REVIEW ARTICLE

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Metabolomics in critical care medicine: a new approach to biomarker discovery

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Abstract

Purpose: To present an overview and comparison of the main metabolomics techniques (¹H NMR, GC-MS, and LC-MS) and their current and potential use in critical care medicine.

Source: This is a focused review, not a systematic review, using the PubMed database as the predominant source of references to compare metabolomics techniques.

Principal Findings: ¹H NMR, GC-MS, and LC-MS are complementary techniques that can be used on a variety of biofluids for metabolomics analysis of patients in the Intensive Care Unit (ICU). These techniques have been successfully used for diagnosis and prognosis in the ICU and other clinical settings; for example, in patients with septic shock and community-acquired pneumonia.

Conclusion: Metabolomics is a powerful tool that has strong potential to impact diagnosis and prognosis and to examine responses to treatment in critical care medicine through diagnostic and prognostic biomarker and biopattern identification.

Manuscript submitted 25th June, 2014

Manuscript accepted 5th October, 2014

Clin Invest Med 2014; 37 (6): E363-E376.

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Metabolomics refers to the systems level analysis of the metabolism and metabolites in response to physiological stimuli, such as disease or drug administration [1]. The application of clinical metabolomics to the complex illnesses of critical care medicine is relatively new [1,2]. Metabolomics, which is most commonly based on nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and/or liquid chromatography-mass spectrometry (LC-MS) platforms, provides an accurate reflection of the metabolic processes and pathways at play within the human body at a particular moment in time [1].

History of metabolomics

While the terms metabolomics and metabonomics are now used interchangeably, their original definitions focused on the level of analysis and the type of analytical technology used. This paper uses the term metabolomics as the terms are often treated as interchangeable and it is more commonly used in the literature than the term metabonomics [1, 2]. Nicholson, who first defined metabonomics in 1999, described it as a systems-based strategy to “measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation” [2]. Conversely, metabolomics was defined as the identification, analysis, and quantification of each metabolite within a biosample [3]. Metabolomics and metabonomics can also be defined by their method of analysis: metabolomics was originally described as a GC-MS-based approach in the study of plant metabolomes [3] and metabonomics was originally defined as NMR-based study of mammalian systems [2]. Although metabolomics and metabonomics were not defined until the late 1990s, biofluid analysis by GC-MS was first reported in the 1960s and 1970s [4]. The basis of today’s metabolomics field started in the early 1980s, when NMR technology became adequately sensitive for biofluid metabolite identification. Combined with the use of statistical analysis for more complex pattern recognition, the advance in NMR technology led to the realization that the broad metabolic patterns found in NMR and GC-MS biofluid analysis could indicate disease pathophysiology and drug reactions [5].

Applications of metabolomics in critical care medicine

Metabolomic analysis aims to define the metabolome, the collection of small biological compounds present in a particular biological sample [6]. While serum or urine samples are typically used, an array of biological samples, including plasma, bronchoalveolar lavage fluid, cerebrospinal fluid, and tissue samples are also suitable for metabolomic analysis [6]. Given

the relatively small number of metabolites and the relative ease of sample collection, metabolomics presents an efficient and cost-effective alternative to genomics and proteomics analyses. Many fields in critical care medicine have already applied metabolomics as a means of biomarker identification, disease profiling, drug development and quantifying response to therapy [7]. Biomarker identification and disease profiling are particularly relevant to intensivists as many of the diagnostic and prognostic biomarkers used in the intensive care unit (ICU) are neither sensitive nor specific enough to act as diagnostic gold standards for specific pathophysiologic processes [8]. Given that metabolomics allows for the untargeted identification of tens to hundreds of metabolites at one time with one sample, the identification of metabolic fingerprints (i.e., multiple biomarkers that form a biopattern) associated with a particular pathophysiology is achievable [7]. Biomarker and biopattern identification and disease profiling have been the focus of clinical metabolomics studies on multiple diseases and conditions, including various forms of cancer, sepsis, diabetes, inflammatory bowel disease, cardiovascular disease, hepatitis, and neurological disease [1, 4, 7].

Nuclear magnetic resonance spectroscopy

For more than 30 years NMR spectroscopy has been applied to study metabolic composition of different biofluids, cells and tissues [9-11]. Most NMR-based metabolomics studies measure the magnetic properties of ^1H , ^{31}P or ^{13}C nuclei [4]. Basically, the sample is placed inside a static magnet field, where it is irradiated with high-frequency pulses. The response of the sample is then detected by an induced current. The intramolecular magnetic field around the NMR-sensitive nuclei affects their resonance frequency, creating very specific spectral intensity patterns for every chemical compound. Therefore, each metabolite can be recognized and assigned through its unique peak pattern observed in the ^1H NMR spectrum (Figure 1). Moreover, the concentration of an individual compound is proportional to its peak area; thus, absolute concentrations can be determined by comparing with the peak area of an internal standard of known concentration [12]. Detailed explanations of NMR theory, spectral acquisition conditions and the origin of the chemical shifts are available elsewhere [13].

It has already been demonstrated that biological sample collection is a crucial step for all metabolomics studies [4]. Usually plasma samples are obtained by venipuncture and collected into standard vials with anti-coagulants such as ethylenediaminetetraacetate (EDTA), citrate or lithium heparin [14]. It should be noted that EDTA creates several additional resonances in the ^1H NMR spectrum due to formation of com-

FIGURE 1. A simplified representation of ^1H NMR spectrum of a biofluid (serum) with the chemical shifts of typical and expected metabolites in the spectral region of 1.0–1.5 ppm.

plexes with Ca^{2+} and Mg^{2+} ions that are present in blood [9]. These signals can obscure metabolite signals and hence the use of EDTA is normally avoided. Moreover, many biological samples contain high molecular weight metabolites (i.e., lipid particles and proteins), which give rise to broad signals in the NMR spectra. Such broad spectral resonances from lipids and proteins are unwanted as they overlap with the NMR signals from the low molecular weight metabolites, making detection and measurement of the low molecular weight metabolites very difficult. Several approaches that facilitate the observation of narrow resonances from small metabolites have been described, such as using the Carr-Purcell-Meiboom-Gill (CPMG) NMR pulse sequence, extraction with organic solvents and perchloric acid or ultrafiltration (e.g., using 3 kDa molecular weight cut-off filters) [4, 15]. The CPMG experiment improves the detection of small metabolites but is not able to completely suppress resonances from mobile lipids and there is still considerable overlap in the NMR spectra. The extraction and ultrafiltration methods have been studied elsewhere and it has been shown that ultrafiltration provides high reproducibility and is superior in removal of high molecular weight metabolites compared to extraction methods [16]. Additionally, ultrafiltration increases the resolution of the NMR spectrum and provides an opportunity to observe aromatic signals, which are not always visible in the CPMG spectrum; therefore, ultrafiltration provides a valuable tool for quantitative metabolomic NMR measurement. It should be noted that filters need to be washed free of glycerol with distilled and deionized water to prevent glycerol contamination (glycerol is often used for storage in commercially available filters).

Plasma and serum can be measured directly with minimal sample preparation. Generally only $\text{H}_2\text{O}/\text{D}_2\text{O}$ and buffer for pH adjustment are required to prepare the sample [17]. Also, the addition of sodium azide is recommended to limit bacterial growth in biological samples. More information regarding sample preparation of different biofluids (urine, serum and plasma) or tissue samples for NMR-based metabolomics experiments can be found in the literature [18, 19].

Usually NMR samples are loaded into 5 mm diameter NMR tubes, which require approximately 600 μl of sample volume. There are also smaller NMR tubes available with diameters of 1–3 mm [20] for which less than 200 μl of sample is needed. The number of detected metabolites in biofluids detected by ^1H NMR ranges from 50 to 200 [15, 21, 22] and is mainly dependent on the type of biofluid, the acquisition time of the spectra and the magnetic field strength of the NMR spectrometer. Generally, 500 or 600 MHz NMR instruments are used in metabolomics studies [19] but the use of more powerful (800 MHz) instruments has been reported [22].

Although one can argue that NMR spectroscopy is a low sensitivity technique compared with other analytical tools such as GC-MS or LC-MS, it has been shown in various studies to be very specific, quantitative and highly reproducible [4]. Clearly, NMR-based metabolomics is an appropriate tool for high-throughput analysis of different body fluids as a broad range of metabolites can be quantified simultaneously and no complicated sample preparation protocol is required [23].

Application of NMR-based metabolomics

In recent years, NMR-based metabolomics has been applied extensively to find specific biopatterns for the diagnosis and prognosis of different human diseases, such as diabetes mellitus [24], cardiovascular disease [25], cancer [26] various infectious diseases [27] and Alzheimer's disease [28]. Although the mechanisms of these diseases may be understood and a significant number of strategies for patient treatment are available, there is still a lack of sufficiently sensitive biomarkers for early disease detection; therefore, identification of new biomarkers and the development of prognostic methods are extremely valuable tools for improving health and patient lifestyle. Diabetes and diabetic complications (e.g., diabetic nephropathy and cardiovascular disease) are causes of significant morbidity and mortality in modern society [29]. In a recent study, researchers investigated serum samples collected from 326 adult subjects with type 1 diabetes (T1DM), with and without complications, in order to identify markers of kidney injury [30]. Using ^1H NMR techniques, the authors detected 58 potentially important metabolites of which sphingomyelin appeared to be the strongest predictive variable for diabetic kidney disease. NMR-based metabolomics studies have also been performed in pediatric cohorts. Deja *et al.* showed that the application of ^1H NMR spectroscopy to urine samples collected from T1DM children with different levels of glycated hemoglobin allowed for the monitoring of metabolic changes in the patient groups [31]. Moreover, the authors distinguished specific metabolic patterns between healthy children and T1DM pediatric patients with normal glycated hemoglobin levels, indicating that NMR-based metabolomics can be used as an additional sensitive and comprehensive source of information about progression of T1DM in children.

In a recent study, we investigated whether a metabolomics approach could be useful in the diagnosis and prognosis of septic shock in pediatric intensive care units (PICUs) [32]. We were able to successfully separate metabolic profiles of PICU septic shock patients from non-infected PICU patients (patients with systemic inflammatory response syndrome but not suspected of having an infection) and healthy children. Additionally, by using an NMR-based metabolomics approach, we could predict septic shock patient mortality more accurately than by applying conventionally used markers such as procalcitonin levels or Pediatric Risk of Mortality (PRISM) III – Acute Physiology scores. Currently, we are expanding our research to pediatric emergency department patients to investigate early metabolic signs that could potentially be important for following early sepsis development and progression. It is well known that the first hours after patient admission are the

most crucial for patient survival [33]; thus, developing diagnostic and prognostic tools that might accelerate disease recognition is extremely important.

Gas Chromatography-Mass Spectrometry

GC-MS analysis is a robust, widely used and important technique for the separation and identification of compounds within biological samples in metabolomics studies. The GC-MS methodology has been developed as a highly sensitive and high-throughput method to detect hundreds of metabolites. This analytical technique is employed by coupling a gas chromatograph to a mass spectrometer in order to separate small molecules in the GC column and then detect these by the mass spectrometer. New developments in GC-MS techniques have led to its adoption as one of the main analytical tools used in metabolomics [34, 25]. GC-MS can be used for the detection of different classes of biochemical compounds, especially both volatile and nonvolatile compounds following derivatization resulting in high sensitivity with excellent reproducibility. GC-MS is well suited for the detection of a variety of metabolites including most amino acids, organic acids, sugar alcohols, sugars, phosphorylated metabolites, aromatic amines and fatty acids. Compared with conventional GC-MS, GC-Time of Flight (TOF) –MS techniques provide better resolution, allowing for higher scan rates. This increase in scan rate also allows for the deconvolution of overlapping peaks in the spectra [36–38]. Today, in conjunction with NMR, GC-MS has become a popular analytical tool in metabolomic studies investigating a variety of biological functions in mammals, plants, fish, bacteria and fungi, investigating various aspects of foods and flavours and contributing to both environmental and clinical studies [39].

Briefly, the metabolites are initially separated in the column by gas chromatography and the separated compounds are then ionized and detected by a mass spectrometer. The derivatized sample is volatilized by rapid exposure to high temperature (200–300°C) into a column. Compounds in the samples are then carried through the chromatography column in the gas phase using N_2 or He. Afterwards, separated compounds reach the ionizer source for ionization in the mass spectrometer and are then detected based on their mass to charge (m/z) ratio [40, 41]. As its name indicates, gas chromatography is inherently more efficient with more thermally stable and volatile or semi-volatile compounds; therefore, non-volatile and thermally stable compounds must first be converted to volatile and thermally stable forms by a chemical derivatization procedure [42]. Accordingly, amino- and non-amino organic acids must be converted to volatile derivatives to be detected using GC-MS

analysis. Highly polar, ionic and high molecular weight compounds are not compatible with GC analysis [43]. Derivatization causes significant alterations in the compound's physical properties, including boiling point, melting point, thermal stability and volatility [44]. During the derivatization reaction, the compounds are separated based on their different affinities with the stationary phase of the columns: compounds showing higher affinity move more slowly, while lower affinity compounds move more quickly through the column. The separated compounds are introduced for ionization sequentially inside the ionizer chamber. Electron ionization (EI) is a commonly used ionization technique. Standard EI uses an electron energy of 70 eV to ionize molecules, giving rise to an electric signal proportional to the amount of a molecule. These electronic signals are then detected based on their m/z , leading to reproducible mass spectra [36, 43]. The impact of chromatographic resolution is influenced by various operational parameters, including the nature of the stationary phase, mobile phase, temperature, column length (L), inner diameter (ID) and the thickness of the stationary phase (df). As the number of components and their structural similarities increase in the mixture (isomerism), longer columns are required for complete compound separation [43]. Compound identification is based on two types of data: chromatographic retention time (RT) and the unique spectral fragmentation based on m/z , which are obtained by GC and MS, respectively. Retention indices can be used as highly reproducible data for orthogonal confirmation of identified compounds by the mass spectrometer as it increases the resolution of overlapping compounds with the same mass but different retention times [36, 45]. Recent developments and innovations offer dramatically increased separation efficiencies and peak identification [36]; for example, in the tandem GC (GC/GC) technique, two capillaries are used for chromatography with stationary phases of differing selectivities. Compounds are initially separated through the longer non-polar column and the separation is then completed in the second polar (or semi-polar) column. GC/GC presents a significant improvement in terms of peak sharpness when compared to conventional GC [46, 47]. MS alone is used by direct injection of samples to the analyzer as a very rapid technique to detect higher molecular weight metabolites for metabolic fingerprinting. In addition, recent developments in MS are likely to further increase the sensitivity and the power or specificity of compound identification [47, 48]. TOF-MS and quadrupole mass spectrometry (Q-MS) are common analytical techniques that are coupled to different types of GC. GC-TOF-MS is more suitable for metabolomics as it has higher accuracy in the deconvolution of overlapping peaks, high resolution and ultra-

fast scanning for complex samples. It is currently the most popular analytical technique used in profiling human and plant samples [36]. GC/GC-TOF-MS is associated with high resolution and sensitivity and is well suited for more complex samples as it is capable of detecting thousands of peaks. Gas chromatography-ion trap mass spectrometry (GC-IT-MS), gas chromatography-single quadrupole-mass spectrometry (GC-SQ-MS) and gas chromatography triple quadrupole mass spectrometry (GC-QQQ-MS) are typically used for metabolomics investigations in laboratories. Metabolite identification is complex and is often done using different database libraries. The detection of components from chromatographic peaks is based on their retention time and mass data. The accurate identification of components is determined by internal reference compounds added to the sample that provide unique ion chromatograms with single, sharp peak as models for quantification [49].

The greatest advantage of GC-MS is the high level of sensitivity as a selective technique to detect many features [34]. GC-single quadrupole-MS (GC-Q-MS) is associated with a good dynamic range [36] and these instruments are widely available, reliable and affordable, making it a popular and robust platform for metabolomics studies. Other mass analyzers, such as TOF-MS and triple quadrupole-MS (3Q-MS), are currently considered the most powerful high resolution tools when coupled to GC. Two dimensional methods, such as GC/GC-MS or GC-MS/MS, are associated with great increases in chemical separation leading to detection of many compounds compared with conventional GC-MS [47]. The database development of most useful libraries HAS made GC-MS an effective analytical tool [50].

GC-MS is a semi-quantitative method that uses internal standards, while NMR can provide absolute quantification of metabolites in biofluid samples. Sample preparation for GC analysis requires the initial extraction of small compounds using various techniques. Derivatization is required for the sample preparation procedure for GC-MS; however, it also presents a potential source of variation via production of byproducts and the degradation of several compounds during the reaction. Due to limitations in the application of GC-MS, this technique is usually used in isolation to obtain truly global metabolomic analysis. The use of a combination of analytical techniques, such as NMR, GC-MS and LC-MS, can identify more metabolites, opening up a larger portion of the metabolome for analysis (Figure 2).

FIGURE 2. The most commonly used analytical platforms in metabolomics. Schematic representation of the limits of detection for each technique to detect small molecules in the metabolome; the size of each individual circle does not accurately approximate the number of detectable metabolites. LDL represents the lower detection limit of the method in molar concentration. Cpd, compounds; NMR nuclear magnetic resonance spectroscopy; LC-MS, liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry

Application of GC-MS-based metabolomics

Recent metabolomics profiling studies have shown significant potential for clinical approaches to develop diagnostic and prognostic biomarkers for various diseases. Metabolic profiling using GC-MS analysis can provide information on comparisons of different metabolic features between two cohorts of patients and/or between patients and healthy controls. This analytical technique is highly developed for qualitative and quantitative analysis of endogenous metabolites to define different stages of a disease or for prognosis of mortality. Hundreds of metabolites, including amino acids, carbohydrates, fatty acids, organic acids and steroids, are detectable by GC-MS. Multivariate data analysis can reveal the metabolites with the greatest variation to discriminate between different cohorts. Metabolomics approaches in critical care medicine are still in their infancy with only a few studies having applied a GC-MS analytical platform to date. Nonetheless, GC-MS has been applied to plasma metabolomics as a prognostic tool in sepsis to discriminate between survivors and non-survivors at 28 days [51], for prognosis of mortality in critically ill adult

patients [52] and for prediction of early and late onset neonatal sepsis [53]. Quantitative analysis of human exhaled gas showed differences in metabolite concentrations between patients with acute respiratory distress syndrome (ARDS) and healthy controls [54].

As an example of the sensitivity of GC-MS metabolomic analysis of patient plasma, our unpublished data show significant differences in plasma metabolomic profiles in different types of control patients using GC-MS of normal, healthy controls *vs.* ICU controls (Figure 3). ICU controls were patients admitted to the ICU post-operatively from an elective procedure (e.g., posterior fossa or neck spinal surgery or off-pump cardiac surgery patients) where there was no suspicion of infection.

Liquid chromatography-mass spectrometry

The LC-MS analytical platform also provides a powerful and sensitive approach for metabolomics analysis with certain advantages compared to GC-MS. LC-MS uses a lower temperature for the separation of compounds and there is no derivatization required for their detection [55-57]. High pressure liquid chromatography mass spectrometry (HPLC-MS) is a common and powerful technique used to separate and quantify compounds and biochemical pathways in metabolomics research. LC-MS is a highly sensitive and precise analytical method for the identification of low concentration compounds (< 1 picogram (10^{-12} g), can be detected. LC-MS is well suited to analyze the non-polar, non-volatile and thermally-sensitive compounds that require derivatization prior to GC-MS analysis and are difficult to detect using GC-MS. LC-MS can detect a wide range of semi-polar compounds; for example, LC-MS has been used to investigate lipid metabolism and signaling in different disorders such as neurodegenerative diseases [36]. Several approaches, including high pressure liquid chromatography-mass spectrometry (HPLC-MS), ultra high pressure liquid chromatography-mass spectrometry (UHPLC-MS) and hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) have been established to improve the separation and quantification of compounds. Typically, LC-MS metabolomics analysis uses both normal phase (NP) and reverse-phase (RP) columns. First, RP columns are used to separate polar compounds. The NP column then provides complementary separation for mostly non-polar molecules. Conventionally, LC-MS includes butyl (C4) and octadecyl (C18) covered small, porous silica particles with diameters of 3-5 microns that help separate the compounds in the sample based on affinities to the stationary phase and the speed of flow passing through the column [36]. In fact, the separation of

FIGURE 3. OPLS-DA plot based on GC-MS data analysis shows a good separation with high prediction ($Q^2 = 0.59$) between normal controls (■) and ICU controls (●). There is even clustering between ICU samples for CV (cardiac patients who were operated for the coronary artery bypass) and ASN (posterior fossa or neck spinal surgery). This suggests a high sensitivity of the technique to separate patient group.

compounds occurs between a mobile (solvent) and stationary phase (column packed small coated particles). In RP chromatography, the retention is caused by hydrophobic interactions of the solute with the non-polar stationary phase. The recently developed ultra-high pressure liquid chromatography (UHPLC) uses a reduced particle size of 1.5-2 μm in reverse phase packing material and high linear flow rates under 12,000 psi. UHPLC can significantly improve the chromatographic resolution compared with conventional LC. The ultra high pressure conditions mean that UHPLC is ten-fold faster and three- to five-fold more sensitive compared with a conventional stationary phase [58]. HPLC-MS is commonly applied as a gold standard analytical tool in pharmaceutical qualitative and quantitative analysis of potential metabolites involved in many metabolic pathways associated with the discovery of new agents [59].

The combination of UHPLC with MS makes it an even more powerful and higher resolution technique capable of detecting a number of polar metabolites. More precise mass detection can be obtained with a combination of a modern MS technique such as Q-TOF-MS with UHPLC. It works as a powerful analytical platform to measure accurate masses [47]. Many important parameters can change the performance of LC-MS [60]. Tandem liquid chromatography (LC/LC) relies on two-dimensional LC analysis and two retention times to provide higher resolution compared with one-dimensional LC. On the other hand, an LC-MS/MS approach has been tailored for targeted analyses for more accurate quantitative measurement. LC-MS is more effective in quantification of larger and highly polar compounds than GC-MS. It is also a powerful technique for detection of a broad range of metabolites, particularly those with similar masses, because HPLC separations are better suited for the analysis of labile and nonvolatile polar

TABLE 1. Strengths and weaknesses of NMR and MS methods used in metabolomics studies.

Technique	Strengths	Weaknesses
NMR	<ul style="list-style-type: none"> – Non-destructive technique (several analyses can be conducted on the same samples) – Minimal sample preparation prior to the experiment (adding deuterated solvent and a buffer) – Versatility for analyzing metabolites in biofluids, tissues or <i>in vivo</i> – Reproducibility and repeatability – High quantification 	<ul style="list-style-type: none"> – Low sensitivity (only metabolites with relatively high concentration (μg) can be detected) – Overlap in peaks and high chemical degeneracy (different metabolites have resonances in the same spectral region) – Mainly identifies polar compounds
GC-MS	<ul style="list-style-type: none"> – High-resolution capacity – High spectral resolution – Very sensitive – Low limit of detection (ng) – Required technical skill – High mass accuracy to detect compounds – Reproducible retention time – Highly developed compound libraries – Linear dynamic range – High separation efficiency – Ideal for thermostable and volatile and non-polar metabolites 	<ul style="list-style-type: none"> – High molecular weight analytes – Derivatization required – Lower reproducibility (within and across labs) – Fragmentation in MS – Extensive sample preparation steps – Poor quantification – Possible variation due samples preparation – Matrix effect – Compounds degradation (high temperature)
LC-MS	<ul style="list-style-type: none"> – Ideal for non-volatile compounds – Short separation time – High resolution – Very sensitive (pg quantities) – Reasonable robustness – Selectivity – High mass accuracy to detect compounds – Simple sample preparation – Detects a wider range of metabolites than GC-MS – - Very small sample size (10 μl) 	<ul style="list-style-type: none"> – Ideal for non-volatile compounds – Short separation time – High resolution – Very sensitive (pg quantities) – Reasonable robustness – Selectivity – High mass accuracy to detect compounds – Simple sample preparation – Detects a wider range of metabolites than GC-MS – - Very small sample size (10 μl)

and non-polar compounds in their native form. LC-MS offers high-resolution and reproducible results but quantification of compounds often requires the use of isotopes to be truly quantitative [61]. Similarly to GC-MS, the sensitivity of chromatographic resolution can be improved by decreasing the column's internal diameter and by using ionization suppression and smaller column particles. In addition, LC-MS has the potential benefit of analyte recovery by fraction collection and/or concentration, which is more challenging when using GC separation [57]. Importantly, because of inherent variations in different parts of LC-MS such as columns, gradient and collision energies, data are generally not directly comparable between different laboratories. Despite some limitations, many use LC triple quadrupole MS for quantitative purposes. There are sig-

nificantly fewer data libraries developed for LC-MS compared to GC-MS, so compound detection is more problematic in LC-MS, although this gap is rapidly diminishing due to the introduction of METLIN [62] and other databases for metabolomic profiling. Different libraries are necessary because different types of chromatography columns are used and a large number of compounds cannot yet be identified so they are listed based on their mass spectral tag data, retention times and ion spectra.

Application of LC-MS-based metabolomics

Depending on the instrument and technique used, LC-MS can provide ultra-sensitive detection - detecting lower concentration compounds (< 1 pg) compared with GC-MS (ng to pg).

FIGURE 4. The PCA score scatter plot of the first two principal components (PC1 and PC2) for septic shock samples (red triangles) and ICU controls (green triangles). The plot shows a reasonable separation between septic shock samples and ICU controls without highlighting any outliers. The dashed elliptic line indicates the 95% confidence interval of the Hotelling's T-squared distribution. Adapted from [15].

Because of rapid and precise analysis of LC-MS, this technique can be widely applied using an untargeted or targeted approach. Untargeted LC-MS, is not quantitative as the readout is typically relative intensities providing putative identification of metabolites. In untargeted metabolomics, LC-MS is used as a non-biased analysis to examine a large number of metabolites in biofluid samples; therefore, it could yield information about unexpected metabolic responses to different stimuli or diseases to unravel novel or unknown metabolic pathways. It could be a powerful approach to understand a data-driven hypothesis. On the other hand, using a targeted approach, LC-MS is well suited for the analysis of a specific group of known metabolites of interest. Different commercial kits, containing stable isotope-labeled compounds, have been developed to quantitatively analyze 100 to 200 known metabolites in a targeted fashion [64-66]. Importantly, this targeted approach is quantitative as it compares metabolites to isotope-labeled compounds that have been added to the tested samples. LC-MS can be applied in drug development, food science, forensic science for studies of drug abuse and to answer various clinical questions. LC-MS could be used to provide a biosignature for therapeutic drug monitoring in the treatment of infectious diseases and for the quantitative measurement of a number of mediators involved in cytotoxicity. Although LC-MS has been applied for biomarker identification for several cancer types [67], few studies

to date have been conducted with LC-MS for metabolic profiling in infectious diseases. Metabolic profiling was used to show a separation between ARDS and healthy controls based on bronchoalveolar lavage fluid samples using LC-MS [55]. This study showed some alterations in various biochemical pathways, such as amino acid metabolism, glycolysis and gluconeogenesis, fatty acid biosynthesis, phospholipids and purine metabolism. LC-MS has also been used to show that the serum metabolome undergoes alterations in adult patients with primary dengue infection [68] and in a cohort study in pneumonia and sepsis [69].

It is fully expected that LC-MS will be used in critical care medicine for diagnostic and prognostic approaches because of its high sensitivity for detecting small molecules using a rather small sample volume.

It seems obvious that, with further development, both GC-MS and LC-MS could become highly efficient analytical tools to provide a comprehensive view of the development and progression of various diseases in critical care from a healthy state to the disease state to show the perturbations in homeostasis. It is anticipated that quantitative isotope-based LC-MS will be utilized in the near future to detect biomarkers through metabolomic profiling in different stages of disorders that lead to the establishment of new biomarkers for diagnosis and prognosis, as well as using LC-MS for early and late detection of diseases and therapeutic progression. The cost of isotope labels continues to limit the pace of the advancement in this area.

Strengths and weaknesses of NMR and MS methods used in metabolomics studies

Given the strengths and weaknesses of the experimental methods outlined in Table 1, it is important to correctly assess and choose the appropriate methodology for the particular biomaterial and type of study. While NMR requires a relatively large sample size (e.g., 200 μ l of serum) and can only identify metabolites with concentrations greater than ~ 10 μ g/ml, it is the most robust and reproducible of the three methods discussed here [22]. Accordingly, the majority of peaks found in NMR biomaterial spectra can be matched to metabolites within NMR metabolome libraries, such as the Human Metabolome Database [22]. Due to this high level of quantification, NMR is the most reproducible (within and across labs) of the three techniques discussed. ^1H NMR mainly identifies compounds of high concentration and high proton abundance, and polar compounds with a typical spectrum containing 50-200 features [15, 22].

While MS methods are excellent for detecting small quantities of metabolites, they are generally less reproducible than NMR. Both GC-MS and LC-MS require smaller sample sizes (50 μ l and 10 μ l of serum or plasma, respectively) and are capable of identifying more features than NMR [22, 59]. That said, both GC-MS and LC-MS libraries are not yet as developed as NMR libraries, so a large percentage of features found by GC-MS and LC-MS remain unknown [22]. GC-MS can be used to identify volatile compounds in the nanogram range and requires a lengthy preparation process because of the need for sample derivatization [22, 59]. As previously mentioned, highly polar, ionic and high molecular weight compounds are not compatible with GC analysis [43]. LC-MS identifies non-volatile compounds in the picogram range and is capable of finding more known and unknown features than GC-MS and NMR [22, 59].

Data analysis

Given the tremendous amount of data that is generated in metabolomics experiments, metabolomic statistical analysis is normally done using multivariate data analysis (MVDA) [5]. While univariate analyses such as t-test or analysis of variance (ANOVA) can be used for metabolomics statistical analysis, this review will focus on principle components analysis (PCA) and partial least squares-discriminate analysis/orthogonal partial least-squares-discriminate analysis (PLS-DA/OPLS-DA), the most popular forms of MVDA in metabolomics [70]. MVDA can be supervised or unsupervised and is typically done using statistical programs, such as SIMCA-P+ (Umetrics, Sweden), Matlab (MathWorks, USA) or R-packages (Vienna University, Austria). The process of data analysis usually begins with an unsupervised principle components analysis (PCA), which is followed by a supervised partial least squares-discriminate analysis (PLS-DA), or orthogonal partial least squares-discriminant analysis (OPLS-DA) [71]. PCA is an important first step for metabolomic data analysis as it enables the user to observe patterns, trends and groups within the unordered dataset. PCA is also essential for the visualization and subsequent removal of any data point outliers. PCA is unsupervised, meaning no information is provided when the dataset is entered into the statistical program. Rather, the program is simply looking for any variation within the dataset. PCA finds the variable that accounts for the highest amount of variation within the unordered data and sets that variable as principle component (PC) one on a coordinate axis. PC two, the variable that accounts for the second largest amount of variation, is then set on an axis orthogonal to that of PC one, allowing for the visualization of dataset variation in two dimensions. Gen-

FIGURE 5. The receiver operating characteristic curve plot obtained for the supervised analysis of metabolomics data collected for septic shock patients and ICU controls (the area under ROC (AUROC) = 0.96 ± 0.02).

erally, clustering is observed between the data points for each principal component. Tighter clustering is observed when the PCA model is better able to account for the variation within the dataset (Figure 4). PLS-DA and OPLS-DA are supervised statistical analyses, meaning that class information is entered into the statistical program along with the dataset (e.g., patient outcome, diagnosis and treatment provided; Figure 3). OPLS-DA and PLS-DA aim to define the relationships between the known variables and class information, showing which variables account for the differences between patient groups [71]. More detailed information about PCA and supervised models (PLS-DA, OPLS-DA) is presented in Table 2.

Assessment of supervised models is generally done using R^2 and Q^2 metrics [71]. The R^2 parameter refers to the percentage of variation explained by a model, while Q^2 describes the predictive ability of a model. Strong and reliable supervised models will have similarly high R^2 and Q^2 values (values of 0.6 are typically very good for human samples) indicating good ability in predicting the class of an unknown sample. The difference between R^2 and Q^2 values is an indicator of the model's

TABLE 2. Comparison of principal component analysis and orthogonal partial least squares/partial least squares analysis.

Type of analysis	Principal Components Analysis (PCA)	Orthogonal Partial Least Squares/Partial Least Squares Analysis (OPLS-DA/PLS-DA)
Level of supervision	Unsupervised	Supervised
Purpose	<ul style="list-style-type: none"> – Observe patterns, trends, and groups within unordered dataset – Outlier identification 	<ul style="list-style-type: none"> – Define relationships between variables – Classification – Biomarker discovery – Diagnostic and prognostic information

goodness-of-fit. As an alternative means to analyze the discriminatory power of supervised models, the area under a receiver operating curve (AUROC) could also be calculated (Figure 5). An AUROC = 1.0 represents a perfect test while 0.5 means that the test does not separate the two groups [72].

Pathway analysis

Pathway analysis, an integral part of metabolomics, uses programs such as Metaboanalyst [73] or Ingenuity Pathways Analysis [74] (IPA) (Ingenuity Systems, USA) to ascertain which metabolic pathways are up and downregulated by the conditions being studied [65, 63]. Pathway analysis consists of enrichment analysis to find the significantly affected metabolic pathways and metabolite mapping to visualize and map out the affected pathways [75]. This analysis should be particularly important for critical care medicine, as many ICU patients have multiple comorbidities and pathway analysis may allow for discrimination between the pathways affected by these different conditions.

Summary and conclusions

The diseases and syndromes seen in critical care medicine are often multi-faceted and extraordinarily complex, meaning that any method that allows for earlier diagnosis or more successful prognostication would be important for patient care. Metabolomics may well represent a highly efficient and powerful future means for diagnostic and prognostic biomarker and biopattern identification in critical care medicine [4]. NMR, GC-MS and LC-MS analyses have shown that metabolomic fingerprints (metabolic biopatterns) can be used to differentiate between patients with various diseases and healthy subjects [31, 51-54, 68, 69, 76]. Additionally, ¹H NMR metabolomic analysis has also been shown to be capable of predicting mortality in children and adults with sepsis [15, 32]. These studies show that the metabolomic identification of new biomarkers or biopatterns for critical care diseases and syndromes is achievable. While the future of diagnostic and prognostic biomarkers may

involve metabolomics studies, some problems remain to be resolved in current methodology.

Clearly, a single analytical tool is not sufficient to detect all possible metabolites, therefore, a combination of different techniques can quantify more metabolites from different classes. Figure 2 shows the approximate number of metabolites that can be identified by NMR, GC-MS, and LC-MS in the human metabolome. GC-MS is well suited to detect volatile and less polar compounds, but LC-MS can detect more polar compounds at a lower concentration (nM-pM) compared with NMR and GC-MS. NMR can identify polar and non-polar metabolites but only at high concentration (>μM). The shared areas also show an estimate of the metabolites that can be identified by both or all three techniques; for example, amino acids are often detected by all three techniques [21].

Currently, while we are currently in the discovery phase of this research, all three analytical techniques must be performed to more fully capture the metabolomic fingerprint of an individual. See Table 1 for a comparison of the advantages and disadvantages of NMR and MS techniques. This means that a significant amount of time must be invested performing and analyzing the results from each technique. Accordingly, it is clear that additional advances of the metabolomics methodology and subsequent analysis methods may be necessary before these techniques can be fully effective in the creation of new gold standard diagnostic and prognostic measures in critical care medicine.

Acknowledgments

We would like to thank the staff of the Bio-NMR-Centre, Department of Biological Sciences, University of Calgary, and Josée Wong and CCEPTR (ICU tissue bank) for assisting with the analysis of ICU and normal control samples.

Financial Support

Financial support from the Alberta Sepsis Network. MB and SD were funded by a grant from the Cumming Faculty of

Medicine, Alberta Health Services, and Alberta's Health Research Innovation Strategy.

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