

The Metabolomic Approach to the Diagnosis of Critical Illness

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Introduction

Advances in molecular and cell biology over the last two decades, including most notably the sequencing of the human genome, have undoubtedly determined a better understanding of disease pathophysiology, and a more precise identification of populations at risk for certain conditions. However, many questions remain unanswered using the genomic approach alone, including what the gene products and the cell responses to certain insults are, given a certain genetic abnormality. The study of the metabolome offers a unique opportunity to answer some of these questions. The metabolome represents a combination of all the metabolites and intermediate products of metabolism in a biological organism. The study of the metabolome, further down the line from gene structure and function, more closely reflects the activities of the cell at the functional level and magnifies events that occur at the level of the genome, transcriptome and proteome (Fig. 1).

The development of chromatographic separation techniques in the early 1970s marked the origin of the metabolite identification field [1]. The ^1H -nuclear magnetic resonance (NMR) technique was first used for metabolic studies in 1977, when a range of compounds, such as lactate, creatine and alanine, was detected in a red blood cell suspension [2]. During the 1980s, analysis of the plethora of metabolites detected in ^1H -NMR spectra boosted the development of new techniques to classify samples according to their biological status [3, 4]. Thus, the

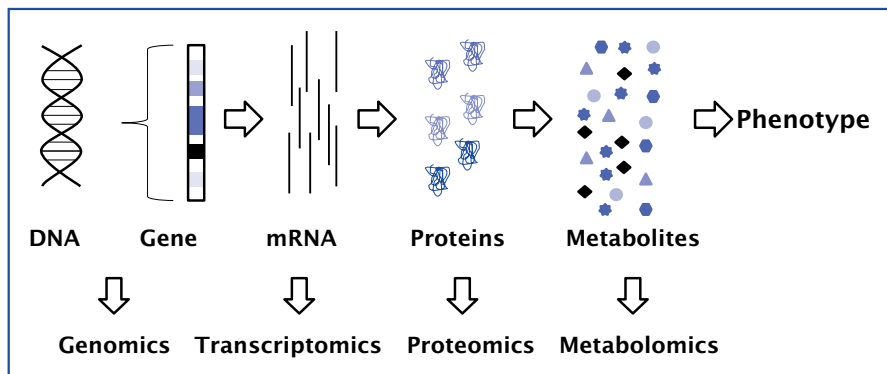


Fig. 1. General view of “-omics”.

concept of metabolomics was born, when complex data from biological samples were interpreted using multivariate statistics. Metabolomics is the scientific study of the metabolic response of living systems to pathophysiological stimuli or genetic modification. Transcriptomics, proteomics, and metabolomics comprise what is referred to as systems biology.

The potential for metabolomic analysis in critical illness is based on its ability to detect changes in phenotypes that may be helpful for early diagnosis, prognosis or prediction of response to therapy. In the present chapter, we first describe the principles of metabolomic analysis, including different analytical platforms and data interpretation tools, and then focus on the application of metabolomic sciences in critical care medicine.

Analytical Techniques

Metabolomic data sets are currently generated mainly through NMR spectroscopy (MRS), mostly ^1H -MRS, and mass spectrometry (MS). Other structural or analytical methods, such as Fourier transform infra-red (FTIR) spectroscopy or high performance liquid chromatography (HPLC), have the disadvantage of yielding a low level of detailed molecular identification.

NMR Spectroscopy

MRS is a non-destructive technique that provides detailed information on molecular structure as well as information on absolute or relative concentrations. NMR is a phenomenon in which magnetic nuclei in a magnetic field absorb and re-emit electromagnetic radiation. MRS is based on the fact that similar isotopes do not resonate at the same frequency because nuclei are surrounded by a cloud of diamagnetic electrons which generate a local magnetic field. Depending on the local chemical environment, different protons in a molecule resonate at slightly different frequencies. The resultant molecular detection and quantification are acquired as a spectral data set (Fig. 2). The horizontal axis is NMR frequency (or chemical shift in parts per million (ppm), relative to a reference chemical at 0 ppm (rather than in the absolute Hertz units), and the vertical axis is signal strength (in arbitrary units). Based on their unique chemical structure, biochemical groups (CH_3 , CH_2 , OH , etc.) of each molecule originate different peaks in the spectrum which appear at a known frequency. MRS using high-resolution magic angle spinning (HR-MAS) [5, 6] allows the acquisition of high resolution NMR spectra on intact tissue samples with minimal sample preparation. The main limitation of MRS is that this technique is much less sensitive than MS. Recent development of cryogenic probes has provided an improvement in resolution by reducing the thermal noise in the electronics of the spectrometer. MRS, by means of HR-MAS, is the only technique available for studying intact tissues.

Two-dimensional MRS can be very useful for elucidating the connectivity between signals, resulting in unbiased metabolite identification. Experiments using correlation spectroscopy (COSY) [7] and total correlation spectroscopy (TOCSY) [8] provide ^1H - ^1H spin-spin coupling connectivity, informing about which proton signals are close in chemical bond terms. Other heteronuclear correlation NMR experiments can be important to help assign NMR peaks. The interaction between ^{13}C and ^1H nuclei [9] is very useful for identification purposes.

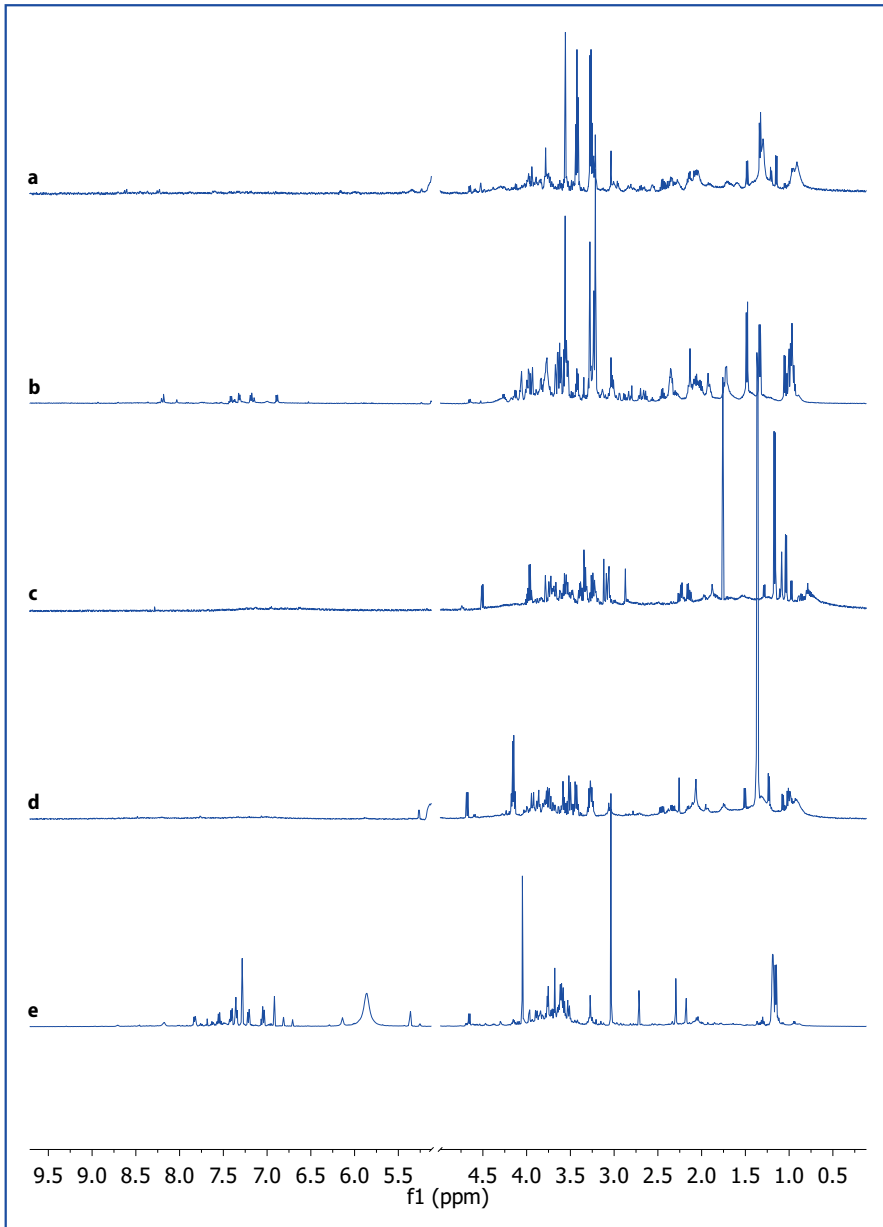


Fig. 2. Representative high-resolution ^1H -NMR spectra. Spectra from different samples are shown: **a** intact lung tissue; **b** intact renal cortex; **c** bronchoalveolar lavage fluid; **d** serum; **e** urine. Spectra **a** and **b** were obtained by high-resolution magic-angle-spinning ^1H -MRS. Spectra **c**, **d** and **e** were obtained by high-resolution liquid ^1H -MRS. All spectra were acquired using a Bruker 500 MHz spectrometer.

Mass Spectroscopy

MS is a destructive analytical technique that measures the mass-to-charge ratio of charged particles. Although the sample preparation requirements of this technique are usually more intensive, MS is considerably more sensitive than MRS, thus allowing the metabolomic analysis of low concentration samples, such as exhaled breath condensate fluid [10]. In general, this technique has been coupled to other pre-separation methods such as gas chromatography (GC) [11], capillary electrophoresis (CE) [12], ion mobility or FTIR spectroscopy [13]. The most common tandem in MS-based metabolomics is liquid chromatography (LC)-MS, which includes HPLC [14] and ultra-high-pressure LC (UHPLC) [15]. LC-MS provides three-dimensional metabolic data (Fig. 3), where at each sampling point in the chromatogram (retention time) there is a full mass spectrum (mass vs. intensity).

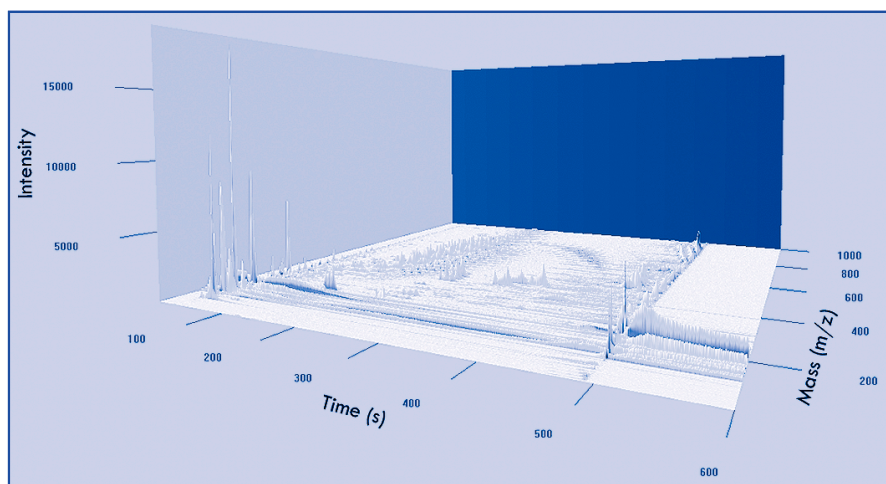


Fig. 3. Ultra-high pressure liquid chromatography (UHPLC). Three-dimensional mass matrix of an exhaled breath condensate from a healthy subject. UHPLC is coupled to a time-of-flight mass spectrometer. m/z : mass to charge ratio.

Spectral Data Analysis

Regardless of the analytical platform used, the spectral data can be thought of as a complex multi-dimensional set of metabolic coordinates. Interpretation of metabolic data is complicated and requires the application of unsupervised and supervised chemometric tools (Fig. 4). Prior to statistical analysis, spectral data have to be preprocessed. Data preprocessing is a necessary step between the acquisition of raw spectra and multivariate analysis of a metabolomic data set. For metabolomic studies, the preprocessing methods are designed to reduce variances and possible influences that might interfere with data analysis (such as possible variations in sample concentrations or variable sample dilutions), phase distortions and rolling baselines in the NMR spectrum and retention time misalignment in LC-MS data.

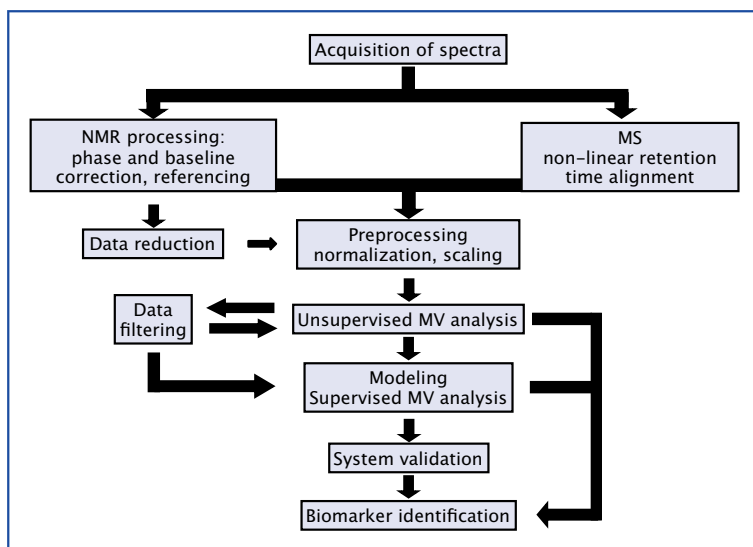


Fig. 4. Basic workflow for metabolomic analysis. Analysis includes: Spectral acquisition by nuclear magnetic resonance spectroscopy (MRS) or mass spectrometry (MS), raw spectra pre-processing, spectral data post-processing, unsupervised multivariate (MV) statistical analysis and modeling.

The multivariate statistical or pattern recognition methods provide a means of collecting relevant information on differences or similarities between the metabolic pathways [16, 17]. These methods include multivariate projection methods, in which principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) are the most widely used techniques.

PCA is one of the most common exploratory techniques in multivariate analysis [18]. Its most important use is to represent multivariate data in a low-dimensional space (Fig. 5a). The first principal component is defined by the spectral profile (loading) in the data which describes most of the variation. The second principal component, orthogonal to the first one, is the second best profile describing the variation, and so on. The principal components are composed of so-called scores and loadings. Loadings contain information about the variables (chemical shifts) in the data set and the scores hold information about sample classes. Score plots (Fig. 5b) are used to distinguish the metabolite profile of control from diseased subjects, or to evaluate the progression rate of the disease. Loading plots (Fig. 5c) highlight the most significant variables. In other words, the chemical shifts (metabolic signals) with the highest loading values in the right direction would be selected as potential biomarkers (Fig. 5d).

PLS-DA is a supervised linear regression method whereby the latent variables constructed with the multivariate variables corresponding to the observations (spectral descriptors) are associated with the class membership for each sample [19]. The aim of PLS-DA is to find in the spectral regions those components that significantly describe relevant variations in the spectra and have maximum covariance with the class information vector. PLS-DA can also be expressed by scores and loadings matrices, and results interpretation is similar as for PCA.

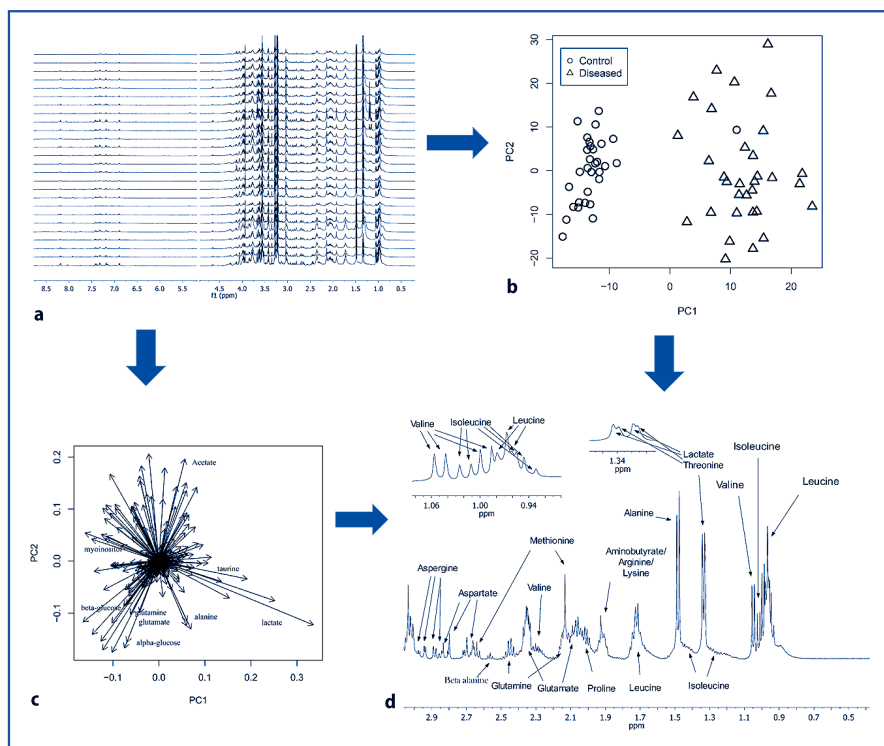


Fig. 5. Example of principal component analysis. Panel **a** shows the basic metabolomic NMR spectral data that can be expressed by a linear combination of a scores matrix **b** and a loadings matrix **c**. The scores matrix summarizes the sample variation (sample classification) and the loadings matrix summarizes the spectral variable variation (biomarker detection). The analysis of both plots will help identify the potential biomarker of the condition under study in the original NMR spectrum.

Metabolomic Analysis and Critical Illness

^1H -MRS analysis of biofluids provides a tool for the understanding of biochemical processes associated with critical care illness [20]. ^1H -MRS, by means of HR-MAS, is an excellent option to study unprocessed fluids [15–17]. A number of ^1H -MRS metabolomic studies of different tissues can be cited as examples for diagnosis in acute animal models and in human illness [21–23].

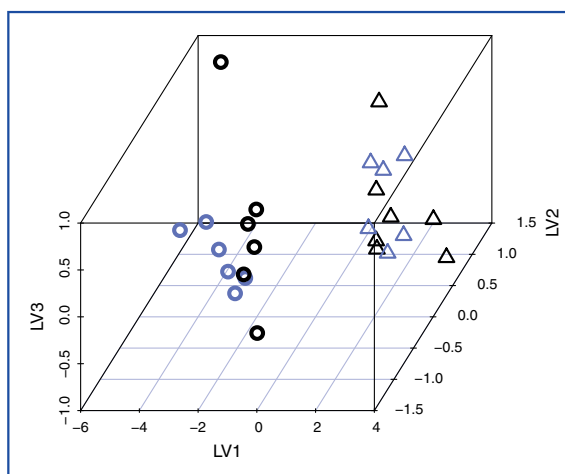
Critical illness is characterized by a severe disruption of metabolic homeostasis and signal transduction pathways. Metabolomics can define specific phenotypes of acute syndromes that potentially may help understand pathophysiology, perform early diagnosis, identify patients at risk, determine disease severity and prognosis, and predict response to therapy. In this way, metabolomics in critical illness can change the concept of biomarkers. For instance, metabolomics can define a specific metabolomic biomarker associated with progression to acute respiratory distress syndrome (ARDS) in patients at risk, or with infections in patients with severe burns [20, 24].

Sepsis

Xu et al. [25] constructed a prognostic model based on serum analysis by HPLC-MS-based metabolomics in rats undergoing cecal ligation and puncture (CLP). HPLC-MS analysis discriminated between rats that survived and those that did not after CLP and sham-procedure with an accuracy of 94 %. Six metabolites were related to outcome, including linoleic acid, linolenic acid, oleic acid, stearic acid, docosahexaenoic acid and docosapentaenoic acid. In a subsequent study performed by the same group with a different technique, serum samples from septic rats were analyzed using ^1H -MRS [26]. Six characteristic metabolites involved in energy metabolism (lactate, alanine, acetate, acetoacetate, hydroxybutyrate and formate) changed in septic rats, more markedly in non-survivors. A prognostic predictive model constructed with these metabolites showed an accuracy of 87 %. In another study [27], LC-MS analysis of plasma samples from thermally injured and septic rats revealed nine characteristic metabolites (hypoxanthine, indoxyl sulfate, glucuronic acid, gluconic acid, proline, uracil, nitrotyrosine, uric acid, and trihydroxy cholanoic acid) discriminating septic from non-septic animals. These biomarkers are mainly related to oxidative stress and tissue damage.

We have recently reported that ^1H -MRS of lung tissue, bronchoalveolar lavage (BAL) fluid and serum samples showed different concentrations of characteristic metabolites in rats undergoing CLP as compared to control rats: Alanine, creatine, phosphoethanolamine and myoinositol concentrations increased in lung tissue; creatine increased and myoinositol decreased in BAL fluid; and alanine, creatine, phosphoethanolamine, acetoacetate and two unidentified fatty acids increased whereas formate decreased in serum [23]. We constructed a model that was 100 % predictive of the diagnosis of sepsis, using all the samples combined or the serum samples only (Fig. 6). One of the novel findings of our study was the different metabolite behavior in the various samples analyzed.

Fig. 6. Partial least square (PLS) discriminant analysis score plot performed on ^1H -NMR spectra. Analysis was performed on serum samples (black) from sham operated (Δ) and CLP (\circ) rats. The model was validated by a new set of samples (blue), showing 100 % sensitivity and specificity.



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