FRONTIERS IN METABOLOMICS FOR CANCER RESEARCH

Nutritional Science Research Group and Cancer Biomarkers Research Group
Division of Cancer Prevention
National Cancer Institute

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EXECUTIVE SUMMARY

WORKSHOP HIGHLIGHTS

Metabolomics—the study of all the metabolites in a cell, tissue, or organism—is an emerging scientific discipline that, by necessity, involves a multidisciplinary approach for discovery, development, and translation. Metabolomic research is focusing on "functional metabolites," those that have been shown to operate within the pathways associated with disease processes. This research suggests that metabolomics may provide benefits for assessing the cellular state in normal, predisease, and diseased cells and tissues. Presentations at the *Frontiers in Metabolomics for Cancer Research* workshop sought to address what is known in metabolomics regarding cancer research, to describe challenges in metabolomics for cancer research, and to assess strengths and gaps in current metabolomic technologies and models.

Challenges for the use of metabolomics in cancer research are enormous. Nevertheless, recent advances in experimental methodologies, technologies, and the ability to process large amounts of data are allowing metabolomics to be applied to cancer research. To this end, an area of future research may be in addressing how to determine what a metabolic profile looks like in normal, precancerous, and cancerous cells or tissues. The use of metabolic profiling for elucidating changes that occur downstream from genomic and proteomic alterations associated with disease-related biochemical reactions, which precede changes in cell morphology that predict disease, may hold significance. However, the adequate measurement of the level of metabolites to draw conclusions regarding the profile and disease risk remains a challenge. For example, even if specific profiles are identified as being associated with disease risk, variability of response to bioactive food components would make it difficult to assess and to develop a global understanding of metabolic interactions. An encouraging aspect of metabolomic research is that research on metabolic pathways for one chronic disease is likely to provide knowledge that can be applied to other chronic diseases. For example, metabolic profiling of lipids in the study of obesity might provide an understanding of phenotypes that increase the risk for cardiovascular disease, diabetes, and cancer. Metabolic profiling also is being used in drug discovery and development. This research is focused on identifying molecular targets in gene-regulatory pathways that can be modified by modulating biochemical intermediates that increase or decrease the levels of metabolites. An example is an investigation in Zucker diabetic rats, where manipulation of the levels of a pyruvate cycling intermediate dimethylmalate has been

shown to reduce the toxicity of lipids on beta cells, thus reducing the loss of glucose-induced pyruvate cycling.

In the past decade, the use of technology has allowed the identification of metabolic signatures secreted from every tissue and cellular compartment. The unique patterns of metabolites are being used to characterize pathologic states of disease. Technologies such as nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and positron emission tomography (PET) allow the identification of differences in the levels or expression of metabolites associated with normal and disease states. Because metabolites can be modified rapidly with increased or decreased exposure to external stimuli (e.g., nutritional exposure, environmental changes, or drug treatment), metabolomic studies have a broad application in research investigating changes throughout the disease continuum. Gaps in research regarding technology include the inability to access well-annotated, well-controlled human samples; a need to create better informatics models to aid in data interpretation; and the need for accessible databases that link disease states and metabolism.

Metabolomics in cancer research offers the opportunity to better understand variations in individual disease risk and individual response to environmental influences, such as diet. There still are many metabolites that need to be characterized and defined regarding their association with disease. Emerging technologies will continue to be applied to the study of tumor metabolomics to better understand characteristics of initiation, promotion, progression, and metastatic processes. Multidisciplinary research, using knowledge gained in the study of all chronic or nonchronic diseases, will be assimilated into translational research for helping patients in the clinic.

SUMMARY REPORT

MONDAY, OCTOBER 24, 2005

SESSION I: OPENING REMARKS AND WELCOME

Welcoming Remarks

Peter Greenwald, M.D., D.P.H., Director, Division of Cancer Prevention (DCP), National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD, welcomed participants to the workshop. He expressed interest in learning the state-of-the-science in metabolomics, an emerging field that explores the structure and function of low molecular weight compounds beyond DNA and protein. Metabolomics is an integrative science that depends on modeling and systems approaches that are anchored in facts derived from discovery research. Dr. Greenwald expressed the hope that this workshop would include discussions of simpler models that could be informative for future research. Because metabolomics is an emerging field, there is a need for discovery science from diverse fields of inquiry. Early efforts in experimental, quantitative, and integrative research and new technologies will be used to help comprehend complex biology. The need for mechanistic knowledge and leads for risk assessment, nutritional and other means of prevention, early detection tests, and better therapies are areas that were addressed by the workshop.

John Milner, Ph.D., Chief, Nutritional Science Research Group (NSRG), DCP, NCI, Bethesda, MD, thanked participants for attending and provided a nutritional perspective for metabolomics. He expressed the hope that ongoing research, such as in metabolomics and other "omics" research (e.g., genomics, proteomics, and transcriptomics) will be able to elucidate the reasons for variability in response to nutritional interventions. He recognized that the definition of metabolomics is inconsistent across scientific fields and that there is a need to broaden the definition to include metabolites of bioactive food components and their impact on cell energetics. From a nutritional viewpoint, quantity and duration of exposure to a food can determine a response; this is an important aspect of nutrition that could be addressed by workshop participants. Dr. Milner also asked speakers to suggest metabolomic biomarkers that are needed in nutritional science, keeping in mind that it is unlikely that one biomarker alone will identify a disease.

Sudhir Srivastava, Ph.D., Chief, Cancer Biomarkers Research Group (CBRG), DCP, NCI, described biomarker research at NCI and the role of the Human Genome Project in making it possible to investigate biomarkers for detection and treatment. The omics field is changing the way researchers approach disease. It is possible that this century will be the century of small molecules. Instead of research focusing on DNA, RNA, and protein, this century's researchers are likely to focus on aptamers in glycomics and micro-RNA in metabolomics. The focus on small molecules resulted from research indications that small molecules may be amenable structurally to targeting by drugs, are likely to produce valuable information on the pathophysiological state of cells, are likely to be less complex than proteins, and are limited in

number (i.e., approximately 3,000 functional metabolites). The key investigational concept will be to determine at what point in the disease process the metabolite is present.

Setting the Stage

Padma Maruvada, Ph.D., Program Director, CBRG, DCP, NCI, Bethesda, MD, provided background for the workshop and questions to be addressed by speakers. Early planning for the workshop developed basic concepts to address the following:

- Applying high-throughput technologies to biological questions in the post-genomic era;
- Supporting the NIH Roadmap initiatives that promote technology development in metabolomic platforms; and
- Investigating metabolomic approaches to cancer research.

Metabolomics has been slow to develop in cancer prevention, but it is hoped that by addressing the following questions, metabolomics can be applied to cancer research.

- Can tumor-specific metabolic alterations be reflected in body fluids? If yes, can such alterations be used for cancer detection, diagnosis, prognosis, or nutritional intervention?
- What are the factors (e.g., physiological factors such as age, sex, and polymorphisms) that would influence metabolic fluxes?
- What molecules should be included in the definition? What small molecules should be investigated for metabolomics?
- What metabolomics technologies are appropriate for cancer research?
- How can meaningful information be derived?
- What are the needed sensitivity, reproducibility, and dynamic range for clinical utility?

Young Kim, Ph.D., Program Director, NSRG, DCP, NCI, provided an overview of the workshop objectives and thanked each of the sponsoring agencies for making the workshop possible. The workshop objectives are to:

- Discuss the unique challenges involved in applying metabolomics to cancer research;
- Identify insights provided by metabolomics that are not found in genomics and/or proteomics studies; and
- Discuss strengths and gaps in current metabolomic technologies and models.

Workshop cosponsors included the Division of Cancer Prevention, Division of Cancer Biology, Division of Cancer Epidemiology and Genetics, and Center for Cancer Research within NCI as well as the NIH Office of Dietary Supplements (ODS). Dr. Kim described the format of the workshop, which included formal presentations, discussions, and breakouts of small groups.

SESSION II: OVERVIEW OF METABOLOMICS

What Is Metabolomics/Metabonomics?

Julian Griffin, Ph.D., Department of Biochemistry, University of Cambridge, United Kingdom, presented the objective of the session, which was to compare and contrast the evolution of metabolomics, including its definitions, with its predecessors and counterparts, and to discuss convergences and divergences. He described key literature that brought the issue of metabolomics to the attention of research scientists. These included studies in yeast, studies using gas chromatography-mass spectrometry (GC-MS) for plant phenotyping, and studies on coronary artery disease (CAD) and screening patients using blood plasma.

Nicholson et al. presented a definition of metabonomics in 1999 as "measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification." Oliver et al. (2002) defined metabolomics as "...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." These definitions fit into the broader field of "omes" that has arisen in the last decade. For clarity and to set the stage for discussions in the workshop, Dr. Griffin presented the following definitions.

- Genomics—Study of genes and the only -ome that is not context dependent.
- Transcriptomics—All of the mRNA in a cell, tissue, or organism.
- Proteomics—All of the proteins in a cell, tissue, or organism.
- Metabonomics/Metabolomics—All of the metabolites in a cell, tissue, or organism.

Estimates of the number of metabolites are substantial, with approximately 69,000 metabolites having been identified in fats, which is just one class of compounds that produce metabolites. Technologies used to measure metabolites include nuclear magnetic resonance (NMR), GC-MS, liquid chromatography-mass spectrometry (LC-MS), and various custom assays. Procedures have been proposed for use in metabolomics. These include measuring small-molecule concentrations through a global approach, using pattern recognition to define metabolism in a multidimensional space, defining a metabolic phenotype (metabotype), and using this information to determine an endpoint (e.g., drug toxicity, disease state) or to mine data in another omics technology. Each technology has its advantages and disadvantages, depending on the outcome desired. Dr. Griffin described the use of these procedures in the previously mentioned studies.

The future of metabolomics largely will depend on the development, adaptation, and integration of technologies. Specific needs include development of rapid phenotyping tools that can correlate with genotype information. This is being investigated in a diabetic mouse model at the Medical Research Council Mammalian Genetics unit at Harwell. The challenge will be to build a more powerful database system to make this information available to researchers. A second future need is to develop improvements in metabolomic technologies. Dr. Griffin described the possible use of cryoprobes, LC-NMR, high-resolution magic angle spinning (HRMAS) methodology, as well as LC-MS and GC-MS. He stated that significant integration of these and

additional technologies is needed to assist in analyzing metabolites of significance in disease states. Some integration currently is being investigated in rat urine studies; primary ciliary dyskinesia (PCD), a rare inherited disease of the lung; and non-alcoholic steatohepatitis (NASH), often called fatty liver disease.

SESSION III: METHODOLOGICAL AND TECHNOLOGICAL ISSUES AND CONCERNS

Sharon Ross, Ph.D., Program Director, NSRG, DCP, NCI, NIH, Bethesda, MD, moderated Session III, presented the session's objective, and introduced the speakers. The objective of the session was to describe prevailing technologies, including those with dual purposes, reagents, instrumentations, and required infrastructures for the study of metabolomics.

State-of-the-Art Technologies in Metabolomics

Stephen Brown, Ph.D., Research Fellow, Department of Chemistry, Esperion Therapeutics, Division of Pfizer Global Research and Development, Ann Arbor, MI, described current technologies used for the study of metabolomics. He began by offering the term "metabolic profiling" to avoid the metabolomic/metabonomic distraction. Strategic issues include determining whether high-throughput technologies can be used where separations are required and finding universal detectors that have similar response factors to all the analytes of interest. Another issue is whether to measure levels of metabolites or fluxes through pathways; fluxes are more accurate but are more expensive to measure. A general question is how accurate and precise the data must be to achieve a level of confidence. In the absence of a "universal detector," it could be better to measure subsets of analytes having similar physico-chemical properties, such as organic acids. Biomarker discovery is most efficiently done by multivariate analyses of data accumulated for hundreds of analytes in each sample.

Technologies for metabolic profiling include both bioanalytical and data analysis methodologies. Bioanalytical methods—such as mass spectrometry (MS), which has the highest sensitivity though variable quantitation ability; Fourier transform ion cyclotron resonance (FT-ICR) or FT mass spectrometry (FTMS), which has the highest mass resolution and mass accuracy; NMR, which has low sensitivity but high quantitation ability; and LC/MS/MS, and GC/MS/MS—are being used in investigative studies. In addition, several investigators use LC-Coulometric Electrochemical Array detection, or metal cation measurement by X-ray fluorescence. Multivariate analytical methods are borrowed from the field of "Chemometrics," which has been successful at characterizing and classifying complex samples.

Dr. Brown presented information on a study in Wistar rats designed to show metabonomic differentiation of organ-specific toxicants. A key finding was that metabolic profiles differ with different insults (i.e., α-Napthylisothiocyanate or p-aminophenol). These profiles are reproducible enough to build classification models and lay the groundwork for a metabonomics-based toxicity-screening paradigm.

In a study of FTMS metabolic profiling, Dr. Brown described the use of FTMS with high resolution to distinguish multiple peaks with finer detail than other spectrographic studies. Testing ESP 55016 (omega-hydroxy-alkanedicarboxylic acid), which favorably alters serum lipid variables, in rats was described. Studies of the livers of rats treated with ESP 55016 compared to untreated rats indicated that free fatty acids (e.g., 16:0 and 18:1) are downregulated; bile acids are upregulated, whereas glycine bile acid conjugates are suppressed. These metabolic effects could be ascertained in a small sample set (five animals per group), even though the variance in exposure to ESP-55016 and its coenzyme-A (CoA) adduct was high.

Discussion

Dr. Brunengraber asked if there was a CoA-trap and alpha-oxidation responsible for the findings on ESP 55016. Dr. Brown said that there was an enhancement of beta-oxidation, but no discernible effects on other major acyl-CoA adducts in livers of these rats. Another participant asked Dr. Brown to discuss the disadvantages of FTMS, such as the inability to detect differences in isomers that is an advantage of separation technologies, especially in lipids. In response, Dr. Brown stated that there always is a tradeoff when using one technology over another.

A discussion ensued regarding measurement of fluxes and the statement that measuring fluxes is more valuable than measuring concentrations. Metabolic control analysis researchers would say the opposite. Dr. Brown stated that he has conducted time-course studies in hamsters with compounds that indicate that metabolism is stressed long term, and this has made him curious about fluxes. A participant asked if outliers had been studied. Dr. Brown responded that his group looked at this but did not find any animals that were outliers in the studies that he presented.

Pathway Discovery through the Association of Metabolomics and Mass Isotopomer Analysis

Henri Brunengraber, M.D., Professor and Chair, Department of Nutrition, Case Western Reserve University, Cleveland, OH, provided the historical perspective that biological research started by identifying metabolites, following their concentrations, and isolating isotopic techniques to determine their dynamic components. This may be needed in the field of metabolomics as well. Metabolomics identifies the modulation of one pathway by an intermediate of an apparently unrelated pathway, without transfer of carbon, and by the unknown transfer of carbon between two pathways.

Dr. Brunengraber offered three strategies for stable isotopic labeling of the metabolome: (1) focused labeling of one metabolite with its [U-^{13}C] mass isotopomer, (2) regional labeling with [^{13}C] bicarbonate, and (3) general labeling with $^{2}H_2O$. He presented details of each strategy in various pathways involving gluconeogenesis. Studies showed that there are unknown carboxylating reactions that imply, when measuring gluconeogenesis in control and diabetic animals, the possibility of overestimating gluconeogenesis by comparing the labeling of phosphoenol pyruvate (PEP) and pyruvate.

Dr. Brunengraber described investigations of metabolism of azelate in the liver to highlight the association of metabolomics and isotopomer analysis and the differences in information provided by high-impact assays, such as the use of CoA esters, carnitine esters, and proxies of the mass isotopomer distribution (MID) of acetyl-CoA pools. These investigations indicated that there is dual metabolism between peroxisomes and mitochondria, and that there are multiple metabolites that interact in different ways that are dependent on precursors and the cellular environment. For refilling the citric acid cycle, there are three processes, including pyruvate, glutamate, or from the up-chain fatty acid through propionyl-CoA or methylmalonyl-CoA.

To investigate whether these compounds are anaplerotic, Dr. Brunengraber reviewed an experiment with labeled acetate, which is not anaplerotic, and measured the labeled acetyl-CoA and succinyl-CoA just before and just after the entry of anaplerotic carbon from propionyl-CoA. Results indicated that, for acetyl-CoA, the labeling curves (before and after) are identical, but for succinyl-CoA, just after the entry of propionyl-CoA, azelate labels more succinate-CoA than acetate for the same labeling of the mitochondrial acetyl-CoA. This demonstrates that azelate and methylmalonyl-CoA are anaplerotic and that the final steps of azelate metabolism occur in the mitochondria. Conclusions of these experiments suggest that there are unknown reactions that occur in the cellular environment and affect metabolites. Although these experiments show that the association between metabolomics and isotopomer analysis yields information on unknown reactions that are controlled by unknown genes, or genes of unknown function, more studies are needed to determine the mechanisms responsible for these reactions. In summary, mass isotopomer analysis can be used to identify links between pathways and features of the compartmentalization of metabolism in cells and organs, and provide the potential for applying these techniques in imaging.

Discussion

A participant asked how these methods could be applied to human studies. Dr. Brunengraber responded that he uses fully labeled substrates that would not be appropriate in people. To study azelate in people, there would have to be lower levels of radiolabeling administered by mouth or intravenous administration and measuring of targeted metabolites in urine or serum. Another participant asked why it would not be useful to use yeast mutants in these experiments. In reply, Dr. Brunengraber said that fully labeled substrates need to be used in mammalian systems and then in transgenic animals.

Evolutionary Optimization in Metabolomics

Douglas Kell, Ph.D., Professor, School of Chemistry, The University of Manchester, United Kingdom, described evolutionary optimization in metabolomics, with a focus on a computational evolutionary technique known as genetic programming, and how it can be used to understand the metabolome. He listed a series of items that could be used to improve metabolomic methods, including straightforward optimizations that are possible to produce good local optima as well as advanced, multi-objective methods. He described the basis of inductive methods of reasoning in which observations or data are used in a principled way to optimize the generation of hypotheses.

Dr. Kell described the ideal analytical method for omic research as having a global scope, as well as the common methods of statistical sensitivity, specificity, and reproducibility. The datasets accumulated during research should also be presented in easy to understand formats and graphical representations. In other words, we require two things from our methods: that they give the correct answer and that the answer is easy to understand.

Dr. Kell described details of the computational principles of evolutionary computing. These include a population of individuals, each encoding a particular solution to a problem and a fitness function or functions, to evaluate the adequacy of a solution. Together, these can be said to represent the evolutionary "landscape." To illustrate the principle, Dr. Kell presented an experiment in plant wounding and salicylate. Exposure to infection in a wild-type (WT) plant is accompanied by changes in the concentration of salicylate, and prewounding gives rise to resistance to wounding elsewhere in the plant; a plant with an implanted transgenic bacterial gene with salicylate hydroxylase (SH-L) can reduce or eliminate the production of salicylate, thus reducing the protection to the plant. Deductive conclusions from this experiment that apply to the study of metabolomics include: (1) the plants that contain SH-L are more sensitive than the WT to subsequent wounding; (2) the salicylate concentration in SH-L is much lower, which is consistent with its involvement in the normal defense response; and (3) although the data produced by liquid chromatography (LC) show changes, they do not show which changes are essential for causing the wounding. Dr. Kell described methods for data analysis that might help to explain which changes do matter, and these kinds of inductive reasoning led to the identification of molecules—not noticed in the original analyses—that are significantly more predictive of wounding resistance.

A unique approach to developing a principle for hypothesis generation is the use of what is known as the "robot scientist." This allows researchers to decide which experiments to conduct, given a large or even unlimited number of possibilities. Dr. Kell described an experiment using the robot scientist method to identify which areas of a metabolic pathway are likely to provide answers to questions of metabolic lesions based on growth experiments. The basis of this method is to use intelligent search methods to identify the most likely hypotheses. These basic ideas in the robot scientist can be applied to evolutionary optimization for metabolomics.

Dr. Kell's conclusions included the following:

- Many areas of metabolomics (and of science more generally) involve optimization.
- The search spaces are necessarily large as they scale exponentially with the number of variables, and heuristic methods are required.
- Evolutionary computing provides a variety of useful heuristic methods and has the advantage that, by selecting input variables, results can be interpreted quickly and easily.
- Examples include automated chromatographic optimization and data mining to discover novel biomarkers.

Discussion

A participant asked if optimization for the peaks in the GC could result in overlooking biomarkers. Dr. Kell said this is so, but with more peaks one was more likely to find them (as

had been shown in a study with pre-eclampsia); the important concept is to determine what to select for and to investigate those biomarkers using more classical hypothesis-driven methods. Another participant asked whether an analysis was conducted on the three biomarkers for blood pressure described in the presentation and whether selectivity and sensitivity were assessed. Dr. Kell responded that using machine learning methods and conventional analyses allowed the researchers to find the biomarkers easily and to determine selectivity and sensitivity, both of which were above 90 percent.

Group Discussion: Sessions III

Moderator: Douglas Burrin, Ph.D., Associate Professor, Department of Pediatrics, Baylor College of Medicine, Children's Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Houston, TX

It was determined that little is known in this area other than how concentrations change in a few pathways. There may be some way to detect subtle changes in ratios or fluxes, for example, pyrimidines and purines; this would, however, confirm the predictive model rather than enzyme changes. These studies are needed to help define some of the measurements. At the present time, the use of tracers is being applied to net flux and isotopic exchange, which vary in controls and intervention. Carbon-13 as a tracer has been found to be effective in determining metabolic load on a system and fluxes, provided that there is a steady state and that parameters can be measured before and after the steady state. Interpreting results still is difficult, especially if it is not clear what cellular compartment or organ is being investigated.

In discussing techniques used for metabolomics, it was determined that there is a need for standards for methods, such as extractions. A recent National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) conference reviewed this problem, and researchers are in the process of developing standards for metabolomics methods. The outcomes and presentations from this August 2005 meeting, entitled "Metabolomics Standards Workshop," are posted on the Metabolomics Society Web Site at http://www.metabolomicssociety.org/. Many different methodologies are being used in laboratories—making it difficult to compare results—which is not unique to metabolomics. In addition, the number of natural and unnatural compounds being used in methodologies makes it complicated to compare results.

Dr. Burrin asked participants to address the limits of technology or other context-specific variables, such as whether the focus should be on biopsies, urine, serum, or other tests. Discussion of these issues resulted in the recognition that few assays are available for metabolomics, but procedures being used in other fields can be adapted to metabolomics. The technologies exist, but applications and standards are needed. On the positive side, smaller tissue samples are producing results using current technologies. Recent advances in luminous technologies in proteomics do not appear to be applicable in metabolomics; however, because the dynamic range of commonly used technologies in metabolomics, such as MS, are as much as 1,000-fold above what is needed to detect small molecules. The NIH Roadmap initiative is funding the development of a luminous probe for fatty acids and other metabolites.

SESSION IV: USE OF METABOLOMICS FOR CANCER RESEARCH

Moderator Paul Wagner, Ph.D., Program Director, DCP, NCI, NIH, Bethesda, MD, described the objective of Session IV as the identification of issues that surround the application of metabolomics in cancer research.

A Crossroad to Tumor Metabolome: Tumor M2 Pyruvate Kinase

Sybille Mazurek, Ph.D., Institute of Biochemistry and Endocrinology, Veterinary Faculty, University of Giessen, Germany, provided background on the role of the pyruvate kinase (PK) isoenzyme type M2 (M2-PK) within tumor metabolism. PK is responsible for net ATP production within the glycolytic sequence. In contrast to mitochondrial respiration, energy production by PK is independent of oxygen supply. Dependent upon the metabolic duties of the tissues different isoenzymes of PK are expressed (PK type L in liver and kidney; PK type M1 in brain and muscle; M2-PK in the lung on the one hand and in all proliferating cells and especially in tumor cells on the other hand).

Dr. Mazurek presented information on immunohistological stainings of the PK isoenzymes with monoclonal antibodies in different tissues. During tumor formation, a shift in the PK isoenzyme configuration always takes place in such a manner that the tissue specific isoenzyme disappears and M2-PK is expressed. In contrast to tumor cells, the M2-PK isoenzyme of lung tissues is not stained with the monoclonal antibody against M2-PK. The reason for this differentiation was found in the quaternary structure. In normal lung, M2-PK was found to exist as a tetrameric form, whereas in tumor cells, M2-PK is mainly dimeric. In tumor cells, the dimerization of M2-PK is induced by direct interaction of M2-PK with different oncoproteins. The pp60v-src kinase phosphorylates M2-PK in tyrosine. The E7 oncoprotein of the human papillomavirus type 16 directly binds to M2-PK.

The tetrameric and dimeric forms of M2-PK are characterized by different kinetic behaviors. Dr. Mazurek showed that the tetrameric form of M2-PK has a high affinity to its substrate, phosphoenol pyruvate (PEP), and is highly active at physiological PEP concentrations. The dimeric form is characterized by a low PEP affinity and is nearly inactive under physiological conditions. The tetrameric form of M2-PK, but not the dimeric form, is associated with other glycolytic enzymes within the so-called glycolytic enzyme complex. In normal proliferating cells, M2-PK is mainly in the tetrameric form. Consequently, glucose is mainly converted to pyruvate and lactate accompanied by the production of energy. In tumor cells, the dimerization and inactivation of M2-PK leads to an expansion of all phosphometabolites above pyruvate kinase, which are then available as precursors for synthetic processes, such as DNA, amino acid, and phospholipid synthesis. When M2-PK is in the inactive dimeric form energy is provided by glutaminolysis.

In addition to ADP, M2-PK also can use GDP as substrate. Accordingly, the tetrameric form correlates with a high (ATP + GTP) to (UTP + CTP) ratio. The dimeric form of M2-PK correlates with a low ratio between purines and pyrimidines. Tumor M2-PK is released from tumor cells into the blood and from gastrointestinal tumors also into the stool of tumor patients.

Dr. Mazurek provided results from clinical studies showing that the amount of tumor M2-PK increases in the EDTA-plasma of patients with different tumor entities as well as in stool samples of patients with colorectal cancer and correlates with tumor size and stages. In follow-up studies, measurement of tumor M2-PK in EDTA-plasma allows monitoring of the success or failure of therapy.

Discussion

A participant asked for an explanation of some of the data on glutamate. Dr. Mazurek responded by describing the results of metabolic studies that were not presented and confirmed the data shown in the presentation. Another participant commented that he had confirmed that fatty acids are released from breast cancer tissue and asked if this would allow the escape of immune surveillance. Dr. Mazurek agreed that this may be true. In response to a question on biomarker sensitivity and specificity, Dr. Mazurek answered that her group did not conduct the studies on the measurements of tumor M2-PK in EDTA-plasma and stool samples and that the studies derive from papers of the diverse clinicians mentioned on the slides. There was discussion of the clinical studies on tumor M2-PK that showed that it is important to test and retest in order to enlarge clinical studies and to confirm the general principles of studies.

Metabolomics by Magnetic Resonance: From Molecules to Man

John Griffiths, M.D., Ph.D., Professor, Division of Basic Medical Sciences, St. George's Medical School, University of London, United Kingdom, presented information on the use of magnetic resonance spectroscopy (MRS) in metabolomics. He described some of the methods using NMR and a list of metabolites that can be measured from tissues, genetically modified organisms, and patient biopsies or during the use of novel drugs. The general strategy is to take a metabolic profile from the abnormal cell or tissue and compare it with the corresponding metabolic profile of WT cells, control organisms, or tissues. This method is even more effective if the corresponding transcriptomic or proteomic profiles also are available.

Dr. Griffiths described the use of methodologies in hypoxia-inducible factor (HIF), which is the major oxygen sensor in cells. The HIF-1 pathway has been studied for its role in metabolomics in cancer cells and tumors and for its ability to induce hundreds of genes and enzymes for glucose transport. Two mechanisms upregulate the action of HIF-1, and certain carcinogens such as c-myc switch on HIF-1 as they cause carcinogenesis. Mechanisms of action of HIF-1 and its α and β subunits were described.

Dr. Griffiths presented data from an experiment based on the premise that cultured HepaC4 tumors, which fail to form active HIF complex, would not be able to upregulate glycolysis under hypoxic conditions. This proved not to be true; in fact, the tumors produced the same amount of lactate and other glycolytic products as WT cells. Further studies showed that the HepaC4 tumors actually had a greater uptake of oxygen than the WT cells. In addition, after 3 weeks, the HepaC4 tumors grew faster than the WT cells, even with ATP in the HepaC4 tumors at 20 percent of normal. In further studies of the HIF pathway in metabolic profiling studies on the role of succinate and fumarate, each was found to be involved in cancer promotion by upregulating HIF.

Discussion

A participant asked why it was necessary to upregulate glycolysis when ATP was reduced. Dr. Griffiths responded that the amount of lactate was the same and glycolysis was upregulated under hypoxic conditions. A participant asked if there are models in which ATP synthesis might be regulated by the availability of glycine. Dr. Griffiths responded that he was not sure if there have been such studies. A participant suggested that the same pathway patterns of degradation of betaine and choline have been seen in studies of fatty acid disease, and these can be reversed by orotic acid by adding adenosine. Dr. Griffiths said that this was an interesting hypothesis that could be investigated.

Metabolomic Profiling of Human Cancer With *Ex Vivo* Tissue MR Spectroscopy

Leo Cheng, Ph.D., Assistant Professor, Departments of Radiology and Pathology, Massachusetts General Hospital, Harvard University, Charlestown, MA, spoke on metabolic profiling in human cancer. He placed metabolomics in the context of genomics, proteomics, and pathology, and said that metabolomics might provide the best opportunity to identify profiles for use in clinical studies. He defined metabolomics in cancer as the study of the global variations of metabolites, and a measurement of global profiles of metabolites from various known metabolic pathways under the influence of oncological developments and progressions. He reviewed the technologies used in metabolomics and expressed the need to identify integrated approaches for the technologies that can be used with clinical relevance and for developing risk prediction algorithms.

Dr. Cheng described experiments using MRS that identify metabolites in *ex vivo-in vivo* correlations and that use NMR and MAS. An experiment using quantitative histopathology and MRS showed that the amount of information available on specific metabolites might be used for diagnoses using Gleason scoring in prostate cancer patients. Using principal component and linear regression analyses, Dr. Cheng discussed how to use these analyses to determine which prostate cancer patients would need specific treatments, although more research needs to be conducted to bring this into clinical practice.

One approach using metabolomics is to assess metabolites in prostate cancer growth by concentrations of spermine and ornithine decarboxylase (ODC). Results indicated that spermine was not correlated with prostate cancer growth, as measured by prostate-specific antigen (PSA); ODC was found to be correlated. The important lesson from this and previous studies is that changes in tumor metabolomics, downstream from genomic and proteomic transformations that reflect disease-related biochemical reactivity, may precede histologically observable changes in cell morphology and, thus, might offer an early means of predicting disease behaviors.

Discussion

A participant commented that one of the most pressing problems in prostate cancer research is discerning between indolent and fatal disease; it appears that PSA is not a good indicator of the solution to this problem. He said that PSA is used to select those who undergo a biopsy and those who do not, but there is no way to determine whom, and how, to treat based on PSA. A

few participants questioned the number of variables listed in Dr. Cheng's studies. Dr. Cheng responded that this would be reviewed at a later time.

A participant asked for an explanation of the differences in spin and nonspin MR. Dr. Cheng responded that there are resolution differences based on differences in concentrations and distributions. Another participant asked if there is a value in conducting experiments that use prostatic milking (i.e., prostate massage) for metabolomics rather than biopsy. Dr. Cheng said that this is a valid question that should be pursued.

Group Discussion: Session IV
Moderator: Sudhir Srivastava, Ph.D., Chief, CBRG, DCP, NCI, Bethesda, MD

Dr. Srivastava introduced the discussion period by commenting that, since the completion of human gene sequencing, the focus has shifted to the function of sequenced genes and their roles in disease detection and treatment. Proteomics, along with other omics, are revolutionizing the approach to disease detection. Cancer detection remains a high priority for NCI, and numerous initiatives on proteomics and nanotechnology have been launched. In addition, this may be the century of the small molecules, which structurally are amenable to targeting by drugs; likely to provide much information on the pathophysiological state of cells; likely to be less complex than proteins; and limited in numbers (i.e., approximately 3,000 "functional" metabolites). He asked panel members to address the issue of improving metabolomics to study the behavior of tumors for diagnostics.

Predicting the diagnosis more accurately in prostate cancer can prevent unnecessary surgeries, but it will take advances in statistical methods to improve predictive power. Recent research also indicates that the metabolism of stromal cells adjacent to breast cancer cells might determine the cancer cell's ability to become invasive. This area could use immediate and focused research. One approach might be to study cancer cells by allowing cytokines to invade stromal cells and conducting metabolic studies. Another approach could use xenographs of stromal cells to determine the proliferation of cancer cells. The field of metabolomics could address this, especially with regard to the impact of nutrition on stromal cells that might influence cancer invasion.

Using differential displays, it may be possible to identify the additive or antagonistic response of metabolites, which leads to the concept that there is not enough information from metabolomics—or the sensitivity— to discern a determinant of tumor growth. Current technologies, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), probably could be applied to metabolomics. Bringing metabolomic findings out of the pathology laboratory and into the clinic is an issue worth investigating. Imaging may be the next step, but identifying the specific problem of concern in metabolism might be problematic.

There was a discussion of increasing ketone bodies, resulting in exaggerated fatty acid metabolism, which could increase the growth of a tumor. Because metabolites accumulate mutations in genes and their enzymes, the effect might not be the same on the growth of a tumor or metabolite concentrations. Most cancers have a high glucose uptake that is theorized to be

caused by glycolytic metabolism, but this has not been proven in the laboratory, at least with the C4 experiments.

Dr. Srivastava asked participants to address the issue of technology in metabolomics. Current methods are not easy to implement, and there is a need to develop more appropriate and less invasive means. Using metabolites can help avoid some of the invasive technologies. In addition, using data analytic methods with metabolomics can add to the ability to create diagnostic tests.

Dr. Srivastava commented that one of the main problems in proteomic technologies is reduction of "noise" in diagnostics. He asked for comments in this area. In the discussion, it was recognized that this is a problem and that, in any technology or laboratory assessment, there will be areas that cannot be assessed or interpreted by the results given.

SESSION V: IMPACT OF CANCER MODIFIERS ON METABOLIC PROFILING

Elizabeth Yetley, Ph.D., Senior Nutrition Research Scientist, ODS, NIH, Bethesda, MD, introduced Session V. The objective of this session was to discuss metabolomic arrays, spectroscopic profiles, and other metabolite-based phenotyping methods in relation to their application for patients and stratification for responders and nonresponders to dietary components, drugs, and toxic agents.

Dynamics of Glucose Metabolism and Cancer

Wai-Nang Paul Lee, M.D., Professor, Department of Pediatrics, David Geffen School of Medicine, University of California at Los Angeles, Torrance, CA, discussed glucose metabolic phenotypes in cancer. Dr. Lee defined metabolic phenotype by physical composition (e.g., metabolite profiling or metabonomics) or functional properties (e.g., tracer-based metabolomics and constraint-based models). He described situations to elucidate phenotyping by physical composition and functional properties. The objective of metabolic profiling is to distinguish between different phenotypes. To define phenotypes by fluxes in metabolic profiling, there must be an assessment of the steady state substrate flux of pathways of a metabolic network to provide a set of dynamic parameters that represent the metabolic phenotype. These measurements, however, together with metabolite concentration, are inadequate to provide a functional description of the phenotype.

Dr. Lee described an "Uncertainty Principle in Biology," which is the basis for biological variability in phenotype, as: (1) the addition of metabolic intermediates (metabolic), as a change in the kinetics or a branch in the pathway (genomic) that increases the uncertainty of the knowledge of the behavior of the system; and (2) the more metabolites that can be measured, the less certain we are of the functions of the system.

Constraint-based modeling may be used to solve a solution space and potential extreme pathways involved in an investigation. Extreme pathways are all of the pathways that are involved in the conditions of the investigation. Tracer-based metabolomics includes all

substrates that take up the tracer—and many might not be targets for study—but tracer distribution reflects the metabolic function of the cell and defines the metabolic phenotype. Dr. Lee described experiments that illustrated these concepts and how each could provide information for metabolic and flux measurements.

Dr. Lee described Phenotypic Phase Plane Analysis, a method to identify the type of metabolic phenotype and how the phenotype can be manipulated for a desired end. An example is glucose/butyrate utilization phenotypic phase plane analysis, in which the labeling of glucose and butyrate allowed the identification of cell growth with variable concentrations of glucose and butyrate.

Principles can be described with regard to gene-environment interaction. They are:

- Genetic factors confer to cells a specific set of metabolic functions that correspond to a metabolic phenotype under a given nutrient environment; and
- Changes in the nutrient environment select cells with the actual observed metabolic phenotypes that function best under such environments.

Dr. Lee said that nutrient-gene interaction in the evolution of cancer cells can be understood in the context of metabolic selection, with genetic mechanisms as the source of phenotypic variation; the final observed species is the result of metabolic selection.

Discussion

A participant asked about the Uncertainty Principle and if there are a number of parameters beyond which a system cannot be defined. Dr. Lee responded that the principle is applied to the prediction of what a cell will do and provides no indication of the biological behavior of the cell. No matter how many parameters are measured, it is not possible to predict a cell's behavior.

Lipidomic Profiling Eicosanoid Changes in Carcinogenesis

Edward Dennis, Ph.D., Professor, Departments of Chemistry/Biochemistry and Pharmacology, University of California at San Diego, CA, discussed lipidomic profiling for carcinogenesis, which depends on establishing techniques, database systems, and development of infrastructure to detect and quantitate the individual molecular species of lipid. He described a systematic approach, known as Lipid Metabolites and Pathways Strategy (LIPID MAPS), for meeting these requirements in the study of eicosanoids. The goals of LIPID MAPS are to:

- Separate and detect all of the lipids in a specific cell and discover and characterize any novel lipids that may be present;
- Quantitate each of the lipid metabolites present and the changes in their levels and location during cellular function; and
- Define the biochemical pathways for each lipid and develop lipid maps that define the interaction networks.

Dr. Dennis described a National Institute of General Medical Sciences Large Scale Collaborative Grant (i.e., a "Glue Grant") to investigate lipids using LIPID MAPS. Information may be found at http://www.lipidmaps.org. A suitable classification for lipids is being developed, and progress toward its development was described. Each lipid compound is being classified using the classification system, which involves an international group of lipid scientists. Dr. Dennis provided an example of the classification, nomenclature, and drawing structure for the system. PubChem has accepted the drawing system, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) in Japan (see http://www.genome.ad.jp/brite/) has accepted the classification.

Dr. Dennis described the six core groups that are working on specific areas of lipids: lipids, genes, proteins, metabolic pathways, annotations, and MS lipid standards. He used arachidonic acid (AA) to exemplify the approaches being developed; even within the AA category, there are thousands of lipids. LC-MS multiple reaction monitoring (MRM) is being used to develop a library of more than 70 eicosanoid standards, which can be viewed at the LIPID MAPS Web Site, including Web links for more information.

The critical question is how this data should be used. Dr. Dennis described an example of RAW 264.7 cells with lipopolysaccharide (LPS) stimulation that illustrated the use of the data. His group identified numerous prostaglandins in samples that were assessed using LC-MS MRM. They also found that small quantities of novel eicosanoids played important biological roles. Detecting these minor components was investigated by supplementing RAW 264.7 cells with deuterated AA (AA-d_8), which creates a more complex LC-MS pattern and allows the identification of lower level AA metabolites.

Discussion

A participant asked if the lipids that were found were natural lipids. Dr. Dennis responded that they were naturally made lipids; and supplementation with AA also produced natural lipids, but in more detectable doses. Another participant commented that current research indicates that studies of former smokers show that there are almost as many lung cancers among them as in current smokers; this is because the return to lower risk takes many years. He said that a focus of research is to find medical interventions that can lower the risk in former smokers more quickly over time. A related idea is whether leukotriene inhibitors (i.e., LOX-inhibitors) could be useful because inflammation is involved. He asked Dr. Dennis whether there was anything in the research or classification system that could be useful in focusing drug development for this problem. Dr. Dennis responded that LPS downregulates the lipoxygenase enzyme, which means that leukotrienes are not included in the information presented. In other studies of agonists, however, one or more may be useful, but more research is needed.

Metabolic Profiling in Drug Discovery and Development

Christopher Newgard, Ph.D., Director, Sarah W. Stedman Nutrition and Metabolism Center, Duke University Medical Center, Durham, NC, presented information on approaches in metabolic profiling that define targets and potential therapeutic approaches for drug discovery and development. He described technological platforms being used in his investigations; including GC-MS and tandem MS (MS-MS) to measure 100 targeted metabolites in four

chemical classes (i.e., free fatty acids, acylcarnitine, organic acids, and amino acids). Standards are being developed to measure other metabolites. He said that it is important to use profiling and flux measurement in analyses. The focus of his investigations includes basic research in mechanisms of glucose-stimulated insulin secretion and its impairment in type 2 diabetes and lipid-induced insulin resistance, as well as clinical research in metabolic profiling during weight loss in people. Another project being conducted in association with cancer researchers is metabolic profiling of oncogenic pathways.

Dr. Newgard described research on Zucker diabetic fatty (ZDF) rats that have a mutation in their leptin receptor and become grossly obese and insulin resistant. He described the progression of pancreatic beta-cells over time as the rats become obese. Results of studies on these rats have indicated that chronic exposure of beta cells to lipids induces a loss of glucose-induced increment in pyruvate cycling; beta-cell "lipotoxicity" is reversible by adding a pyruvate cycling intermediate, dimethylmalate. An important concept is that the pathways have to be understood before beginning the search for drugs that modulate disease states.

Dr. Newgard described investigations using the tools noted above to study gene regulatory pathways that are related to cancer. By perturbing oncogenes, expression profiling has been used to discriminate between the "*ras*-ness" versus the "*myc*-ness," referring to the effect of the *ras* and *myc* oncogenes in the carcinogenesis of a tumor. This method also can be used to discriminate between tumors that have different degrees of aggressiveness.

In acylcarnitine and organic acid profiling investigations, there have been early indications that low levels of long-chain acylcarnitines and increases of short-chain acylcarnitines could suggest increased rates of fatty acid oxidation and decreased rates of glucose oxidation, or alteration in lipogenesis from glucose. In addition, increased α-ketoglutarate might indicate an increase in amino acid oxidation in E2F-transduced cells (e.g., increased conversion of glutamate to α-ketoglutarate through glutamate dehydrogenase or transaminase reactions), and initial testing of these ideas has involved the measurements of: (1) glucose oxidation, (2) glucose incorporation into lipids, (3) fatty acid oxidation, and (4) amino acid oxidation.

Discussion

A participant asked where transproteomic profiling enters the discussion of metabolic profiling. Dr. Newgard said that he intends to increase profiling. Another participant commented that insulin resistance was described but did not explain the shrinkage of the pancreatic islets in the ZDF rats. Dr. Newgard responded that expression profiling may have identified a transcriptional network that controls beta-cell replication and glucose-stimulated insulin secretion. One of the genes controlled by the transcription factor involved is isocitrate dehydrogenase, a cytosolic protein.

Another participant commented that this field might be useful not just for drug development but to help clinicians determine when a drug will be useful and when it might not be useful. Because obesity may be responsible for approximately 90,000 cancer deaths per year, insulin resistance is being studied in cancer (e.g., breast cancer). He asked whether there were approaches that could help determine the risk benefit without some of the adverse side effects seen with current drugs.

Dr. Newgard responded that he agrees with the potential that multiple chronic diseases can have common mechanisms. In addition, there may be different risks associated with individuals from different ethnic and racial backgrounds.

Group Discussion: Session V
Moderator: John Milner, Ph.D., Chief, NSRG, DCP, NCI, Bethesda, MD

Dr. Milner asked Dr. Newgard about the alanine shuttles and what happens to intracellular ammonia concentrations when glucose is shifted. Dr. Newgard responded that he has not studied these, but he can conduct amino acid profiling and can follow that in beta-cell extracts. In response, Dr. Milner commented that the article by Calle and colleagues tied together cancer, diabetes, and obesity at some cancer sites. It appears, however, that obesity might be protective against premenopausal breast cancer but harmful in postmenopausal breast cancer. He asked whether metabolomics could be used to help understand this problem. Dr. Newgard said that there still is much to be learned about cytokines and insulin resistance, and it is known that hormones such as leptin are involved in many processes. It makes sense to learn as much as possible through profiling. Dr. Lee added that studies in breast cancer cells indicate that there are differences with a fatty acid assault or inflammation. Dr. Dennis added that there is a correlation between inflammation and most chronic diseases.

Dr. Milner asked the panelists to address the issue of differences between normal and cancerous cells and how studies are being characterized relative to metabolomics. The panelists responded by explaining that looking at rate-controlling enzymes in a pathway can affect the metabolic pool unless there is extra capacity. This impacts the use of fluxes and makes it difficult to determine the effects. They also said that cell lines have different metabolic profiles and will lead to different results in proliferation and apoptosis depending on changes in metabolism. In addition, oncogenes do not necessarily change the metabolic activity of all cell types.

Dr. Hanash asked panelists to speculate on the way to proceed in metabolomics in relation to cancer research. This discussion resulted in the following suggestions:

- Use simple methods for clarity, with careful interpretation of results.
- Design rate- and concentration-controlling investigations that are appropriate for metabolomics, bearing in mind that it is easier to measure concentrations than fluxes.
- Follow threads of research that are well known, and apply these tools and procedures to metabolomics.
- Use a variety of platforms, but do not restrict the use in any single investigation to only one type.
- Ascertain if molecular signatures can be used for metabolomics by looking at multiple pathways.
- Integrate metabolomics in mouse or other animal models.

Dr. Milner asked participants to suggest the types of research that NCI should conduct or support. Participants responded that NCI could support metabolomics research by developing standards (e.g., compound-specific) and collaborating with industry to support the development of assays and encourage commercialization. Another challenge will be to develop informatics

that can support metabolomic data. At this point, the increase in the knowledge base for metabolomics will direct future research directions.

Dr. Maruvadu asked if participants could address the issue of metabolic fluxes. The discussion highlighted the concept that there are differences in fluxes of individual reactions versus input and output in the pass-through of a cell. Differences can be measured in pathways using tracer-based metabolomics, but it is important to identify the substrate involved in the flux. It also is detrimental to consider any issue in isolation of other processes that occur within a cell, and there is support for the study of concentrations in addition to flux. Each discipline feeds on the other, and it is important to recognize this critical issue.

A discussion ensued about applying metabolomics to the clinic and how this can help track changes in individual patients. For cancer prevention, are there fluids (e.g., blood or urine) that can be collected to inform how to treat a patient or prevent a cancer? It also is important to remember that one can treat a patient without knowing the mechanisms involved.

A participant asked if there is a statement that can characterize the state-of-the-science for metabolomics in real tumors. Discussions included the need to understand the metabolic transitions that result from controlled manipulations. That context can begin the search for understanding, although there are no answers at this point. Many issues need to be addressed, such as whether it is more appropriate to use cell lines or primary cells for reproducibility and accuracy; there are issues with cell lines that are not receiving adequate attention. It also is important to remember that tumors often are individual and react differently, even within the same tumor type.

Dr. Harold Seifried, DCP, NCI, Bethesda, MD, informed the participants that NCI is working to make samples available if they were collected under federal funding. A repository is being developed, and standards are being made available in some areas. NCI has funding mechanisms that can be used for smaller metabolomic studies. Issues that the repositories address continually involve patient privacy and differences in sample collection. A participant added that the Human Cancer Genome Project may be developing additional applications of the data that go beyond sequencing, and it is important to follow this development to see if any of these efforts could be used in metabolomics.

TUESDAY, OCTOBER 25, 2005

SESSION VI: MODELS FOR STUDYING METABOLOMIC APPROACHES

Moderator David Goldstein, Ph.D., Chief, Center for Cancer Research, Office of Science and Technology Partnerships, NCI, NIH, Bethesda, MD, introduced Session VI. The objective of the session was to identify challenges in the application of metabolomics in cancer research.

Analysis of Metabolic Phenotype Changes in Response to Therapy in Cancer Animal Models

Risto Kauppinen, M.D., Ph.D., Professor, School of Sport and Exercise Sciences, University of Birmingham, United Kingdom, presented information on research on the detection of metabolites using proton-MRS on tumors and changes in response to therapy. He reviewed NMR spectroscopy results in rat brain tumor that indicate several assigned peaks from various metabolites. His interest is in the biochemistry associated with apoptosis. Dr. Kauppinen described past experiments that showed that glioma cell death was associated with the accumulation of lipid droplets that normally were not present in the cells. He described gene transfected BT4C glioma cell lines that were used as the tumor model in his experiments. Results showed the accumulation of fat droplets during gene therapy induced apoptosis *in situ*, as seen in previous studies using cell cultures as well. Also in this model, the cellular pathways of apoptosis can be identified using immunocytochemical methods, such as the DNA *in situ* nick end-labeling (TUNEL) assay.

Dr. Kauppinen presented a series of slides that showed MRS differences between treated and untreated gliomas, and illustrating that there is an approximately 70 percent increase in polyunsaturated fatty acids (PUFAs) in gliomas and that ^{1}H NMR techniques can be used to quantify and characterize the lipid profile *in situ*. Within lipids, there are significant shifts among lipid type, even if the total lipids in the tumor remain the same. He described investigations of lipid droplet size using diffusion NMR spectroscopy in various cell compartments, with larger droplet sizes found to be associated with cell death. Other experiments, such as lipid extractions analyzed with ^{1}H MRS, were conducted to determine where these fats originated. The ^{1}H NMR detectable lipids appear to come from the cell membranes. Whether these techniques can be used for other tumor types is uncertain; several cancer cell lines *in vitro*, however, have been shown to produce ^{1}H NMR visible lipids during apoptosis.

Recent NMR studies of lymphomas and heptocellular carcinoma have indicated lipid accumulation in response to anti-cancer therapy. Dr. Kauppinen concluded that ^{1}H MRS shows that PUFAs are highly correlated with cell eradication and lipid body formation/repartitioning that occurs *in situ*. The PUFAs originate from the cell membranes, possibly in the mitochondria. Lipids in eradicated tissue become visible using ^{1}H NMR spectroscopy.

Discussion

A participant commented that PUFA is released from breast cancer tissue and not from matched normal breast tissue. The question of whether the event of PUFA accumulation could be involved in apoptosis also was raised. Dr. Kauppinen responded that this has not been the case in his studies. Another participant commented that, when proton-MRS studies are conducted in breast tissue, it is important to be aware that the surrounding cells affect the test results. This is not the case in brain tumors. Dr. Truman Brown mentioned that he has conducted studies on phosphorus in non-Hodgkin's lymphoma that indicate that the phosphocholine and phosphoethanolamine peaks in ^{31}P NMR are strong predictors of treatment response. This indicates that metabolites, associated with membrane biosynthesis, might be involved in these processes.

Other Models To Understand the Cancer Cell Metabolic Flux

Zoltan Oltvai, M.D., Associate Professor, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, commented that in the disease state no single change occurs to cause the disease, but there is reorganization from a normal to a pathophysiological state at the organ and organism levels. To develop an omics platform to understand pathophysiology, biomarkers must be identified for normal, precancerous, and malignant states, which can lead to the identification of drug intervention points. This will take an understanding of systems biology and the integration of all omics platforms.

Dr. Oltvai discussed mathematical matrixes and flux-balance analysis to describe the effect of metabolites and solution spaces that identify potential organism states. Identifying the parameters of metabolism is critical to this process. He described experiments in *Eschericia coli* (*E. coli*) and flux states during growth that indicate that these are measurable and will be the same with different substrates. In addition, flux patterns can be diagrammed to show that pathways are reorganized when suboptimal conditions apply.

Dr. Oltvai described pathways with highlighted reactions that are essential to proper functioning (core reactions) and their reorganization under different growth conditions. Metabolic flux rates of essential and nonessential reactions indicate that nonessential reactions interact with the core reactions, but this is complex. A transcription regulatory (TR) graphic illustrated the complexity; it also is possible to map the specificity of signal recognition and the hierarchy of information flow that define TR subnetworks, known as origons. Dr. Oltvai described types of origons and their topological relationship in the *E. coli* TR network and the origon concept using microarray analysis. The purpose of studying the origon concept is to separate signals and develop computations that describe actual and expected signal interactions.

Discussion

A participant asked about the concepts of "optimization of growth" and "plasticity with respect to signals" and what is being optimized in these systems evolutionarily. In addition, he asked Dr. Oltvai to comment on issues of "boom-and-bust" cycles that can lead to extinction with regard to optimization. Dr. Oltvai responded that he was not sure how to answer the question,

but what makes a system robust in an unfavorable environment is not the same as what is needed to provide maximal growth. Systems must be viewed from an evolutionary perspective, whether for a single-cell or multi-cell organism. The participant added that some of the stability properties that are associated with scale-free networks have been related to these ideas. Dr. Oltvai characterized these as interesting but noted that there are different methods to determine the same properties of networks.

Metabolomics in the Study of CNS Disorders

Rima Kaddurah-Daouk, Ph.D., Research Associate Professor, Department of Psychiatry and Behavioral Science, Duke University Medical Center, Durham, NC, presented background information on central nervous system (CNS) disorders and the state-of-the-science of CNS research. She discussed the complexity of understanding the human brain and the use of genomics, proteomics, and technologies that have begun to show benefit in this research; she noted that a global approach must be part of future approaches. Dr. Kaddurah-Daouk described common biochemical pathways that are of interest in the study of neurodegenerative diseases such as Parkinson's disease, schizophrenia, and drug abuse. Future metabolic pathways are of interest in understanding these diseases. There are few animal models for CNS diseases, which makes it difficult to know what is common and what differs between animals and humans.

Dr. Kaddurah-Daouk provided two examples of CNS disorders—schizophrenia and biomarker research in motor neuron diseases (MND) such as ayotrophic lateral sclerosis—to highlight metabolic approaches for these diseases. In schizophrenia, new research is investigating lipid profiles because there appear to be lipid perturbations, and finding differences in the metabolome of these patients enables understanding of the disease and could lead to potential treatments. Initial results using metabolic signatures indicate that there are significant differences in lipid profiles between controls and treated patients. In schizophrenic patients at baseline, phosphatidylcholine and phosphatidylethanolamine concentrations are reduced, and there is a clear pattern toward decrease in long-chain PUFAs, which suggests impairments in membrane structures. The effect of three antipsychotic drugs—Risperidone, Aripiprazole, and Olanzapine—used in these patients showed a decrease in fatty acids and an increase in triglycerides, although there are differences among each of the three drugs. This suggests that there is a problem in turnover in fatty acid metabolism.

Dr. Kaddurah-Daouk described the initiative for biomarker discovery in MND, which involves collaboration among various public and private entities using proteomics and metabolomic technologies. She described MND diseases and initial experiments for metabolomics using LC to determine oxidation potential for quantification. Thirty patients were selected, and metabolites were analyzed using data mining tools. Results indicated that metabolites differed among controls and patients; and characteristic metabolic profiles existed even among patients with subclasses of MND and those who were on or off medications. This exciting result established tools that can be used to identify potential biomarkers.

Dr. Kaddurah-Daouk added that there is an effort underway to establish pharmacogenetic research networks that will use metabolomic tools, technologies, and informatics to explain whether metabolomics can add to the understanding of drug response.

Group Discussion: Session VI
Moderator: Bruce Kristal, Ph.D., Associate Professor, Dementia Research Service, Burke Medical Research Institute, Weill Medical College of Cornell University, White Plains, NY

Dr. Kristal presented three questions for the panelists to address during the discussion period:

- Which, if any, criteria for models for metabolomics differ from those for other areas of cancer research?
- What are the general lessons to be learned from models?
- What types of data can be retrieved from models and how can they be used?

In discussion, it was noted that a wealth of information has been gathered from model research. Experimental work on lipids has influenced clinical studies and therapy monitoring. Research on apoptosis also has been valuable and can be applied to the general field of cancer research, although there is disagreement as to whether research in these areas is equivalent. Applications for metabolomics are beginning to emerge from different fields of research and being integrated and assimilated for use in analyzing entire systems. Important issues related to model use include the following:

- Mouse models with a fixed genotype are used to model diseases that occur in humans with innumerable genotypes. A suggestion was made to study WT mice rather than in-bred mice.
- Studies in animal models will produce different results, depending on the strains used. This is problematic for developing conclusions from the results of these experiments.
- Models can be beneficial, but they must be based on mechanisms that are mimicked in humans and that produce similar metabolites.
- Animal models should be used, but there should be tight controls and focused research in specific metabolite research.

The participants noted that the easiest part of research is acquiring data; the difficult part is determining what to do with the data. The issue of understanding whether the metabolite that is being measured is related to the disease can be a roadblock unless sound approaches are used from the beginning of the research. Participants stressed the need to understand mechanisms and pathways so that changes in cells, tissues, and tumors can be related directly to identified metabolites. Understanding mechanistic relevance is a significant challenge in cancer research.

An issue that was discussed throughout the session was the need to understand the state of the animal or human at the time that samples are taken for the study of metabolites. It is difficult to account for the numbers of variables unless it is known which variables are relevant to the disease state. This is different than in biomarker research, in which a biomarker can be identified and tested for in small numbers of human patients. There is, of course, a difference in numbers of variables between biomarker and metabolomics research.

A problem that surrounds securing research funding in metabolomics is writing grant proposals so they do not sound like “fishing expeditions.” Because it is never just one metabolite that

determines a cancer type, there must be a way to direct grant applications without being trapped in the area of hypothesis-generation, which NCI often does not view as a valid use of funds.

It was stressed during the session that none of the research in metabolomics will be worthwhile unless there can be relevance for the clinic. It is difficult to translate results from experimental studies quickly for use in clinical studies that can benefit people.

SESSION VII: INTEGRATION OF METABOLOMICS INTO SYSTEMS BIOLOGY

Moderator: Lee Moore, Ph.D., M.P.H., Epidemiologist, Occupational and Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics, NCI, NIH, Bethesda, MD, introduced Session VII. The objective of the session was to discuss a systems biology approach to mapping small molecules in metabolic pathways and biochemical networks in relation to phenotypic changes.

Integration of All "Omics"

Pedro Mendes, Ph.D., Associate Professor, Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA, described the integration of omics (i.e., systems biology) and computational proofs for pursuing omics approaches. He described his investigations at Virginia Tech, using plant and yeast systems, including an early investigation in cancer cell lines. Dr. Mendes commented that fluorescence tools may be more exact tools for identifying expression profiles transcripts, proteins, and metabolomics, but the integration of various technologies is needed to determine the most information possible. He presented slides of procedures used in his investigations and examples of expression profiles and different types of information available using integrative technologies.

Challenges involved in collecting the large amounts of data that result from these investigations include organizing the data to allow intelligent analyses and integrating data from different technologies. Dr. Mendes described the DOMES (i.e., a database for "–omes") database and the types of analyses that are possible with integrative data. Information in the DOMES database then is processed using a DOMES-2 program (i.e., a "wizard" program) that guides the user to select the important data for analyses. He provided a demonstration of the Web-based DOMES systems using structured query language (SQL) for identifying and reporting the data from integrative research.

Dr. Mendes discussed a nomenclature used in his laboratory to describe compounds identified during his investigations. He does not think that there is enough knowledge about biochemical pathways on which to base investigations in metabolomics. He used glycolysis to show that many pathways are not well understood; some are simple and some are complex. Using an approach based on developing mathematical models of biochemical networks that use various computational techniques, Dr. Mendes described investigations that produce results on the levels of metabolites and proteins that are associated with specific cellular environments.

Dr. Mendes discussed the use of his techniques with yeast and malignant cell lines. Discriminant analysis is used to determine results from MS data and to find differences among normal cells,

cancer cells, and blanks (i.e., samples that have undergone the same procedures as the normal and cancerous cells but do not contain cells). He commented that this could be a potential biomarker discovery strategy, although more samples would be needed.

Discussion

A participant asked if the experiments in breast cancer included cell lines or tissue extracts. In response, Dr. Mendes said that they used cell lines; the concept is to use cell lines that emulate different stages of transformation by oxidative stress. Another participant requested an explanation of normalization of the blanks. Dr. Mendes explained that the internal peak of a standardized compound normalizes the data.

Another participant asked for an explanation of the choice of masses selected by the DOMES-2 program and whether it is possible to know which compound the peak identifies because the compound could come from different metabolism pathways. Dr. Mendes answered that the software does not recognize an individual compound from a specific pathway and only can find compounds of interest, not the answers to everything. Chromatograms can be used to identify the specific compounds.

Systems Approach to Cancer

Michael Liebman, Ph.D., Executive Director, Windber Research Institute (WRI), Windber, PA, presented information on a systems approach to cancer research and how omics, although providing a logical approach to understanding biological function and activity, presents only one perspective to solving complex problems. As a foundation in systems biology, omics studies represent a bottoms-up approach, which requires describing normal physiology to enable its comparison with abnormal physiology. To fully understand these differences requires incorporating all available perspectives (e.g., gene expression and proteomics) and might be limited by existing technologies. A contrasting approach is top-down and based on "personalized disease," by recognizing and using the extensive clinical information that already describes patients with "abnormal physiology." Dr. Liebman specifically noted that a challenge to disease research is making translational medicine work in two directions, by enabling physicians to identify the problems that they deal with daily in treating patients and focusing on solutions to those problems in driving the molecular research.

Dr. Liebman discussed the Clinical Breast Care Project, which is a collaboration between the WRI and Walter Reed Army Medical Center. He reviewed patient characteristics, the repository guidelines, and standards for sample collection. The advantages of using military personnel in the project include equal access to health care and the amount of data that can be collected from each patient. Dr. Liebman reviewed a case to show the types of data collected and the types of tests used (e.g., genomic sequencing, genotyping, comparative genome hybridization, loss of heterozygosity, gene expression, and proteomics). These data can then be used for data mining, data modeling, and disease analyses.

Dr. Liebman used the example of the 1918 influenza pandemic to exemplify our lack of understanding of environmental assaults through exposure to disease over time. Studies have

indicated that the offspring who were *in utero* during the pandemic, and potentially were exposed to the infection, have more disabilities and other physical factors (e.g., chronic diseases) than women born in the adjacent time period. This implies that we frequently do not have or utilize all of the information needed to make proper associations regarding causal factors.

One of the projects being conducted at WRI is a data mining activity focusing on 45 scientific journals to develop an ontology of normal breast development, identify gene and protein regulation and pathway changes, and to identify when and where these changes are occurring. Biomarkers also are being studied but can be misleading. For example, *Her2/neu* testing might not be measuring the functional form of *HER2/neu* that needs to be addressed for the correct treatment. Dr. Liebman presented information showing that data gathered at different points in the progression along the cancer continuum can provide different information and affect treatment. He discussed T,M,N staging and how treatment paradigms are created, and some of the difficulties in accurately using the staging system, including subtyping of breast tissue, to prioritize treatment.

Dr. Liebman also provided information on differences in pre- and postmenopausal women regarding breast diseases. An overall approach to disease identification and characterization is based on the fact that many chronic diseases are related to aging; in women, this relates to the menopausal transition. Using data analytic methods and modeling, it may be possible to improve patient diagnosis and the treatment of chronic diseases.

Discussion

A participant asked about a breast cancer histology slide that showed the presence of different types of tumor cells. Dr. Liebman responded that his group is working with tumor blocks and finding each type of breast cancer tumor type in a large number of samples.

SESSION VIII: REPORTS ON BREAKOUTS

Participants met in breakout sessions to discuss specific topics and address questions developed by the workshop organizers and session chairs. After the smaller group meetings, reports were presented on the discussions in the breakout groups. The following are reports presented by session chairs.

Breakout I: Metabolomics versus Other Omics

Chair: Lance Liotta, M.D., Ph.D., Professor of Life Sciences, Department of Molecular and Microbiology, George Mason University, Manassas, VA

Co-Chair: Samir Hanash, M.D., Ph.D., Member and Department Head, Department of Molecular Diagnostics, Fred Hutchinson Cancer Research Center, Seattle, WA

Metabolomics: Profiling the Ongoing Pathophysiology of the Whole Organism

Metabolic signatures secreted or shed from every tissue and cellular compartment exist. Hundreds of known identified metabolites can be measured. Thousands more correlate with biologic state (MS patterns) but have not yet been identified.

Opportunity: Subsets of these identified metabolites, or patterns of unknown metabolites, if validated, may constitute patterns of information concerning the pathologic state of diseases such as early stage cancer.

Challenges:

- Reliability and reproducibility of the analysis platform.
- Complexity of the data and rate/risk of false discovery.
- Design of clinical study sets to avoid bias.
- Independent blinded validation across laboratories and platforms.
- Population Screening versus focused "intended use" trials in which metabolomics is used to individualize therapy.
- Visualization and integration of metabolomics data with other omics data to create a systems biology approach.

Question 1: What information can metabolomics provide that genomics and proteomics do not address for cancer research?

Opinions:

- This is a nonissue; the information from metabolomics is distinct from other omics and is complementary.
- Metabolomics uniquely provides an amplified and integrated record of the ongoing molecular/chemical events in a tissue or organ. Does it provide a higher level of specificity?
- Example applications are: (1) response to therapy (e.g., toxicity versus efficacy); (2) radiation sensitivity; or (3) screening of small molecule inhibitors.
- Metabolomics has a risk of uncovering stress response or other nonspecific signatures reflecting dietary or inflammatory changes.

Conclusion: A focused success story in cancer is needed.

Question 2: Can a metabolomics approach serve as a functional annotation that links to the system biology?

Opinions:

- Systems biology should be hypothesis driven, with a targeted approach using lead molecules from genomics, proteomics, or metabolomics.
- There should be no preconceived assumption about the identity or existence of diagnostic metabolites. Start with comparison sets of large, well-characterized, well-annotated matched clinical sets. Start with the perfect study sets and then expand to field studies.
- It should not be required to have matched genomics and proteomics with metabolomics.

Breakout II: Looking Forward: Applying Metabolomics to Understanding Tumor Cell Behavior

Chair: Dr. Kristal

Co-Chair: Gilbert Omenn, M.D., Professor, Departments of Internal Medicine and Genetics, University of Michigan, Ann Arbor, MI

Background

The NCI of the NIH convened a meeting on October 24-25, 2005 in Rockville, Maryland, for the purpose of facilitating the application of metabolomics approaches to challenging problems in cancer research. This report emerged from a breakout session that was specifically charged with opening dialogues into issues relating to the application of metabolomics to understanding tumor cell biology.

Metabolomics and tumor biology

At the risk of preaching to the choir, it is worth noting that some of the advantages in metabolomics relative to other approaches in biology may enable metabolomics to make unique contributions to the field of tumor biology. Three advantages appear to stand out:

- The metabolome responds to stimuli within seconds or less, far faster than other omics. This speed makes it possible to follow rapid changes that occur in a tumor in response to environmental changes, such as drug treatment or nutrient deprivation. Many such changes may not require similar changes in transcription or translation. Several groups have shown that metabolomics is capable of discerning subtle changes in metabolic pathways and shifts in mechanistic aspects of homeostasis prior to the ability to detect a phenotypic change or macromolecular changes. Thus, we may be able to pick up the earliest changes involved in temporal aspects of tumor biology.

- The metabolome is the end-product of both nature (including the mutational changes inherent and implicit in carcinogenesis) and nurture. This gives researchers the best chance of finding signals dependent on gene-environment interactions or just environmental influences, which can be deciphered subsequently.

- Metabolic pathways may represent “final common pathways” for multiple receptor and signaling pathways, integrating information that is difficult to evaluate in lists of genes or proteins whose expression levels are altered by a particular perturbation.

Together, these aspects of metabolomics investigations suggest that they will be useful for investigating currently limiting questions in tumor biology

Molecular characterization of changes during carcinogenesis and tumor progression has focused on gene expression and protein expression patterns. Increasingly, there is attention to interpretation of the functional consequences of up- or downregulation of various specific genes or proteins or pathway-related genes and/or proteins. Just as mRNA levels do not always predict protein levels, however, neither protein nor mRNA levels can consistently predict metabolite

levels or flux, a major remaining piece of the systems biology puzzle. Indeed, many perturbations are known that act directly on metabolic pathway targets or intermediates without requiring changes in mRNA or protein levels. In the regulation of energy metabolism, for example, pyruvate dehydrogenase activity is modulated by calcium through phosphorylation, phosphofructokinase via feedback inhibition by ATP and citrate, and mitochondrial electron transport by the membrane potential. The kinase and second messenger cascades critical for cell signaling, survival, and growth provide another example. Direct analysis of metabolites and metabolic pathways thus offers much-needed and complementary molecular information both for understanding carcinogenic processes and for evaluating therapeutic and adverse effects of therapies.

Can metabolomics address the limiting factors in tumor biology research?

Any consideration of the potential for metabolomics to influence research in tumor biology must begin by asking what the limiting questions are. That is, what are the bottlenecks? What are the most important/relevant models? How do we know and how sure can we be? What are the conditions under which we should study these phenomena, and what time points should be studied?

Two related major foci for research were highlighted: (1) What is the molecular or metabolic etiology of a given tumor? (2) What metabolic changes underlie metastasis, the main route to lethality? Together these questions reflect an underlying query: How different are tumors that are classified together but may be highly heterogeneous?

We concluded that metabolomic approaches can help discover and further develop metabolic biomarkers that are distinct in precancerous tissues or in the tumor cell environment of premetastatic lesions. In both cases, limitations are how early changes can be detected, and whether the changes that make a cell cancerous or metastatic are unique to that cell or depend upon the microenvironment. If the changes are early and transient, the signal early in the process of carcinogenesis or metastasis would likely be swamped by the noise of the remaining tissue/tumor. A related question lies in understanding the metabolomic correlates of shifted balance among growth, stasis, reduced apoptosis, and increased proliferation.

What are the key metabolites relevant for tumor cell behavior?

The observation that tumor cells and non-neoplastic cells have differing metabolism dates back at least to Warburg. The advent of imaging tools such as NMR and PET has enabled observation of some of these differences in living patients. From these observations, on which metabolites should we need to focus on?

The discussion group agreed that there are probably few or no metabolites that are consistently informative across all cancers. This has a functional implication that all metabolites are thus potentially important, and the above question probably cannot be directly answered for a general case. Furthermore, we need to specify the various attributes of tumors that are the focus of study; which cells, fluids, or tissues are available for study; and the manner in which tumor heterogeneity will be addressed.

These complications and confounders put a premium on performing nontargeted analysis, even if the primary analysis is targeted (e.g., based on the Warburg hypothesis). With two very different analyses, one can test well-developed hypotheses, and the other can generate hypotheses for future testing. The two are not mutually exclusive (although resource and statistical limitations of the combination must be considered in the design). This systematic uncertainty suggests that experiments which might be termed data driven, hypotheses generating, or discovery-based by their proponents and "fishing" by their detractors may have an important part to play over the coming period in cancer research.

We conclude that it is possible, indeed probable, that there are limited numbers of metabolomics phenotypes for any given tumor type. Functionally, following the tumor phenotypes rather than individual metabolites should reduce the dimensionality of the "key metabolite" problem substantially, perhaps by 1,000-fold. Within the realm of classification, we might classify tumors and characterize them metabolically both with regard to response to drug (stasis, death, and resistance) and to basal states. Simultaneous measurement of the tumor metabolome, plasma proteome, and tumor transcriptome would strengthen the studies. This is because the genetic changes in the tumor may be associated with particular attributes and behaviors of the tumor cells.

Not only are tumors different from normal tissues, they are both externally and internally heterogeneous. Metabolomic queries and signatures may help us to address ever-broadening questions, such as:

- Which differences are related to the mechanism in the individual tumor, which are related to site of origin, and which are general properties of tumor cells?

- How does diet affect tumor metabolism, and how does this correspond, or not, to the changes seen in equivalent, nontransformed cells?

- How do comorbidities and "on-board" medications affect tumor metabolism?

- How do chemoprevention regimens affect tumor metabolism?

- How does metabolism differ as one moves into a solid tumor across the oxygen diffusion gradient?

- Are there efficient ways to separate signal from noise without requiring unfeasibly large studies?

Can metabolomics help us understand tumor responsiveness to chemotherapy?

Yes. For most tumors, lethality is a function of metastases, not growth of the primary tumor. As surgical resection becomes less efficacious, chemotherapy and radiotherapy become the patient's best, and often only, hope. With the exception of a several recent molecularly targeted drugs (e.g., such as Herceptin, Gleevec, Avastin, and Iressa), chemotherapy approaches have changed little conceptually since their initial introduction. The concept of attempting to kill a tumor

before killing the patient is inherently reliant on the nature of the tumor's response to chemotherapy. This problem is ideally suited for metabolomic approaches. There are at least five specific questions that need to be addressed:

- Can we better understand the metabolic changes induced by these agents in specific tumor types? In more detail, can we use metabolomics to better understand the mechanism of action, and side effects, of chemotherapy agents?

- Can we predict the response of an individual patient and individual tumor to a drug (pharmacometabolomics)?

- Can we understand the tumor cell pathways that are critical to the prediction of continued growth versus cell cycle arrest and of differentiation versus cell death?

- Can we understand the mechanisms of resistance? Can we use these data to better model tumor metabolism *in vitro*?

- Can we understand how drug efficacy is or is not altered as the drug moves into a solid tumor across the oxygen diffusion gradient?

The questions above represent only a fraction of the ways in which metabolomics as a field can potentially hope to influence and aid work in translational cancer research. Given the potential of these approaches, it also seemed appropriate to consider the technical bottlenecks most likely to slow these advances. It was agreed by the majority of the participants that the most important limitation was access to well-annotated, well-controlled human samples. It was specifically suggested that improvements of access to already available samples—and to the metadata about these samples and their availability—and the *de novo* creation of additional samples collected under well-defined conditions, would be the most important advance in the area. A second major area of agreement was the need for informatics models to aid in data interpretation with respect to both generalized analysis (e.g., a data pipeline for classification informatics) and pathway analysis. A related need is for a single, broadly accessible database that links disease states and metabolism. Another common resource requested was for more analytical standards to be generally available. From the analytical side, advances in *in vivo* analytical capacity were seen as an avenue for future development. Finally, it was noted that metabolomics must eventually integrate seamlessly into the realms of hypothesis driven research, other "-omics," and systems biology; work that, in part, is being addressed as standards are being set in the field (http://www.metabolomicssociety.org).

Breakout III: Evaluation of Technologies
Chair: Dr. Brunengraber
Co-Chair: Alan Kleinfeld, Ph.D., Member, Department of Membrane Biology, Torrey Pines Institute for Molecular Studies, San Diego, CA

Questions 1: What are the strengths and weaknesses of the most commonly used metabolomic technologies?

Opinions:

- NMR is faster, cheaper, and very reproducible; MS is usually more sensitive.
- Probes are usually univalent, but array of probes might be useful.
- The number of isomeric compounds complicates assays.
- When using stable isotopes, NMR and MS yield complementary information: mass isotopomers and positional isotopomers, which each yield different information.
- Some NMR techniques can be used *in vivo*.

Question 2: Which bioinformatic tools can/should be used for the metabolomics data analysis?

Opinions:

- It depends on the question: unsupervised or supervised.
- New tools (bioinformatic and statistical) need to be developed; "machine learning" and "multivariant" analyses should be emphasized.
- We need new methods to build and link metabolic pathways automatically.
- The number of isomeric compounds complicates assays.

Question 3: How can imaging be used to detect metabolites in cells or organs?

Opinions

- Imaging methods are already in use; MRS and MS imaging, fluorescent probes, specific contract agents, isotope probes, and molecular probes have been demonstrated for metabolites.
- New cancer disease-relevant targets need to be identified; and example is tumor hypoxia signature.

DISCUSSION OF BREAKOUT SESSIONS

During the discussion of the breakout sessions, specific statements that could guide future directions in the field of metabolomics were considered.

A participant began the discussion by commenting that, because metabolomics is such a new field, it may not be wise to constrain investigations because there is not a clear understanding of how results from such investigations can be used in the clinic. Risk assessment and diagnosis are worthy goals, and each has its own possibilities for potential breakthroughs through metabolomic research.

Another participant commented that individualized monitoring and therapy was the closest clinical application that could be visualized. Screening is unlikely to be a goal because the number of people who would have to be screened for some cancers would be substantial to find only one cancer. Ovarian cancer, for example, occurs in only 1 of 2,500 women; screening, even with 99 percent sensitivity, may potentially miss 25 of the 2,500 women. It may make more sense to start with drug toxicity or drug response studies with metabolic profiling. It appears that

it will be a long time before testing will be available. Another participant added that this exemplifies the need to combine technologies, such as imaging and profiling, and apply this to an individual. An example of this strategy could be in confirming suspicious mammogram findings.

Dr. Milner suggested that participants could find out about ongoing clinical studies by visiting the DCP Web Site (http://www.cancer.gov/prevention/). There also is a Human Nutrition Research Information Management (HNRIM) system Web Site (http://hnrim.nih.gov/) that lists ongoing studies in nutrition. He said there is no need to reinvent a research entity from scratch when there are ongoing studies that may be relevant that could include basic metabolomic research. Another issue Dr. Milner raised was the types of funding mechanisms (e.g., R03, R21) available for exploratory studies in metabolomics; if the studies were not funded at a high enough level, NCI would need to be informed of that. Dr. Kristal noted that many people in the metabolomic community feel that the NCI study sections often are not open to approving grants in such exploratory studies. Dr. Seifried interjected that R03 grants have been available for interdisciplinary research that relates to issues being discussed. Dr. Milner responded that it is important to generate interest in the types of studies discussed at the workshop and find support by providing hypothesis-driven grant proposals.

Dr. Couch said that NCI is interested in omic research; approximately $5 million has been invested in proteomics and standards. Dr. Milner added that metabolomic researchers should consider piggybacking on some of the ongoing research. He said that NIH might consider a working group to look at these issues. Another participant commented that the NIH Roadmap Initiative is an appropriate place to look for ongoing initiatives that include metabolomics research issues.

FUTURE DIRECTIONS

Drs. Kim and Maruvada

Dr. Kim thanked participants and presented slides summarizing the main points raised by workshop participants. Those included:

- **Characterization of individual's response to cancer preventive dietary components or chemotherapeutic agents using metabolomic profiling;**

- **Metabolite-based phenotyping of various tumors to assess the functional significance of metabolites in cancer prevention; and**

- **Establishment of a tumor metabolome database for the entire human metabolites to reflect various stages of cancer development.**

Dr. Maruvada thanked the chairs and co-chairs, speakers, and participants for making the workshop a success. She said that the proceedings from the meeting would be published in the *Journal of Metabolomics*. Dr. Maruvada will contact speakers to ask permission to place slide presentations on the meeting Web Site (http://www.cancer.gov/prevention/frontiers/index.html).

She asked participants to contact Dr. Kim or her if they have questions about the meeting. She adjourned the meeting by again thanking participants for attending.