

Review

How close is the bench to the bedside? Metabolic profiling in cancer research

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Published: 20 January 2009

Genome Medicine 2009, **1**:5 (doi:10.1186/gm5)

The electronic version of this article is the complete one and can be found online at <http://genomemedicine.com/content/1/1/5>

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Abstract

Metabolic profiling using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) is integral to the rapidly expanding field of metabolomics, which is making progress in toxicology, plant science and various diseases, including cancer. In the area of oncology and metabolic phenotyping, researchers have probed the known changes in malignant cellular pathways using new experimental techniques to gain more insights, and others are exploiting these same cellular pathways for therapeutic drug targets and for novel cancer biomarkers, with the ultimate goal of translation to the clinic. Here, we discuss the challenges and opportunities in metabolic phenotyping for discovering novel cancer biomarkers, and we assess the clinical applicability of MS and NMR.

The National Cancer Institute recently posted a Request for Information (RFI) proposing to bring together experts from academia, government and industry with drug-development skills and expertise 'to address unmet needs in therapeutic oncology' [1]. This RFI calls for a cross-disciplinary and multi-center approach to speed up the translation of new molecular therapeutic agents directed against novel targets to assist people with cancer. These meetings will bring together scientists from basic research and those from the various 'omic' fields. Although genomics, transcriptomics and proteomics will undoubtedly be well represented, metabolomics may bring the most excitement to the meetings. Metabolomics is the comprehensive analysis of the low-molecular-weight molecules, or metabolites, that are the intermediates and products of metabolism. The aim of metabolomics is to understand the underlying biochemical processes that are altered in disease states, toxicological progression, recovery, and so on. The potential for discovery and utilization of novel metabolites to aid early cancer screening and to improve diagnosis, prognosis and therapeutic regimens has led to an upsurge of research studies in the field.

The search for cancer biomarkers

The reduction of the burden of disease on society depends on early screening and detection to enable timely therapeutic regimens when these therapies are likely to be most effective. The ideal biological marker(s) for cancer risk assessment and early detection must have high sensitivity and specificity, be found in a biosample obtained using minimally invasive procedures, and be analyzed using a high-throughput, cost-effective assay. In lieu of early detection biomarkers, prognostic biomarkers can empower physicians in the selection of the most effective therapy for treating an active tumor. The importance of studying the cellular metabolome for the discovery of biomarkers has been shown throughout studies conducted over the last few decades. Tumor cells are adept at evading the host's immune surveillance, apoptosis and anti-growth checkpoints to sustain angiogenesis and limitless replication [2]. Some of these characteristics can be traced back to the reprogramming of cellular bioenergetic pathways. The key pathways that behave differently between tumor and normal cells include the glycolysis and pentose phosphate pathways, nucleotide and protein biosynthesis, lipid and phospholipid turnover,

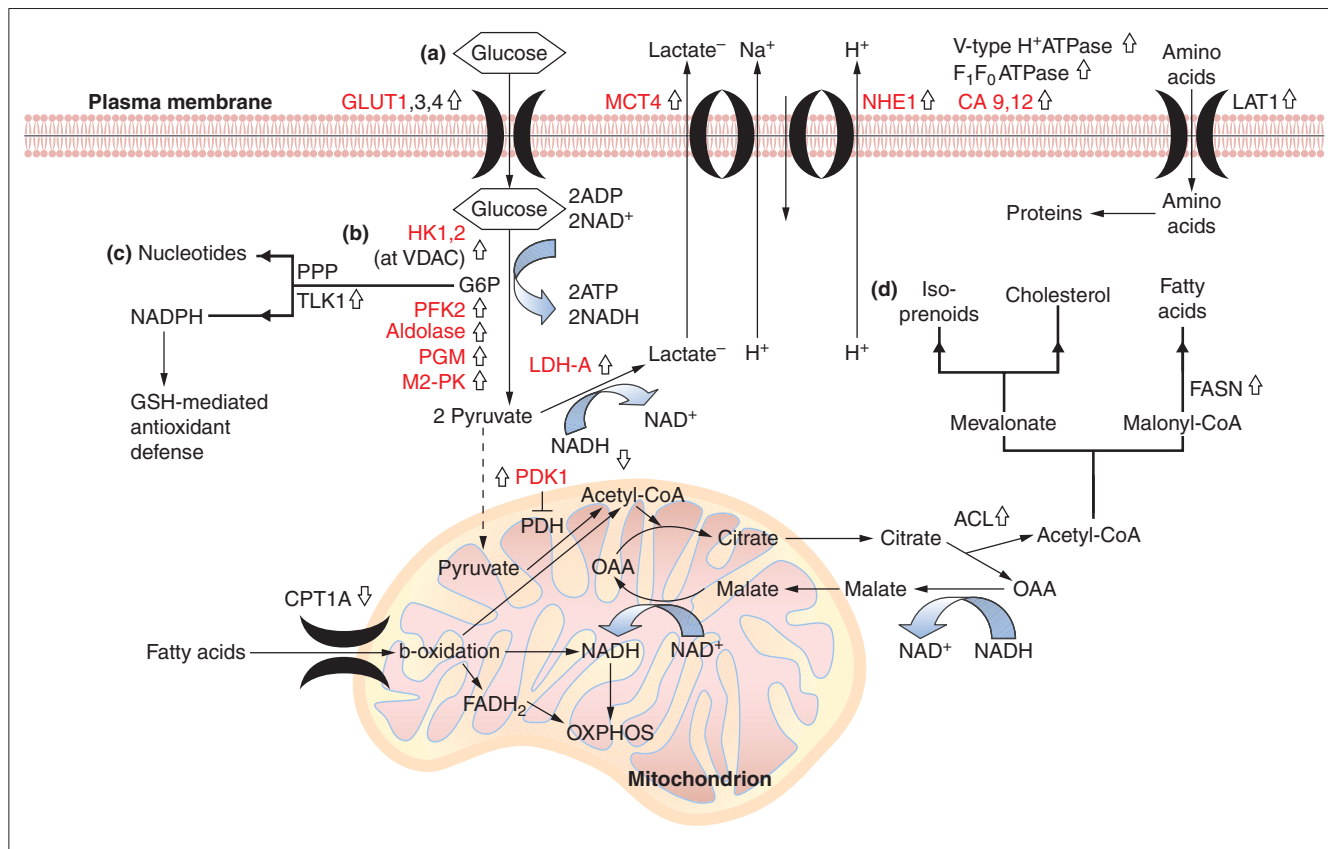


Figure 1

Metabolic reprogramming in tumor cells. The alteration of bioenergetic pathways in tumor cells is evident from their increased glucose uptake (a) through glycolysis (b), the intermediate metabolites of which are also shuttled into biosynthetic pathways (synthesis of nucleic acids from glucose 6-phosphate through the pentose phosphate pathway (c), amino acids from glycerate 3-phosphate (not shown) and lipogenesis from pyruvate (d) that are necessary for cell growth and proliferation. In tumor cells, pyruvate in the mitochondria is shuttled into a truncated tricarboxylic acid cycle where it is converted to acetyl-CoA by pyruvate dehydrogenase and combined with oxaloacetate for export into the cytosol as citrate for the synthesis of isoprenoids, cholesterol and fatty acids. Open up and down arrows indicate upregulation and downregulation of enzymes, respectively. Enzymes upregulated by activation of HIF-1 are in red. Abbreviations: ACL, adenosine triphosphate citrate lyase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CA9 and CA12, carbonic anhydrases 9 and 12; CPT1A, carnitine palmitoyltransferase 1A; FASN, fatty acid synthase; G6P, glucose 6-phosphate; GLUT1,3,4, glucose transporter 1, 3 and 4; GSH, glutathione; HIF-1, hypoxia-inducible factor 1; HK1,2, hexokinase I and 2; LAT1, L-type amino acid transporter 1; LDH-A, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4; M2-PK, pyruvate kinase isoform M2; NAD⁺, nicotinamide adenine dinucleotide oxidized; NADH, nicotinamide adenine dinucleotide reduced; NADPH, nicotinamide adenine dinucleotide phosphate; NHE1, Na⁺/H⁺ exchanger 1; OAA, oxaloacetate; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PFK2, phosphofructokinase 2; PGM, phosphoglycerate mutase; PI3K, phosphatidylinositol 3-kinase; PPP, pentose phosphate pathway; TLK1, transketolase 1; VDAC, voltage-dependent anion channel. Reproduced with permission from [2].

the citric acid cycle and redox stress pathways (Figure 1) [2,3]. Essentially all pathways needed for cellular growth and proliferation are affected, and many of these have been targeted for drug research and development [2,4]. Several metabolites are commonly found to be elevated in tumors, including lactate, choline-containing compounds, nucleosides, myoinositol and lipids [5]. As investigators continue to drill down from gene expression to metabolite end products, new hypotheses emerge concerning common metabolites. For example, recent evidence shows that lactate may act as a regulator of glycolysis and mitochondrial physiology, and not simply be a waste product of glycolysis [6].

There are several reasons why metabolomics has great potential in bringing the 'bench closer to the bedside'. One of the major reasons is that metabolites are already being used in a number of diagnostic and therapeutic tests. For instance, innate errors in fatty-acid, organic-acid and amino-acid metabolism are measured using tandem mass spectrometry (MS) from blood samples obtained from newborn babies [7]. Physicians also use LipoScience's commercial nuclear magnetic resonance spectroscopy (NMR)-based LipoProfile® test to estimate a patient's cardiovascular risk by measuring the number and size of low-density lipoprotein (LDL) particles and of the other subclasses of such particles,

high-density lipoprotein (HDL) and very-low-density lipoprotein (VLDL) [8]. Just as microarray technologies have transformed genomics and transcriptomics, new instruments with higher sensitivity and throughput have had a major impact on the metabolomics field.

Analytical platforms for metabolomics: MS and NMR

Although MS and NMR are the main technology platforms used for metabolic profiling, each of these technologies has its own specific advantages and disadvantages when used in the search for disease-specific metabolite biomarkers (Table 1). The main advantage in using MS is its sensitivity. State-of-the-art mass spectrometers can detect analytes routinely in the femtomolar-attomolar range. When coupled with liquid chromatography (LC), MS can easily detect hundreds of individual species in a single clinical sample. Many mass spectrometers also have high mass accuracy and can do real-time tandem MS to identify detectable metabolites.

Unfortunately, metabolites do not provide the complex fragmentation patterns that peptides do, making their identification somewhat more difficult. Quantitation, which is critical for recognizing potentially useful biomarkers, is one of the weaknesses of MS. There are several factors that influence the signal intensity of a compound in MS, including the composition of the mixture in which it is located (for example, its molecular environment). Addition of a known amount of internal standard(s) is generally useful only for the compound(s) it is targeted against and does not translate to the rest of the compounds in the complex mixture, making it impractical for metabolite biomarker discovery. Most investigators have relied on the comparison of peak area or intensity to measure differences in the relative abundance of specific metabolites between samples. These measurements, however, have suffered from a lack of accuracy and precision.

The main strengths of NMR spectroscopy are reproducibility, the ability to quantitate compounds in mixtures and the ability to identify unknown metabolites. Dumas *et al.* [9] have shown that the analytical reproducibility of NMR is >98% and Keun *et al.* [10] have observed <3% variance in identical samples acquired at two different sites using spectrometers of two different field strengths. Given that peak area in the NMR spectrum relates directly to the concentration of specific nuclei, quantitation of compounds in a complex mixture is very precise. Once detected, various NMR pulse-sequences can be used to help determine the structure of the molecule that gave rise to the peak of interest; these include total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC). Another underappreciated character of NMR is its versatility for analyzing metabolites in the liquid state (serum, urine, and so on), in intact tissues (for example, tumors) or *in vivo*. However, NMR's major limitation is

Table 1

Comparison of MS and NMR spectroscopy for metabolomics

Criterion	Best technique
Equipment cost	Neither
Maintenance cost	MS
Cost per sample	Neither
Sensitivity	MS
Quantitation	NMR
Resolvable metabolites	MS
Identification of unknown metabolites	NMR
Inter-laboratory reproducibility	NMR
Potential for sample bias	NMR
Technical skill requirement	MS

sensitivity. Although recent technological advances in cryogenically cooled probe technology, higher field-strength superconducting magnets and miniaturized radiofrequency coils have increased sensitivity, MS is still orders of magnitude more sensitive than NMR.

Both MS and NMR suffer in throughput for the discovery of metabolite biomarkers, even if sample preparation methods are not considered. To maximize metabolomic coverage, MS relies on LC separation of the metabolites before analysis. This need dictates that each sample will require hours per MS analysis. Although a single high-resolution, one-dimensional ^1H spectrum is obtainable in minutes, true global profiling requires higher-dimensional data to maximize the number of metabolites detected. Further development of two-dimensional methodologies to increase throughput while retaining high data resolution for global profiling is needed. New data acquisition schemes to reduce the time required for multidimensional data are required to make two-dimensional NMR practical. Techniques such as non-linear data sampling and the filter diagonalization method show great promise [11,12]. Combining non-linear data sampling with maximum entropy reconstruction is another option being explored by the Wagner group at Harvard [13].

What is a normal metabolic profile?

An important consideration in any metabolomic study aimed at identifying diagnostic or prognostic biomarkers is that there are many modifiers of metabolic phenotype, such as gender, age, genetics, hormonal cycle, life style, diseases and drugs. Aside from a clear understanding of the metabolic phenotype of cancer at different stages of the disease, there is also a need to define the 'normal' metabolic signature as a point of comparison. Specifically, what needs to be defined is the 'normal range' of inter- and intra-individual

metabolite variance [14]. The range of what is considered normal, however, can be quite large. For example, work completed in our laboratory [15] has focused on measuring estrogen metabolites in urine samples obtained from a sub-population of women. This class of compounds is believed to have an important role in the genesis and progression of cancers of the reproductive system. Our results show that the levels of these metabolites typically vary 10- to 100-fold between individuals depending on their menstrual status [15]. What is needed in the future are efforts analogous to that underway in the Clinical Breast Care Project [16]. This joint collaboration between the Windber Research Institute and the Walter Reed Army Medical Center is developing a tissue bank and database detailing the ontology of normal breast development through the mapping of temporal changes in gene and protein expression. Perhaps in the future metabolic phenotyping will play a part in any such large endeavors.

Cancer detection

Several groups have aimed to exploit the metabolic difference between normal and cancerous cells to diagnose cancer. The following select examples illustrate the use of MS and NMR to find diagnostic or prognostic biomarkers.

Prostate cancer diagnosis by NMR

Prostate-specific antigen (PSA) is a serine protease produced by cells in the prostate gland that can be detected in blood samples using monoclonal antibody assays [17,18]. The detection of serum PSA has increased early diagnosis of prostate cancer but has suffered from mediocre sensitivity and specificity because other conditions, such as prostatitis (inflammation of the prostate) and benign prostatic hyperplasia (BPH; enlarged prostate), can raise the level of PSA beyond the current 4 ng/ml cutoff that is thought to indicate cancer. Unlike other tissues, citrate is found in very high concentrations in the prostate epithelium, where a high level of zinc prevents the oxidation of citrate in the Krebs cycle [19]. The citrate concentration declines in malignant cells but remains high in BPH and normal prostate tissues. Using ^1H NMR spectra of unprocessed semen and prostatic secretions from healthy controls and patients with prostate cancer, Kline *et al.* [20] were able to show that quantitation of citrate concentration outperformed PSA for detecting prostate cancer. Recent work by Serkova *et al.* [21] showed that the absolute concentrations of citrate, myoinositol and spermine were age-independent in human prostatic secretions. In addition, the ratio of the concentrations of choline plus creatine to citrate from *in vivo* imaging has been shown to be a good correlation to the Gleason score, which indicates prostate cancer aggressiveness [22].

Ovarian cancer diagnosis using MS

The prevalence of ovarian cancer is low and accounts for only about 3% of all cancers in women, but it is the most

lethal of all the gynecological malignancies [23]. Walsh *et al.* [24] and Yemelyanova *et al.* [25] both pointed out that in order to reduce ovarian cancer mortality, assays are needed to screen for stage I, high-grade serous carcinomas, which are most responsible for the high mortality rate. The five-year survival rate of 90% for diagnosis at stage I may be skewed because the tumors commonly detected at this stage are non-serous tumors that have good prognosis. Screening of women at high risk for ovarian cancer is currently performed using ultrasound and the measurement of the serum cancer antigen CA-125. Similarly to PSA for prostate cancer, the CA-125 assay lacks sufficient sensitivity and specificity. CA-125 is elevated in only 50% of stage I ovarian tumors and in 80% of late-stage ovarian cancers [26].

In a large MS-based effort, Olivier Fiehn's group profiled 66 invasive ovarian carcinoma tissues and 9 borderline tumors of the ovary [27]. Principal component analysis modeling and clustering analysis were able to distinguish 88% of the borderline tumors from the carcinomas. Almost 300 metabolites were consistently detected in 80% of the tissue samples, of which 114 could be identified. The 51 metabolites found to be significantly different in the two samples include some involved in purine and pyrimidine metabolism, glycerolipid metabolism and energy metabolism. The next challenge is to determine which of these potential markers survive a rigorous validation study and become useful clinical biomarkers for invasive ovarian carcinoma.

Finding the metabolic signature of radiation using MS and NMR

Radiation therapy, surgery and chemotherapy are three traditional treatments for cancer. To understand why some cancer patients respond better to ionization therapy and drug treatment, several groups have investigated the underlying cellular response of tumor cells to such treatments. Rainaldi *et al.* [28] acquired one-dimensional ^1H NMR spectra of whole leukemia cells (HL60) to compare the apoptotic metabolic signature induced by radiation, the antineoplastic drug doxorubicin and heat-induced necrosis. The results showed similar profiles for irradiation and doxorubicin-treated leukemic cells, but the necrotic cells had different metabolic profiles. Many of the same metabolites were found to be depleted in the irradiation and doxorubicin-treated cells, including reduced glutathione (GSH). In contrast, GSH remained at low levels whereas all other metabolites showed an increase in abundance in the necrotic cells.

Another group analyzed cell extracts from lymphoblastic cells (TK6) treated with radiation using MS [29]. Similarly to the above NMR study [28], they discovered GSH to be depleted in the irradiated cells, but there were differences in the metabolites found to be downregulated by MS [29]. This difference may be due to the fact that the NMR group [28] used a radiation dose that was ten times higher than the biologically relevant ionizing radiation dose of <10.0 Gy to

ensure that all cells underwent apoptosis synchronously. This observation highlights the fact that metabolomics, being a young and developing field, lacks standard protocols for sample preparation [30]. Perhaps in the future such disparities in sample preparation will be overcome to allow the combination and interpretation of scientific findings obtained from different methodologies and their confirmation on independent platforms.

The outlook for clinical applicability

Changes in metabolic composition are likely to be subtle in the early stages of any disease. Many key metabolites from different pathways have a role in tumor development, and the ability to simultaneously detect and quantitate all these metabolites would give a more global analysis of the state of the tumor. Metabolomics is currently at the stage that proteomics has occupied for the past decade: the search for disease-specific biomarkers. It is important, however, to look forward to the time when useful metabolite-based biomarkers are discovered using NMR and/or MS. What will be the role of these technologies then? Will both, either, or neither have a role in the routine analysis of these biomarkers?

In our opinion, MS will have a greater clinical role in the future than NMR for several reasons. The main reason is simply that MS has historically been more widely applied for routine clinical tests than NMR [31]. This historical 'head start' has allowed more effort to be expended on developing MS methods for clinical applications. Secondly, a clinical test will not be required to measure as much of the entire metabolome as it can, but simply to focus on quantifying a single analyte or a small number of analytes. This type of analysis is one of the major strengths of MS. Methods such as isotope dilution (ID)-MS have been used routinely in clinics for decades. A recent study compared the accuracy and precision of three methodologies - enzyme-linked immunosorbent assay (ELISA), LC with fluorescence detection and LC with ID-MS - to analyze aflatoxin-albumin adducts in human serum as a measure of aflatoxin exposure [32]. Aflatoxins have been shown to be carcinogenic in humans and animals and are linked to an increased risk of hepatocellular carcinoma. The ID-MS method not only showed the lowest standard deviation, but also the greatest sensitivity. ID-MS has also shown exquisite precision and sensitivity in a variety of clinical tests.

Currently, most clinical MS assays are conducted in reference laboratories and not within hospital-based clinical laboratories. This fact is a result of the expertise required to prepare and analyze samples using MS and to interpret the results. As the technology becomes simpler to use and the number of scientists familiar with this technology continues to increase, the use of MS-based tests within the hospital clinic will certainly expand. Clinical NMR spectroscopy is currently more focused on the *in vivo* imaging area, and

although its role in metabolite biomarker discovery will continue to increase, it faces more obstacles than MS before becoming a routine clinical tool. Although LipoScience is currently working on developing a fully automated clinical NMR analyzer, in general the lack of NMR spectrometers within clinical laboratories and NMR's low intrinsic sensitivity contribute to the greater challenge of making this technology routine when compared with MS.

Advances in MS and NMR instrumentation will also be crucial in bringing these technologies from the bench to the bedside. For MS, this will require simplification of the entire analytical method, starting with sample preparation and continuing through to the final data analysis and presentation. The major NMR instrumentation breakthroughs needed can be best illustrated by a recent article describing a miniaturized diagnostic magnetic resonance (DMR) system [33]. This chip-based DMR system uses multichannel detection for the analysis of unprocessed biological samples approaching sensitivity limits of 10^{-12} M. This multichannel miniaturized NMR has been shown to be able to detect bacteria, profile circulating cells and identify biomarkers in sera obtained from cancer patients. Chip-based systems, such as the DMR, may provide the low-cost, high-throughput platforms necessary for point-of-care translational medicine.

The ability to translate biomarkers quickly for cancer diagnosis, prognosis and risk assessment from their discovery to their application within the clinic would have an enormous impact on public health. The entire concept of 'bench-to-bedside' has been discussed for several years and has been used in both genomics and proteomics. This concept is now firmly entrenched within the field of metabolomics as well.

Metabolomics has the opportunity to learn from the issues that have primarily plagued proteomics investigators over the past few years. One of these issues is standardization. The ability to compare results from various labs working on similar problems could certainly shorten the time for biomarker discovery. Fortunately, the Metabolomics Society has organized a Chemical Analysis Working Group to develop standards for metabolomic analysis [34]. Adherence to these standards will assist in inter-laboratory data comparison and hopefully will also expedite the finding of clinically relevant metabolite biomarkers.

Abbreviations

BPH, benign prostatic hyperplasia; DMR, diagnostic magnetic resonance; ELISA, enzyme-linked immunosorbent assay; GSH, reduced glutathione; HDL, high-density lipoprotein; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; ID-MS, isotope dilution mass spectrometry; LC, liquid chromatography; LDL, low-density lipoprotein; MS, mass spectrometry; NMR,

nuclear magnetic resonance spectroscopy; PSA, prostate-specific antigen; RFI, Request for Information; TOCSY, total correlation spectroscopy; VLDL, very-low-density lipoprotein.

Competing interests

The authors have no competing interests to report.

Acknowledgements

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

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