The Emerging Field of Quantitative Blood Metabolomics for Biomarker Discovery in Critical Illnesses

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Metabolomics, a science of systems biology, is the global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity, and the physiological landscape. Metabolite detection, either individual or grouped as a metabolomic profile, is usually performed in cells, tissues, or biofluids by either nuclear magnetic resonance spectroscopy or mass spectrometry followed by sophisticated multivariate data analysis. Because loss of metabolic homeostasis is common in critical illness, the metabolome could have many applications, including biomarker and drug target identification. Metabolomics could also significantly advance our understanding of the complex pathophysiology of acute illnesses, such as sepsis and acute lung injury/acute respiratory distress syndrome. Despite this potential, the clinical community is largely unfamiliar with the field of metabolomics, including the methodologies involved, technical challenges, and, most importantly, clinical uses. Although there is evidence of successful preclinical applications, the clinical usefulness and application of metabolomics in critical illness is just beginning to emerge, the advancement of which hinges on linking metabolite data to known and validated clinically relevant indices. In addition, other important aspects, such as patient selection, sample collection, and processing, as well as the needed multivariate data analysis, have to be taken into consideration before this innovative approach to biomarker discovery can become a reliable tool in the intensive care unit. The purpose of this review is to begin to familiarize clinicians with the field of metabolomics and its application for biomarker discovery in critical illnesses such as sepsis.

Keywords: sepsis; acute lung injury; pneumonia; trauma

Critical illness due to trauma, sepsis, and septic shock remain clinically challenging problems for a number of reasons, including poorly understood complex physiology and patient heterogeneity. In addition, these illnesses are likely to remain relevant as their incidences continue to rise as the population ages. In fact, sepsis affects more than 750,000 patients annually in the United States and remains a leading cause of death worldwide (1, 2). This significant

hazard to human health is coupled with an economic burden to the healthcare system. The mean total hospital charges for sepsis can be in excess of \$190,000 based on 2008 data released by the Healthcare Cost and Utilization Project of the Agency for Healthcare Research and Quality (3). Despite this level of spending, little progress has been made in improving outcomes, as 25 and 30.5% of patients with sepsis or respiratory failure, respectively, admitted to hospital, die before discharge (3), and long-term morbidities adversely affect functionality and quality of life (2).

The absence of biomarkers that predict early disease onset, progression, and severity have had a negative impact on our ability to identify and develop effective pharmacotherapy aimed at improving morbidity and mortality in patients with critical illness. Nevertheless, biomarker discovery is no small undertaking and typically requires years of validation testing before the application phase is reached (Figure 1) (4). An ideal biomarker will not only be sensitive and predictive, it should be measurable in a readily accessible biofluid and cost effective. Importantly, the usefulness of a biomarker in critical illness will be based on its ability to differentiate what may initially be subtle changes in phenotype, such as the transition of a patient from systemic inflammatory response syndrome to multiple organ dysfunction syndrome or the early development of acute lung injury (ALI) in a septic patient. In addition, its validation will require that it be linked to established clinically relevant indicators of disease severity (e.g., Acute Physiology and Chronic Health Evaluation score) or outcome (e.g., mortality) (5). As such, it is likely that no single biomarker will accurately represent or distinguish these or other differences in patients in the intensive care unit (ICU) because these patients are fundamentally heterogeneous and there is significant uncontrolled variability in this patient population. This does not dismiss the possibility of meaningful biomarker identification in these patients because this inherent variability is not necessarily due to disease but rather to normal physiological processes, such as those related to age, diet, time of day, and sex. Therefore, it is not unreasonable to expect that a profile or pattern of indices is more likely to demarcate disease severity and progression than any one parameter.

In this context, the sequencing of the human genome has moved systems biology into the forefront of biomarker discovery. However, the fields of genomics and proteomics have yet to deliver any biomarkers that are clinically applicable, and none have gone beyond the discovery phase (6). This lack of translation and validation could stall progress toward biomarker qualification in critical illnesses and hinder the usefulness of systems biology in the process. It has also brought about the realization that the sequencing of the human genome alone cannot provide the insight that is needed to unravel the complexity

(Received in original form March 15, 2011; accepted in final form May 28, 2011)
Supported by the National Institutes of Health grants P30 CA046934–14 and UL1 RR025780.

Author contributions: All authors have made substantive intellectual contributions to the manuscript.

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Am J Respir Crit Care Med Vol 184, pp 647–655, 2011
Originally Published in Press as DOI: 10.1164/rccm.201103-0474CI on June 16, 2011
Internet address: www.atsjournals.org

Figure 1. Identification of clinically relevant biomarkers requires a rigorous multistep process that can take years and includes analytical and prospective validation to demonstrate accuracy and prognostic value of the marker(s). The qualification phase includes the validation of the marker. This also encompasses the assessment of assay accuracy and the association of the marker with a clinical feature or outcome. Presently, the use of metabolomics for this purpose in critical illnesses is in the exploratory phase.

of disease, particularly acute, severe illnesses. This is a scientific challenge of the systems biology approach and highlights the need for new knowledge of the relationships and interactions between the components of the system (Figure 2). Monitoring fluctuations of certain metabolites (endogenous low-molecular weight molecules) in body fluids, such as blood (including plasma and serum) and urine, is an important way to detect various human pathologies, including cancer, cardiovascular disease, diabetes, and drug toxicity (6–15). These data are also needed to construct powerful top-down systems biology tools that link the “omics” disciplines (16). This top-down idea is based on the principle that small molecule metabolites are at the top of systems biology continuum and that metabolites reflect and magnify (several thousands of times) events that occur at the level of the genome, transcriptome, and proteome (Figure 2) (17, 18). Undoubtedly, metabolomics could have very meaningful applications in critical care medicine because acute illness most often causes significant disruption in biochemical homeostasis. Unfortunately, it is still in its infancy, which may be due, in part, to a lack of understanding of metabolomics science by clinical researchers. In the present article, we describe principles of metabolomics with a special emphasis on the existing challenges of its application to critical care medicine, including sample collection and handling, advantages and disadvantages of available analytical platforms, and data interpretation.

METABOLOMICS AS A COMPONENT OF A SYSTEMS BIOLOGY APPROACH TO BIOMARKER DISCOVERY

Metabolomics (also known as metabonomics), is the global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity, and the physiological landscape (19–21). Metabolomics is also a broad term for a range of different methods, including: (1) metabolic finger- or footprinting, which measures a subset of the whole profile followed by multivariate spectral analysis (e.g., principal component analysis on nuclear magnetic resonance [NMR] spectroscopy or mass spectrometry [MS] spectra) with little differentiation or quantitation of metabolites; (2) metabolic targeted profiling, the quantitative analysis of patterns of metabolite identities and concentrations followed by classical statistical or multivariate analysis on defined metabolite concentrations; and (3) target isotope-based analysis or targeted metabolomics, which focuses on a particular segment of the metabolome by analyzing only a few selected metabolites

that compose a specific biochemical pathway. Generally speaking, for preclinical animal studies (especially using urinary markers for drug toxicities), metabolic fingerprinting has been applied due to the large number of samples that have to be analyzed. This provides a rather “black box” classification approach (e.g., toxic versus nontoxic) with no precise biomarker discovery. On the other hand, for the mechanistically oriented basic science approach, targeted metabolomics is ideal, because it precisely (quantitatively) evaluates a specific metabolic pathway. Targeted metabolomics is also preferred because it allows for cross-validation of quantified metabolic markers between multiple laboratories for assessment of metabolite predictiveness as well for creating quantitative metabolite databases for diverse populations. Thus, because most clinical studies are seeking more

Figure 2. Schematic representation of the “top-down” relationship between the components of a systems biology approach. Metabolomics is part of the continuum of systems biology and represents a read of gene function and the physiological landscape. Pathological events can be measured by changes in the genome, transcriptome, proteome, and metabolome. Integration of these systems biology components can be viewed as host-specific rather than tissue- or site-specific. Bioinformatics is a key element in data management and analysis of collected data sets arising from genomics, transcriptomics, proteomics, and metabolomics. Identified proteins and metabolites can be assessed by fast and highly specific clinical laboratory assays in the body fluids or, ultimately, by tracer-based molecular imaging. Magnetic resonance imaging/spectroscopical imaging (MRI/MRSI) and positron emission tomography (PET) provide attractive noninvasive platforms for biomarker detection in organs. (Partially adapted from Reference 29.)

precise biomarker identification, quantitative metabolic profiling is beginning to emerge as the preferred strategy (10, 19).

Initial differences in metabolites may be predictive of disease severity, and changes over time may be useful in characterizing therapeutic response, disease progression, or clinical outcome. These differences and changes can also be linked to biological events that provide clues to the pathogenesis of disease or mechanisms of drug action or toxicity using computational or bioinformatics models that can map and visualize network interactions (21–24). Alternatively, as a complimentary approach to genomics and proteomics, metabolic imaging techniques (such as positron emission tomography or magnetic resonance spectroscopic imaging) can be used to translate noninvasive imaging protocols in humans (Figure 2). Therefore, systems biology is a logical strategy for biomarker discovery in critical illnesses because the complexity of these diseases lend themselves to a multipronged and integrated approach that links genes, proteins, and metabolites (Figure 2). However, the advancement of the field is dependent on the generation of data sets in each discipline. Regrettably, the active and advancing research in genomics and proteomics has not been met with a similar effort in metabolomics (6).

Most routine clinical chemistry assays are only qualitative and measure a single metabolite in patient samples (20). These tests are not sensitive, predictive, or specific to any particular disease and often have to be taken in context with other analytical or clinical assessments (20). Alternatively, metabolomics is the acquisition of multiple metabolites (sometimes more than 100) from a single sample without preconception. When coupled with the significant advances that have been made in computational technologies it permits the measurement of complex metabolic profiles in biological fluids.

The field of metabolomics is not new. In principle, it has existed for centuries and is based on the premise that changes in tissues and biological fluids reflect disease processes (25). Diagnostic “urine charts” were initially used in the Middle Ages to link the color, smell, and taste of urine to a range of medical problems. It is now recognized that these changes are due to metabolic abnormalities and we now use contemporary analytical techniques to measure them. Modern-day metabolomics began in the 1960s when metabolic-control analysis was developed using a mathematical approach to model cellular metabolism (25). This strategy used gas chromatography (GC) or GC-mass spectrometry (MS) to quantify metabolites. In parallel, nuclear magnetic resonance (NMR) spectroscopy, developed by the mid-1980s, was sensitive enough to identify metabolites in unmodified biological fluids. This led to the discovery that changes in metabolite profiles were due to certain diseases or drug toxicity (25). Techniques in MS and NMR have improved (in both specificity and sensitivity), but metabolomics advanced most as a science with the introduction of pattern-recognition methods (e.g., chemometrics) that were pivotal in the interpretation of the complex data sets that are generated by these studies.

Analytical Platforms for Metabolite Detection and Quantification

Either individually or grouped as a metabolomic profile, detection of metabolites is usually performed in cells, tissues, or biofluids by either NMR spectroscopy or MS (6, 7, 26). In general, NMR spectroscopy, mostly ^1H -NMR, and MS, particularly, GC, liquid chromatography (LC)-MS and Fourier-transform (FT) ion cyclotron resonance (FT-MS), are the primary spectroscopic techniques used in metabolic analysis.

NMR exploits the behavior of molecules when placed in a magnetic field allowing the identification of different nuclei

based on their resonant frequency when exposed to magnetic fields. The resultant metabolite detection and quantification is acquired from a data set called a spectrum (Figure 3). In NMR spectra, the *x*-axis is given as parts per million (ppm), which refers to the small variation of resonance frequencies (referred as “chemical shift”) of individual chemical entities of various metabolites. Based on unique chemical structure, each chemical group (CH , CH_2 , CH_3 , etc.) of each metabolite will have a unique chemical shift (in ppm), which will allow for metabolite identification by NMR. Moreover, NMR is a highly quantitative technique, because each metabolite peak will have an integral value that is directly proportional to the metabolite concentration (a distinct advantage over MS analysis). MS determines the composition of particles based on the mass-to-charge ratio in charged particles. Each technique has distinct advantages and disadvantages (13, 27). The major advantages of ^1H -NMR include its unbiased metabolite detection, quantitative nature, and reproducibility (6, 7, 26). ^1H -NMR can be used for liquid or solid samples, using high-resolution magic angle spinning (HR-MAS) techniques, with minimal sample preparation (Table 1, Figure 4). In addition, nearly all major classes of metabolites have characteristic NMR spectra, which makes this technique very useful for metabolite fingerprinting. Recent advances in NMR technology have improved its capability to measure small amounts of metabolites in reduced sample volumes, which makes sensitivity less of a concern (20). However, the main limitation of NMR is that it is relatively insensitive to very small amounts of metabolites, as it requires concentrations of about 1 to 2 μM in comparatively large sample volumes (~ 0.5 ml; Table 1).

The MS analysis requires more labor-intensive (and destructive) tissue preparation than NMR, but its advantage is higher sensitivity for metabolite detection. This results in detection typically in the picogram level. It also allows for specificity in metabolite identification at low concentrations, which is useful for biofluids like bronchoalveolar lavage fluid, in which metabolite levels are low, and for more compounds to be screened compared with NMR. Although polar molecules may be detected when electrospray ionization is used, nonpolar molecules may require atmospheric pressure chemical ionization. Similarly, the methods of extraction, quenching, and sample storage conditions can affect and potentially modify metabolite structure, thereby confounding already complex data sets and introducing more sample-to-sample variability. Although high-resolution profiling methods exist for GC-MS, detectable compounds are limited to those that can be derivatized, which can be time consuming, costly, and carries a risk of metabolite loss. Conversely, LC-MS does not require derivatization, but it has only recently begun to be applied to metabolic profiling due to recent major advances in chromatography, instrumentation, ionization capabilities, and software. In spite of a rich history of discovery- and targeted-based methods in small molecules using MS, a widely adopted and validated methodology for sensitive, high throughput discovery-based LC-MS metabolomics is still lacking.

Overall, although there are advantages and disadvantages to both MS and NMR, ^1H -NMR spectroscopy has more potential as a screening tool in translational research because it can provide comprehensive information on the degree of organ dysfunction and putative mechanisms of injury and has been proven to be more robust, reliable, and reproducible across a range of laboratories (13, 28, 29). More recently however, the coupling of NMR and MS to the quantitative measurement of small molecule metabolites enhances sensitivity and specificity and permits the rapid identification and quantification of metabolites from a single patient sample (10, 11, 17, 20). This improves the likelihood of detecting subtle changes in metabolites and metabolite profiles.

Figure 3. Representative high-resolution ^1H -nuclear magnetic resonance (NMR) spectra obtained by a Bruker500 MHz spectrometer. Spectra peak sizes are directly proportional to metabolite concentration, which is an advantage of NMR compared with MS. Peak positions on the x-axis are indicative of the chemical properties of metabolites and allow for metabolite identification. Body fluids containing water-soluble small-molecule metabolites do not require any sample extraction such as (A) human expressed prostatic secretions (EPS), or (B) rat urine. Blood products that contain large lipoproteins and other hydrophobic molecules will yield more metabolic information if extracted: (C) nonextracted human serum versus (D) hydrophilic fraction of the whole rat blood after dual methanol/chloroform extraction. Finally, tissue samples require either extraction (E) hydrophilic fraction of a human brain biopsy (9 mg) after dual acid extraction using a 1-mm Bruker microprobe, or (F) use of high-resolution magic-angle-spinning (HR-MAS) NMR on intact rat muscle. Selected peak assignments: (B) rat urine: 1, valine, leucine, isoleucine; 2, lactate; 3, CH_3 -acetyl groups; 4, succinate; 5, 2-oxoglutarate; 6, citrate; 7, creatinine (and creatine); 8, trimethylamine-N-oxide (TMAO with betaine and taurine); 9, trans-aconitate; 10, hippurate; 11, allantoin; 12, urea; 13, trans-aconitate. (D) Extracted blood: 1, valine, leucine, isoleucine; 2, hydroxybutyrate; 3, threonine; 4, lactate; 5, alanine; 6, arginine and lysine; 7, acetate; 8, CH_3 -acetyl groups; 9, glutamate and hydroxybutyrate; 10, glutamine; 11, total glutathione; 12, reduced glutathione (GSH); 13, creatine and creatinine; 14, trimethylamine-N-oxide (TMAO with betaine, taurine, glucose and arginine). (E) Extracted brain biopsy: 1, valine, leucine, isoleucine; 2, threonine; 3, lactate; 4, alanine; 5, N-acetyl aspartate (NAA); 6, CH_3 -acetyl groups; 7, glutamate; 8, glutamine; 9, xenobiotics; 10, phosphocreatine and creatine; 11, cholines and taurine; 12, glucose; 13, myo-inositol. Abbreviations: Cho = cholines; Cr = creatine; Lac = lactate; Tau = taurine; TSP = d-trimethyl-silyl-propionic acid; UFA = unsaturated fatty acids; (Adapted from Reference 13).

Regardless of the analytical platform used, the basic workflow for metabolomics studies is: quenching/extraction of metabolites $\rightarrow$ data collection $\rightarrow$ data processing/analysis (7, 13). As our expertise and experience is with NMR-based metabolomics, we will address issues related to sample handling and preparation and data analysis from this perspective.

Sample Handling and Preparation

Information on sample requirements and handling for metabolomics analysis has been previously published (7). Briefly, all biological samples, collected for metabolic analysis, require

careful sample handling. This includes maintaining the sample on ice immediately after collection and, if applicable, during centrifugation and the storage of samples at a uniform temperature (ideally at -80°C) before assay. Presently, there are no data that address metabolite stability during storage, which makes consistent sample handling within a study all the more important. Urine may be more susceptible to changes in storage conditions than blood or plasma because of the possibility of bacterial contamination (30). Use of a consistent extraction process is also crucial. These latter two points can make comparisons across data sets or studies from different laboratories problematic.

TABLE 1. SUMMARY OF BIOMATERIALS USED FOR ¹H-NUCLEAR MAGNETIC RESONANCE METABOLIC ANALYSIS IN VARIOUS AREAS OF BIOMEDICAL RESEARCH

Biofluid	Required Sample Handling
Urine	Add deuterated phosphate buffer to 0.5–3 ml urine
Blood	For 0.5 ml of heparinized blood product:
Plasma	only deuterium oxide addition (lock)
Serum	acetonitrile addition (protein precipitation) methanol/chloroform extraction (lipid separation)
CSF	Addition of deuterium oxide to 0.5 ml CSF
EPS	Add deuterium oxide to 0.03–0.10 ml EPS
Bile	Add deuterated methanol to 0.5 ml bile
Saliva	Add deuterium oxide to 0.5 ml saliva (usually obtained after chewing a polyester tampon for 60 s)
Sputum	Add deuterium oxide (0.1 ml) to 0.5 ml sputum
BALF	Add deuterium oxide to 0.5 ml BALF
EBC	Volatile substances in 3–4 ml condensates (sampled using an EcoScreen condenser) removed by nitrogen stream; deuterium oxide (0.1 ml) is then added to 0.6 ml of EBC
Tissue	Add 0.01 mL deuterium oxide to 3–10 g tissue in MAS rotor Perchloric acid extraction on 20–200 mg frozen tissue Methanol/chloroform extraction on 20–200 mg frozen tissue

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; CSF = cerebral spinal fluid; EBC = exhaled breath condensate; EPS = expressed prostatic excretions; MAS = magic angle spinning.

Although diet and time of day may influence endogenous and exogenous metabolite levels, these are less likely to be discriminating metabolites of pathological relevance, particularly in the assay of blood. Compared with blood products, urine is more prone to the influence of diet and diurnal variation, but it has an important role in the acquisition of metabolite data in certain patient populations. In particular, urine metabolomics is well suited for young children and in patients in whom venous access is problematic or for those who are averse to blood sampling. Notably, pathogen-specific changes in urine metabolites were detected in an experimental model of pneumonia (31). This was mimicked in the human situation, in which this approach successfully differentiated bacterial and viral pneumonias (32).

Inherent variability can be minimized by collecting samples in the morning (08:30–09:30) preferably before food intake (33, 34). For biofluids, the optimal sample volume is 0.1 to 0.5 ml. For NMR, minimal sample preparation is required for urine and other low-molecular weight metabolite-containing fluids (Table 1). Blood, plasma, and serum can yield more metabolic information if extraction (using acid, acetonitrile, or two-phase methanol/chloroform protocols) or NMR-weighted techniques are applied to separate (or suppress) polar and lipophilic metabolites (Figure 3). Cancer cells and/or tissue biopsies usually undergo acidic extraction (7–12% perchloric acid, for example) or dual-phase methanol chloroform extraction. The extraction step and consequent lyophilization of the extract allows for (1) separation of lipid and water-soluble metabolites to prevent peak overlapping, (2) precipitation of proteins and other macromolecules to avoid peak contamination and line broadening, (3) acquisition of the liquid state spectra from tissue specimens with high-resolution and low line broadening, (4) use of deuterated solvents to suppress water signal. Based on our experience, acid extraction allows for better recovery of energy-rich phosphate metabolites, whereas dual-phase extraction has a better recovery rate for lipid-containing metabolites. Lyophilized extracts (body fluids, cells, tissues, cell culture media) are then usually redissolved in deuterated solvents and analyzed by conventional 5-mm or ultra-small (micro-) 1-mm high-resolution NMR probes at the magnetic field strength of 7.0 and 14.1 Tesla (300–600 MHz for proton frequency) (19, 35, 36). In general,

however, there is presently no standardized protocol for sample preparation for NMR analysis, and the protocols presented here are based on literature reports and our experience in the field of NMR-metabolomics over the past 15 years (19, 35, 37, 38).

Spectral Interpretation and Data Analysis

One reason the clinical application of metabolomics is challenging is because of the requirement for the ready availability and accessibility of an analytical laboratory and an investigator capable of performing the spectral interpretation and data analysis. Interpretation of metabolite data is complicated by a number of variables, one being that because humans require nutrients from the environment (i.e., we are unable to manufacture our own), the metabolome is composed of both exogenous (e.g., by-products of food consumption) and endogenous metabolites (17). Endogenous metabolites are those generated by enzymes encoded by the host's metabolome. Nevertheless, endogenous metabolites (including blood lipids, carbohydrates, lactate, and amino acids, just to mention a few) are also affected by food intake, daily routine, and sample handling to a greater extent than protein or gene compositions. As such, it is essential that the investigative team include a knowledgeable and experienced metabolomics researcher to perform the initial metabolic fingerprinting, namely ¹H-NMR, because it is technically very complex and expensive and requires expertise to discriminate these spectra. These requirements are well known and described for the use of metabolomics in clinical oncology, nutrigenomics, and toxicology studies and therefore do not preclude the execution of metabolomics studies in critically ill patients despite that some variables (e.g., food intake) may be more difficult to control.

Metabolomic data analysis can be accomplished using a chemometric (e.g., principal component analysis, partial least squares discriminant analysis) approach either on spectral data sets or on quantitative metabolic data sets. A chemometric spectral approach generates information about trends in spectral patterns (Figure 4), often without metabolite identification/classification, and along with other multivariate statistical analyses it is the most common approach used because it permits the interpretation of large metabolite data sets without requiring the precise measurement of metabolite concentration. In this way, pattern recognition is used to distinguish the metabolite profiles of control subjects from diseased subjects. It can also be used to determine which specific metabolites are responsible for the pattern deviation (Figure 4), filtering the metabolite “noise” that may be unrelated to disease. However, chemometric analyses are not perfect, and there is still the risk of the introduction of bias. As such, the use of a chemometric strategy on spectral data alone without further metabolite validation is not recommended. It should be followed by quantification of metabolites and/or a bioinformatics analysis with validation in subsequent studies (Figure 4).

Quantitative metabolomics requires the identification of specific metabolites and their association with biological functions. The advantage of quantitative metabolomics is that it is unbiased, is performed without preconception, and is suitable for the identification and quantification of all detectable metabolites from a single sample, which are found using a reference spectral library of known endogenous metabolites (9, 28, 29, 39) (Figure 4). Ideally, a classic metabolomics studies should consist of three major steps: (1) data collection (acquisition of NMR or MS spectra) followed by chemometric classifier for “normal” versus “abnormal” (Pattern Recognition) → (2) identification of spectral regions and corresponding metabolites, which are responsible for group separation (Identification) → (3) quantitative validation of distinguished metabolic markers (Quantification)

Step 1. Pattern Recognition

Step 2. Identification

Step 3. Quantification

Figure 4. A representative three-step scheme for nuclear magnetic resonance (NMR)-based metabolomics analysis using human transplantation (Tx) as an example. Step 1: Analysis begins with NMR spectral data processing and pattern recognition. (A) From raw spectra (B) spectral patterns and intensities are recorded and compared (compounds are not initially identified). (C) This finds relevant spectral features so that sample groups (e.g., treatment failure versus success) can be distinguished. Step 2: Metabolite identification typically starts with an extension of pattern recognition using principal component analysis (PCA). (A) Metabolites can be subjected to analysis by (B) PCA software to (C) identify which metabolites are responsible for the patterns observed in step 1. Step 3: To quantify metabolites, spectra are matched to those in a spectral library. Two examples of metabolite quantification ("validation") are shown here in which longitudinal changes in amounts of (A) lactate and (B) glutamine were indicators of outcome. (Partly adapted from Reference 13.)

(Figure 4) (13). However, the first step (chemometric spectral classification) can be ignored, the metabolites can be directly identified and quantified, and then multivariate analysis can be applied to the quantitative data sets. This "targeted" quantitative data set strategy can be applied when various analytical techniques and their combinations (NMR, GC-MS, LC-MS) are used to produce an expanded data set for a single sample. This strategy also has application in translational research because it includes the quantification of subtle changes in metabolites that may occur but are not recognized by pattern trends and permits the monitoring of changes in metabolite concentrations over time. Importantly, quantitative metabolomics is not necessarily dependent on the identification of a specific metabolite but rather differences in known metabolite concentration that result from disease or are due to disease severity. Changes in metabolite concentration over time may be predictive of disease susceptibility or progression or drug response or toxicity. Subsequently, these data can be linked to biological pathways

using bioinformatics analysis (Figure 2). Ultimately, quantitative metabolomics may be more likely to lead to biomarker discovery via the generation of hypotheses relevant to mechanisms of disease, drug action, or toxicity. Furthermore, as data are generated from proteomic, genomic, and metabolomic studies they can be integrated using a broad systems biology approach to generate applicable information for the clinic. This makes quantitative metabolomics a powerful tool for the discovery of biomarkers for numerous diseases, particularly those with a rapid onset and high mortality rates, such as those of the ICU, including sepsis and ALI/acute respiratory distress syndrome (ARDS) (19).

Clinical Application of NMR-Based Blood Quantitative Metabolomics

To date, most metabolomic data have been generated in experimental models, including sepsis (24, 40, 41) and inflammatory lung injury (42–44). These preclinical animal data provide clear

evidence that tissue-based as well as serum-based metabolic markers are closely linked to inflammatory processes (heat shock protein-related pathways), oxidant stress (glutathione), increased anaerobic metabolism (lactate, alanine), and dysregulated lipid metabolism (acetate, fatty acids) that result in loss of energy homeostasis. These data should not be dismissed as inapplicable to humans because it seems that the endogenous metabolome of mammals does not vary much across species (17). In fact, rats, mice, and humans have nearly identical constituents and show only small variations in concentrations across species.

Human metabolomics studies have been conducted on intact organs, extracted tissues, or biopsies and on virtually every biofluid (12, 17, 33, 45, 46). However, the great majority of metabolomics data are generated from studies of biofluids, not tissues (17). Advantages of the use of blood (plasma or serum) include that it is readily available and does not require elaborate processing or preservation schemes to acquire accurate metabolite information. It is also a uniquely uniform and very homeostatic biofluid and is therefore less affected by confounding factors such as age, sex, diet, fluid consumption, diurnal cycles, and stress (17). Proteins and other macromolecules that can interfere with metabolite spectra are effectively removed using the appropriate extraction procedures (see the section on sample handling).

We and other investigators have used blood, plasma, or serum as the biofluid of choice based on the principle that because it interacts with all tissues, it represents a physiological “average” of the host’s biochemical information and has practical application in the clinical situation (19, 35, 47, 48). For example, it is not reasonable to acquire serial lung biopsies from patients with ALI/ARDS, and it has been our experience that bronchoalveolar lavage fluid contains mostly proteins and low levels of metabolites (19, 49). Therefore, we contend that use of a “host” approach rather than a compartment- or tissue-specific approach to metabolomics (and systems biology in general) is more likely to enhance the clinical applicability of these sciences to biomarker discovery (Figure 2) (50, 51). In this regard, the clinical application of metabolomics could be enormous. For example, blood metabolite profiles could be used for the early detection or diagnosis of disease. In the ICU, temporal changes in metabolite concentrations may serve as indices for disease severity, could be used to direct care including the optimization of pharmacotherapy, and could lead to more efficient use of healthcare resources. The fact that metabolite levels change over time suggests that they may also have value as prognostic markers or to chart the progression of disease. Finally and most importantly, metabolomics is likely to provide much-needed insight into the pathogenesis of critical illnesses, including the mediators and pathways involved. In this way, new and novel therapeutic targets could be identified, which may lead to more effective pharmacotherapy for these challenging diseases.

Despite these potential uses, to date, the clinical application of metabolomics in critical illnesses has not yet been realized. Presently, its use is primarily evident from the fields of nutrition, cancer, cardiovascular disease, and diabetes. The ability to determine nutritional imprinting on metabolism, whether beneficial or detrimental, is easily detected with metabolomics. Imbalances in carbohydrate, lipid, and amino acid metabolism are associated with nutritional imbalance and predisposition to obesity (52–54). Using a ^1H -NMR platform, two sophisticated pattern-recognition techniques distinguished patients with coronary artery disease or hypertension from control subjects (55, 56). However, both studies had several limitations and are therefore unlikely to achieve clinical acceptance because differences in metabolites were mainly attributed to the differences in lipoprotein particle composition. This was due to the use of

nonextracted blood samples, which resulted in lipoprotein as the dominant signal compared with other low-concentrated (water-soluble) metabolites. This illustrates the importance of appropriate extraction/quantification protocols so that more specific markers can be detected, as has been shown for metabolic plasma markers (lactate, AMP catabolism products, citrate) of myocardial ischemia in an MS-based quantitative metabolic profiling study (57).

Clinical cancer research is ideally suited for quantitative NMR-based metabolomics (7). The comprehensive work of both clinical and basic science studies has demonstrated a clear proof of concept for “*in vitro* discovered–*in vivo* validated” biomarkers in translational metabolic profiling research and the ability of this application to identify distinguishing metabolites for specific brain tumor types (N-acetyl-aspartate and choline for astrocytomas; inositol for gliomas; creatine and glutamine for meningiomas) (58). This work eloquently shows the potential of metabolomics to differentiate brain tumor phenotype, a principle that would be extraordinarily useful in demarcating critical illness phenotype in the ICU.

The growing wealth of evidence of the usefulness of metabolomics for biomarker discovery in oncology is not being met to a similar extent in critical care. Presently, there are only a limited number of metabolomics studies in acutely ill patients. An important element that is needed to establish the clinical usefulness of metabolomics in critical care patients is the association between metabolites and clinical outcome markers. Our most recent work has introduced the potential applicability of quantitative metabolomics in both trauma- and sepsis-induced ALI (19, 35). In trauma patients, multivariate data analysis of 43 quantitative metabolic parameters identified three lipid metabolites, triacylglycerol (TAG), glycerol-heads of phospholipids, and monounsaturated fatty acids (MUFA), as being the most discriminative markers to separate survivors versus nonsurvivors at the time of admission (35). Glucose and glutamate were intermediate predictors, followed by lactate and hydroxybutyrate as two low-weight predictors. Thus, in nonsurviving trauma patients, changes in circulating metabolites in the blood were characteristic of decreased lipid synthesis and urea cycle activity in the liver, as well as for increased hyperglycemia, lactic and ketoacidosis. In another preliminary study we showed that total glutathione, adenosine, phosphatidylserine, and sphingomyelin differentiated sepsis-induced ALI from health (19). In addition, myoinositol (a pulmonary osmo- and volume regulator) was inversely associated with ventilator-free days ($\rho = -0.73$, $P = 0.005$) and both myoinositol and total glutathione were positively associated with acute physiology score ($\rho = 0.53$, $P = 0.05$; $\rho = 0.56$, $P = 0.04$, respectively). These studies used small samples sizes but sound sample handling and analytical principles to generate meaningful proof of concept data to demonstrate the usefulness of metabolomics in acutely ill patients. Certainly, larger more elaborate studies are needed to validate these findings and anchor metabolite profiles to phenotypes to move the field beyond the biomarker discovery phase (Figure 1) (4).

CHALLENGES AND FUTURE DIRECTIONS

The field of metabolomics is well positioned in systems biology to play a pivotal role in biomarker discovery in patients with critical illness, but much work has yet to be done. First and foremost, the human metabolome needs to be defined (59). More studies are needed to quantitatively characterize the normal blood metabolome to precisely describe the “normal range” of multiple endogenous metabolites in blood (mostly by NMR). Although rapid progress in analytical instrumentation is being made, the

“normal” ranges of metabolites from NMR spectra have not been established. In addition, the absence of analytical standards and blood or plasma metabolite reference ranges derived from a large database of control subjects makes the classification of metabolite concentrations as normal or abnormal particularly difficult. This is one of the challenges being tackled by the Human Metabolome Project (<http://www.hmdb.ca>), which is compiling reference range data and developing bioinformatics tools to assist in mapping the human metabolome (23, 59–61). Progress is being made, but the available data have not obviated the need for within-study healthy control subjects.

Other challenges that metabolomics faces are metabolite identification and interpretation. No single analytical technique presently exists that can identify all the metabolites in the metabolome, even though improved sensitivity of ^1H -NMR spectroscopy permits the detection of many metabolites that are representative of important pathways that are altered by disease processes (16). An important component of the analytical aspect of metabolites is spectral peak identification. Therefore, spectral reference databases are necessary for confident metabolite identification and quantification (29). This is being addressed by a few public and private spectral databases, such as the Human Metabolome Database (<http://www.hmdb.ca>), PubChem (pubchem.ncbi.nlm.nih.gov), the Madison Metabolomics Consortium Database, Metlin (<http://metlin.scripps.edu>), and the Chenomx database (4, 29). In addition, unlike the information we have from the Human Genome Project, the number of endogenous metabolites that exist in the human metabolome is not known (29, 62). Progress is being made in this area as databases are expanding (<http://www.metabolomicsociety.org/database.html>) and metabolic pathway databases are available (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/>). So although the field of metabolomics holds great promise for advancing translational research, it is still developing and evolving, and a number of gaps in our knowledge exist that will need to be filled before its potential can be fully realized.

Unlike the human genome that has been formally defined as 23,300 genes, we do not yet know how many endogenous metabolites constitute the human metabolome (63). Estimates of 1,500 up to 100,000 compounds, depending on expected analytical sensitivity, have been made (17). Moreover, our interpretation of what is referred to as “biologically relevant” is limited by the current state of our biological knowledge. This makes the integration of metabolomics data with other data from systems biology and the linkage of these findings to clinically meaningful end points all the more important as we move forward in this field.

CONCLUSIONS

Metabolomics applications are still in progress, but it is already clear that modern metabolomic technologies could have a great impact on the diagnosis, prognosis, and discovery of biomarkers in critically ill patients. Presently, the clinical application and usefulness of NMR-based quantitative metabolomics in critical illnesses is limited due to the lack of supportive evidence. Undoubtedly, biomarker discovery in critical illness is challenging because these patients are a heterogeneous group and there are numerous variables that cannot be controlled. These factors however, do not negate the feasibility of metabolite biomarker discovery in this patient population and in fact may make metabolomics, which is less prone to these influences compared with genomics and proteomics, potentially the ideal tool for the task.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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