004), 5-oxoprolinuria for disruption to glutathione metabolism (Waters et al. 2006) and medium chain dicarboxylic aciduria for dysfunction of mitochondrial fatty acid metabolism (Mortishire-Smith et al. 2004). A combination of markers was also shown to give site-specific information with regard to nephropathy and was sensitive to the severity and recovery of the lesion (Gartland et al. 1990; Holmes et al. 1992; Anthony et al. 1994).

Information derived from metabonomics could provide toxicological input in lead selection and optimisation where the amount of compound available is relatively low. By using urine that might already be collected for metabolism studies and by sampling continuously, early hazards can be detected efficiently, i.e. without extended dosing using extra animals. Using a multivariate regression model, five structurally similar compounds could be ranked based on NMR urinalysis, revealing a specific interruption of renal choline uptake that was not detected using classical methods of assessing toxicity (Dieterle et al. 2006). In this instance, metabonomics was able to detect the potential for an adverse event prior to the appearance of histopathology as well as provide clues as to the mechanism of toxicity, thus aiding risk assessment. All else being equal, the compounds producing a normal metabolic profile could be the better candidates for further development.

An important factor in interpreting changes in metabolic profile is the time course or trajectory. Urine sampling is noninvasive and effectively allows continuous monitoring over time. As variation in the dynamics of these metabolic perturbations also coincide with variation in the rate and severity of toxicity between individual animals, metabolite trajectories are important for understanding the specificity of a biomarker response (Nicholson et al. 2002). In principle, even a single molecule could be affected by several processes throughout an experiment, such as an adaptive or stress response, the loss of function or compartmentation, or significantly, regeneration (Fig. 2). It is difficult to correlate such changes directly to other endpoints that may be undersampled or have completely different time courses such as histopathology or gene expression (Fig. 3). We may wish to filter out the other factors and focus on the adaptive changes that might be the most relevant for predicting the chronic outcome from an acute study. One way we can tackle the

problem is to look at the relationship between the time courses of several metabolites. Metabolites that share the same trajectories presumably reflect the same underlying process, and understanding the collection of metabolites that respond together can help to refine the definition of these processes. For example, it has frequently been observed that the excretion pattern of hippurate and the Krebs cycle intermediates are frequently coincident in toxicological studies (Fig. 4). While several explanations of this phenomenon could exist for any given treatment, such as a mitochondrial specific response, it is known that all these molecules are similarly reduced by reduced food intake (Connor et al. 2004). We can then use this correlation of metabolite excretion to infer the process occurring, even when the magnitude of the effect is different from study to study. We can also use the pattern of correlations as a model from which to discern deviation from the influence of these processes and begin to attribute further biological significance to metabolite changes.

Many of these key ideas were developed within the Consortium for Metabonomic Toxicology (COMET) project (Lindon et al. 2003, 2005), which during its initial phase between 2001 and 2004 generated a database of over 35,000 biofluid metabolic profiles from 147 exposures in rodents to toxicological and physiological stressors. These data mostly included acute exposures to a wide variety of liver and kidney toxicants, but physiological stressors such as food restriction and partial hepatectomy were also examined, as was toxicity at other target organs such as testicular and pancreatic toxicity. This project was able to demonstrate that metabonomic responses to toxicity were (a) robust analytically and biologically using a multi-centre approach and high-throughput profiling (Keun et al. 2002, 2004) and (b) were sufficiently specific to the site and mechanism of toxicity to allow detection and classification of adverse events using a statistical model alone, the COMET expert system (Ebbels et al. 2007).

A large element of the expert system was the efficient handling of multivariate data via pattern recognition techniques. It had been shown previously that techniques such as principal components analysis (PCA) were potentially very valuable in visualisation and classification of metabonomics data (Holmes et al. 1992; Keun et al. 2004). Within this approach is the implicit assumption that allegedly similar profiles represent similar states and hence the same responses to toxin expo-

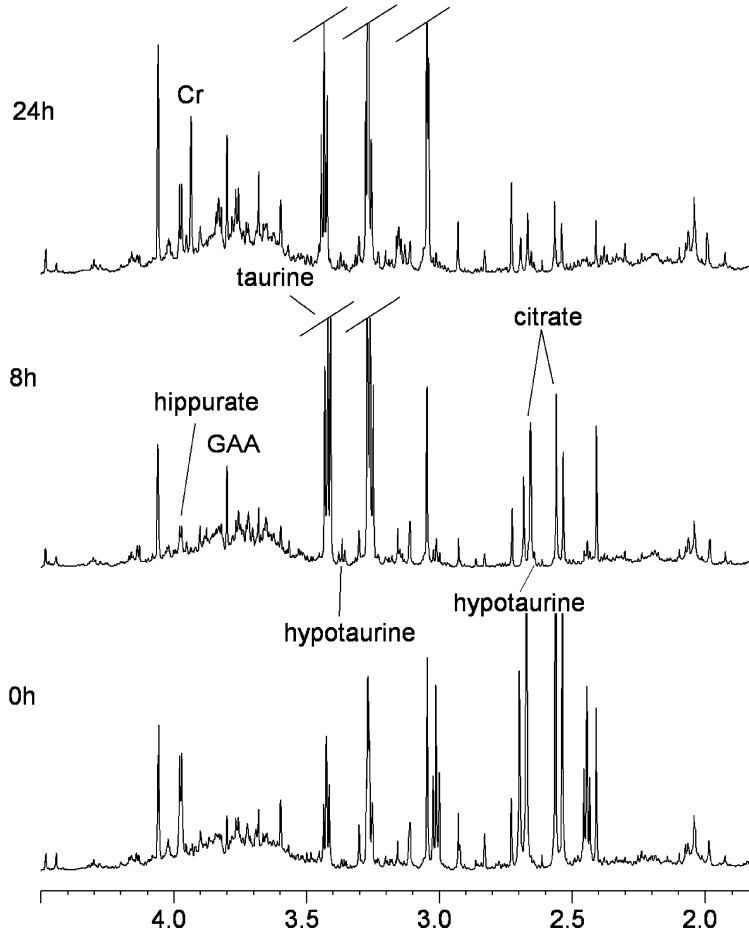

Fig. 2. Early effects to the aliphatic region of the ^{1}H NMR spectrum of rat urine after partial hepatectomy, a model of liver regeneration

sure, i.e. a compendium approach to toxicity classification. Putting this idea into practice across many studies required that highly multivariate metabolic trajectories be modelled. PCA allows trajectories to be visualised not in just one or two dimensions but using an infinite number

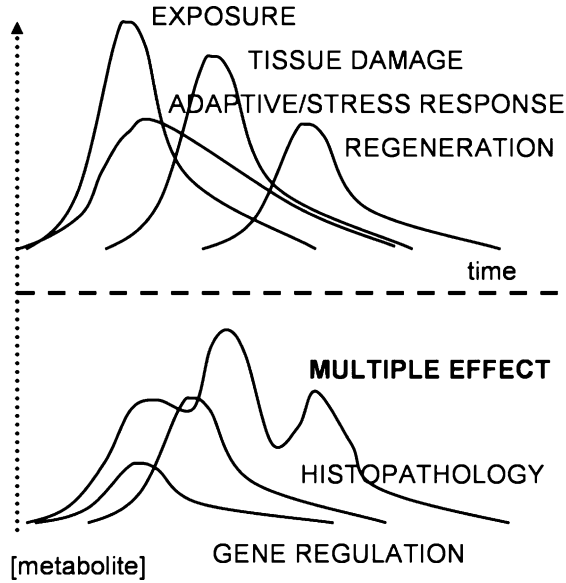

Fig. 3. Biomarker dynamics after acute toxin exposure

of measurements, summarising the variation into a lower dimensional space. It was subsequently shown that, after appropriate adjustment for the severity of response and the baseline metabolic profile, coincident metabolic trajectories result from the appearance of the same lesion via different chemical compounds (Keun et al. 2004). This led us to the homothetic trajectories hypothesis: it is the shape of the trajectory, i.e. how metabolite changes correlate to each other, that encapsulates the metabolic response in a manner best suited to classifying the toxicity.

For the COMET expert system, it was also necessary to compare trajectories easily and objectively. A nonlinear density estimation approach called CLOUDS was used (Ebbels et al. 2003). Related to Parzen density estimation, CLOUDS allows toxin-likeness to be assessed by superposition of trajectories. The overlap integral generated indicated the similarity of response while also taking into account the variability in response. In a training set of urinary NMR data from 80 studies, the

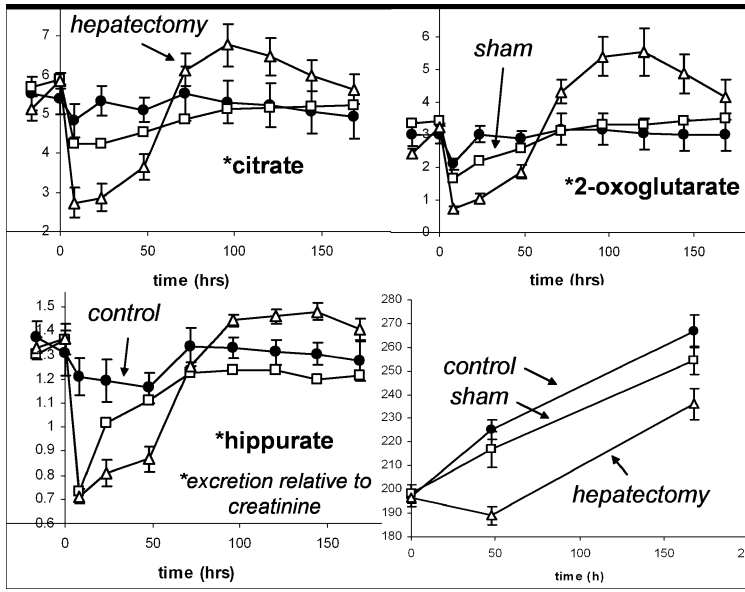

Fig. 4. Selected metabolite excretion and body mass trajectories after partial hepatectomy

approach was show to be able to cluster treatments according to target organ and even sub-organ specificity (Ebbels et al. 2007). In a predictive analysis it was possible to assign the correct target organ to the majority of treatments with 92% accuracy.

In a shift from observational to mechanistic application of metabonomics in toxicology the second phase of the COMET project will attempt to (a) establish biomarkers for renal papillary necrosis, an otherwise silent lesion and (b) define factors that contribute to the hypervariability of response to galactosamine (galN), a model for idiosyncratic hepatic toxicity. Metabonomic studies are already providing new insight into the protective role of glycine in galN toxicity, providing an example of the mechanistic role of the platform. In a ^{1}H NMR spectroscopic study, the level of *N*-acetylglucosamine (glcNAc) in the post-dose urine was found to correlate strongly with the degree of galN-induced liver

damage, while the urinary level of glcNAc was not significantly elevated in rats treated with both galN and glycine (Coen et al. 2007). Treatment with glycine alone was found to significantly increase hepatic levels of uridine, UDP-glucose and UDP-galactose. Uridine is also protective to galN toxicity, suggesting that the protective role of glycine against galN toxicity might be mediated by changes in the uridine nucleotide pool rather than by preventing Kupffer cell activation, as currently presumed.

In addition to correlating to the presence of toxicity, there is also evidence that metabolic profiles can directly predict the susceptibility of individual organisms to pathological events following toxicant exposure. Proof-of-principle studies have shown that the severity of drug hepatoxicity and drug metabolism in rodents can be predicted from pretreatment urinary metabolic profiles (Clayton et al. 2006), and a validation of the latter of these observations in a clinical trial is currently underway. In light of the analogous concept of pharmacogenetics, this model has been described as pharmaco-metabonomics. Interestingly, the prognostic urinary metabolites identified appeared to be diet-related compounds generated by commensural gut microbes, highlighting how metabonomics provides a unique viewpoint on extra-genomic interactions.

As metabolites from exogenous and endogenous sources are measured simultaneously in an unbiased manner, metabonomics is generally well suited to the generation of novel predictive biomarkers that link environmental exposures to human health via a meet-in-the-middle approach (Vineis and Perera 2007). The risk of developing cancer is clearly linked to dietary exposure to carcinogens, such as in the meat-derived heterocyclic amines (Gooderham et al. 2006), and exposure to chemopreventative agents, such as resveratrol found in red grapes (Aziz et al. 2003). Many more factors, either directly active toxicants or modulating agents could be discovered by a metabonomic approach, which will be a more common part of prospective biomarker studies, both epidemiological and clinical.

3 Metabonomics in Oncology

Irrespective of biomarker discovery, there is strong evidence for a common metabolic phenotype associated with cancer. As long ago as the 1920s, Otto Warburg described the phenomenon of aerobic glycolysis, the apparently greater tendency of tumour cells to convert glucose to lactate in the presence of normal oxygen conditions. Evidence exists to suggest that the glycolytic phenotype confers selective growth advantages to transformed cells (Gatenby and Gillies 2004) and the function of the tumour suppressor p53 has been linked to this phenomenon (Matoba et al. 2006). Other aspects of the tumour metabolic phenotype centre around the observation that growth of tumour cells in culture is often unusually dependent on the availability of common substrates, such as glutamine, methionine, cysteine and arginine (Wheatley 2005). In oncology, the altered metabolic phenotype of tumours is routinely exploited in diagnosis (e.g. FDG-PET) and in therapy (e.g. 5-FU, an antimetabolite). Metabolic profiling offers a number of opportunities for discovery and development of noninvasive biomarkers in cancer studies based on both screening and functional genomics strategies.

Metabolic profiles have been shown to be able to subclassify cancer phenotypes in number of solid tumours, including those of the brain (Tate et al. 2006), prostate (Cheng et al. 2005), ovary (Denkert et al. 2006) and breast (Katz-Brull et al. 2002). A key pathway involved in this discrimination is choline metabolism. The ubiquitous presence of elevated choline metabolites in tumour cells (Fig. 5) has been detected by magnetic resonance both *in vivo* and *in vitro*, translating across species and present across a wide range of primary and secondary tumour sites (Glunde et al. 2006). This, together with an increase in the phosphocholine/glycerophosphocholine (PC/GPC) ratio, has been shown to be a general marker for rapid proliferation and tumorigenicity, but in conjunction with other metabolite measurements could be predictive of the invasiveness of a tumour and useful in the clinical staging of disease. While effort continues to be invested in determining the mechanism behind this phenomenon, it would appear to be in part due to increased choline transport into the cell and upregulated choline kinase activity in response to the demand for phosphatidylcholine and membrane synthesis (Glunde et al. 2004, 2005). The observation has proved

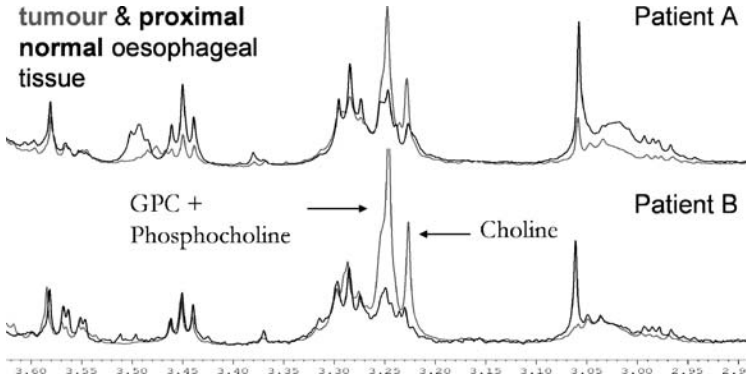

Fig. 5. Perturbations to choline metabolism detected by ^{1}H MAS-NMR spectroscopy of intact tissue

of particular value in the development of choline kinase inhibition as a therapeutic strategy, which has been shown to be successful in both HT29 and MDA-MB-231 xenograft models (Al-Saffar et al. 2006). This work tells an important story about the value of biomarker research via exploratory clinical studies in the translational research setting. Since the identification of choline kinase as a drug target has derived in part from the visible impact of its activity in tumours, we can immediately turn around the result and use the choline NMR signal as a pharmacodynamic marker, safe in the knowledge that it has clinical relevance (Fig. 6). It also provides a phenotypic anchor with which to help evaluate results in animal models.

We were interested in understanding how the metabolic phenotype of tumours in an autochonous model of prostate cancer (Transgenic Adenocarcinoma of Mouse Prostate; TRAMP) compared to the known human tumour profile. We found that while prostate-specific features of the human phenotype were preserved, such as a depletion of the unusually high levels of citrate in the prostate, the more general feature of elevated choline metabolites was not (Teichert et al., in press). Tumour tissue from the TRAMP model did not exhibit any upregulation of ChoK at either the transcriptional or protein level. These results helped to rationalise the lack of sensitivity of certain *in vivo* MRS parame-

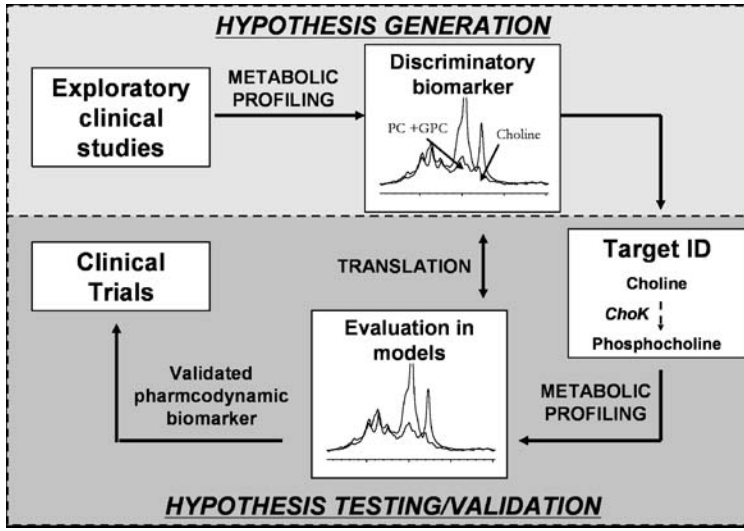

Fig. 6. The potential life cycle of a metabolic biomarker

ters such as the total choline/citrate ratio, which have known significance as a detection and progression marker in human disease, in the TRAMP model (Fricke et al. 2006). It is an example of how metabonomics can be used to evaluate preclinical models of cancer and suggests that the TRAMP model will behave differently pharmacodynamically to metabolically targeted therapies such as ChoK inhibition. These differences between the TRAMP tumour metabolic phenotype and the human disease may well originate from the specific form of oncogenic transformation (SV40 t & T antigen) used to generate the model that affects the products of p53 and Rb genes. It is interesting to note that the loss of the PTEN tumour suppressor, a more relevant event to human prostate cancer than the consequences of SV40 transfection, produces the expected metabolic response *in vivo* MRS data to suggest that it may be a more appropriate model for biomarker studies (Fricke et al. 2006). Such experiments do not only support biomarker development, but simultaneously further our understanding of the link between metabolism and malignant transformation.

To what extent the metabolic phenotype of cancer is causal or consequential to carcinogenesis and disease progression is still widely debated; however, key examples such as the pseudohypoxia effect of high succinate and fumarate levels in HIF activation show how metabolism can directly promote tumour development (Pollard et al. 2005). In light of this, metabolic profiling has clear potential to reveal new relationships between metabolic perturbation and transformation, and there is a good evidence base already available. Fibroblast cell lines progressively transformed from a primary to a cancerous state using telomerase, and oncogenic Ras show increasing sensitivity to glycolysis inhibitors while simultaneously becoming resistant to inhibition of oxidative metabolism. The choline metabolic phenotype is also mediated by both the loss of tumour suppressor function and oncogene activation, specifically p300 (Bundy et al. 2006), oncogenic Ras (Ratnam and Kent 1995), and telomerase (Iorio et al. 2005). While there are not yet clear examples of metabonomics predicting the future occurrence of cancer, there is *in vivo* evidence to support the hypothesis that some of the metabolic features of cancer can arise with premalignant transformation of cells. The presence of a biochemically abnormal field surrounding a tumour and either originating the neoplasm or caused by it can be defined by p53 mutation (Ito et al. 2005) and epigenetic changes (Ushijima 2007). It has been suggested that such a field could be detected by metabolic profiling, since correlations to the stage of disease were observed even in histologically normal tissue from patients with prostate cancer (Cheng et al. 2005).

Whether any of these effects manifest themselves in biofluids is not known, but clearly the discovery of systemic metabolic biomarkers specific to cancer is of enormous value in terms of patient management and detection screening. Although there is evidence that metabolic profiles of sera or serum lipids can detect the presence of ovarian (Odunsi et al. 2005) and pancreatic cancer (Beger et al. 2006), biomarker screening in serum or plasma has a difficult past with both NMR-based (Fossel et al. 1986; Okunieff et al. 1990) and SELDI-MS-based (Petricoin et al. 2002; Baggerly et al. 2005) protein marker profiles being challenged due to high normal variability and sample bias (Ransohoff 2005; Teahan et al. 2006). Thus despite the enormous potential of all "-omics" technologies, it is important to exercise some caution and to work hard

to avoid historical pitfalls. Even in exploratory clinical studies, it is valuable and probably necessary to rationalise any putative metabolite biomarker firmly in the context of the tumour metabolic phenotype.

Acknowledgements. I would like to thank Mr. Danny Yakoub and Dr. Mary Bollard for their help in the production of the figures, and Miss Alexandra Backshall for proof-reading the manuscript.

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