METABOLIC PROFILES OF CANCER CELLS

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In the post-genomic era, several profiling tools have been developed to provide a more comprehensive picture of tumour development and progression. The global analysis of metabolites, such as by mass spectrometry and high-resolution ^{1}H nuclear magnetic resonance spectroscopy, can be used to define the metabolic phenotype of cells, tissues or organisms. These 'metabolomic' approaches are providing important information about tumorigenesis, revealing new therapeutic targets and will be an important component of automated diagnosis.

Since the completion of the sequencing of the human genome, the main goals of functional genomics have been to determine the function of the products of newly identified genes, as well as to determine those that might be therapeutically targeted. So far, functional genomic strategies have largely centred on gene-expression studies (transcriptomics) or protein profiles (proteomics)[1–3]. These approaches have led to several successes in the field of cancer biology, such as the identification of new tumour subtypes, as well as transcriptional and protein biomarkers for certain types of cancer[4–8]. Metabolic activity can also be quantified, as various analytical tools have been developed to measure concentrations of low-molecular-weight metabolites (FIG. 1). This is a particularly challenging task as low-molecular-weight metabolites represent a diverse range of chemicals. Perhaps the best description of this approach was offered by Steve Oliver of Manchester University, who used the term 'metabolomics' to describe "the complete set of metabolites/low-molecular-weight intermediates, which are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism"[9]. This definition arose from the term that was originally used to describe a powerful tool for phenotyping yeast mutants[9].

Although '-omic' technologies are complementary, analysis of the metabolome is an especially useful approach for identifying pathways that are perturbed in a given pathology, when compared with the transcriptome and proteome. Measuring metabolite concentrations is a more sensitive approach than following the rates of chemical reactions directly. Metabolic control analysis has demonstrated that although changes in enzyme concentrations and activities ('the proteome') have a small impact on metabolic fluxes (the rate of which material passes through a given metabolic pathway), changes in flux have a significant impact on metabolite concentrations[10–12]. This is because the control of the metabolic flux of a pathway is spread across all the enzymes present in the pathway, rather than being controlled by a rate-determining step. Furthermore, there is not necessarily a good quantitative relation between mRNA concentrations and enzyme function, but as metabolites are downstream of both transcription and translation, they are potentially a better indicator of enzyme activity[13]. So, metabolomics offers a particularly sensitive method to monitor changes in a biological system, through observed changes in the metabolic network.

Metabolomic strategies present several practical advantages, including being relatively cheap on a per-sample basis, high throughput and fully automated. For example, after the initial purchase of a nuclear magnetic resonance (NMR) spectrometer or mass spectrometer, (MS) samples can be analysed for a cost of about US $1 apiece, with analytical acquisition times typically taking 10 minutes (NMR) to 30 minutes (MS-based approaches). This compares very favourably with transcriptional and proteomic approaches. Furthermore, the metabolome of one species can easily be compared with another. Whereas

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Summary

- Metabolomics is the study of the complete metabolic compliment of the cell, organ or organism.
- The technique involves the combined use of multivariate statistics and an analytical technique such as nuclear magnetic resonance spectroscopy, gas chromatography–mass spectrometry or liquid chromatography–mass spectrometry.
- A wide range of metabolites have been shown to be useful in distinguishing tumours from healthy tissue and in monitoring cellular activities such as cell-cycle progression or apoptosis.
- Metabolomic approaches have been used to study the function of hypoxia-inducible factor 1 in tumour growth and shown that this transcription factor is involved in increasing glucose metabolism, rather than inducing angiogenesis, in hepatomas.
- *In vivo* studies have shown that magnetic resonance spectroscopy can be used to identify tumour types, especially brain tumours, by their metabolic profiles.
- As both nuclear magnetic resonance spectroscopy and mass spectrometry are high-throughput technologies, these tools can be used to profile systemic metabolism in tumour diagnosis and prognosis, through analysis of urine and blood plasma.

gene and protein sequences vary between species, many metabolites are conserved between species, and the analytical tools used to detect these in one organism can be applied to another without the need for recalibration.

Understanding disease processes through metabolic profiling is not a new concept — ^{31}P, ^{1}H and ^{13}C NMR spectroscopy, along with gas chromatography–mass spectrometry (GC–MS), have been widely used as metabolic profiling tools since the early 1970s[14–16] (BOX 1). NMR spectroscopy has also been used to differentiate between different cancer cell lines[17,18] and to monitor metabolic processes that occur in cancer cells during events such as apoptosis[19–21]. Metabolomics differs, however, in that rather than analysing a single class of compounds, it involves an attempt to measure all the metabolites that are present within a cell simultaneously. However, because of immense technical challenges, many studies still have a long way to go towards this ideal. Using NMR-based approaches, 20–40 metabolites can typically be detected in tissue extracts, and 100–200 can be detected in urine samples. Using the more sensitive approach of GC–MS, around 1,000 metabolites can be detected in these samples types (BOX 1). Various other analytical tools have also been used for metabolomics — these are listed in TABLE 1. Multivariate pattern-recognition techniques are then used to distinguish normal metabolic variations from those patterns induced by a disease process, a genetic manipulation or a drug intervention (BOX 2). Furthermore, as multivariate analyses are 'black-box' approaches, often it is not necessary to identify all the metabolites detected to classify a sample.

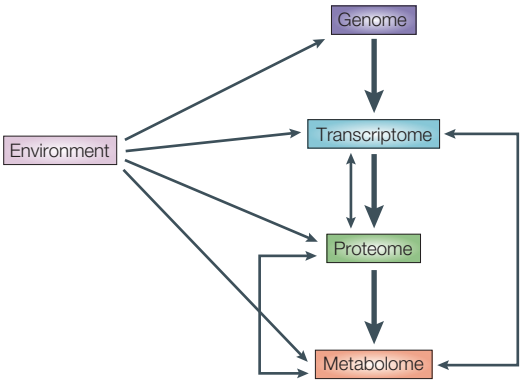

Figure 1 | **The biological organization of the '-omes'.** The classical view of biological organization is to consider the flow of information from the genome to the transcriptome, to the proteome and then the metabolome. However, each tier of organization depends on the other, so a perturbation in one network can affect another. Furthermore, the environment has a crucial impact on not only expression and concentrations of transcripts, proteins and metabolites, but also on the genome by selecting for adaptive changes in subpopulations of cells within a tumour. Metabolomics can potentially probe much more than classical metabolism and metabolic disorders — it can also be used to monitor changes in the genome or to measure the effects of downregulation or upregulation of a specific gene transcript.

Such approaches have found applications in phenotyping mutant and transgenic yeast, plants and mice[22–25], as well as assessing drug efficacy and safety[26,27]. Part of the success of metabolomics as a tool in toxicology is the ability to readily identify temporal changes in a cellular or organism's phenotype, making the approach ideal for following rapidly changing phenotypes. The use of metabolic profiling to follow systemic changes in multicellular organisms has led Jeremy Nicholson to coin the word 'metabonomics'. He defines metabonomics as "the quantitative measurement of the multivariate metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification"[27]. In addition to the terms 'metabolomics' and 'metabonomics', researchers have felt it necessary to distinguish the types of analytical techniques used in these approaches[28]. 'Metabolic profiling' has been proposed as a means of measuring the total complement of individual metabolites in a given biological sample, whereas 'metabolic fingerprinting' refers to measuring a subclass of metabolites to create a 'bar code' of metabolism[23,24]. In this approach, only a limited number of metabolites are quantified and used to distinguish between different samples, such as those of different disease or physiological states. However, there is significant overlap in the definitions and uses of these terms, and throughout this review the term 'metabolomics' will be used for all approaches in which an analytical tool is used in conjunction with pattern-recognition approaches to follow metabolic changes in a biofluid, tissue or organism.

Despite the successful use of metabolomics to investigate phenotypes of transgenic animals and plants, and its use in the pharmaceutical industry, most functional genomic studies of cancer have focused on transcriptomics and proteomics. However, metabolomics can be used to monitor tumour growth and regression, and can therefore increase our understanding of pathogenic mechanisms as well as improve monitoring of treatment regimens. It has already been used to analyse the function

Box 1 | **The main tools used in metabolomic studies**

Nuclear magnetic resonance (NMR) spectroscopy

Certain isotopes possess the property of magnetic spin, causing their nuclei to behave in a similar manner to a tiny bar magnet. When they are placed in a magnetic field, the magnets either align or oppose the external magnet field. By applying a radiofrequency to the nuclei, one can cause the nuclei to flip into the other magnetic state and the differences in the populations between these two magnetic energy states can be detected as a radiowave as the system returns to equilibrium. NMR analysis for metabolomics has centred on ^{1}H and ^{13}C NMR spectroscopy, although ^{31}P NMR spectroscopy has been used to measure high-energy phosphate metabolites and phosphorylated lipid intermediates. Although this is a relatively insensitive technique, the approach can be used in a non-invasive manner, making it possible to metabolically profile intact tissue using either high-resolution magic angle spinning ^{1}H NMR spectroscopy of small pieces of intact tissue[25,31] or *in vivo* spectroscopy of the whole organ[72]. Current detection limits for ^{1}H NMR spectroscopy are of the order of 100 μM in a tissue extract or biofluid. Typical acquisition times are about 10 minutes. NMR-spectroscopy analysis of biofluids has been shown to be highly reproducible, as samples analysed by this method have produced similar results to those measured on other types of spectrometers[73].

Gas chromatography– and liquid chromatography–mass spectrometry (MS)

Both approaches involve an initial chromatographic stage in which metabolites are separated either in the gas or solution phase, respectively. Subsequently the metabolites are ionized and then separated according to their mass to charge ratio, which can be used to identify the metabolites. MS-based approaches are more sensitive than NMR spectroscopy, and so can potentially detect metabolites at a concentration two orders of magnitude below that of NMR. However, not all metabolites can be ionized (converted to a positively or negatively charged species suitable for mass spectrometry) to an equal extent, potentially biasing the information produced. This approach is the method of choice for plant metabolomics[23,24] where the challenge of profiling all the metabolites in a given tissue is even greater than that in mammals and yeast. In spite of the fact that plant genomes typically contain 20,000–50,000 genes, 50,000 metabolites have been identified in the plant kingdom with the number predicted to rise to about 200,000 (REF. 74), compared with 30–600 metabolites identified in mammalian cells. The current detection limits for MS-based approaches are of the order of 100 nM, allowing the detection of about 1,000 metabolites, with typical acquisition times of about 30 minutes.

of hypoxia inducible factor-1β (HIF-1β) in tumours and to monitor the progression of therapy-induced apoptosis in gliomas. Looking to the future, the technology could be applied as a minimally invasive screening technique and as a bioprospecting tool to identify new anticancer drugs.

Metabolomic profiles of cancer cells

NMR spectroscopy, including *in vivo* magnetic resonance spectroscopy (MRS) and high-resolution solution-state analysis of tissue extracts, have been widely used for several years to distinguish between different cell lines and tumour types. Although NMR spectroscopy detects only a fairly small number of metabolites, it can still be used to monitor the activity of many cellular activities, because so many metabolic pathways are connected. So changes detected in the metabolome can be used to follow several seemingly unrelated pathways. MRS has been used to analyse several tumour types in humans and in animal models of cancer[29,30], and despite limitations in sensitivity and the ability to measure a broad range of metabolites, metabolomic profiles have been successfully used to distinguish between tumours types and between cell lines.

In vitro metabolomic studies have also demonstrated several differences between tumour types, in terms of various biochemical pathways[17,18] (TABLE 2). One technique, called high-resolution magic angle spinning (HRMAS) ^{1}H NMR spectroscopy, can produce high-resolution spectra from intact tissue[31–34]. A biopsy or post-mortem sample of intact tissue is spun at an angle to the applied magnetic field. The spinning results in a significant improvement in the resolution of the spectrum obtained. This approach has several advantages over NMR spectroscopy of tissue extracts. Both aqueous and lipid-soluble metabolites can be observed simultaneously *in situ*, whereas solution-state NMR would require separate extraction procedures. One of the first applications of this technique was to distinguish between normal lymph nodes and those that contained malignant cells[31]. Information about the metabolic environment of the tumour can also be obtained using HRMAS ^{1}H NMR spectroscopy, which can be used to identify metabolites with a range of physical properties. These approaches have also been used to follow the effects of various therapeutics on tumour cells *in vitro* and *in vivo*.

Metabolomics has been used to study the changes that occur in cancer cells in hypoxic regions of tumours, which develop when a tumour outgrows is vasculature. In these studies, the effects of HIF-1β deficiency on tumour metabolism and growth were anaylsed *in vivo* and *in vitro* using NMR spectroscopy[35,36]. The HIF-1 transcription factor is a heterodimer that consists of HIF-1α and HIF-1β subunits[37,38]. HIF-1 is upregulated in several cancer cell types, resulting in the increased expression of proteins involved in a range of metabolic pathways, including glucose transporters, glycolytic enzymes and growth factors such as vascular endothelial growth factor (VEGF). So the rate of glycolysis is increased in these tumours, along with angiogenesis induction.

HIF-1β-deficient hepatoma cells grown as solid tumours in mice were found to have reduced rates of growth compared with wild-type hepatoma cells. It was not clear, however, whether this was a result of

Table 1 | **Different spectroscopic methods used in metabolomic analysis**

Technique	Description	Advantages	Disadvantages
Fourier-transform infrared (FT-IR) spectrometry	Uses vibrational frequencies of metabolites to produce a fingerprint of metabolism	Cheap and good for high-throughput first screening; used to differentiate yeast respiratory-chain mutants from wild-type strains[77]	Very difficult to identify which metabolites are responsible for causing changes; very poor at distinguishing metabolites within a class of compounds
Gas chromatography–mass spectrometry (GC–MS)	The method of choice for plant metabolomics; uses gas chromatography to separate metabolite mixtures prior to mass spectrometry to identify the different metabolites	A relatively cheap and reproducible method that also has a high degree of sensitivity	Sample preparation can be time consuming; not all compounds are suitable for gas chromatography
Liquid chromatography–mass spectrometry (LC–MS)	A similar approach to GC–MS, except separation occurs during liquid chromatography	This method is increasingly being used in place of GC–MS as sample preparation is not as time consuming; similar in sensitivity to GC–MS	More costly than GC–MS and depends on the reproducibility of the liquid chromatography (potentially more difficult to control than gas chromatography); also can suffer from ion suppression, where metabolites are poorly ionized when in the presence of cations and anions
Metabolite arrays	These devices use a 96-well plate assay system for phenotyping; such arrays have been used to phenotype *Escherichia coli* by 700 different assay mixtures ('assay-on-a-chip')[78]	Good as a screening tool when produced for a given situation	The number of metabolites that can be measured is limited by the number placed on the chip; difficult to screen for unknowns and follow metabolism of xenobiotics
Nuclear magnetic resonance (NMR) spectroscopy	This approach has been widely used by the pharmaceutical industry and in the screening of patient urine and blood plasma samples	A non-invasive technique — the use of NMR spectroscopy demonstrates that metabolomic analysis of tissues in humans is possible; it can be fully automated and has a high degree of reproducibility; relatively easy to identify metabolites from simple one-dimensional spectra	Lower sensitivity than mass spectrometry; co-resonant metabolites can be difficult to quantify; drug metabolites can be co-resonant with metabolites of interest
Raman spectroscopy[79]	An extension of FT-IR and ultraviolet/visible-light spectroscopy; relies on light scattering following irradiation with a laser	Has the advantage over FT-IR in that water has only a weak Raman spectrum and, therefore, many functional groups can be observed (for example, better distinction of carbon–carbon bonds)	Very difficult to determine which metabolites are responsible for causing changes; very poor at distinguishing classes of compounds
Thin-layer chromatography (TLC)	Used to follow the metabolic fate of ^{14}C-glucose in *E. coli* under different culture conditions[80]	Inexpensive	Inter-assay variation, limited in terms of the metabolites that can be quantified

the reduced vascularity because of decreased tumour production of VEGF, or the failure to increase the rate of glycolysis flux because of a lack of upregulation of the glucose transporters (GLUT1 and GLUT3). Intriguingly, T_2 RELAXATION MEASUREMENTS made by magnetic resonance imaging (MRI) and post-mortem histological analysis showed no difference in vascularity between the wild-type and HIF-1β-deficient tumours[35]. However, in terms of high-energy phosphate metabolism, the mutant tumours had a fivefold decrease in total ATP content and a threefold increase in the ratio of phosphodiester (PDE) to inorganic phosphate (P_i). The increased ratio of PDE to P_i indicates an increase in phosphorylated cell-membrane constituents, whereas the decrease in total ATP indicates a reduction in the bioenergetic status of the tumour. So the primary contribution of HIF-1 to the growth of this tumour type seems to be an increased rate of glucose metabolism, rather than the induction of angiogenesis. This example illustrates how metabolomics can be used to monitor the functions of a gene product in tumours.

Further experiments on HIF-1β-deficient and wild-type hepatoma cell extracts were performed using NMR-based metabolomic analysis. This approach demonstrated a significant decrease in phosphocholine, choline, betaine and glycine[35,36], indicating the cause of the reduced ATP content in the mutant cells. Glycine is formed from the glycolytic intermediate 3-phosphoglycerate and is an important source of one-carbon units for the synthesis of nucleotides, through serine. So if HIF-1β-deficient tumours had a reduced rate of glycolysis and, therefore, glycine content, this would impair nucleotide synthesis and reduce the concentration of ATP. Glycine can also be produced through choline and betaine, which explains the decreases detected in these metabolites. Furthermore, increased choline metabolism would produce an increased turnover in lipid membranes, explaining the high ratio of PDE to P_i detected. Therefore, metabolomic studies of this type might be used to identify metabolic pathways that could be targeted therapeutically, to undermine the bioenergetic status of the tumour.

T_2 RELAXATION MEASUREMENTS The nuclear magnetic resonance (NMR) signal decays by several physical processes, one of which is T_2 relaxation. This rate of relaxation is faster for metabolites that are slowly moving in the cell. NMR analysis can exploit this property, to selectively detect fast-tumbling molecules, which include many of the metabolites that are found in the cytosol. These spectra are referred to as 'T_2 weighted'.

Box 2 | **Multivariate statistics**

Pattern-recognition tools are a vital part of the process of metabolomics and are being used to fully analyse the large multivariate data sets that are produced by other '-omic' technologies[75,76]. Both unsupervised and supervised techniques can be used to derive metabolic profiles. Supervised techniques use the information of class membership, such as disease status, to classify a given data set and therefore should be tested by data that are not used to build the pattern-recognition model. To investigate the innate variation in a data set in an unsupervised manner, techniques such as principal components analysis or hierarchical cluster analysis have been applied. However, in situations in which specific questions are being posed, supervised techniques might be more appropriate to either force classification (such as in determining which metabolites distinguish between groups) or regress a pattern against a trend (such as correlating a temporal progression with metabolic changes). Methods for supervised pattern recognition include prediction to latent structures through partial least squares, genetic programming and neural networks. Supervised approaches, such as orthogonal signal correction, can also be used as a means of data filtering. For all the supervised techniques it is necessary to test the sensitivity and reproducibility of the models produced, although the biological function of the metabolites identified can also indicate the success of the particular pattern-recognition tool.

In addition to analysis of glycolysis, several studies of tumour metabolism have focused on lipid metabolism, particularly during apoptosis and necrosis[19,20,39]. One such study involved a combination of *in vivo*, *in vitro* and HRMAS ^{1}H NMR spectroscopy in conjunction with principal components analysis (BOX 2) to examine polyunsaturated fatty acids (PUFAs) that accumulate in BT4C gliomas in rats during gene-therapy-induced apoptosis[21]. In this study, apoptosis was induced in rat gliomas that carried a herpes simplex thymidine kinase following administration of ganciclovir. Gliomas were analysed post mortem using both MRS and HRMAS ^{1}H NMR spectroscopy. This analysis demonstrated that the concentration of PUFAs increased threefold, as determined by increased CH=CH and CH=CHCH$_2$CH=CH resonances (FIG. 2). This increase in PUFA levels, which was confirmed chromatographically using lipid extracts of tissue, was much more pronounced than the general increase in lipids previously detected in apoptotoic cells, indicating that apoptosis in glioma cells might be specifically associated with an increase in the amount of unsaturated lipids. Furthermore, by examining the LINE WIDTHS of the resonances of these lipids under different physical conditions, such as temperature and SPINNING RATE, these lipids did not seem to be membrane associated. These biophysical data, alongside NMR measurements of the diffusion rates of these lipids, indicate the PUFA lipids were most likely to be the constituents of cytoplasmic lipid vesicles. These cytoplasmic lipids are easy to detect *in vivo* using MRS and could be used in the future to monitor the efficacy of gene therapy in patients with glioma. Furthermore, although histology and TUNEL STAINING could be used to follow the rate of apoptosis in excised tumours, the changes observed using NMR spectroscopy revealed specific pathways by which glioma cells undergo apoptosis. It remains to be determined whether this characteristic rise in polyunsaturated lipids is a feature of other types of cancer cells as they undergo therapy-induced programmed cell death.

LINE WIDTHS
The distance between the two sides of a NMR signal (resonance) at the half height of the resonance. Each resonance will have a line width that is inversely dependent on the rate of T_2 relaxation for that resonance. Therefore, metabolites that are slowly moving have broad line widths.

SPINNING RATE
During high-resolution magic angle spinning ^{1}H nuclear magnetic resonance spectroscopy experiments samples are spun at an angle (the so-called magic angle) to the magnetic field to reduce line-broadening effects.

TUNEL STAINING
(Terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling). A procedure for identifying apoptotic cells based on the detection of DNA cleavage.

One challenge to the use of one-dimensional ^{1}H NMR spectroscopy to investigate changes in lipid biochemistry is that this tool distinguishes the chemical groups present — for example, the total amount of saturated lipid — rather than the actual chain length of the lipids contained in the sample. This complicates the process of delineating which metabolic pathways are perturbed during apoptosis. However, using two-dimensional NMR techniques, in conjunction with HRMAS ^{1}H NMR spectroscopy analysis of tumour lipid extracts, the PUFA resonances observed in the glioma study were shown to result from the accumulation of 18:1 and 18:2 lipids. These lipids were likely to be released as part of the apoptotic process during the breakdown of membrane-bound vesicles. For example, disruption of the mitochondrial membrane would release cardiolipin, a lipid that is rich in PUFAs. So, monitoring the early stages of PUFA accumulation, using MRS, could provide a non-invasive way to monitor the early effects of cancer treatment in patients *in vivo*.

One shortcoming of this approach is that in regions of the body outside the brain, it is difficult to detect subtle changes in lipid profiles, because of the presence of fatty tissues. Nonetheless, in analysis of breast ductal carcinomas, which are surrounded by tissues that contain large amounts of lipid, metabolic profiles that were derived from HRMAS ^{1}H NMR spectroscopy were shown to accurately distinguish between normal tissue and carcinomas. This was done by measuring alterations in the T_2 relaxation times of cellular metabolites, which revealed that the concentrations of phosphocholine, lactate and various lipids were correlated with histopathological grade of tumours[40].

This metabolomic approach has also been used to analyse changes in low-molecular-weight metabolites following apoptosis induction in the same rat glioma model[41]. The metabolites investigated in this approach included choline-containing metabolites, amino acids, organic acids, osmolytes, nucleotides and sugars. Both *in vivo* and *in vitro* NMR-spectroscopy studies demonstrated that *myo*-inositol, glycine and taurine concentrations were correlated with the cell density of tumours, whereas the overall concentration of choline-containing compounds was unaffected by cell loss. However, analysis of the same tumours using HRMAS ^{1}H NMR spectroscopy revealed separate peaks of choline, glycerophosphocholine, phosphocholine, taurine and *myo*-inositol. Furthermore, when these choline-containing metabolites were measured using the clinical MRS systems, their resonances were detected as a single composite peak. So, although several MRS studies have reported changes in levels of choline-containing metabolites, it is difficult to determine which specific metabolites are altered — this is a key problem with the interpretation of *in vivo* data.

The analysis of intact tissue by HRMAS ^{1}H NMR spectroscopy in conjunction with pattern-recognition techniques has also been used to study cervical biopsies. Metabolomic profiles derived from this technique have

Table 2 | **Metabolic biomarkers of tumours**

Metabolite*	Metabolic function	Associated tumours/characteristics
Alanine	In conjunction with lactate, increases in tissues during hypoxia; made by transamination of pyruvate to prevent further increases in lactate[81]	Hepatoma and brain tumours, including astrocytomas, metastases, gliomas, meningiomas and dysembryoplastic neuroepithelial tumours
Saturated lipids	An important constituent of cell membranes, although membrane lipids are poorly resolved by NMR; lipid peaks detected by NMR are believed to either be present in cell-membrane microdomains or in cytoplasmic vesicles	Alterations in levels associated with proliferation, inflammation, malignancy, necrosis and apoptosis[20,29,33,39,82]
CCMs	Include choline, phosphocholine, phosphatidylcholine and glycerophosphocholine; these are key constituents of cell membranes	Levels change during apoptosis and necrosis; the tumour types that these changes have been found in include brain, sarcomas, prostate and hepatoma[31–34]
Glycine	An amino acid and an essential precursor for *de novo* purine formation	Decreased following disruption of the HIF-1 signalling pathway[36]
Lactate	An end product of glycolysis	Increases rapidly during hypoxia and ischaemia; poorly vascularized tumours have a low intracellular pH as a result of increased lactate production; increased rates of lactate production have been associated with a range of tumours and, in particular, certain types of neoplasms[83]
Myo-inositol	Involved in osmoregulation and volume regulation	Increased in colon adenocarcinoma, glioma, schwannomas, ovarian carcinoma, astrocytoma and endometrial cells[43,55]; decreased in breast tumours[84]
Nucleotides	Used to manufacture DNA and RNA; also key metabolic intermediates in fatty-acid and glycogen metabolism; changes in ATP concentration also indicate the energetic status of the tumour	Increased in glioma during apoptosis[41]; CDP-choline is also increased during apoptosis[19,85]
PUFAs	Constituents of cell membranes, especially mitochondrial	Increased in glioma cells undergoing apoptosis[20,21], and in dedifferentiated and pleomorphic liposarcomas[86]
Taurine	Important in osmoregulation and volume regulation; hypotaurine is also an antioxidant and might protect cells from free-radical damage	Increased in squamous-cell carcinoma[87], prostate cancer and liver metastasis[88]

*Metabolites identified by NMR spectroscopy of tissue extracts or magnetic resonance spectroscopy *in vivo*. CCM, choline-containing metabolites; CDP-choline, cytidine diphosphocholine; HIF-1, hypoxia-inducible factor 1; NMR, nuclear magnetic resonance; PUFAs, polyunsaturated fatty acids.

correlated with histopathology results. For example, lactate concentration was associated with metastatic potential[42]. Features such as choline, amino-acid and glucose levels were also correlated with patient outcome.

Metabolomic analysis is therefore a promising approach to identify biomarkers that could be used in the non-invasive monitoring of anticancer therapies, particularly those that induce apoptosis. This could also be used as a non-invasive tool for monitoring tumour progression in animal models, which can not be achieved using histology, transcriptomics or proteomics. One drawback is that the number of metabolites that can be detected *in vivo* is relatively small at present, making it difficult to determine exactly which metabolic pathways underlie a detected alteration.

Pattern recognition

Although there are many different approaches to collecting metabolic profiles of cells and tumours, pattern-recognition software is needed to associate specific profiles with different cell types, tumour types or a stage of treatment. Large sets of MRS spectra, analysed using intelligent pattern-recognition systems, have already been used in patient diagnosis for various tumour types for more than a decade[43–48]. Furthermore, these approaches have also been used to identify 'metabolic fingerprints' associated with breast and brain tumours[49,50]. Gray and colleagues used an approach based on genetic programming to classify biopsy samples of human tumours[49]. This approach has the advantage over technologies that are used at present, which are based on NEURAL NETWORKS, in that it is able to classify metabolites such as glutamine, glutamate and alanine, rather than weighting a large series of resonance intensities. Similarly, Gribbestad and colleagues have been able to identify low-molecular-weight markers, including increases in choline and reductions in glucose levels, for breast tumours[50]. These studies, however, just scratch the surface of the metabolic network, focussing on high-concentration metabolites that dominate the NMR spectra. However, they do provide proof of principal that metabolomic profiles can be applied to tumours, and the sensitivity of this approach will increase as research progresses.

Several cross-centre studies, such as the Interpret NMR database, are being developed as part of a collaboration between European hospitals to store and analyse metabolic profiles of tumours[46,51–53]. An automated pattern-recognition approach has also been implemented to help radiologists categorize MRS data of brain tumours according to histological type and grade. Using metabolic profiles, this system can successfully distinguish meningiomas, low-grade astrocytomas and aggressive tumours such as glioblastomas and metastases. This pattern-recognition process was even successful at predicting tumour type using data acquired at separate hospitals using different model

NEURAL NETWORKS
Pattern-recognition processes that iteratively search for the best solution using a network construction that is similar to neurones in the brain.

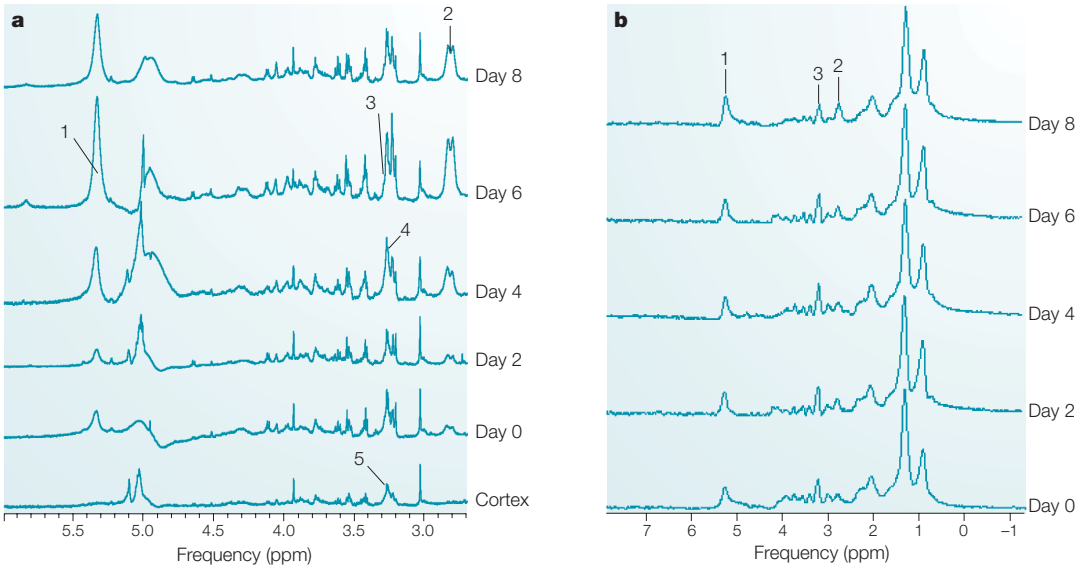

Figure 2 | **An *in vivo*, *in vitro* and *in situ* study of apoptosis in tumours.** One of the main benefits of nuclear magnetic resonance (NMR) spectroscopy is its versatility. These spectra are taken from a study of apoptotic cells in rat glioma. Spectra obtained from excised tumours using high-resolution magic angle spinning ^{1}H NMR spectroscopy (**a**) were compared with spectra acquired *in vivo* (**b**), to identify the key metabolic events that accompanied apoptosis. The main metabolic changes were associated with polyunsaturated lipids (peak 1, CH=CH; peak 2, $CH=CHCH_2CH=CH$) and choline levels (peak 3, phosphatidylcholine; peak 4, phosphocholine; peak 5, choline). The *x* axis represents the chemical shift range displayed and the *y* axis indicates the time after initiation of gene-therapy-induced apoptosis in tumours. Spectra reproduced with permission from REF. 21 © (2003) American Association for Cancer Research.

MRI instruments. So, pattern-recognition algorithms are less sensitive to acquisition parameters than had been expected.

These findings provide support for the creation of central databases for high-resolution metabolomic data, which would make it possible for researchers at different locations to analyse and cross compare data using different platforms. Such databases are already being generated for transcriptional and proteomic data, allowing the rapid transfer of information between research sites. The development of metabolomic databases will also lead to the production of better predictive software packages, allowing this type of data to be used in patient prognosis and diagnosis.

Drug development

Metabolomic tools can be used to monitor the effects of anticancer drugs, and also to detect specific genetic alterations in tumour cells. Pattern recognition models and NMR spectroscopy have been used for several years to follow metabolic changes that occur in tumours in response to therapy. For example, neural networks have been used to identify metabolic profiles of chemotherapy-resistant gliomas in patients before treatment[54]. In this regard, metabolic profiles could be used to predict which tumours are most likely to respond or become resistant to a specific type of therapy.

In a similar manner, high-resolution MAS ^{1}H NMR spectroscopy of intact Ishikawa cells was used to investigate the action of tamoxifen and other specific oestrogen-receptor modulators (SERMs). Ishikawa human endometrial adenocarcinoma cells are hormone responsive and are therefore ideal for investigating drugs that modulate the oestrogen receptor[55]. This study collected metabolic fingerprints, made up of about 20 metabolites, in intact cells and generated pattern-recognition models that correlated metabolic changes with varying doses of different SERMs[56]. FIG. 3 shows the advantages for using several pulse sequences to identify metabolites. By making T_2 relaxation measurements and using this process to weight the spectra, the contribution of broad resonances, such as those from lipids, can be reduced, allowing the detection of CO-RESONANT, low-concentration metabolites. The metabolites analysed in this model included ethanolamine, *myo*-inositol, uridine and adenosine, revealing alterations in both membrane turnover and transcription levels (TABLE 2). Furthermore, the metabolic effects of other oestrogen modulators could be monitored using this pattern-recognition model. This identification of specific metabolomic fingerprints that are associated with various drug types and dosages will allow researchers to determine how well certain tumour cells respond to different doses of drugs such as tamoxifen.

Metabolomic approaches have also been used to identify surrogate biomarkers for the pharmacodynamic monitoring of tumour responses to therapy in human colorectal xenografts grown in mice[57,58]. The drug 17-allylamino-17-demethoxygeldanamycin prevents tumour-cell growth by inhibiting the action of heat-shock protein 90. Although the exact *in vivo* mechanism of action of this drug is yet to be determined, ^{31}P NMR spectroscopy and *in vitro* metabolomic analysis have indicated that one of its functions is to perturb

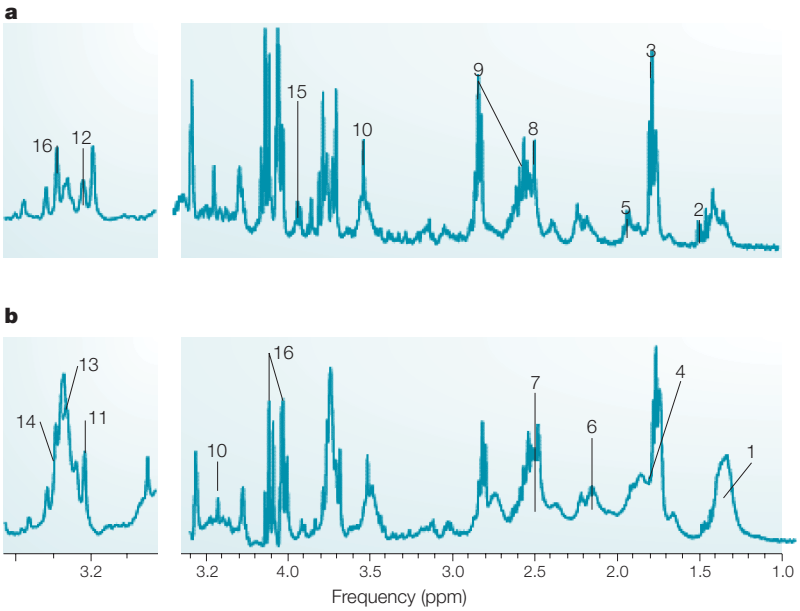

Figure 3 | **Monitoring the action of tamoxifen on endometrial cells.** High-resolution magic angle spinning 1H NMR spectroscopy was used to analyse endometrial cells that were treated with tamoxifen. This approach allows the use of pulse sequences that can measure the effects of the cellular environment. **a** and **b** show the aliphatic region of two T_2-weighted spectra. Spectra were acquired with 10 ms (**a**) and 320 ms (**b**) total spin echo time. This sequence does not show large lipid resonances and macromolecules, improving the detection of aqueous metabolites. T_2-weighted spectroscopy allowed the monitoring of nucleotide metabolism in these cells. The increase in a number of nucleotides indicated that tamoxifen increased the proliferative capacity of the endometrial cells. Peaks are numbered as follows: peak 1, CH_3CH_2 lipid groups; peak 2, leucine, valine and isoleucine; peak 3, lactate; peak 4, CH_2CH_2 lipid groups; peak 5, alanine; peak 6, $COCH_2CH_2$ lipid groups; peak 7, $C{=}CHCH_2CH_2$ lipid groups; peak 8, acetyl groups; peak 9, glutamate; peak 10, creatine; peak 11, choline; peak 12, phosphocholine; peak 13, glycerophosphocholine/phosphatidylcholine; peak 14, phosphoethanolamine; peak 15, taurine; peak 16, *myo*-inositol. Spectra reproduced with permission from REF. 55 © (1998) Marcel Dekker.

cell-membrane metabolism. These types of characteristic metabolic perturbations can also be used to follow treatment efficacy *in vivo*. The biochemical actions of R-roscovitine (CYC202) — an inhibitor of the cyclin-dependent kinases-1, -2 and -7 — is also being investigated in a similar manner, using a combined *in vivo* and *in vitro* metabolomic approach[59]. This approach has shown that CYC202 decreases the bioenergetic status of the tumour, produces intracellular acidosis and decreases the glycerophosphocholine, glutamate and glycine concentrations of the cell. Further studies are required to determine how this information relates to the inhibition of cylin-dependent kinases and the treatment of tumours.

Phospholipid metabolism has been investigated in breast cancer cells during exposure to the antimitotic drugs paclitaxel, vincristine, colchicine, nocodazole, methotrexate and doxorubicin using ^{31}P NMR spectroscopy[60]. Intriguingly, these studies found that antimitotic drugs cause a large increase in glycerophosphocholine that was not associated with changes in cell-membrane composition and that this change could be further increased by synchronizing cells in the G2–M phase. This indicates a correlation between microtubule status and cellular phospholipid metabolism in these cell lines. So, ^{31}P NMR spectroscopy can be used both as a tool to identify proliferative breast cancer cells, based on the concentration of glycerophosphocholine levels, as well as to identify new drug targets associated with specific metabolic changes.

Finally, metabolomics is also being used as a bioprospecting tool. For example, Joshi and colleagues[61] have used high-performance liquid chromatography (HPLC)-based separation methods to investigate the metabolic pathways responsible for saponin production in the plant *Acacia victoriae*. Saponins have been shown to be selectively toxic to tumour cells at very low doses, although *Acacia victoriae* produce very small amounts of these compounds. Further analysis of the metabolomic profile of *Acacia victoriae* might lead to the identification of ways to upregulate saponin production in these plant cells, to provide enough material for clinical applications. Metabolomics might also be used to identify the mechanisms by which these compounds are specifically toxic to cancer cells, by analysing the metabolomic profiles of cells treated with this and similar drugs.

Diagnosis

The ease of automation for NMR-based metabolomics also makes it an ideal technique for screening human populations for common metabolic disorders. Systems have already been developed to identify patients with coronary artery disease using blood-plasma samples[62]. If such systems can be applied to the clinic, they would be much more cost-effective than highly invasive techniques such as angiography, which is used to diagnose coronary artery disease at present. High-throughput analysis of biological fluids, which are obtained in a minimally invasive manner, could also be used to diagnose cancer or follow therapy responses.

In a study of 52 patients, Bathen and colleagues[63] used neural networks to examine the lipid profiles and lipoprotein composition of blood plasma in patients with a range of cancers compared with controls. Their models were 83% accurate at predicting which patients had tumours, based on the blood-plasma lipid profile, and only 8% of patients were identified as false positives. A similar approach has also been proposed for the diagnosis of colon cancer using NMR spectroscopy of whole blood followed by analysis using neural networks[64]. This method accurately distinguished between patients with and without colon cancer in this small-scale study. However, despite these promising initial results, no large-scale clinical trial has validated the use of ^{1}H NMR spectroscopy of blood plasma as a tool for diagnosing cancer. Furthermore, the limited number of metabolites that can be detected using this approach probably precludes the identification of specific biomarkers for cancer.

High-throughput metabolomics is not confined to NMR spectroscopy, however. Although FOURIER-TRANSFORM INFRARED (FT-IR) SPECTROSCOPY is generally poor at identifying specific metabolites, the metabolic fingerprints produced by the approach can be used to identify cells that are in specific phases of the cell cycle, as well as to distinguish between different cell types by measuring changes in spectral features that result from differences

CO-RESONANT METABOLITES
Nuclear magnetic resonance (NMR) spectroscopy detects the chemical groups that make up a molecule. Some metabolites have regions that are chemically very similar and therefore occur in the same position in the NMR spectrum. When the individual peaks (resonances) from two or more metabolites can not be distinguished, they are said to be 'co-resonant'. This confounds direct quantification.

in DNA. FT-IR has also been used to follow the maturation of cervical cancer cells, through changes in glycogen metabolism[65], as well as for dividing chronic lymphocytic leukaemia samples into different subgroups, based largely on DNA content[66]. In a similar manner RAMAN SPECTROSCOPY, which relies on the measurement of scattered light as it passes through a sample, is being used to monitor neoplastic colon tissue[67].

Metabolomic approaches are also being used by nutritionists who are interested in examining the link between nutrition and resistance to certain cancers[68]. A range of systems are being used, including cell-culture strategies and samples from human population studies. Large data sets must be examined to understand the relationships between genetic polymorphisms, demographics, environment, diet and cancer risk. The analysis of biofluids by NMR spectroscopy and approaches based on liquid chromatography–mass spectrometry (LC–MS) provide an unrivalled tool for analysing samples from large populations in that these techniques are both high throughput, reproducible and cost effective. Current research includes studies of nutrients that modulate DNA damage and repair, DNA methylation, gene expression, antioxidant production and oxidative stress, signal transduction, cell-cycle control, apoptosis and anti-angiogenic processes.

Future directions

Most of the recent research into tumour metabolomics has come from NMR-based studies, reflecting the potential of MRS in terms of clinical diagnosis. However, given the greater sensitivity of mass spectrometry, the combined use of NMR spectroscopy with either GC–MS or LC–MS for the analysis of tissue and cell extracts will clearly increase. New technological advances will also improve the information that can be obtained using NMR. Increased automation will allow the rapid generation of metabolomic databases to assist in patient screening. Improved sensitivity will also be possible using cryogenically cooled NMR probes, known as CRYOPROBES. This approach reduces the amount of electronic noise in the NMR signal and therefore significantly improves sensitivity. Miniaturization will also allow the examination of smaller, and potentially more homogeneous, samples, particularly when used in conjunction with laser-capture techniques[69].

The advent of cryoprobe technology also makes it possible to study nuclei such as ^{13}C in tissue extracts. Carbon-13 NMR is less sensitive than ^{1}H and ^{31}P NMR, as a result of a lower LARMOR FREQUENCY at a given field strength and its lower natural abundance (BOX 1). Despite this lower level of overall sensitivity, the sensitivity in biological samples is greater, as a result of the larger chemical shift range of ^{13}C compared with ^{1}H — this reduces co-resonances and improves the detection of key markers. One study that shows the potential of ^{13}C NMR spectroscopy was carried out by Boros and colleagues[70], and described a technique for stable isotope-based dynamic metabolic profiling. The approach relies on using [1,2-$^{13}C_2$]glucose to label a proportion of the metabolome and therefore identify which metabolic pathways metabolize glucose. Proliferating tumour cells have a distinct metabolic phenotype, characterized by increased and preferential utilization of glucose through the non-oxidative pathway of the pentose cycle for nucleic-acid synthesis, with a reduced rate of *de novo* fatty-acid synthesis and tricarboxylic-acid-cycle glucose oxidation. By using steady-state labelling of intermediates of the PENTOSE-PHOSPHATE PATHWAY as biomarkers of cell proliferation, these approaches could be extended to assess the efficacy of therapeutics that aim to inhibit cell proliferation.

The results from the HRMAS ^{1}H NMR spectroscopy investigations of tumours have demonstrated the importance of being able to separate out co-resonant metabolites, and indicate that the best approach might be to use methods such as GC–MS and LC–MS or LC–NMR for the analysis of heterogenous samples such as biofluids and tissue extracts. These techniques can separate many chemically similar metabolites for analysis by chromatographically separating metabolites before analysis. This both concentrates the metabolites and eases their detection. However, one study[21] has shown that HRMAS ^{1}H NMR spectroscopy of intact tissue can be used to detect 'active' metabolites, as opposed to components of the cell membrane and other compartments where metabolic turnover is very slow. Therefore, HRMAS ^{1}H NMR spectroscopy is a useful technique for following metabolic changes in the cytosol, whereas tissue-extraction procedures often dilute these metabolites, as they also include cell-membrane constituents. As it seems that many of the key metabolic events associated with apoptosis occur in the cytosol, HRMAS ^{1}H NMR spectroscopy will become increasingly important as a metabolomic tool. It is also being used to confirm the metabolic events measured, *in vivo*, using MRS[71].

NMR- and mass-spectrometry-base metabolomic approaches have the unusual property, in terms of '-omic' approaches, of being both high throughput and relatively inexpensive. Like gene-expression profiling, this approach allows researchers to screen large populations and identify interesting subsets for further analysis. Unlike DNA microarrays, there are few costs on a per-sample basis after the initial equipment purchase. Metabolomic approaches also generate highly reproducible data sets. One disadvantage is that the number of metabolites that exist in a mammalian tissue is probably far smaller than the number of transcripts that are present in the mammalian transcriptome. Therefore, a given metabolite pattern can reflect several genomic changes. It is also not a trivial matter to connect the genome to the metabolome, complicating attempts of using metabolomics as a functional genomic tool in cancer research. Metabolomic approaches, however, will become increasingly popular in disease diagnosis and will have an important role in improving our understanding of the mechanisms of cancer.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY
Spectroscopic technique based on examining the vibrational frequencies of given molecules. When a molecule absorbs infrared radiation of a defined energy, vibrations are induced in the molecule. However, these vibrations must involve an electrical dipolar change in the molecule. In general, this technique is poor at discriminating metabolites from a similar class of compounds.

RAMAN SPECTROSCOPY
When a metabolite is irradiated by light from a laser, the light is scattered with either the same amount of energy (Rayleigh scattering), or with more (Stokes) or less (anti-Stokes) energy because of changes in the vibrational energy of the metabolite. This Stokes and anti-Stokes scattering is observed in Raman spectroscopy.

CRYOPROBE
Nuclear magnetic resonance (NMR) probes for which the coil and pre-amplifier have been cryogenically cooled to reduce the amount of electronic noise in the NMR signal. They increase the signal-to-noise ratio by a factor of 3–4, compared with conventional probes. This can reduce experiment time 16-fold or required sample concentration by up to 4-fold.

LARMOR FREQUENCY
When a magnet or dipole is placed in a magnetic field, a torque is placed on it, called a 'magnetic moment', causing it to align with the magnetic field. For an electron, however, the magnetic moment is produced by the orbital motion of the electron about the nucleus. This produces a force that causes the magnetic moment to process around the direction of the magnetic field at a frequency termed the Larmor frequency.

PENTOSE-PHOSPHATE PATHWAY
An anabolic pathway that uses the six carbons of glucose to generate five-carbon sugars. The roles of this pathway are to generate NADPH for biosynthesis reactions, to provide cells with ribose-5-phosphate for nucleotide synthesis and to metabolize pentose sugars.

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Competing interests statement

The authors declare competing financial interests: see web version for details.

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