Development and Application of Methodologies for Non-targeted Metabolomics in Animal Models of Lung Injury

by
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PhD Dissertation

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This work was conducted at the Centre of Metabolomics and Bioanalysis, Department of Chemistry and Biochemistry, Faculty of Pharmacy, University San Pablo CEU, under the direction supervision of Dr. Coral Barbas, Professor of Analytical Chemistry and co-supervised by Dr. Antonia García, Senior Lecturer of Analytical Chemistry.

Dr. Coral Barbas and Dr. Antonia García, supervisor and co-supervisor of this work, express their agreement to present the same on the ground that meets the requirements and the original contribution to the specified subject.

Fdo. Dr. Coral Barbas **Fdo. Dr. Antonia García**

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DEDICATION

To my husband Zia and my son Zarif, their unwavering love and support made this endeavor possible.

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LIST OF ABBREVIATIONS

ALI: Acute lung injury

ARDS: Acute respiratory distress syndrome

CE: Capillary electrophoresis

CE-MS: Capillary electrophoresis - mass spectrometry

ESI: Electrospray Ionization

GC: Gas chromatography

GC-MS: Gas chromatography - mass spectrometry

ICU: Intensive care unit

LC: Liquid chromatography

LC-MS: Liquid chromatography - mass spectrometry

LLE: Liquid Liquid Extraction

LOD: Limit of detection

LOQ: Limit of quantitation

MS: Mass spectrometry

MVDA: Multivariate data analysis

NMR: Nuclear magnetic resonance spectroscopy

OPLS-DA: Orthogonal partial least square- discriminant analysis

PCA: Principal component analysis

PLS-DA: Partial least square- discriminant analysis

TOF: Time of flight

QC: Quality control

SI-ALI: Sepsis induced acute lung injury

SPE: Solid Phase Extraction

VILI: Ventilator induced lung injury

Introduction

Metabolomics background:

The term “metabolome” has been defined as the complete set of metabolites synthesized by an organism, reflecting the closest non-structural phenotype. To understand the mechanism of a biological system and their potential dysregulation in any diseased conditions, the study of metabolism plays a dynamic role. However, “metabolome” comprises a huge number of compounds (are mainly of low molecular mass, usually < 1,500 Da) but differ in many other chemical and physical properties (1, 2). The field metabolomics, which is defined as the study of metabolites, is growing exponentially in the post genomic era with advances in analytical instrumentation, chemometric tools and online database system. Consequently, metabolomics has gained considerable attention in biomedical research as practiced independently to study metabolite interaction or in combination with genomics, transcriptomics, and/or proteomics data to study biological systems in a holistic manner (e.g. systems biology) (3). In the evolution of “omics” cascade, metabolomics helps to answer the questions what has happened and what is going to happen in any altered biological metabolism (Figure 1). Metabolomics involved in monitoring the ultimate product of gene expression which is the metabolite. Metabolites are organic compounds that may not be directly encoded in the genome, and their biosynthesis often involves a diversity of enzymes. Furthermore, metabolites are stoichiometrically interrelated, which results in more complex metabolic networks that do not exist in the case of transcripts or proteins. So the application of metabolomics approach may offer the valuable and functional information that is crucial to system biology (3-5).

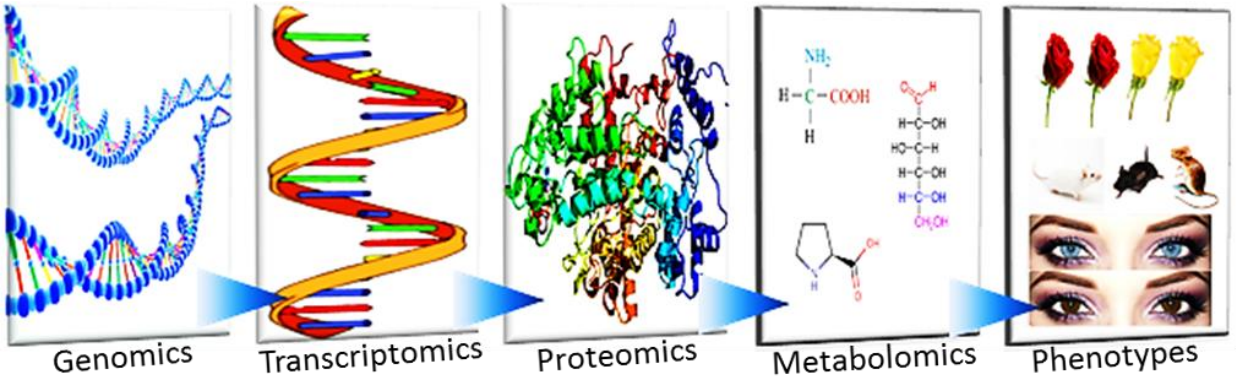

Figure 1: The **OMICS** Cascade. The metabolomics is complementary to transcriptomics and proteomics. Metabolome captures the functional or physiological state of a system and provides a link between genotypes and phenotypes.

Nicholson Group at The Imperial College of London and the Fiehn group at Max-Planck Institute of Molecular Plant Physiology, coined the terms “metabonomics” and "metabolomics" in 1999 and 2002,

respectively (6, 7). However, now-a-days these two terms are being used indistinctly. Different strategies have emerged for tackling the enormous data generated from the metabolomics study. However, the exact definition of each strategy is still in debate. All the available approaches for metabolomics are presented in Table 1. In order to avoid any conflict in this dissertation, the available metabolomics strategies are divided in two major categories such as targeted and non-targeted.

Table 1: Available strategies for metabolomics analysis

Term	Definition
Metabolomics	Complete quantitative and qualitative analysis and identification of all metabolites present in an organism or a biological system, under a given set of conditions (4).
Metabonomics	The quantitative detection of endogenous metabolites that are vigorously altered in a living system in response to pathophysiological stimuli or genetic modification (6).
Global metabolomics profiling	The identification and quantification of a set of metabolites related through their metabolic pathway(s) or similarities in their chemistry (8).
Metabolomics fingerprinting	Unbiased global analysis of all possible metabolites present in crude samples or simple cellular extracts using high-throughput techniques providing all the metabolite information present in the given set of sample (9, 10).
Metabolomics footprinting	The analysis of metabolites secreted from the intracellular complement of an organism (or biological system) into its extracellular medium or matrix. This approach is commonly used in microbial metabolomics (11, 12).
Target analysis	Quantitative analysis of one or few pre-defined metabolites related to a specific metabolic/diseased condition (13).

Targeted approach focuses on some or a group of metabolites to be analyzed. Usually these metabolites are related to specific pathways, or often directly related to genetic perturbations. Targeted approach allows us to quantify the concentration of the metabolite under study, employing robust and sensitive methods. This strategy is very useful in hypothesis testing and addressing well defined biological questions. One of the main limitations of this approach is, it does not provide the global metabolite information of a system. Thus information related to other metabolites can be lost with this approach.

Non-targeted approach attempts to detect as many metabolites as possible in a given set of sample. This approach yields a global picture of the metabolic state of any system of interest. The non-targeted approach measures the signal of all possible chemical entity named as “feature”. The significant increasing or decreasing pattern of these features, in the comparison of two or more groups, is the ultimate goal of non-targeted metabolomics. These differentiating features then can be further interrogated and structurally identified, leading to new ideas regarding the alteration in biochemical processes responsible for the observed differences. Hence, this class of experiment is often referred as “hypothesis generating”.

Both approaches can participate in the quest for novel biomarkers. The level of glucose and cholesterol in blood and creatinine level in serum, describes the value of even a single biomarker. Metabolomics is not only limited to discover biomarkers, however the whole profiling could aid in understanding the mechanism of the specific disease states. For example, evaluating related biochemical pathways in response to drug treatment will give a more complete description of feedback mechanisms than a single biomarker can deliver.

Non-targeted metabolomics workflow:

“Metabolome” of any biological system can be very diverse with metabolites of complex and divergent physicochemical properties. The fundamental of non-targeted approach is to cover a broad metabolic picture through single or in combination of complementary analytical systems along with various extraction protocols. Non-targeted metabolomics approach comprises of four principal stages: (i) sample treatment, (ii) data acquisition employing single or multiplatform techniques, (iii) data processing and statistical analysis using chemometrics followed by compound identification and (iv) chemical marker identification through pathway interpretation. All these four steps are remarkably interrelated and as depicted in Figure 2, each of the steps may contain several sub-steps.

Unlike any other analytical approach, one of the critical steps in non-targeted metabolomics is the transformation of any kind sample to an appropriate solution suitable for instrumental analysis which will contribute in covering an array of metabolite classes. Sample preparation stage constitutes a series of experimental stages, choosing a sample specific to the biological question to be answered, sample collection, applying quenching steps and trying different metabolite extraction protocols. Throughout this step, care must be taken to avoid the introduction of any form of unwanted variability that would significantly affect the outcome of the analysis. No single solvent or extraction procedure can extract all metabolite classes from a given sample. Thus the involvement of two/more extraction protocols and solvents could enhance the range (14-17).

Data acquisition (instrumental analysis) follows the sample preparation step and requires advanced analytical techniques as the ultra-complexity of samples for metabolomics analysis makes it impossible to technologically separate, quantify and identify every metabolite within a biological sample. Several analytical platforms are being used in metabolomics either alone or in combination of two or three (18-20). Prior to any sample analysis, analytical method development and its validation comes in forehand.

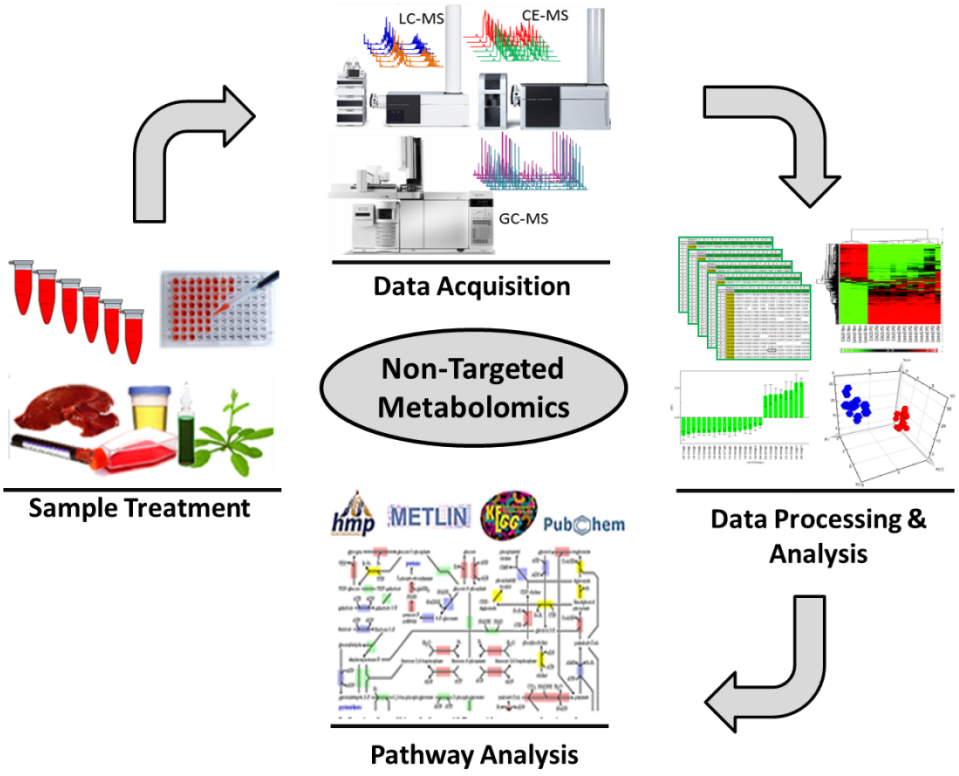

Figure 2: General workflow involved in non-targeted fingerprinting approach, from sample treatment to pathway analysis.

Sample analysis generates huge data sets in terms of molecular feature. Therefore informatics and statistics are essential tools for processing metabolomics data sets (21, 22). Numerous software packages have been developed to aid with data processing (data pre-processing, data pre-treatment and statistical modelling of the primary data) in an automated manner. Data pre-processing and pre-treatment includes centring, scaling, transformation, filtering and above all aid in 'cleaning' the data to focus on biologically relevant information (23). The cleaned data are then subjected to statistical analysis which provides model-based descriptions of the biological variation in the system under study. These statistical models specifically single out representative metabolites of interest which can further be chemically or structurally identified through online based database information and finally in a definitive manner by MS/MS or injecting authentic standards.

The final step in non-targeted approach is relating these identified metabolites with their respective pathways. Though not 100% complete but online data bases provides metabolite information along with their related pathway information. All the metabolites are very much related in the biological system however in a complex manner. So the alteration in one of them could alter the entire network and thus the reason behind any biological dysregulation can be uncovered.

Selection of sample type:

Non-targeted approach has been applied on several sample types from non-invasive to highly invasive, such as urine, blood, plasma/serum, saliva, cerebrospinal fluid, cell lines, exhaled breath condensate, plants, or various tissues (20, 24-33). The biological question being asked influences the chosen type of sample. Each of the matrices has their own strengths and weaknesses. Most widely used sample types are, urine and plasma/serum due to their ease of collection and minimally invasive nature (17, 34). These sample type has been used extensively in metabolomics study so their protein, lipid and metabolite composition are very much well known. Urine is being the most attractive due to its low levels of proteins and cellular material and normalization of total metabolite content using creatinine is well described (17, 35, 36). Urine and plasma/serum are integrated bio-fluids. This offers the simultaneous advantage of reflecting both localized and systemic changes; however, it can be difficult to identify the origins of the observed metabolic changes. Plasma/serum also possesses a number of analytical challenges, especially higher protein concentrations mean samples require deproteinization prior to analysis (17). Although many metabolites are found in bio-fluids but not all affects or changes in the same direction at tissue level or vice versa which has been established by several studies (32, 37). Metabolic changes in specific disease state are first seen in at the tissue site. Moreover, pairwise comparison of diseased and control tissue regions could provide strong markers. Hence, tissue metabolomics can take a big part in non-targeted metabolomics research (20, 38-40). Sample treatment as well pose a challenging part in non-targeted metabolomics. An ideal sample-pre-treatment protocol for non-targeted approach should be: (i) non-selective, (ii) simple and fast with the minimal number of steps, (iii) reproducible, and (iv) incorporate a metabolism-quenching step, if required. Overall, the goal of the sample-preparation procedure is to transform the sample reproducibly into a format that is compatible with the given analytical platform while maintaining the original metabolite composition of the sample as unique as possible. The characteristics and applications of the mostly used biological samples are as follows-

Urine

In mammals, the kidneys extract the soluble waste from the body system and the excess of water, named as urine. Apart from waste and water, urine contains a lot of other low molecular weight compounds in

high concentration such as urea, inorganic salts, creatinine, ammonia, organic acids and various others. Since ancient period, urine has given considerable attention as a diagnostic bio-fluid. Bioanalysis of urine are being performed in clinical diagnosis routinely for glucose, bilirubin, ketone bodies, nitrates, leukocyte esterase, specific gravity, hemoglobin, and protein. Being relatively simple, typically protein free and easily accessible bio-fluid, urine has been applied to various non-targeted metabolomics approach using one or multiplatform techniques such as nuclear magnetic resonance spectroscopy (NMR), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), capillary electrophoresis-mass spectrometry (CE-MS) and so on (24, 41-44). Several non-targeted analysis also used to study a variety of renal conditions, such as bladder, ovarian and kidney diseases (45-48). In non-kidney related diseases urinary metabolomics has also been applied (49). Recently a mostly complete urine metabolome has been explained by *Bouatra S. et. al.* using quantitative multiplatform metabolomics (50). Almost 3079 detectable metabolites in human urine (both exogenous and endogenous) were proposed. Relative to other bio-fluids, such as cerebrospinal fluid or saliva, urine contains significantly more compounds with significant chemical diversity (51, 52). Moreover, the human urine metabolome is a subset of the human serum metabolome, both in terms of composition and chemical diversity (26). The use of metabolomics through examination of patient urine is an ideal means to study disease prognosis and diagnosis. Urine metabolomics has potential utility in non-targeted metabolomics as well for biomarker discovery (47). Since urine has potential importance in non-targeted metabolomics, so urine sample preparation is also being studying (42). Once urinary biomarkers are discovered and validated, they could conceivably be used for prognosis as well as to predict response to targeted therapies as obtaining urine is always more feasible than gaining access to tumor tissue.

Blood (plasma/serum)

Blood serves as a connecting bridge in all body systems through tissue and cells, as all the molecules that are being secreted, excreted or discarded by different tissues and cells are transported to blood in response to different physiological needs or stresses. Blood contains a wide variety of chemically diverse low molecular weight compounds of the metabolome. Most of today's diagnosis are based on blood (plasma/serum) analysis because it is evident fact that tissue lesions, organ dysfunctions and pathological states can alter both the chemical and protein composition of blood plasma/ serum (53). Though plasma and serum are derived from blood, however compound composition is of little different (54). Plasma is the liquefied component of unclotted blood usually obtained with EDTA treatment and contains clotting agents and other components (55). On the other hand, serum is the fluid component of clotted blood (contains proteins, cells and other components, except clotting factors and fibrinogen). Both plasma and serum are aqueous solutions containing a variety of substances including proteins and peptides (such as albumins, globulins, lipoproteins, enzymes and hormones), nutrients (such as

carbohydrates, lipids and amino acids), electrolytes, organic wastes and variety of other small organic molecules suspended or dissolved in them. Blood plays a critical role in transporting and exchanging nutrients, gases, hormones, regulates the pH and ion composition, excerts defensive mechanism against toxins/pathogens and most of all stabilize body temperature (55). Being an important and easily accessible biological fluid, plasma/serum has been applied to various non-targeted metabolomics approach using one or multiplatform techniques such as NMR, LC-MS, GC-MS, CE-MS and so on (18, 19, 56-58). Several non-targeted analyses also used to study a variety of diseases. A database of the human serum metabolome (a complete set of 4229 compounds) has already been introduced (26). Accordingly efforts have been made to develop proper sample treatment in order to get reliable and fruitful results (17). Unlike urine, serum/plasma metabolomics has also potential utility in non-targeted metabolomics for biomarker discovery. The identified biomarkers can be used for prognosis as well as to predict response to targeted therapies as obtaining blood is minimally invasive and more feasible than gaining access to tumor tissue.

Tissue

Tissue metabolomics has many advantages over bio-fluids. The metabolic modifications and the upstream regulations are first seen in tissue. Moreover, the pairwise comparison of tissue taken from diseased region and non-diseased region could reflect the interactions despite of any individual differences. Global determination of metabolite concentrations in tissues provide novel anatomical aspects of pathological conditions that cannot be obtained from target-specific fluid measurements. Providing more relevant information than systemic bio-fluids, tissue metabolomics has a great importance in biomedical research. So far, many studies have already shown the applicability on a variety of human/animal tissues for metabolomics including liver, kidney, lung, brain and spleen from both rodents and other models (20, 32, 59-61). One of the main challenges lies in tissue metabolomics is its collection and treatment to make it appropriate for instrumental analysis. An extensive review of the up-to date published researches based on the challenges related to MS based non-targeted animal/human tissue metabolomics is one of the focuses of this present study. The accepted review manuscript, describing the non-targeted tissue metabolomics has been attached with Chapter-3.

Analytical platforms:

The expanding field of non-targeted metabolomics is growing exponentially in recent years by the advances in analytical methods of high-resolution NMR, MS coupled to separation techniques such as LC, GC, CE, Fourier transform-infrared spectroscopy or direct infusion (62-67). Of these, NMR and MS coupled to LC and GC are the most extensively applied.

NMR is a rapid, non-destructive technique, requires minimum sample preparation and thus lessens the chance of sample loss or the introduction of variability during sample preparation (68). Prior NMR analysis, sample derivatization or analyte separation is not required. Hence NMR analysis is independent of analyte polarity. In NMR, the signal intensity for all compounds is dependent on the molar concentrations and reproducibility is high. However the sensitivity is relatively low (micromolar range) and required sample amount is of very high (69). Although NMR can yield detailed information on the quantities and identities of metabolites present in a biological sample, the chemical elucidation of NMR-detected compounds can be highly complex as a result of overlapping signals. Moreover, NMR analysis is sensitive to the chemical environment (pH, ionic strength, temperature, etc.) of the sample and the differential sensitivity of metabolites to the chemical environment, which hampers the quality of NMR analyses for complex samples (68). However, recent development of cryogenic probes, higher strength superconducting magnets, miniaturized radio frequency coils and multidimensional techniques are being developed in order to improve the sensitivity and resolution of NMR (68-70).

Compared to NMR, MS methods are more prevalent in metabolomics for measuring metabolites in complex biological samples, because they allow reliable metabolite identification with high sensitivity (typically pg level). The developments in LC, GC and CE have significantly broadened the applicability of MS-based metabolomics. However, the comparatively long sample preparation steps and analytical separation of the metabolites before it is directed into the mass analyzer can make the analysis time consuming. Fourier transform coupled to MS provides extremely high resolution and mass accuracy (71). However, this instrument is relatively very expensive which has limited its use to date. Current challenges involved in the development of MS-based metabolomics include the development of more robust methods for chromatographic separation and reduction of matrix effects, including ion suppression, which can cause widely varying signal intensities.

Single analytical approach is not sufficient to cover the entire metabolome. Thus multiplatform applications are necessary to extend the coverage. To obtain this goal, MS detection coupled to separation techniques are becoming the most relevant tools along with NMR. Multiplatform non-targeted metabolomics has been applied on different biological specimens for non-targeted analysis. The importance of multiplatform approach has been well demonstrated by several studies (19, 20, 26).

The main objective of non-targeted metabolomics study is to obtain as much as analyte signals as possible. In order to attain this goal, advanced analytical tools with characteristics of high degree of sensitivity, selectivity, throughput ability and comprehensiveness are desired. The features of most widely used analytical techniques are presented in Table 2. One particular technique is not sufficient for the analysis of all compounds, thus a selection of two from the table would give more coverage than a single one. Otherwise any one form of separation will inherently introduce a bias towards the analytes being detected.

Table 2: Most widely used analytical techniques used in non-targeted metabolomics analysis.*

Technique	Sensitivity	Throughput	Comprehensiveness
NMR	Low	Low–high	Low–high
LC-MS	High	High	High
GC-MS	High	High	High
CE-MS	Medium	Medium	High

- *Information obtained from (72)*

Method development and validation

The development of a suitable method is the pre-requisite for any analytical platform and non-targeted metabolomics is not out of this benchmark. Prior analyzing, in any instrumental platform, a valid and robust method needs to be developed and validated. Analytical procedures play a critical role in acquiring corresponding analyte signal for further data analysis in non-targeted approach. Validation of analytical method should demonstrate that they are suitable for their intended use. Validation should be founded on method development performed beforehand that suggests the suitability and robustness of the method. The steps of method development and method validation depend upon the type of method being developed. A proper method should have the following characteristics-

- *Specificity:* ability to measure desired analyte in a complex mixture.
- *Accuracy:* agreement between measured and real value.
- *Linearity:* proportionality of measured value to concentration.
- *Precision:* agreement between series of measurements.
- *Range:* concentration interval where method is precise, accurate, and linear.
- *Detection limit:* lowest amount of analyte that can be detected.
- *Quantitation limit:* lowest amount of analyte that can be measured.
- *Robustness:* reproducibility under normal but variable laboratory conditions.

Method for non-targeted metabolomics are being developed mainly taking into consideration the most important criteria, which is broader metabolite coverage. Although for targeted metabolomics there are several guidelines available, for non-targeted metabolomics analysis scientists are using alternative approaches to validate the method following one/two from the using-

a. Validation criteria used in targeted metabolomics in terms of linearity, accuracy, precision, limit of detection and limit of quantitation.
b. One of the main limitations in high-throughput analysis is the drift in both chromatographic and MS performance. To control and monitor this type of drawbacks use of quality control samples (QCs) has been introduced in non-targeted analysis. QCs are a pool of sample made from the biological test samples to be studied, or a representative bulk control sample which should be assessed against predefined criteria to enable acceptance or rejection of the analytical run.
c. Use of external standards, are very common in pharmaceutical industry in order to check the reliability of the methodology.
d. An alternative validation strategy is the statistical model validation. The acquired data from instrumental analysis generate huge data sets which are usually expressed in class models applying chemometrics tools. Two approaches are available for validation: test-set and re-sampling method. Another widely used method validation strategy is the prediction of QCs in the chemometrics models. The clustered prediction of QCs describes that the separations between the groups are not due to the instrumental variations but due to the sample itself.

One of the primary objectives of this dissertation is to develop and validate analytical methodologies for serum and lung tissue which has been described in chapter 1 and 3 respectively. The available strategies involved in MS based non-targeted metabolomics till date has been discoursed. A review has been accomplished on the available validation strategies for non-targeted metabolomics methods and included in Chapter-1 pointing the objective of this dissertation.

Data analysis

High-throughput non-targeted metabolomics analysis generates extremely large volume of data. Hence automated software is needed in order to understand and handle these large data sets. The automated software helps to identify peaks from raw data, align them among different samples and identify each metabolite. Afterwards, informatics and statistics are essential tools for reprocessing metabolomics data sets (55). Metabolomics data analysis comprises of data pre-processing, data pre-treatment and statistical analysis (modeling) of the primary data. The main target of metabolomics study is to obtain variations in the data sets due to their biological means. However non-biological, technical or instrumental variations are as well very prone in any analytical platforms. In order to clean these unexpected variations, data mining (pre-processing and pre-treatment) plays an important role. Most frequently applied tools in metabolomics data mining are: alignment, normalization, centering, scaling, filtering and mathematical transformation of the raw data (23). Alignment helps in assigning the obtained peaks, which appears in all samples at the same mass (m/z) and time of the same feature, finally listing all possible components as represented by spectral data. Peak alignment is necessary to perform in order to

compare data across samples. Data normalization minimizes the differences in the detection between samples arising from variation in the concentration. Several data normalization strategies are available such as the use of internal standards, MS total signal, MS total useful signal, sample volume and median fold change normalization (35, 73). The aim of logarithmic data transformation is to reduce the influence of potential outliers and to transform the data matrix into a more Gaussian-type distribution (74). Moreover, transformation increases the weight of low-intensity metabolites and compresses the upper end of the measurement scale (75). Scaling of the data is desirable to adjust the importance assigned to the elements of the data in fitting the model. By scaling, the weight of each variable is adjusted with a scaling factor estimated by either a dispersion criterion or a size measure. Unit variance scaling and Par-scaling are two of the most prevailing scaling types used for non-targeted metabolomics studies (76, 77). Generally centring is applied in combination with scaling. Mean centring converts concentrations to fluctuations and variables are centred around zero (23). The final step in data mining is the data filtration. Applying data filtering, potential biomarkers can be selected by retaining only masses that appear in all samples within the groups. The frequently used filtering allows features to be filtered out based on their presence/absence. It is a crucial point for studies carried out on experimental groups under various conditions such as treatment or diet, because metabolites coming from the treatment, which contribute to discriminate the groups but are not interesting for the study, can be eliminated by retaining only those compounds present in all samples in all groups. Depending on the analytical technique, different software packages are available to perform data mining in an automated manner (23).

The cleaned data are then subjected to statistical analysis with the help of chemometric tools, which provides model-based descriptions of the biological variation in the system under study. The chemometric tool uses mathematics, statistics and formal logic (a) to design or select optimal experimental procedures, (b) to provide maximum relevant chemical information by analyzing chemical data and (c) to obtain knowledge about chemical systems (78). Two different statistical analyses are being used in non-targeted metabolomics data analysis: univariate and multivariate data analysis (MVDA).

The univariate data analysis assumes that the response of variables is influenced by only one factor. In a complex disease, different biologic pathways simultaneously governed by multiple variables are involved and compromised. The traditional statistical analysis tends to transform all problems into univariate problems, even those that are inherently multivariate. For this reason, MVDA is a proper statistical tool for the interpretation of data coming from a metabolomics study. It summarizes data tables with many variables and few observations and works reducing the number of variables and classifying the data into groups (55, 79). MVDA provides statistical models specifically single out representatives of metabolites of interest (annotated peaks), which can further be chemically or structurally identified in a definitive manner. The first step of MVDA is the creation of the X matrix, where the samples are included in rows, and all the variables (the compounds) in columns. Subsequently, it is always necessary a data pre-treatment (scaling and transformation) for improving relevant information. After this, data are analyzed through the

unsupervised principal component analysis (PCA) and the supervised partial least squares regression-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS -DA).

To avoid the risk of over fitting, as the results found after MVDA are sensitive to chance-correlations, statistical model needs to be validated. Validation tools, such as (a) permutation test and (b) cross-validation, are most widely used to provide an objective assessment of the performance and stability of a model.

(a) Permutation Test: A permutation test is used to assess the significance of a classification. The class assignment can be permuted several times and for each permutation, a model between the data and the permuted class-assignment can be built. The discrimination between classes of the model based on the permutated class-assignment is compared to the discrimination of the model based on the original classification (55).

(b) Cross-Validation: In cross-validation the first step is to divide the samples into several groups. These groups are then sequentially withheld while the remaining samples are used to build cross validated models. The withheld samples for each model are predicted and the predictive ability is measured and this process repeated until all the samples are withheld and predicted.

The data statistics provides statistically significant masses for a corresponding compound which is/are involved in the differentiation of study group. These significant masses are then finally search against online data bases, which provides information about the mass with their related corresponding compound(s). Several online databases (free and commercial) are available which provides identification of the significant masses, chemically or structurally in a definitive manner. Two types of databases are available: (i) databases that contain metabolic pathways based on information from the literature which has been integrated and curated manually and (ii) databases that contain raw or processed data from analytical system. Examples of some online databases are: Kyoto Encyclopedia of Genes and Genomics (http://www.genome.jp/kegg/), MetaCyc (http://MetaCyc.org), MetaCrop (http://metacrop.ipk-gatersleben.de), Reactome (http://www.reactome.org), The Human Metabolome Database (http://www.hmdb.ca), Small Molecular Pathway Database (http://www.smpdb.ca), Golm Metablome Database (http://csbdb.mpimp-golm.mpg.de/gmd.html), METLIN (http://metlin.scripps.edu), MassBank (www.massbank.jp), Metaboanalyst 2.0 http://www.metaboanalyst.ca and Mouse Multiple Tissue Metabolome Database (http://mmmdb.iab.keio.ac.jp) (61, 80-89). There are also several commercial pathway databases available which contain integrated knowledge and well-studied pathways, e.g. Cell Signal pathways (www.cellsignal.com), Sigma– Aldrich pathways (http://www.sigmaaldrich.com/lifescience/ cell-biology/learning-center.html), Ambion pathways (http://www.ambion.com), and ProteinLounge (http://www.proteinlounge.com/).

Application to pulmonary diseases

Pulmonary diseases are prevalent and one of the major causes of global morbidity and mortality (90-92). The principle pulmonary diseases are acute respiratory disease syndrome/acute lung injury (ARDS/ALI), chronic obstructive pulmonary disease, asthma, pulmonary arterial hyper tension, pulmonary embolism, cystic fibrosis, lung cancer and particularly infectious disease including tuberculosis. The exact mechanism of disease onset are still unknown, however environmental exposures have been found to be directly involved in the development of lung diseases (93, 94). At present several quantitative and semi-quantitative tests are involved in pulmonary disease diagnosis such as radiological examinations, spirometry, sputum analysis and, more recently, exhaled nitric oxide, carbon monoxide testing etc. (95-98). A range of markers are also available to improve clinical diagnosis but unfortunately they have very low specificity and they are often incapable of identifying and diagnosing specific disease sub-phenotypes (34, 99). However, MS-based techniques have been used to measure leukotriene B4, a marker of inflammation and 8-isoprostane, a marker of oxidative stress (100, 101). A number of challenges are still there which need to be addressed to improve diagnosis, understand and treatment of lung diseases. The promising technique metabolomics have successfully applied and classified several respiratory diseases, including asthma, chronic obstructive pulmonary disease, ARDS, ALI, pulmonary embolism, lung cancer and cystic fibrosis (102-108). With the application of metabolomics it was possible to tentatively identify distinct areas of metabolism and the pathways that characterize the individual disease metabolic phenotypes, proving the fact that metabolomics approaches can play a central role in diagnosing/characterizing pulmonary diseases.

Acute lung injury

ALI/ARDS, is characterized by the onset of clinically significant hypoxemia and diffuse bilateral pulmonary infiltrations. ALI/ARDS is an important cause of pulmonary and non-pulmonary morbidity in patients who survive hospitalization (109). Due to the inherent heterogeneity of the disease along with the consistent lack of correlation between biochemical markers, pathophysiologic variables and clinical outcomes, the search for ALI biomarkers are being hampered. Figure 3 explains the pathogenesis of lung injury obtained from Ware and Matthay (109). Usually normal lung epithelium is composed on flat and cuboidal type of cells, which comprises 90 % and 10% respectively of the alveolar surface area. Compared to cuboidal, flat type cells are more prone to injury. Upon injury the normal epithelial fluids transport disrupted and contribute to epithelial flooding (110, 111). Following those neutrophils starts adhering to the injured capillary endothelium and marginating through the interstitium into the air space. Air space contains alveolar macrophage which secrets cytokines, interleukin-1, 6, 8, and 10, and tumor necrosis factor α, which act locally to stimulate chemotaxis and activate neutrophils. Neutrophils involves in releasing oxidants, proteases, leukotrienes and platelet-activating factor (109). With the improved

understanding of ALI pathophysiology, interleukin-1β and tumor necrosis factor-α have been evaluated to check their diagnostic or prognostic capability as a biomarker. These inflammatory mediators can be detected in the distal airspaces of the lung in ALI/ARDS patients (112-114). Most recently investigators have documented the influence of the coagulation system in ALI/ARDS. ALI is characteristically heterogeneous, depending on the origin it can be several types such as endothelial injury, epithelial Injury, neutrophil-dependent lung injury, ventilator induced lung injury (VILI) and sepsis induced lung injury (SI-ALI) (115-119). Following the aim of this dissertation, a brief discussion about VILI and SI-ALI are as follows.

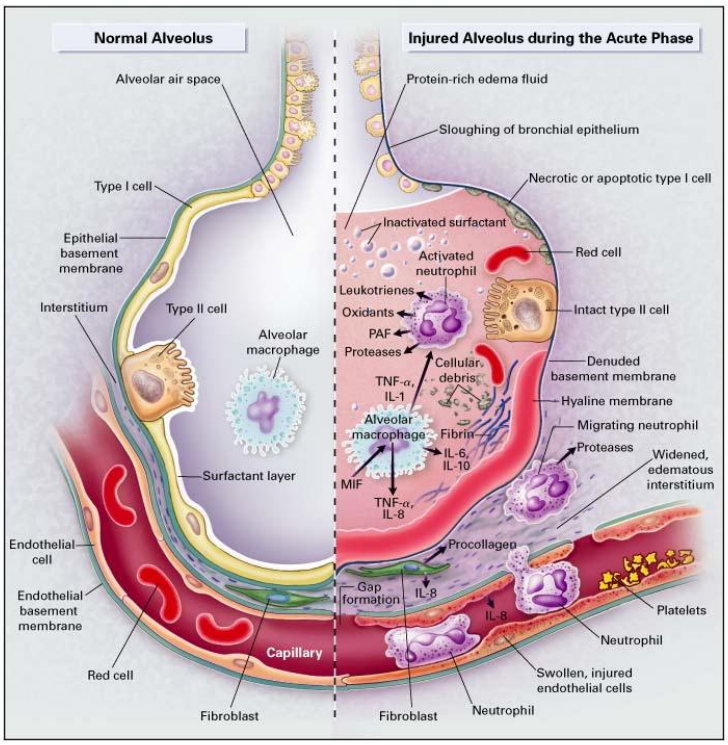

Figure 3. The normal alveolus (left-hand side) and the injured alveolus in the acute phase of acute lung injury and the acute respiratory distress syndrome (right-hand side). Obtained from Ware and Matthay (109).

(i) Ventilator-Induced Lung Injury (VILI): In clinics, from several decades mechanical ventilation has been used as the important part of basic life support. However several potential complication has been identified with the use of mechanical ventilation (120). There are evidences that mechanical ventilation at high volumes and pressures can injure the lung, causing increased permeability pulmonary edema in both uninjured and injured lung (121, 122). Thus, VILI has received much attention in both experimental and clinical field. This harmful effect of ventilation has been hypothesized by the fact that

the capillary stress failed due to alveolar over-inflation. More recently, cyclic opening and closing of atelectatic alveoli during mechanical ventilation have been shown to involve in developing lung injury independently of alveolar over-inflation. Alveolar over-inflation together with the repeated collapse and reopening of alveoli can initiate a cascade of pro-inflammatory cytokines (123). In intensive care units (ICU) patients with ALI/ARDS might require ventilation at traditional tidal volumes. The use of ventilation may affect the uninjured alveoli along with the injured one, promoting further lung injury and finally contribute to multi-organ failure (123). Several other techniques has been applied in order to evaluate the exact molecular mechanism of ALI, however there are no evidences of non-targeted metabolomics application on VILI models of ALI. One of the aims of this dissertation is to apply non-targeted metabolomics methodologies on serum samples of VILI and healthy rat models applying CE-MS and LC-MS. The application and findings of the applied methodologies are described in Chapter-1 and Chapter-2.

(ii) Sepsis-Induced Lung Injury (SI-ALI): Sepsis is a potentially deadly medical condition associated with an extremely complex chain of systematic inflammatory and anti-inflammatory processes due to the presence of a known or suspected severe infection, trauma, blood loss, and perforated neoplasms (124). The characteristic effects of sepsis are extreme inflammatory changes, profound hypotension, hypoxemia, and lethal tissue damage, which ultimately proceed to organ dysfunction (125, 126). Despite extensive research, sepsis remains one of the causes for death and the mortality rate remains higher (approximately 30%) in ICU (127). Generally sepsis affected patients are treated in ICU and to support their lung function they may require mechanical ventilation. There are evidences that approximately 74% of sepsis affected patients admitted in ICU consider sepsis as the primary risk factor for ALI (119). The accurate progression of ALI in patients whether induced by mechanical ventilation or sepsis is still not known. One of the reasons is the cause of ALI which is extremely heterogeneous, leading to difficulties in phenotyping patients as well as monitoring and treatment. Currently there are several cell-specific biomarkers available such as cytokines and their receptors, surfactant protein D, coagulation factors such as protein C and plasminogen activators (128). Nevertheless, these biomarkers mainly help to understand the pathogenesis of the disease but not the progression. On the other hand the role of mechanical ventilation during sepsis is not known: whether the lung injury is the effect of ventilation or sepsis by itself. The molecular mechanism behind SI-ALI is still under investigation. Several metabolomics study has been performed on sepsis and ALI induced by sepsis, separately (129, 130). The molecular changes could aid in finding the involvement of ventilation or sepsis in SI-ALI. Moreover, the proved fact is that tissue provides more site specific information. Considering all these, another aim of this dissertation is the application multiplatform tissue metabolomics on the lung tissue of sepsis, VILI and SI-ALI, to find out exact mechanisms. Chapter-4 explains the results of the applied multiplatform methodology on lung tissue of rat models of sepsis, VILI and SI-ALI.

Animal models for lung injury

For more than a hundred years, virtually every medical breakthrough in human and animal health has been the direct result of research using animals. The use of animals in biomedical research is essential to the development of new and more effective methods for diagnosing and treating diseases that affect both humans and animals. Animals are being used in biomedical research to learn more about health problems and to assure the safety of new medical treatments. Medical researchers need to understand health problems before they can develop the ways to treat them. Some diseases and health problems involve processes that can only be studied in living organisms. Animals are the good research subjects for a variety of reasons. Animals are biologically similar to humans. In fact, chimpanzees share more than 99% of DNA and mice share more than 98% DNA with humans. Therefore, animals are susceptible to many of the same health problems as humans. Animals have a shorter life cycle than humans and as a result, they can be studied throughout their whole life span or across several generations. In addition, scientists can easily control the environment around animals (diet, temperature, lighting), which would be difficult to do with humans. A variety of animals provide very useful models for the study of diseases afflicting both humans and animals, including rats, mice, birds, rabbits, guinea pigs, sheep, fish, frogs, pigs, birds, dogs, cats, primates, among others. Approximately 95% of the animal model based research includes the use of rats and mice.

Ideally, an “animal model of ALI” should be a model in which this pathologic triad is reproduced. Many different modeling strategies have been developed in an attempt to reproduce the features of human ALI in animals. From a practical standpoint, there are four general types of model systems:

1. Direct induction of lung injury using a noxious stimulus. These include the intratracheal or intranasal administration of bacteria or bacterial products such as lipopolysaccharides, the administration of an acid such as hydrochloric acid or of gastric particulates to reproduce aspiration, the administration of high inspired fractions of oxygen, depletion of surfactant by serial lavage with 0.9% sodium chloride, the induction of ischemia/reperfusion by clamping the hilum (131-133).
2. Another direct way of inducing lung injury is the exposure to mechanical stretch using mechanical ventilation with high tidal volumes, which is called VILI (18).
3. There are some indirect ways of inducing lung injury in animal model. This category includes models based on reproducing sepsis, such as cecal ligation and puncture (CLP), the administration of intravenous bacteria or lipopolysaccharide and mesenteric ischemia/reperfusion. This category also includes the oleic acid model, with attempts to reproduce the release of oleic acid from bone marrow in patients with multiple bone fractures (134).

4. Combination models. To better reproduce human ALI/ARDS, different injury strategies can be combined; most commonly these include saline lavage followed by mechanical ventilation, or CLP followed by hemorrhage.

No single animal model reproduces all the histopathological elements of ALI satisfactorily. Thus, when choosing an animal model of ALI, it is important to consider the key features of ALI that will be tested by the hypothesis of the study, and then choose a model that reproduces those features. Concentrating on the hypothesis of this dissertation, sepsis, VILI and SI-ALI rat models were chosen as a model for ALI to apply the developed non-targeted metabolomics approaches.

The originality of this dissertation

This dissertation contains several originalities:

(i) An extensive review on methods for human/animal based tissue metabolomics.

(ii) A critical review on the available validation strategies used for MS-based non-targeted metabolomics.

(iii) A CE-MS based methodology for non-targeted serum analysis including sample treatment, and validation. Following method development, a complete fingerprinting of rat serum was performed for the first time using CE-MS.

(iv) A multiplatform method (LC-MS, GC-MS and CE-MS) for lung tissue fingerprinting was designed using a minimum amount of sample. It was developed and validated along with sample treatment. The multiplatform method was then used for a complete mouse lung fingerprinting.

(v) All the developed and validated methods for serum and lung were applied on the rat models of lung injury (VILI+SI-ALI) for the first time.

(vi) Involvement of collagen remodeling, oxidative stress, energy metabolism, carnitine biosynthesis and cholinoenergic pathway in the pathogenesis of ALI.

(vii) Association of ceramide and sphingomyelin pathways in the pathogenesis of ALI.

(viii) The effect of ventilation was more prominent compared to sepsis in ALI.

(ix) Many of the serum and lung metabolites changes in opposite directions for ALI.

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Objectives

Non-targeted metabolomics has already been proved as a tool for generating hypotheses with evidences. The characteristics metabolite changes under certain conditions left this approach attracted to researchers as well as clinicians in recent days. However, new methodologies have yet to be developed and validated to support an effective application. Providing these facts the main objective of this dissertation is to develop and validate analytical methodologies for serum and tissue, followed by a complete fingerprinting of rat serum and mouse lung tissue and application of the developed methodologies on serum and tissues from rat models of lung injury, to find new markers that may be involved in the pathophysiology of ALI. To achieve the overall goal we propose the following specific objectives:

Specific objectives:

- Development and validation of a non-targeted CE-MS method for serum analysis, focusing on both analytical and sample treatment optimization.
- Perform a complete fingerprinting of rat serum using CE-MS.
- Non-targeted metabolomics analysis of rat serum profiles from VILI and control animals using validated CE-MS method.
- Analyze the serum metabolomics profiles of VILI and control groups of rat model of ALI by LC-MS applying non-targeted metabolomics approach.
- Development and validation of a non-targeted multiplatform method for lung tissue based on LC-MS, GC-MS and CE-MS, concentrating on both analytical and sample treatment optimization.
- Evaluate the complete fingerprinting of rat lung tissue using LC-MS, GC-MS and CE-MS.
- Conduct a non-targeted metabolomics analysis of lung tissue from rat models of sepsis, VILI and SI-ALI applying LC-MS, GC-MS and CE-MS.
- Correlate the information obtained from the non-targeted analysis of serum and tissue independent of the study undertaken and identify new potential markers associated to lung injury to understand its pathogenesis and treatment in advance.

Outlines

Metabolomics signifies the targeted and non-targeted analysis of all the small metabolites present in the biological system. The 'metabolome' comprises of low molecular weight compounds (typically <15,000 Da), representing the molecular phenotype of any cell, tissue or whole organism. The fundamental value of non-targeted approach over targeted is, it enables the analysis of all possible small metabolites of the metabolome. Till date numerous analytical platforms have been described for non-targeted metabolomics applications but, no single method has been described that is suitable for an exhaustive metabolomics analysis. To circumvent this problem, integration of different analytical platforms could be a fruitful strategy for non-targeted analysis. Another important prerequisite in non-targeted metabolomics approach is the capability to get reliable and reproducible data along with high number of metabolites. However developing any analytical method consideration in analysis time and sample volume have always been given high priority. Though for targeted analytical method there have been several published guidelines for method development and validation, but very less has been spoken for non-targeted approach. Moreover choosing a suitable sample of interest for analysis (from non-invasive, minimally invasive to invasive) is another important factor for metabolomics approach. Usually non-invasive or minimally invasive sample are the first choice for metabolomics study. Although the first sign for any disease state is seen at the tissue level. Due to the invasive nature they are not being frequently used in metabolomics. While analyzing both sample type could give us an idea about site-specific and secreted metabolite mechanism of any conditions. The aim of this dissertation has been spawned by integrating all these general conditions for non-targeted metabolomics method development, validation and application on both serum and tissue samples. This dissertation consists of four chapters and the independent outlines of each chapter are as follows-

Outline for chapter 1: Method development and validation for rat serum fingerprinting with CE-MS: Application to ventilator induced lung injury study.

One of the major aims of the present work will be the development of a method along with validation which would allow both the fingerprinting and application. The state-of-art used for non-targeted metabolomics based method validation till date will be described. There are several methods available for non-targeted analysis of serum based on LC-MS, GC-MS and NMR. CE-MS based methods has also been described but very few. Moreover the described methods are not adequately validated, involving high sample volume and long sample

preparation steps. CE possesses many advantages over other separation techniques, ability to separate charged metabolites and high separation efficiency is its main focus. The use of TOF-MS along with CE would enhance the analysis providing high mass accuracy and fast scan speed over a wide mass range, which are prerequisites for screening applications. Here in this study a CE-MS based non-targeted method for serum analysis will be developed, following the validation steps used for targeted metabolomics approach. Focusing the metabolites type which can only be identified from CE-MS, a complete fingerprinting study of rat serum will also be performed. Finally the developed method will be used to apply on serum sample of an animal model for mechanical ventilation (VILI).

Outline for chapter 2: A metabolomic approach to the pathogenesis of ventilator induced lung injury.

The discovery of biomarkers by non-targeted metabolomics has a high potential in metabolomics field. ALI/VILI is a major cause of morbidity and mortality in critically ill patients. There are no biomarkers to identify patients who would develop VILI during or following mechanical ventilation. LC-MS represents an important part of the evolving "metabolomics toolbox" with advantages of high sensitivity and good potential for biomarker identification. Thus the broad applicability of LC-MS to metabolites of all classes leads to accept it as a first choice of consideration. Previous studies have described the application of non-targeted metabolomics approach on lung injury induced by hyperoxic, gamma-irradiation and sepsis using NMR and LC-MS. However there are no publications available for non-targeted metabolomics application on VILI models. A non-targeted LC-MS based method for serum samples has already been developed in our laboratory. That developed method will be applied on the serum samples of a rat model of VILI to understand its pathogenesis.

Outline for chapter 3: Multiplatform analytical methodology for metabolic fingerprinting of lung tissue.

Generally non-invasive (*e.g.* urine) or minimally invasive (*e.g.* blood/plasma/serum) bio-fluids are the primary choice for metabolomics study either in targeted or non-targeted due to their ease of collection. But the metabolite information from tissue provides innovative information rather than bio-fluids as they are quite target specific. The main handicap for tissue analysis is the sample pre-treatment protocol specially homogenization, which is usually being overlooked.

In this chapter, up to now the applied tissue metabolomics study will be reviewed extensively, focusing on the non-targeted approach of human and animal tissue. On the other hand, no single analytical technique can represent the whole metabolome picture. Each separation technique (LC, GC and CE) has its own specific metabolite characteristics. So in order to cover as much metabolites as possible the integration of multiplatform analytical approach would be a greater idea which has already been proved by several studies. A very few methodology has been described on lung tissue metabolomics applying MS-based approach. However in all cases biphasic separation, long extraction processes along with high sample quantity were used. Other than that only one/two choosing from NMR, LC-MS, GC-MS and CE-MS platform was/were applied for non-targeted tissue metabolomics. Though, none of the published work has followed any method validation criteria except using internal standard and QCs. Considering all these facts a multiplatform method along with sample treatment (targeting minimum sample volume, single phase extraction and analysis in three platforms from the same extraction) will be developed for LC-MS, GC-MS and CE-MS non-targeted analysis and also a complete validation in terms of linearity, specificity, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) will be followed for the first time. The developed method should then be used for a complete mouse lung fingerprinting, which has not been done before. Eventually the validated method will be applied on rat lung tissue from control and sepsis group in order to prove the potentiality of the developed and validated non-targeted methodology.

Outline for chapter 4: Unveiling the effect of ventilation in an animal model of sepsis through a multiplatform lung fingerprinting approach.

Sepsis is a systemic disorder leaving an inflammation and hypoxic condition at the site of infection, eventually spreading throughout. In severe septic conditions, patients are admitted to ICU, where they are given mechanical ventilation for proper respiration. Although life-saving, but over ventilation could injure the lung and studies have shown that 50% mortality rate are due to SI-ALI. However it is still not clear whether the lung injury is due to mechanical ventilation or due to sepsis. Till date independent studies have been undertaken either focusing on ventilation or sepsis. Combinatorial analysis of sepsis, VILI and SI-ALI could provide the exact reason behind the lethal incident on lung. Moreover lung tissue metabolomics would provide the actual impact any altered condition on lung due to sepsis or ventilation. Through non-targeted approach the molecular mechanism behind this fact could be exposed and thus with a preliminary knowledge

it will be beneficial for clinicians to optimize the treatment for respective patients. Provided that the successful multiplatform (LC-MS, GC-MS and CE-MS) developed and validated method development for lung tissue will be used to analyze tissues sample from rat model of sepsis, VILI and SI-ALI to determine whether lung injury is due to mechanical ventilation or sepsis.

Chapter-1

Method development and validation for rat serum fingerprinting with CE-MS: application to ventilator induced lung injury study

The primary challenges in the field of non-targeted metabolomics are the development and validation of instrumental methodologies along with sample pre-treatment. Fundamentally, the developed methodology must be robust to the typical biological variation in physicochemical properties of the chosen sample type and must be performed in the same manner across all analyses. The reliability of any methodology can be assured upon a proper validation attempt. No specific guidelines have been proposed till date for non-targeted metabolomics approach. Due to that alternatives approaches are being undertaken, however not unique for all cases. To fulfill the objectives of this dissertation an extensive study on available validation criteria for MS-based non-targeted approach was commenced.

Following that an extensive review is accepted and available online in the *Journal of Chromatography A* and included in this Chapter-1.

Title: Method validation strategies involved in non-targeted metabolomics.

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CE coupled to electrospray ionization (ESI)-TOF-MS is a comprehensive technique in non-targeted metabolomics for screening polar and charged metabolites. CE is able to separate complex mixtures with high resolution with minimal sample treatment. The minimally invasive nature of serum has attracted the non-targeted metabolomics field, hence has been used extensively in multiple studies. Several experiments have been conducted to describe serum sample treatment such as using solid phase extraction (SPE), liquid-liquid extraction (LLE) and ultrafiltration. However it is well known that SPE and LLE are very time consuming protocols. The documented ultrafiltration method was highly diluted and very low nominal cutoff filter was used. In order to improve and obtain an easy sample preparation step, a one-step ultrafiltration method using 30kDa protein cut-off filter was applied in this study. To compare, the sample were also prepared following SPE protocol. With sample treatment, sample volume CE-MS conditions were also optimized. The optimized method was then applied on rat serum sample for a complete profiling. In order to validate the optimized methodology a series of compounds were selected from the profiled list. To evaluate the performance of the validated method, the

method was applied to serum samples from rat of VILI (an animal model of ALI) and controls. Patients in ICU may require mechanical ventilation for proper respiration. However it has been recognized that certain mechanical ventilation strategies characterized by the use of high tidal volume and low positive end-expiratory pressure may induce lung injury in both normal or pre-injured, which is termed as VILI. In present the diagnosis of ALI is based on non-specific criteria and there is a need of chemical signature for this. Several metabolomics work have been implemented to find diagnostic markers using NMR and LC-MS but principally in a quantitative manner. The developed and validated method in this present study was applied to the serum sample of VILI for the first time using CE-MS successfully, resulting with significant metabolites discriminating VILI from the normal group.

In continuation, the in-detailed description of the developed methodology and application of this study was published in *Analytical and Bioanalytical Chemistry* Journal.

Title: Method development and validation for rat serum fingerprinting with CE-MS: application to ventilator-induced-lung-injury study.

Authors: Naz Shama, Garcia Antonia, Rusak Magdalena, Barbas Coral

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Resumen

ESTRATEGIAS DE VALIDACIÓN DE MÉTODOS APLICABLES A METABOLÓMICA CON ENFOQUE NO DIRIGIDO

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La metabolómica con enfoque no dirigido se basa en la hipótesis de conseguir el análisis global, no sesgado de todos los metabolitos de pequeño tamaño presentes en un sistema biológico bajo unas determinadas condiciones. Incluye diferentes etapas tales como la selección de las muestras biológicas, pretratamiento de las muestras, condiciones analíticas, adquisición de datos, análisis de datos por quimiometría, búsqueda en bases de datos e interpretación biológica. Los estudios metabolómicos no dirigidos ofrecen el potencial de la aproximación holística en el área de la investigación biomédica a fin de mejorar el diagnóstico de enfermedades y entender el mecanismo de su patología. En literatura científica se citan diversos métodos analíticos basados en RMN y MS acoplada con diferentes técnicas de separación. La clave en el desarrollo de cualquier método analítico está en la validación de cada una de las etapas que lo componen a fin de obtener resultados fiables y reproducibles, pero en la metabolómica no dirigida no se aplican los mismos criterios, ya que los desafíos analíticos a los que se nos enfrentamos son completamente nuevos y diferentes a los de los método clásicos. En este artículo de revisión se describirán las estrategias de validación que se vienen utilizando entre las posibles y también se darán algunas recomendaciones a considerar en el desarrollo de un método analítico de aplicación en metabolómica no clásica.

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Method validation strategies involved in non-targeted metabolomics

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ABSTRACT

Non-targeted metabolomics is the hypothesis generating, global unbiased analysis of all the small-molecule metabolites present within a biological system, under a given set of conditions. It includes several common steps such as selection of biological samples, sample pre-treatment, analytical conditions set-up, acquiring data, data analysis by chemometrics, database search and biological interpretation. Non-targeted metabolomics offers the potential for a holistic approach in the area of biomedical research in order to improve disease diagnosis and to understand its pathological mechanisms. Various analytical methods have been developed based on nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled with different separation techniques. The key points in any analytical method development are the validation of every step to get a reliable and reproducible result and non-targeted metabolomics is not beyond this criteria, although analytical challenges are completely new and different to target methods. This review paper will describe the available validation strategies that are being used and as well will recommend some steps to consider during a non-targeted metabolomics analytical method development.

1. Introduction

Metabolomics was defined by Oliver Fiehn as the global unbiased analysis of the small-molecule metabolites present within a biological system in an identified and quantified manner [1]. In parallel, Jeremy Nicholson coined the term metabonomics, which is defined as the comprehensive and simultaneous profiling of metabolites and their effective changes resulting from different conditions such as diet, life style, genetic or environmental factors [2]. Currently both terms are used interchangeably. This methodology offers the potential for a holistic approach to clinical medicine, as well as improving disease diagnosis and understanding of pathological mechanisms. The exact definition of the metabolome is in some debate, however in general can be thought of as the complete compliment of all the low-molecular weight molecules (<1500 amu) present in the biological compartment in a particular physiological state under a given set of environmental conditions [3]. Metabolomics may also be the methodology for biomarker discovery. Biomarker was defined in 1998 by the National Institutes of Health Biomarker Definitions Working group as: "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" [4]. To make this definition more clear in the year of 2010 the World Health Organization suggested that a biomarker is "*almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The response may be functional and physiological, biochemical at the cellular level, or a molecular interaction*" [5]. A biomarker could be anything such as, physical traits (body temperature, blood pressure, etc.) or presence of biological molecules in tissues or body fluids. The fundamental goal of biomarker identification in biomedical research is the discovery of a molecular signature which can correlate with a specific disease type that can be used as early diagnostic tools in clinical practice [6]. This type of marker requires high sensitivity and specificity. The metabolomics field has a key role in screening chemical markers and this approach can mainly be divided in two categories, targeted and non-targeted. Targeted metabolomics consists of the quantification of one or a set of known metabolites, which are generally related to a specific pathway or biological activity [1]. It enables exact quantification of the metabolite by employing authentic analytical standards and only focus on the changes of the quantitated metabolites [7,8]. On the other hand, non-targeted approach is the data driven, rapid high-throughput analysis of all possible metabolites present in a given set of samples without any prior knowledge of the metabolites [9]. Compared to targeted approach, in non-targeted metabolomics, it is not

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possible to quantify due to the larger number of variables and because the identity of the metabolites is often unknown [10–12]. The key advantage of global approach over targeted approach is, that it enables novel areas of metabolism to be identified [8,13]. Numerous analytical platforms have been used in non-targeted metabolomics applications, such as nuclear magnetic resonance spectroscopy (NMR), Fourier transform-infrared spectroscopy and mass spectrometry (MS) coupled to separation techniques, or using direct flow injection [2,14,15]. The potentiality of NMR in high throughput metabolomics, is the minimal sample pre-treatment requirement, obtaining highly quantitative and reproducible data, however with this technique only medium to high abundance metabolites can be detected and challenges still remain for complex mixtures. MS-based metabolomics offers analyses with high selectivity and sensitivity, moreover positive and negative ionization will increase the variety of metabolites, either using direct infusion or in combination with separation techniques choosing from liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE). Use of separation techniques also reduces the complexity of the mass spectra and delivers additional information on the physico-chemical properties of the metabolites [16]. However, MS-based techniques usually require sample preparation steps and analytical method development, which should be valid enough to get effective results. LC–MS based analysis is the most widely used analytical platform in non-targeted metabolomics due to its high sensitivity and selectivity. Separation with LC can reduce ion suppression caused by co-eluting compounds, isobaric interferences and often can separate isomers. In addition, analytical separation with LC could be benefited from lower detection limits and improved MS data quality due to reduced background noise. The combination of GC with MS provides high-resolution, analyte-specific detection, and quantification of metabolites and as well it has the capability to identify unknowns. However, a major prerequisite for GC–MS analysis is a sufficient vapor pressure and the analytes should be thermally stable. The sample pre-treatment steps for GC–MS analysis are quite long and only volatile metabolites can be analyzed, which are the main limiting factors for this technique. CE is a powerful technique for the separation of charged metabolites, offering high-analyte resolution. The combination of CE with MS makes CE–MS an ideal tool for the analysis of polar compounds present in the metabolome. However, only a few applications have been published as it is not a generally available technique in the laboratory.

Accordingly, a single analytical approach is not enough to cover the entire metabolome. Thus the integration of multiplatform approaches are necessary to circumvent this issue and MS detection coupled to separation techniques along with NMR are becoming the most relevant tools in this aspect. Multiplatform metabolomics has been applied on different biological specimens either for targeted or non-targeted analysis. The multiplatform approach was well demonstrated in a study by Psychogios et al., while establishing human serum metabolome [17]. In order to achieve coverage of 4229 metabolites, six distinct analytical platforms were used, including high-resolution NMR, GC–MS, LC–MS (positive and negative ionization) and direct flow injection in MS.

After instrumental considerations another critical step in metabolomics approach is the biostatistics (univariate and multivariate data analysis) which reflects the differences between metabolomes and characterize specific phenotypic characteristics. However the correct biological interpretation of a specific metabolite difference in non-targeted approach depends on the reliability and suitability of the entire approach (from sample treatment to biomarker identification). Hence, the term validation comes in concern. The objective of any analytical measurement is to obtain consistent and reliable data avoiding false positive and negative discoveries. Validated analytical methods play a major role in achieving this goal. Results from method validation provide the picture of the quality, reliability and consistency of analytical results, which are fundamental for any good analytical practice. Method validation has received considerable attention since many years in the literature. For targeted metabolomics, there are several validation guidelines available, especially focused on instrumental aspects, while for non-targeted approach there are no guidelines available and they will only be promoted by the research community, because regulatory agencies are mainly focused on manufactured products. However, researchers are using several alternative ways to validate non-targeted approaches. The aim of this review paper is to discuss about the different validation criteria that are being used in analytical methods for non-targeted metabolomics and as well proposing validation steps to carry out this type of analysis.

2. Concept of validation

In the mid of 1970s in order to improve the quality of pharmaceuticals, the concept of validation was first proposed by the Food and Drug Administration (FDA) [18]. According to FDA validation is "*Establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes*" [19]. A properly designed system will provide a high degree of assurance that every step, process and change has been properly evaluated before its implementation. Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. It is the process of defining an analytical requirement and confirms that the method under consideration has performance capabilities consistent with what the application requires. Results from method validation can give an overview about the method quality. Quite often method validation evolves from method development and so the two activities are closely tied.

Method validation has received considerable attention in the literature and there are several guidelines available for analytical and bio-analytical aspect and they are as follows:

a. The United States FDA established two industrial guidelines. First one for the validation of analytical methods (this guidance provides recommendations to applicants on submitting analytical procedures, validation, data and samples to support the documentation of the identity, strength, quality, purity and potency of drug substances and drug products) and second one for the validation of bioanalytical methods (this guidance applies to bioanalytical methods used for human or non-human clinical, pharmacological, toxicological studies and preclinical studies-based on bioanalytical procedures such as chromatography, immunology and microbiology) [20,21].
b. ICH developed two guidelines for method validation that were later merged in one: Q2-R1. It discusses the considered characteristics (terminology and definitions) and methodology to be used during the validation of the analytical procedures [22].
c. International Union of Pure and Applied Chemistry published "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis". This guideline provides minimum recommendations on procedures that should be employed to ensure adequate validation of analytical methods [23].

The above-described guidelines are mainly focused on seven common parameters that should be considered during bio-analytical method validation in order to establish the method "fit-for-purpose". The definition for these seven parameters has been included in Table 1.

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Table 1
Parameters for analytical method validation.

Criteria	Definitions
Accuracy	The accuracy of an analytical procedure is expressed as the closeness of an agreement between the value which is accepted as a conventional true value or an accepted reference value and the original value found
Precision	The precision of an analytical procedure is defined as the closeness of agreement between a series of analytical measurements obtained from multiple sampling of the same homogeneous sample under the same developed conditions. Precision can be considered at two levels: repeatability and intermediate precision *Repeatability* Repeatability can be defined as the precision under the same operating conditions over a short interval of time, may be performed in the same day. Repeatability is also termed intra-assay precision *Intermediate precision* Intermediate precision can be defined as the precision performed within-laboratories variations: different days, different analysts, different equipment, etc. Repeatability is also termed inter-assay precision
Specificity	Specificity is expressed as the ability to assess indisputably the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.
Limit of detection (LOD)	The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value
Limit of quantitation (LOQ)	The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy
Linearity	The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample
Range	The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity

3. Non-targeted metabolomics and method validation

Metabolomics is growing rapidly with the major advances not only in analytical tools (mainly NMR and MS) but also in chemometrics. The goal of non-targeted approach is to determine all possible metabolites differentially expressed under the conditions that are compared in a study, with the potential to detect and identify candidate chemical biomarkers involved in a specific condition. The steps involved in non-targeted metabolomics includes: (i) selection of biological sample, (ii) sample pre-treatment, (iii) instrumental analysis of prepared samples using NMR or MS coupled with LC, GC or CE, (iv) data treatment and statistics (univariate and multivariate), and (v) identifying the important discriminating metabolites using commercial database or analytical tools (Fig. 1).

Non-invasive/minimally invasive sample types, principally urine and serum/plasma are often used in metabolomics, as these are the best accessible and it is supposed that the end product of any metabolic changes are seen in biological fluids. Non-targeted metabolomics has been successfully applied on serum/plasma and the authors were able not only to distinguish case and control but also to establish strong biomarkers in many diseases [24,25]. Using non-targeted approach several studies have found urinary markers, in subjects either treated with a drug or a specific dietary consumption or microorganism contaminated or in specific diseased concern [26–28]. Beside urinary and plasma metabolomics, tissue analysis is also important because not all changes affect in the same direction at tissue level or vice versa which has been seen in several studies and that is important mainly for mechanism research and in animal models [29–33]. Metabolic changes in specific disease state are first seen at the tissue site. Moreover, pairwise comparison of diseased and control tissue regions could provide strong markers. Hence, tissue metabolomics can take a big part in non-targeted research [34–36]. Non-targeted approach has also been applied on different other sample type such as saliva, bile extract, cell lines, microbial extract, food extract or even in environmental toxicology [37–43]. To understand a disease mechanism perfectly researchers have analyzed not only a single biological fluid but also a combination of urine, plasma and tissue to see the proper molecular mechanism [31,32,44–46].

An important number of papers have also been published on plant applying non-targeted approaches, including flower plant, herbal medicine, diseased plant, food industry, etc. [47–49], however analytical aspects are quite different and they are out of the focus of this review.

In addition to the selection of the proper type of sample or combination of them, another question appears, as no single analytical technique is suitable for the detection, identification and quantification of all the metabolites with varying physicochemical properties in non-targeted analyses. Thus multiplatform approach has also been applied choosing from NMR, LC, GC or CE and combination of LC × LC and GC × GC are also being used [36,50–55]. Sample pre-treatment is a pre-requisite for any analytical technique. Thus improved sample preparation techniques are as well in concern to get wider metabolite coverage along with easy preparation steps and minimal sample volume [56–58]. After sample treatment and instrumental consideration, data treatment comes in the next row and many research lines try to contribute to establishing or upgrading the data processing algorithms in order to get actual numbers [59–62].

The term validation describes the meaning by itself: “valid for a purpose”. Therefore a valid method needs first and for all a clear description of the purpose of which it is intended to be used. There are no guidelines for validating analytical part in non-targeted approach. The purpose of non-targeted metabolomics is to find statistically significant biomarkers through unbiased differential analysis of as many signals as possible coming from biological samples which will compare different situations being studied. That purpose will determine the validation parameters considering the facts that the accurate quantification is not an aim and standards are not included in the analysis. Comparisons will be valid as long as all the samples are studied under the same conditions under a precise method and the change in the signal should be related to the concentration of the components.

Prior to method validation, the key point is to develop a method including all necessary steps and with special attention on sample pre-treatment, that should be the minimum possible to make it compatible with the instrumental technique. Highly precise, broad metabolite coverage will prove the applicability of the method for non-targeted approach [36].

Subsequently, researchers are using some alternative approaches to validate the analytical methodology. The recent validation strategies are described as follows.

3.1. Quality control samples (QCs)

When using high-throughput techniques for non-targeted approach, some potential limitations arise which should be kept in mind while developing and applying such technology. Such limitations are the potential for drift in both chromatographic and MS performance, the repeatability and reproducibility of the method. These drawbacks should be recognized and controlled in order to eliminate bias due to a gradual change in the performance of the system. To monitor the performance of the instruments researchers have described to randomize the order of the samples and include

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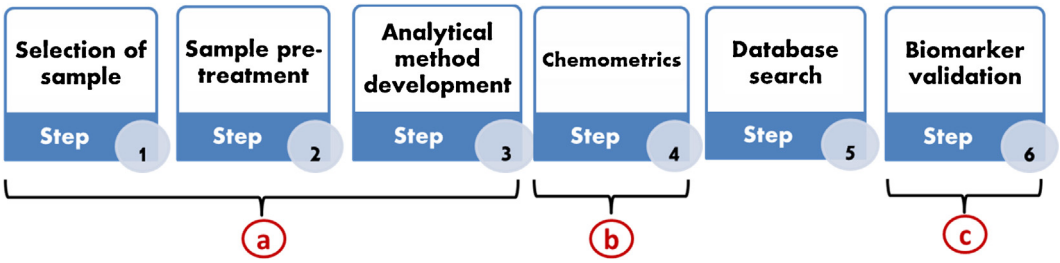

Fig. 1. Footsteps involved in non-targeted metabolomics approach. a, b, and c step has been marked in red where the validation needed to be performed in order to get effective and reliable results.

quality control samples (QCs) [63–65]. QCs are a pool of sample made from the biological test samples to be studied, or a representative bulk control sample which should be assessed against predefined criteria to enable acceptance or rejection of the analytical run. It is also strongly advisable to implement a number of injections at the beginning of the analytical run to stabilize the system and at the end and as well they should be repeatedly analyzed at regular intervals throughout the sequence run. This would subsequently explain whether the system is or not stable along the entire sequence run and will describe the quality and integrity of the analyzed data sets. FDA has proposed other useful criteria for bioanalytical methods validation to calculate the relative standard deviation (RSD) of the molecular features or possible compounds present in QCs [66]. For non-targeted metabolomics analysis by LC–MS values close to 30% RSD could be allowed (clearly highly variable ions, with RSD higher than 30%, would not be good candidates as biomarkers). Along with checking RSD in QCs, researchers are trying to validate the method by checking the linearity of diluted pooled QCs. Matrix effects and recovery were checked as well in the QCs [67,68]. Checking the linearity in diluted QCs helps to get clean data set based on the criteria that only those metabolites which pass a linear trend will go for further data analyses. However, there are some metabolites whose concentrations are very low and will not show a linear trend or sometimes too much diluted that will be under LOD. This can lead us to lose these metabolites. In addition, the dilution of the matrix makes that linearity not fully reliable, because ion suppression will be different to real samples. In any case, the use of QCs in non-targeted metabolomics analysis can be considered as equivalent to the use of standards in routine target analysis, more than validating a method. In a review by Dunn et al. the importance of QC samples in non-targeted MS based metabolomics has been well explained [69].

3.2. Spiked standards

Using the external standard method is very common in pharmaceutical industry in order to check the reliability of the methodology. Several researchers have followed this validation criterion in non-targeted analysis. Pereira et al. used this criteria somehow common with the quantitative method validation in nutritional plasma metabolomics by calculating the matrix effect and recovery after spiking six different xenobiotic standards [70]. However, for large batch analysis the method was validated in terms of the reproducibility of retention times and accurate masses in QCs. In another strategy, analysis of the analytical reproducibility within a run was performed by monitoring sample specific identified metabolites with different physico-chemical properties including known endogenous metabolites along with xenobiotics [71]. Nonetheless it needs to be stated here that although spiking some selected metabolites could give a good approach to the general behaviour of the method, however results cannot be assumed for all the components in the sample. The use of internal standards has also been applied to check instrumental reproducibility [36,72].

Conventional validation guidelines were as well followed in several strategies. Non-targeted metabolomics provides information of huge number of metabolites present in a specific sample type. It is not possible to use all of them to validate the method. However it would be good to target some of them from the non-targeted metabolites (considering different class/category, retention time/migration time, more abundant or less abundant, various physico-chemical properties) and validate the methodology. Though this validated method is not enough for validating a final biomarker for clinical use, which requires a target method with a proper, classical method validation, but results are more reliable compared to non-validated methods at least for further data processing. There are very few papers that used these criteria not only to check method reproducibility but to successfully validated and applied on bio-fluids or tissues [36,72–76].

3.3. Statistical model validation

In disease screening, non-targeted metabolomics results are ended-up with a prediction of class belonging, using multivariate statistical algorithm, usually first applying non-supervised principal component analysis (PCA). PCA provides summery of all the variables entering in the statistical analysis by finding correlation among the variables. Following correlation, it reduces the variables into a smaller number of principal components which is responsible for the possible variance in the observed variables. Finally PCA expressed the whole data sets in a global and qualitative visual pattern, highlighting similarities and differences between and within the sample [77,78]. In metabolomics, either targeted or non-targeted, the quality of the instrumental analysis is usually checked by the tight clustering of QCs in the PCA model. By the poor or good clustering of QCs, a preliminary idea of the data could be obtained. Before making a PCA model the QCs can be excluded and later they are predicted on the same model. The clustering of the QCs after prediction shows the repeatability of the instrumental performances as well it describes that the separations between the groups are not due to the instrumental variations but due to the sample itself [64]. However, an in depth discussion of statistical model validation is out of the scope of this paper as this review article is devoted to non-targeted metabolomics analytical method validation.

3.4. Other parameters

In addition to the criteria described above some researchers are using alternative ways to qualify the analytical method by examining the system performance checking the signal to noise ratio, plate number, retention factor, etc. either in pooled samples or by diluting the pooled samples [67,68,79]. Whereas, using these strategies only relative quantification of metabolites are possible.

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4. Recommended validation criteria for non-targeted approach

The goal of non-targeted metabolomics (which utilizes all the possible metabolites that are changing in specific conditions) includes the analysis of biological samples in an analytical platform and the generation of a predictive model from a collection of detected multiple compounds, which used to classify new samples/persons into specific groups (e.g. control vs. case) with optimal sensitivity and specificity. Finally the significant metabolites should be related to biological pathways in order to identify potential markers and their biological meaning. Thus a thoroughly controlled, validated analytical method should consider while developing a non-targeted approach and the validation should be based on the primary objective of the research. In Fig. 1, the general steps involved in non-targeted approach, the red circle with marked a, b and c steps should be monitored and validated in order to get a reproducible and sensitive result. In this review paper we are only focusing on the validation steps involved in stage "a": From sample selection to analytical method development.

(a) From sample selection to analytical method development:
   (i) *Selection of sample*: In any research study including non-targeted metabolomics to define the biological variation accurately and validly a set of study population is the first requirement, whether it is small or large needs to be monitored carefully. For non-targeted approach, the first step is to select samples minimizing the noncontrolled variations among them as much as possible (such as age, sex, body mass index and any medical conditions), then randomly select a minimally sufficient set of samples. It is necessary to check that groups have equal scores on the dependent variable at the start of the study and are randomly assigned, extraneous variables are controlled so no group is affected by them during the analysis and each group receiving identical treatment.
   (ii) *Sample treatment and analytical conditions*: The need of method development arises the necessity of method validation and proper sample treatment. One of the critical steps in non-targeted approach is the sample preparation with important consequences on the accuracy of the result. The choice of sample treatment depends on the analytical tools and the metabolites of interest. For non-targeted metabolomics, an easy sample pre-treatment should be focused to get broader analyte signals compatible along the techniques with appropriate sensitivity and reproducibility in terms of retention/migration time, abundance, pressure (LC) and current (CE) and minimizing carry over effects (LC). However, for biofluids the sample preparation is relatively easy where as for cell or tissues or extracts, special attentions are needed to cover wider metabolites. For small scale study the sample preparation can be done on the day of analysis, however this is not possible for large scale study. The sample preparation for large scale can be done in two ways – preparing all of them in one day and analysing in different days or dividing them in several batches and prepare every day before analysis. The design of the sample pre-treatment will completely be dependent on the objective of the study. Despite this, careful attention should be taken during the set up of analytical conditions. Consideration should be focused to get reliable and reproducible data acquisition along with broader signal range [80]. Several protocols are available on urine, plasma/serum and tissue metabolomics analysis [81–83].
   (iii) *Analytical method validation*: Non-targeted metabolomics helps to see the changes in all the possible metabolites identified and to interpret for the specific conditions. So a reliable analytical method could give us a primary reliable assumption from the identified distinctive metabolites. The fundamental of any analytical technique is to obtain dependable, consistent and accurate data. As previously described in Section 3, an analytical non-targeted method can be validated by following steps:

First, following the published guidelines for targeted metabolomics, some known compounds that have already been identified in the specific sample should be selected (considering different physic-chemical properties, intensities along the entire chromatogram). If the method is validated in terms of linearity, accuracy and precision for the selected compounds, it can be considered that the non-targeted approach will generate reliable and acceptable data sets for further evaluation. Moreover, sample treatment and method development steps should be optimized.

For routine work it is recommended the use of QCs, which are theoretically identical biological samples, with a metabolic and sample matrix composition similar to those of the biological samples under study. Two types of QCs are being used in metabolomics: (i) pooled QCs where small aliquots (same volume) of each biological sample to be studied are aliquoted and thoroughly mixed and (ii) commercially available biological samples. Pooled QCs are useful as they are very close to the composition of the biological sample to be studied. The signal variations of any metabolite could be reflected in the QCs. However, pooled QCs can only be applicable in small scale studies whereas for large batch analysis it is not possible to aliquote in order to make a pool for QCs. Therefore, a replacement for QCs is required. As the replacement QCs does not completely resemble the original population, there are some metabolites which will not be seen in there. During data processing RSDs for these metabolites will be unknown and they could be removed during data filtering, leading to a loss of metabolites which could be important biomarkers. QCs help to measure the repeatability within an analytical run and allow filtering data before analysis considering the drift in signal variations, retention time and accurate mass. QCs are being used in metabolomics for three reasons: The first is to 'condition' or equilibrate the analytical system with injections of matrix prior the main analytical run, to ensure that reproducible data are acquired. Secondly, it provides data to calculate analytical and technical precision. And thirdly the data from QCs are used for signal corrections within or between analytical run/batch analyses. An analytical method can be validated by diluting the pooled QCs and checking the linearity. By doing so, the metabolites which are showing a linear trend can be considered for further data processing. There are some limitations in this methodology that have been previously commented.

To get a snapshot about the instrumental repeatability before starting any data treatment the total signal from each chromatogram could be plotted against each sample (as per the sequence/run order) as shown in Fig. 2. The dispersion of QCs in the plot could reflect the instrumental repeatability.

Moreover, for validation it is recommended to use blanks during method development. Blanks should contain the solvent resembling the study samples and will be processed following the same procedure along with the real samples. The goal of including blanks is to detect and minimize the carryover during method development and consider the identified features in blanks as background, mainly for GC–MS.

Another indirect way of analytical method validation is to check the signal variation in all identified metabolites in terms of relative standard deviations in QCs and as well filtering data

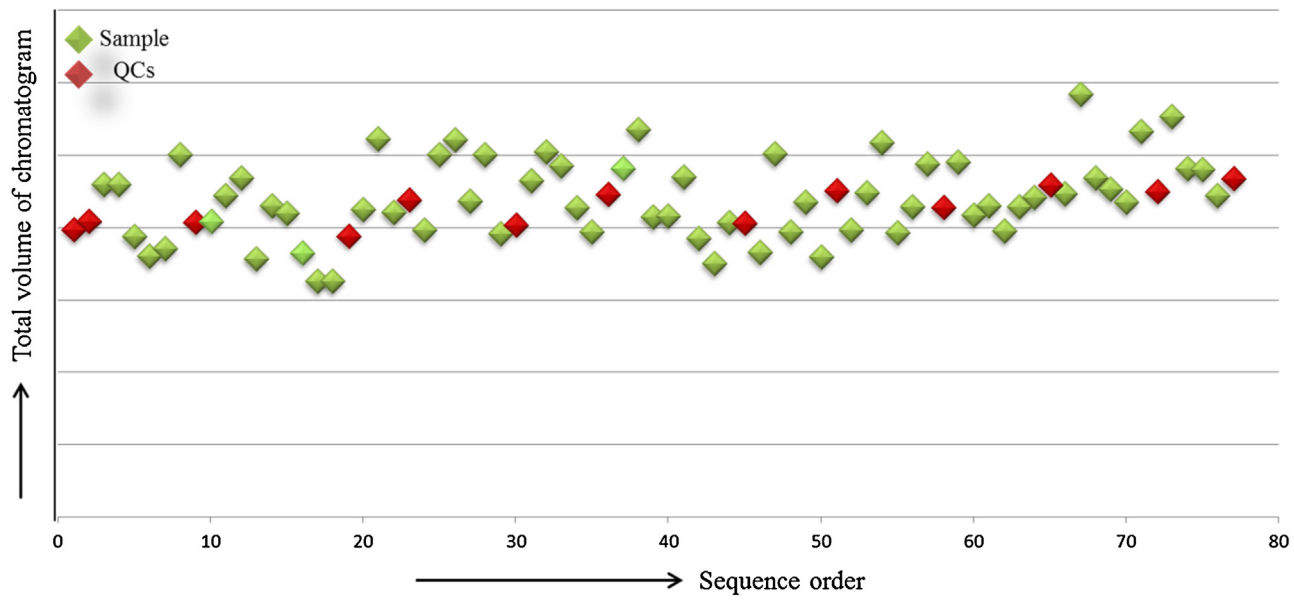

Fig. 2. Plotting the total volume of each chromatogram according to sequence order in order to observe the trend of QCs along a sequence run. This can provide a primary idea about the repeatability of the method.

by frequency present in any study group. Thus the repeatability and sensitivity of the method can be measured.

(b) *Chemometric models validation*: Non-targeted approach generates huge data sets. Using multivariate algorithms these data sets can often be presented as prediction of class separation and it is very important that this prediction is carried out in a relevant way. There are several strategies that are already being used in validating statistical models and they are out of the scope of this work. At the very least, to avoid the overfitting of a multivariate statistical model a cross validation scheme should be employed.

(c) *Biomarker validation*: At the starting point of metabolomics, non-targeted approach was thought to be a diagnostic tool, but scientists and clinicians derived the conclusion that the methodology was better suited for a discovery phase. Therefore, the end result of every non-targeted approach comes with one or more statistically significant compounds relating with specific pathways. That can give an insight into the progress or pathogenesis of specific conditions. However to find out one or several biomarkers from the identified differentiating significant compounds is far beyond and this involves several other validation steps. Steps involved in biomarker validation require target specific analytical methods, the corresponding method validation, reference materials and proficiency tests. The goal of this review article is to discuss about the validation parameters involved in non-targeted approach from analytical point of view but not for the biomarker validation. Already published guidelines are available for biomarker validation by FDA and others [84,85].

5. Conclusion

Non-targeted metabolomics may advance our discovery phase in diagnosis, treatment and prognosis of diseases. Using this approach, disease-related mechanisms may be uncovered, independent of the pathways involved and chemical diagnostic biomarkers in biological samples can be characterized. The analytical methods applied in metabolomics latest approaches need to be qualified in terms of validation. Thus there is a need for a well-defined validation criterion to perform a non-targeted study controlling false discoveries. Different efforts have been made by some research groups in order to define the validation parameters with some accepted ranges after following established methodologies, though up to now validation strategies for non-targeted approach are not well defined. Once the method has been optimized for the broadest physicochemically different types of compounds, the clues for validation of the analytical method in non-targeted metabolomics are obtaining objective evidences about the number of possible compounds following defined criteria of precision and linearity by spiking or by dilution of the QCs. Metabolomics method validation should now be considered as an open discussion among the researchers.

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Resumen

DESARROLLO Y VALIDACIÓN DE UN MÉTODO DE HUELLA METABÓLICA CON CE-MS: APLICACIÓN A UN ESTUDIO DE DAÑO PULMONAR INDUCIDO POR VENTILACIÓN MECÁNICA

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Ante la necesidad de métodos de cribado para detección de biomarcadores fiables y rápidos, se ha desarrollado un método de huella metabólica basado en CE-TOF-MS (electroforesis capilar con detección de espectrometría de masas con analizador de tiempo de vuelo) y se ha aplicado a muestras de suero de ratón. Se ha utilizado un pretratamiento de las muestras basado en la ultrafiltración. Se ha validado el método con el fin de evaluar su capacidad y fiabilidad, para el análisis de carnitina, colina, ornitina, alanina, acetilcarnitina, betaina y citrulina, cubriendo así la totalidad del electroferograma obtenido para un pool de suero de rata. La linealidad obtenida para todos los metabolitos fue >0.99 con una recuperación y precisión adecuada. Se ha confirmado la presencia de aproximadamente 34 compuestos. El método se aplicó satisfactoriamente a muestras de suero de ratas en un estudio real de daño pulmonar agudo (ALI), obteniendo un total de 1163 posibles compuestos. Se realizó una estadística univariante y multivariantes y se encontraron 18 compuestos con diferencias entre los dos grupos estudiados estadísticamente significativas, de los que 5 fueron confirmados. Se conocía que las enzimas arginasa y óxido nítrico sintasa estaban implicadas en otras enfermedades pulmonares, conduciendo a un aumento de dimetil arginina asimétrica (ADMA) y ornitina y una disminución de arginina; en este trabajo se han confirmado también estos hallazgos en consonancia con la literatura publicada. Por último, resaltar que este estudio de huella metabólica ofrece la posibilidad de identificar biomarcadores que podrían analizarse regularmente como parte de un chequeo rutinario. En este entido, además podría ser posible la prevención de la enfermedad ALI en pacientes atendidos en Unidades de Cuidados Intensivos.

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ORIGINAL PAPER

Method development and validation for rat serum fingerprinting with CE–MS: application to ventilator-induced-lung-injury study

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Abstract In the search for a noninvasive and reliable rapid screening method to detect biomarkers, a metabolomics fingerprinting approach was developed and applied to rat serum samples using capillary electrophoresis coupled to an electrospray ionization-time of flight-mass spectrometer (CE–TOF-MS). An ultrafiltration method was used for sample pretreatment. To evaluate performance the method was validated with carnitine, choline, ornithine, alanine, acetylcarnitine, betaine, and citrulline, covering the entire electropherogram of pool of rat serum. The linearity for all metabolites was >0.99, with good recovery and precision. Approximately 34 compounds were also confirmed in the pool of rat serum. The method was successfully applied to real serum samples from rats with ventilator-induced lung injury, an experimental rat model for acute lung injury (ALI), giving a total of 1163 molecular features. By use of univariate and multivariate statistics 18 significant compounds were found, of which five were confirmed. The involvement of arginase and nitric oxide synthase has been proved for other lung diseases, meaning the increase of asymmetric dimethyl arginine (ADMA) and ornithine and the decrease of arginine found were in accordance with published literature. Ultimately this fingerprinting approach offers the possibility of identifying biomarkers that could be regularly screened for as part of routine disease control. In this way it might be possible to prevent the development of ALI in patients in critical care units.

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Introduction

Researchers are currently focusing on the metabolome to understand the control and regulation of complex mechanisms in the human body. It is well known that concentrations of some metabolites change in response to specific diseases. Knowledge of concentration changes of these metabolites may be useful to detect the onset of disease in patients before the observation of symptoms. The best ways to discover and validate biomarkers, and which instrumental methods to use, are current challenges in this field. To meet this challenge, several analytical and chemometric techniques are being used to detect as many components as possible in different biological fluids. The methodology must be robust enough to deal with the typical biological variation in physicochemical properties of samples, and must be performed in the same manner for all analyses. It must also be economical in terms of time, effort, and expense.

Capillary electrophoresis (CE) coupled to an electrospray ionization-time of flight-mass spectrometer (ESI-TOF-MS) is a particularly attractive, although currently uncommon, metabolomics technique for detection of biomarkers. Its application for discovery of biomarkers in urine, serum, cell lines, and cerebrospinal fluid has already been reported [1–6]. CE provides the capacity to separate complex mixtures with high resolution and minimum sample treatment. However, the routine, automated use of CE–MS is not without challenges.

Serum is an integrated bio-fluid with the advantage of reflecting both localized and systemic changes. It is also useful because of its ease of collection and minimally

invasive nature. Serum has been used extensively in multiple studies, so the protein, lipid, and metabolite composition of this matrix is relatively well documented [7, 8]. There are several challenges in serum analysis, including the need to remove its high protein content: to minimize the matrix effect for global metabolomics fingerprinting, it is necessary to perform deproteinization on the serum before analysis. Numerous studies have described the sample preparation procedure for metabolite profiling in serum using CE–MS, mainly with solid-phase extraction (SPE) or liquid–liquid extraction (LLE) and filtration [3, 9, 10]. It is well-documented that SPE and LLE are very time-consuming sample-preparation steps and can cause the loss of some metabolite information. To minimize the preparation time a one-step ultrafiltration method has been tried, initially using ten dilutions [11]; however, this can reduce sensitivity to less abundant metabolites. For this study we used the ultrafiltration method with a nominal molecular weight cutoff of 30 kDa, using the lowest number of dilutions possible, and validated it for metabolic fingerprinting of serum for the first time.

It has been recognized that some mechanical ventilation strategies, characterized by the use of high tidal volume (VT) and low positive end-expiratory pressure (PEEP), may induce lung injury in both normal [12] and pre-injured [13] lungs, a condition termed ventilator-induced lung injury (VILI). In patients with acute respiratory distress syndrome (ARDS) or acute lung injury (ALI), the use of VT of 12 $mL kg^{-1}$ rather than 9 $mL kg^{-1}$ can worsen outcomes [14], and mechanically ventilated patients receiving higher VT are at increased risk of developing ARDS compared with those receiving lower VT [15]. Currently the diagnosis of ALI is based on the presence of non-specific criteria, for example physiological and radiological changes, which are neither specific nor sensitive [16]. There is therefore a need for biomarkers of ALI.

In a pioneering study of an ALI model induced by intratracheal administration of TNF-alfa and IL-beta in mice, it was shown that ^{1}H NMR spectroscopy is useful for detecting and quantifying inflammation-induced lung injury [17]. More recently, ^{1}H NMR spectroscopy was used to identify the metabolome of plasma, revealing the metabolic pathways affected in patients with ALI [18]. Differences in plasma levels of glutathione, adenosine, phosphatidylserine, and sphingomyelin were found in patients with ALI compared with healthy controls. Moreover, these abnormalities had a physiological correlation: levels of myoinositol and total glutathione were correlated negatively and positively, respectively, with acute physiology scores. In some studies oxidative lipidomics has been used for analysis of rat lung by liquid chromatography–mass spectrometry (LC–MS) to characterize cardiolipin and phosphatidylserine in hyperoxic acute lung injury and γ-radiation-induced lung injury [19, 20]. Much of the metabolomics work examining respiratory diseases to date has focused on development and validation of analytical approaches to provide robust and reproducible data from a range of different bio-fluids using NMR and LC–MS. To our knowledge, no metabolomics fingerprinting study has been performed on ALI/VILI serum by use of CE–TOF-MS.

This study was designed to develop and validate a new method for serum fingerprinting using CE–TOF-MS. We developed a very sensitive and robust method for serum profiling and used this global profiling method for the identification of metabolic biomarkers in an experimental model of VILI, a form of ALI not previously studied using this approach. Several compounds were found discriminating VILI from the normal group, and these biomarkers were interpreted to determine the metabolic pathways affected in VILI.

Materials and methods

Reagents

Carnitine, choline chloride, ornithine, alanine, acetylcarnitine hydrochloride, betaine, citrulline, and methionine sulfone (IS) were purchased from Sigma (Steinheim, Germany); HPLC-grade methanol and acetonitrile were from Sigma–Aldrich (Steinheim, Germany); ammonium formate and formic acid for MS were purchased from Sigma–Aldrich (St Louis, USA); and ammonia 30 % and sodium hydroxide from Panreac (Barcelona, Spain). Water purified by reverse osmosis was obtained from a Milli-Q Plus 185 (Millipore, Bedford, USA) was used in preparation of all buffers, standard solutions, and dilutions. For the standard reference solution, purine and HP921 were obtained from Agilent Technologies (USA). For profiling rat serum spermidine, thiamine hydrochloride, lysine, histidine, $N^{G}N^{G}$-dimethylarginine (asymmetric dimethyl arginine, ADMA), aminobenzamide, creatine, *N*2-acetyl-L-lysine, 2-aminobutyric acid, isoleucine, leucine, pipecolic acid, asparagine, glutamine, glutamic acid, tryptophan, phenylalanine, *N*,*N*-dimethylglycine, tyrosine, and aspartic acid, were purchased from Sigma–Aldrich (Steinheim, Germany); and proline, 1-methylhistidine, 3-hydroxyproline, serine, threonine, methionine and valine were purchased from Fluka Chemie (Buchs, Switzerland). All chemicals were analytical or reagent grade.

Apparatus and conditions

The experiments were performed by use of an Agilent 7100 capillary electrophoresis system coupled to an Agilent Technologies (Wilmington, USA) 6224 Accurate-Mass Time of

Flight mass spectrometer system. The coupling was equipped with an electrospray source. A 1200 series ISO Pump from Agilent Technologies was used to supply sheath liquid. The capillary for separation, of diameter 50 μm and length 96 cm, was from Agilent Technologies (Germany). The new capillary was conditioned for 30 min with 1 mol L^{-1} sodium hydroxide, 30 min with 2 mol L^{-1} aqueous ammonia and 30 min with background electrolyte (BGE) (0.8 mol L^{-1} formic acid in 10 % methanol). Before each analysis the capillary was flushed with BGE for 5 min at 950 mbar pressure. Sample injections were done for 35 s at 50 mbar pressure. After injection of each sample the BGE was injected for 10 s at 100 mbar pressure, to improve repeatability. Separation conditions were 25 mbar pressure and 30 kV voltage; the current observed under these conditions was 25 μA. The instrument automatically replaced the BGE before each injection.

The MS was operated in positive polarity, and other conditions were: drying gas 10 L min^{-1}, nebulizer 10 psi, voltage 3500 V, fragmentor 100 V, gas temperature 200 °C, and skimmer 65 V.

The sheath liquid used for detection in the positive mode consisted of 50 % methanol, 50 % water, 4 μL formic acid, and reference standards (10 μL of 5 mmol L^{-1} purine and 30 μL of 2.5 mmol L^{-1} HP922), at a flow rate of 0.6 mL min^{-1} (1:100 split). BGE and sheath liquid were freshly prepared and degassed by sonication for 5 min before use, to ensure proper and reproducible ionization.

Data acquisition in CE was performed with a DE-CE ChemStation B.04.02; in MS it was performed with a MassHunter Work-Station B.05.00 (Agilent Technologies).

Working solutions and standards

Individual 1000-ppm stock solutions of carnitine, choline chloride, ornithine, alanine, acetylcarnitine, betaine, and citrulline were prepared in purified water and stored at −20 °C. From those solutions an intermediate 10 ppm solution of each amino acid was prepared and stored at 4 °C during the working week, and these solutions were appropriately diluted on the day of the analysis. The structures and molecular weights (MW) of these metabolites are presented in Fig. 1.

Validation study

The method for global profiling of rat serum was validated for linearity, accuracy, instrumental precision, method precision (both with standards and samples), limit of quantification (LOQ), and limit of detection (LOD).

The linearity of the response for standards was studied by triplicate assay of at least five concentrations, covering all expected values, ranging from 25 % to 200 % of mean values found in a preliminary assay. Individual ranges are listed in Table 1.

Recovery was estimated by comparing values obtained in triplicate from spiked samples prepared in the linear range, taking into account endogenous concentrations which had been previously measured in the samples.

Instrumental precision was tested by checking for a consistent response to a given analyte in the mid-range of the calibration curve, evaluated by multiple injection (n=10) of homogeneous standard solution.

The intra-day (n=7) precision of the method was tested by injecting individual preparations of standards and samples in the mid-range of the calibration curve. Inter-day (n=14) precision was tested in the same way but on two different days, with the buffer and all the reagents freshly prepared.

The LOQ and LOD were calculated as the amounts for which the signal-to-noise ratios were 3 and 10, respectively, in accordance with the IUPAC method [21].

Serum profiling

A series of standards, a sample of combined serum, and the sample of combined serum spiked with the standards were

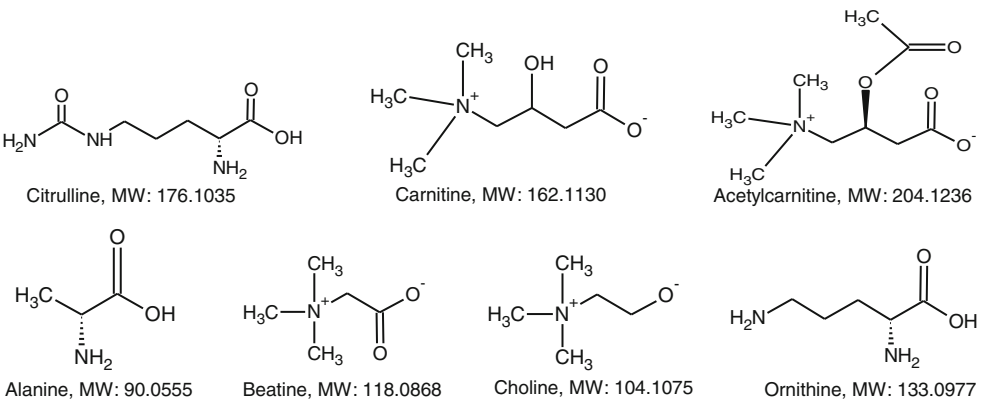

Fig. 1 Structures of compounds selected for method validation in CE–TOF-MS

Table 1 Validation data for selected metabolites in rat serum with the optimized CE–ESI-TOF-MS method

	Carnitine	Choline	Ornithine	Alanine	Acetylcarnitine	Betaine	Citrulline
Linearity							
Slope	$24.9\pm1.2\times10^4$	$25.6\pm1.2\times10^4$	$148.4\pm4.7\times10^3$	$147.1\pm9.3\times10^3$	$27.8\pm2.0\times10^4$	$2.6\pm1.5\times10^5$	$15.1\pm1.0\times10^4$
Intercept	$1.49\pm1.51\times10^5$	$0.42\pm2.24\times10^5$	$1.48\pm1.45\times10^5$	$2.7\pm6.83\times10^5$	$3.11\pm7.45\times10^4$	$2.97\pm5.72\times10^5$	$-0.02\pm2.0\times10^5$
r	0.997	0.997	0.993	0.995	0.993	0.995	0.993
Range (ppm)	2.5–20	3.75–30	6–50	15–120	0.75–6	7.5–60	3.75–30
Recovery (%)	107.5	114.9	92.4	106	111.5	105.4	103.6
RSD (%)	3.5	5.1	4.6	4.6	4.4	3.4	4.7
Instrumental precision							
(n=10), RSD (%)	2.6	2.7	3.3	4.2	1.6	4.1	2.5
Method Precision with standards							
Intra-day (n=7), RSD (%)	5.6	3.2	4.3	5.1	4.3	4.6	3
Inter-day (n=14), RSD (%)	6.2	5.7	7.3	9.4	7.3	6.5	4.8
Method Precision with samples							
Intra-day (n=7), RSD (%)	8.8	5.2	10.9	9.9	3.6	11	7.5
Inter-day (n=14), RSD (%)	9.6	6.1	10.9	10.9	8.8	11.4	8
LOD (ppm)	0.06	0.075	0.19	0.24	0.03	0.50	0.10
LOQ (ppm)	0.21	0.24	0.62	0.80	0.09	1.70	0.33

injected for identification of metabolites through the profile. The standards are listed in Table 2.

Rat serum samples

Animal preparation and experimental procedure

Experiments followed the Principles of Laboratory Animal Care (EU 609/86 CEE, Real Decreto 1201/05 BOE 252, Spain) and the research protocol was approved by the Getafe Hospital, Madrid, Spain. Male Sprague–Dawley rats (Harlan Iberica, Spain) weighing 342.3±5.4 g (mean ± SD) were anesthetized by use of intraperitoneal ketamine (90 $mg\,kg^{-1}$) and diazepam (5 $mg\,kg^{-1}$).

Two ventilation strategies were used: low-VT ventilation (control: VT 9 $mL\,kg^{-1}$, PEEP 5 cm H_2O, n=14) and high-VT ventilation (VILI: VT 25 $mL\,kg^{-1}$, zero PEEP, n=10). For both groups, respiratory rate was 70 bpm, inspiratory time 0.3 s, expiratory time 0.56 s, and FiO_2 0.45. Control and VILI rats were ventilated for an equilibration period of 10 min using the low-VT ventilation conditions, and then assigned to one of the two ventilation groups.

At the end of the experiment, serum was collected and frozen at −80 °C for metabolomics studies.

Sample-treatment procedure

The samples were defrosted on the day of analysis, homogenized by vortex mixing for 1 min, and treated using two different strategies: SPE and ultrafiltration.

SPE procedure

To remove phospholipids and proteins, 225 μL serum and 10 μL methionine sulfone, internal standard (IS), were vortex-mixed with 300 μL methanol–ammonium formate (1:1) and then passed through a Supelco HybridSPE-Phospholipid Ultra cartridge (Supelco, Sigma–Aldrich). The extracts were then dried at 35 °C by use of a SpeedVac and dissolved in 100 μL 0.1 $mol\,L^{-1}$ formic acid. After centrifuging (16000×g, 4 °C, 20 min) the supernatant was transferred directly to a vial (Chromacol, UK) ready for injection.

Ultrafiltration procedure (30 kDa protein cutoff)

Each serum sample (90 μL) was mixed with 10 μL IS and 100 μL 0.2 $mol\,L^{-1}$ formic acid containing 5 % acetonitrile. Each sample was vortex mixed for approximately 1 min, and then transferred to a Centrifree ultracentrifugation device (Millipore Ireland, Eire) for deprotenization by centrifugation (2000×g, 70 min, 4 °C). The filtrate was then transferred directly to a vial for analysis.

Rat samples from control and VILI were prepared by following the filtration procedure.

Quality-control (QC) samples

QC samples were prepared by combining equal volumes of serum from each of the 24 samples. Five samples were independently prepared from this pool of plasma sample,

Table 2 List of compounds identified in combined rat serum sample

Name	Molecular mass	Migration time (min)
Spermidine	145.1579	6.17
Thiamine	264.1045	8.86
Choline	103.0997	9.07
Ornithine	132.0899	9.37
Lysine	146.1055	9.45
Arginine	174.1117	9.79
Histidine	155.0695	9.95
1-Methylhistidine	169.0851	10.31
$N^G N^G$-Dimethylarginine	202.1430	10.69
Carnitine	161.1052	11.24
Aminobenzamide	136.0637	11.86
Creatine	131.0695	12.01
Acetylcarnitine	203.1158	12.10
Alanine	89.0477	12.36
*N*2-Acetyllysine	188.1161	13.06
2-Aminobutyric acid	103.0633	13.15
Valine	117.0790	13.64
Serine	105.0426	13.77
Isoleucine/leucine	131.0946	13.87
Pipecolic acid	129.0790	14.10
Asparagine	132.0535	14.51
Threonine	119.0582	14.71
Methionine	149.0511	14.72
Glutamine	146.0691	14.97
Glutamic acid	147.0532	15.10
Tryptophan	204.0899	15.12
Proline	115.0633	15.24
Citrulline	175.0957	15.28
Phenylalanine	165.0790	15.35
N,*N*-Dimethylglycine	103.0633	15.52
Tyrosine	181.0739	15.61
Betaine	117.0790	16.01
Aspartic acid	133.0375	16.10
3-Hydroxyproline	131.0582	18.56

by following the same procedure as for the other samples. QC samples were analyzed throughout the run to provide a measure of the system's stability and performance [22] and of the reproducibility of the sample-treatment procedure.

Data treatment for VILI and control samples

Raw data collected by the analytical instruments was cleaned of background noise and unrelated ions by use of the Molecular Feature Extraction (MFE) tool in MassHunter Qualitative Analysis Software (B.05.00, Agilent Technologies). The MFE algorithm uses measurement accuracy to group ions by charge state envelope, isotopic distribution, and/or the presence of adducts and dimers. The MFE then creates a list of all possible components, as defined by the full TOF mass spectral data. Each compound is described by mass, retention time, and abundance. For data extraction by MFE the limit for the background noise was set to 300 counts; then, to find co-eluting adducts of the same feature the following adduct settings were applied: +H, +Na, $+NH_4$ in positive ionization, and + HCOO for negative ionization. Dehydration neutral losses were allowed.

Primary data treatment (alignment and filtering) was performed with the mass profiler professional B.05.00 (Agilent) software. Alignment was performed first on all samples with QCs, and later only for the samples under investigation. Corrections applied for the alignment were 6.8 % retention time correction, and±20 ppm mass correction. Differences between control and VILI serum were evaluated for individual metabolites using a *t*-test or a jack-knifed confidence interval. The jack-knifed algorithm was used for a partial least-squares discriminant (PLS-DA) model. Before any statistical calculations, logarithmic transformation of data was performed to approximate a normal distribution. Univariate statistical analysis (*t*-tests) were performed using MS excel (Microsoft), with unpaired unequal variance assumed. Multivariate statistical analysis (jack-knifed confidence intervals) and other multivariate calculations and plotting were performed using SIMCA-P+12.0 (Umetrics). Accurate masses of features representing significant differences were searched against METLIN; KEGG, LIPID MAPS, and HMDB databases.

Validation of the PLS-DA model

To avoid the risk of overfitting for a PLS-DA model used for selection of statistically significant metabolites according to jack-knifed confidence intervals, the model was validated by use of a cross-validation tool [23], using a 1/3 out approach which has been described elsewhere [24]. In brief, the dataset was divided into three parts, 1/3 of samples were excluded, and a model was built using the remaining 2/3 of samples. Excluded samples were then predicted by this new model, and the procedure was repeated until all samples had been predicted at least once. Each time the percentage of correctly classified samples was calculated.

Compound identification

Identification of compounds found to be significant in class separation was confirmed by injecting standards, serum, and spiked serum. Experiments were repeated under chromatographic conditions identical with those in the primary analysis. Comparison of migration times for serum samples, standards, and standard spiked serum samples was used for the final confirmation under identical conditions.

Results and discussion

Method development for sample treatment

The objective of this study was to develop a robust, sensitive, and easily applied procedure for global fingerprinting of serum samples using CE–TOF-MS and to validate it as a method to characterize rat serum metabolic profiles, so it could be used to study a rat model of VILI and control samples.

Direct injection of the sample after precipitation of proteins with organic solvents led to unstable current and frequent breakdowns. To avoid clogging of capillaries by lipids and proteins, and changes because of absorption on the capillary wall, this study tested an SPE method, mainly focused on eliminating phospholipids, whose matrix effect on ionization suppression is well documented [25, 26]. In a second study the serum samples were minimally treated, using just ultrafiltration through a Millipore filter (30-kDa protein cutoff). The results from these two procedures are compared in Fig. 2. Better sensitivity, lower variability, and shorter and easier sample treatment were obtained with the ultrafiltration method. The total number of molecular features extracted was higher with ultrafiltration (1203.66± 3.28, mean ± % RSD) than with the SPE method (659.00± 9.49, mean ± % RSD). Moreover, the total area of the electropherogram was higher for ultrafiltration (300.1± 0.51×10^6, mean ± % RSD) than for SPE ($79.73\pm6.30\times 10^6$, mean ± % RSD). Because of the higher sensitivity, number of molecular features, and total area of electropherogram, ultrafiltration was chosen for further validation and application.

Sample treatment and CE conditions optimized were: initial serum volume, injection time, dilution conditions, and centrifugation time. Initial sample volume was set as low as possible while working with small animal models. The tested volume ranged from 50 to 225 μL; 100 μL was the volume chosen because it provided the appropriate volume for the CE–MS vial after processing. Several dilution solutions were tested: water, 0.2 $molL^{-1}$ formic acid, and 0.2 $molL^{-1}$ formic acid containing 5 % acetonitrile. Use of water as a dilution solution provided only the free metabolites present in serum; those absorbed in proteins were lost. Use of 0.2 $molL^{-1}$ formic acid provided more molecular features than water, and addition of 5 % acetonitrile further increased the number of features in the serum profile by contributing to liberation of absorbed compounds. Therefore 0.2 $molL^{-1}$ formic acid with 5 % acetonitrile was selected as dilution buffer. To optimize dilution, 1:1, 1:2, and 1:3 dilution were tested. The 1:1 ratio was selected, because at this dilution the best sensitivity and acceptable repeatability in migration time were obtained. Centrifugation for 50, 60, 70, or 80 min at $2000\times g$ was tested and 70 min was selected. Injection times of 17 s, 35 s, and 50 s were tested, and the best resolution, sensitivity, and number of molecular features were obtained by use of an injection time of 35 s.

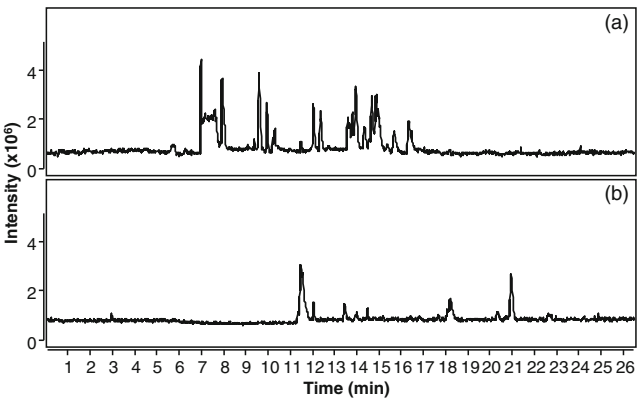

Fig. 2 Comparison of total ion electropherograms from (**a**) ultrafiltration and (**b**) SPE procedures

Validation

Complete validation of migration time over the entire electropherogram was performed with seven different metabolites: choline, carnitine, acetylcarnitine, ornithine, alanine, betaine, and citrulline. A summary of the validation data for these metabolites, including both standards and samples, is shown in Table 1 and will be briefly discussed. The standards fit the linear model ($r>0.99$) for all metabolites and no bias was found, because the confidence limits of the intercept included the zero value.

Recoveries ranged from 92.4 % to 111.5 % and, taking into account their relative standard deviations (RSDs), did not differ statistically from 100 ($p\leq0.05$).

Instrumental precision ranged from 1.6 % to 4.2 % for standards ($n=10$). Intra-assay precision ranged from 3.0 % to 5.6 % ($n=7$) and inter-assay precision from 4.8 % to 9.4 % ($n=14$). When seven samples prepared from the same sample of combined serum were treated and run in the same assay, the daily RSDs ranged from 3.6 % to 11.0 % and from 6.1 % to 11.4 % on different days.

The theoretical LOD calculated by use of the IUPAC method [21] for these metabolites ranged from 0.03–0.24 ppm, and the LOQ was in the range 0.09–1.70 ppm.

Serum profiling with standards

A standard mixture was analyzed with serum for characterization. The presence of metabolites was checked by adding standards to the combined serum sample and comparison with the blank serum profile. Thirty-four compounds were found in blank rat serum. The list of profiled compounds is summarized in Table 2 and includes organic amines, amino

acids, amino acid derivatives, and carnitines. These findings prove the usefulness of CE–MS, showing how it complements other techniques when investigating altered metabolic pathways.

Application on VILI serum samples

The final method of global serum profiling was applied to the VILI model. Results are summarized in Table 3.

Quality control of the method

For quality checking, electropherograms from serum samples and QCs were aligned, revealing 1231 features in total. A PLS-DA model was built for the two groups, taking into account all variables generated from the mass spectra. The robustness of the analytical procedure was tested by prediction of the QC samples in the model, and proved by the clustering of the QC samples in the center of the plot (Fig. 3a). The quality of the model built for two components was acceptable, with variance explained R^2=0.98 and variance predicted Q^2=0.286.

Sample classification and statistical analysis

To perform sample classification, electropherograms from the 24 investigated serum samples were aligned, giving primary datasets with 1163 features. Data filtering was performed by choosing the data present in 100 % of the samples in any group; 118 features remained after filtering. To distinguish between the groups, PCA (Fig. 3b) and PLS-DA (Fig. 3c) models were built for these 118 features. The quality of the model built for two components was very good regarding variance explained (R^2=0.913) and variance predicted (Q^2=0.374). The PLS-DA model was validated as described in the "Materials and methods" section: 1/3 of samples (eight samples after randomization of all) being excluded and predicted until all samples had been predicted at least once. An average misclassification table, summarizing the misclassification tables generated by each prediction of the leaving 1/3 out model, is presented in Table 3, and examples of the model in 2D and 3D are shown in Fig. 4a and b, respectively. All the controls were 100 % predicted with appropriate classification, and from VILI only one out of 10 samples was misclassified.

Table 3 Average misclassification table for validation of the PLS-DA model after applying the leaving 1/3 out method

Group	Members	Predicted	Correct prediction (%)
Control	14	14	100
VILI	10	9	90

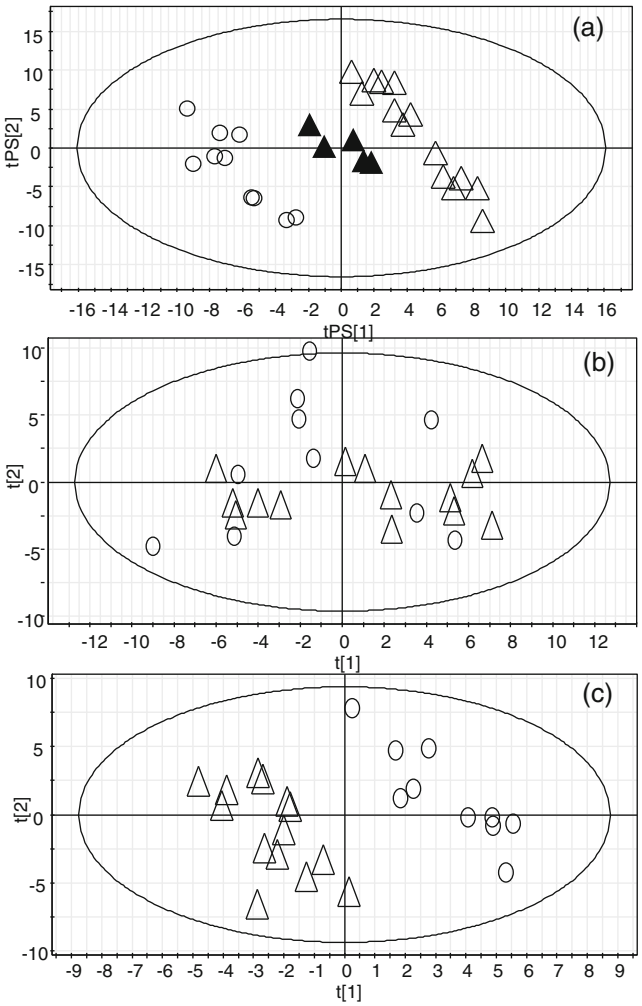

Fig. 3 (**a**) Scores plot for a PLS-DA model built with the whole data set and with prediction for QCs. Quality data for the model: explained variance: R^2=0.98, predicted variance: Q^2=0.286. (**b**) PCA score plot. Quality data for the model: R^2=0.535, Q^2=0.0161. (**c**) PLS-DA score plot. Quality data for the model: R^2=0.913, Q^2=0.374. *Empty triangles*, control; *empty circles*, VILI; *filled triangles*, quality control

By use of univariate and multivariate statistics, 12 statistically significant metabolites ($p \leq 0.05$) were identified. Selection of statistically significant metabolites by use of multivariate statistical tools (jack-knifed confidence interval) was performed by use of the PLS-DA model (Fig. 2c). Eighteen metabolites were found to be significant by use of multivariate statistics; of these, 12 were found to be significant by use of the *t*-test.

Compound identification

Identification of statistically significant metabolites was performed as described in the "Materials and methods" section. Of the 18 statistically significant compounds, five were confirmed by use of standards. The calculated mass error, coefficient of variance according to quality control, *p*-

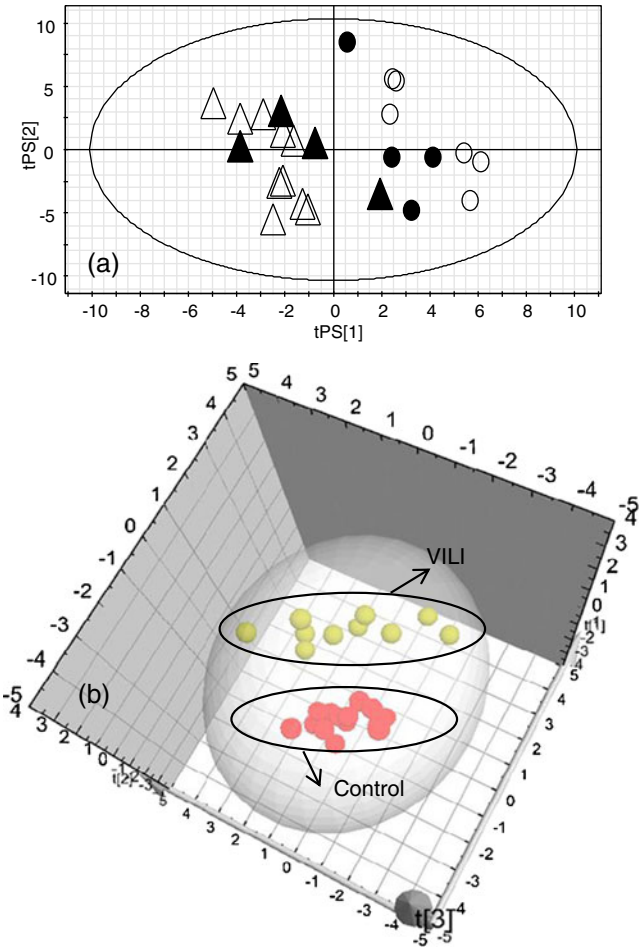

Fig. 4 (**a**) Validation of the PLS-DA model (2D), an example of the leaving 1/3 out model, classified according to their group after prediction. *Empty triangles*, control; *filled triangles*, predicted control; *empty circles*, VILI; *filled circles*, predicted VILI. (**b**) A PLS-DA model in 3D, clearly showing separation of the groups

value, and percentage change among the groups are presented in Table 4, with their migration times.

Biological interpretation of metabolic changes in VILI

Substantial efforts have been made to find characteristically altered molecules in ALI, in the hope of developing an efficient approach for early diagnosis of this disease [27]. Although several plasma proteins have been proposed as diagnostic biomarkers [28], they lack sufficient sensitivity and specificity. In addition, specific biomarkers of mechanical VILI are unknown. In this study, we have tried to show that metabolomics analysis reveals distinctive metabolites that change markedly in serum of rats with VILI compared with that of control rats.

Arginine depletion is linked to ALI, and arginine supplementation reduces inflammation [29]. Arginase converts arginine to urea and ornithine. Ornithine is converted by ornithine aminotransferase to proline, which is an essential component of hydroxyproline and collagen. Ornithine decarboxylase also metabolizes ornithine to putrescine, which forms the polyamines spermine and spermidine that increase cell proliferation [30]. Increased arginase activity may contribute to lung airway remodeling by increasing collagen deposition and cell proliferation [31]. This study is consistent with previous findings. We have seen a reduced level of arginine and an increased level of ornithine, which establishes an increased level of arginase activity in the VILI model.

On the other hand, ADMA is an analog of arginine and an endogenous inhibitor of all three isoforms of nitric oxide synthase (NOS) [32]. ADMA is synthesized by hydrolysis of proteins with methylated arginine residues [33]. In the presence of increased ADMA, NOS can become uncoupled, producing superoxide (O_2^-) [34], and, because ADMA is a competitive inhibitor of NOS, reduction of NO in the presence of O_2^- can result in peroxynitrite production [35]. Thus, in the presence of ADMA, NOS can produce reactive oxygen species and reactive nitrogen species in addition to NO, which may contribute to inflammation. ADMA is involved in the pathophysiology of human diseases such as endothelial dysfunction and cardiovascular risk factor [36, 37]. In recent studies, it has been found that increased ADMA also contributes to polycystic ovary syndrome and pseudoexfoliation syndrome [38, 39]. Bulao has reported that increased intracellular ADMA levels may be particularly relevant to lung disease, because the lung has one of the highest baseline concentrations of intracellular ADMA [40]. Altered metabolism of ADMA has also been found in asthma, pulmonary hypertension, allergic inflammation, and burn and inhalation injury [41–44]. Our study also investigated the effects of ADMA levels on ALI, because we found an increased level of ADMA in the VILI model.

Table 4 Identified significant metabolites discriminating control and VILI rats' serum

Name	Mass (Da)	MT (min)	Mass error (ppm)	CV % for QCs (%)	*p*-Value	Change (%)
Choline	103.1001	9.98	3.8	20.5	0.029	33.5
Isoleucine/leucine	131.0948	15.27	1.3	6.5	0.023	−10.63
Ornithine	132.0899	10.19	0.2	10.4	0.0005	41.75
ADMA	202.1425	11.47	−2.3	10.1	0.025	13.65
Arginine	174.1116	10.64	−0.4	7.6	0.033	−22.95

Choline is essential for structural integrity and signaling in cell membranes and is also involved in cholinergic neurotransmission via acetylcholine synthesis. ALI is among the devastating disorders of pulmonary inflammation. In VILI, the repetitive cyclic stretch and/or over-inflation during mechanical ventilation promote diffuse cellular infiltration, inflammation, loss of membrane permeability, activation of the coagulation system, and cell death, leading to worse outcomes [45]. To limit and/or reverse lung injury, recent evidence suggests that stimulation of the endogenous cholinergic anti-inflammatory pathway may be an attractive way to reduce inflammatory injury [46, 47]. A recent study has demonstrated that stimulation of the cholinergic anti-inflammatory reflex can protect the lung against VILI, and it could be a promising alternative for the treatment of ventilated patients with ARDS/ALI [48]. The increased level of choline found in the VILI animal model in this study confirms that this protective mechanism is active in this pathology.

Conclusion

A rapid and simple method has been developed and validated for global serum metabolic profiling using CE–TOF-MS. Validation data are adequate for bio-analysis and sample treatment is very simple, enabling the detection of a wide range of compounds. The validated method was applied to VILI samples for the first time, and results obtained were in agreement with literature values.

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Chapter-2

A metabolomic approach to the pathogenesis of ventilator induced lung injury

Non-targeted metabolomics offers advantages over the targeted approach mainly due to its wider metabolite coverage present in a biological system. However, it is well known that single platform cannot analyze the entire metabolome. Hence, in continuation with chapter-1, another concomitant non-targeted metabolomics application was performed on VILI and control samples using NMR and LC-MS. LC-MS provides higher sensitivity over the other analytical platform and it covers metabolites from polar to non-polar groups. LC-MS has also been used to define the metabolic profile in hyperoxic and gamma-radiation-induced ALI. The present pilot study was designed to identify metabolic screening markers in VILI, which have not been previously studied using NMR and MS through a data driven non-targeted metabolomics approach. The discovery of biomarkers of VILI is useful for the identification of metabolic pathways involved in VILI and for a better understanding of the pathogenesis of this condition. Previous studies have used only particular sample type, however in this present study, different sample types were analyzed such as lung tissue, bronchoalveolar lavage fluid and serum. The objective of applying different sample type was to determine whether the inflammatory response is confined to the site of injury (the lung) or rather affects other organs. The findings from this pilot study demonstrated that VILI is characterized by a particular metabolic profile and VILI represents alterations in energy and membrane lipids.

The obtained result of this application study was published in *Anesthesiology* Journal.

Title: A metabolomics approach to the pathogenesis of ventilation-induced lung injury.

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Izquierdo-García José Luis, Naz Shama, Nin Nicolás, have equal contribution in this manuscript.

The published manuscript has been attached here with this chapter-2.

This pilot work was accomplished in collaboration with the Hospital Universitario de Getafe, Madrid, Spain. The part with LC-MS was performed by us and thus the focus of chapter 2 is based on the LC-MS method application on VILI models. The applied serum non-targeted methodology using LC-MS was previously developed in our laboratory. In extension with Chapter 1, along with CE-MS, Chapter-2 is providing the application of LC-MS to get distinct metabolite information than the other.

Resumen

ESTUDIO METABOLÓMICO APLICADO A LA PATOGÉNESIS DE LA ENFERMEDAD PULMONAR INDUCIDA POR VENTILACIÓN MECÁNICA

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Antecedentes: El estudio global de huella metabólica basado en Resonancia Magnética Nuclear cuantitativa (MRS) y espectrometría de masas (MS) es útil para el descubrimiento de biomarcadores. El objetivo de este estudio fue el descubrimiento de biomarcadores de ALI (daño pulmonar inducido por ventilación mecánica [VILI]), utilizando MRS y MS.

Métodos: Ratas Sprague–Dawley fueron sometidas a ventilación mecánica con dos condiciones diferentes durante 2,5 h: control (n = 14) con volumen tidal 9 ml/kg, presión positiva al final de la expiración (PEEP) 5 cm H_2O y VILI (n = 10), con volumen tidal 25 ml/kg y PEEP 0 cm H_2O. Se analizaron los espectros de RMN de ángulo mágico y ^{1}H-MRS del tejido pulmonar, lavado broncoalveolar y suero y también los obtenidos por LC-MS-QTOF (cromatografía de líquidos acoplada a detección por espectrometría de masas con analizador Quadrupolo-Tiempo de vuelo). Se realizó un estudio multivariante de los perfiles obtenidos por Análisis de Componentes Principales (PCA) y por Análisis disciminante por Mínimos Cuadrados Parciales (PLS-DA).

Resultados: El estudio de huella metabólica permitió diferenciar las muestras pertenecientes a los dos grupos de animales comparados Control y VILI. Las ratas con VILI presentaron por MRS mayor concentración de lactato y menores concentraciones de glucosa, glicina en tejido pulmonar, además de un aumento en los niveles de glucosa, lactato, acetato, 3-hidroxibutirato, y creatina en tejido broncoalveolar. En suero también se encontraron aumentadas por MS las concentraciones de fosfatidilcolina, oleamida, esfinganina, hexadecenal y lisina y disminuidas las concentraciones de lisofofatidilcolina y esfingosina.

Conclusiones: Los resultados obtenidos en este estudio piloto sugieren que la enfermedad VILI está caracterizada por un perfil metabólico particular que puede ser identificado por MRS y MS. A pesar de tratarse de resultados preliminares y pendientes de confirmación con mayor número de muestras, sugieren alteraciones en la energía y en los lípidos de membrana

A Metabolomic Approach to the Pathogenesis of Ventilator-induced Lung Injury

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ABSTRACT

Background: Global metabolic profiling using quantitative nuclear magnetic resonance spectroscopy (MRS) and mass spectrometry (MS) is useful for biomarker discovery. The objective of this study was to discover biomarkers of acute lung injury induced by mechanical ventilation (ventilator-induced lung injury [VILI]), by using MRS and MS.

Methods: Male Sprague–Dawley rats were subjected to two ventilatory strategies for 2.5 h: tidal volume 9 ml/kg, positive end-expiratory pressure 5 cm H_2O (control, n = 14); and tidal volume 25 ml/kg and positive end-expiratory pressure 0 cm H_2O (VILI, n = 10). Lung tissue, bronchoalveolar lavage fluid, and serum spectra were obtained by high-resolution magic angle spinning and ^{1}H-MRS. Serum spectra were acquired by high-performance liquid chromatography coupled to quadupole-time of flight MS. Principal component and partial least squares analyses were performed.

Results: Metabolic profiling discriminated characteristics between control and VILI animals. As compared with the controls, animals with VILI showed by MRS higher concentrations of lactate and lower concentration of glucose and glycine in lung tissue, accompanied by increased levels of glucose, lactate, acetate, 3-hydroxybutyrate, and creatine in bronchoalveolar lavage fluid. In serum, increased levels of phosphatidylcholine, oleamide, sphinganine, hexadecenal and lysine, and decreased levels of lyso-phosphatidylcholine and sphingosine were identified by MS.

Conclusions: This pilot study suggests that VILI is characterized by a particular metabolic profile that can be identified by MRS and MS. The metabolic profile, though preliminary and pending confirmation in larger data sets, suggests alterations in energy and membrane lipids. **(Anesthesiology 2014; 120:694-702)**

ABOUT one third of patients admitted to the intensive care unit worldwide require mechanical ventilation, of whom more than two thirds have acute respiratory failure as the admitting diagnosis.[1] Although life saving in many patients, it is recognized that mechanical ventilation by itself may cause lung injury by repeated stretching of lung tissue during tidal mechanical breaths (ventilator-induced lung injury [VILI]), initiating or aggravating lung injury.[2–5] In addition, the use of large tidal volumes (V_T) in patients receiving mechanical ventilation may be associated with worse outcomes.[6–8] Unlike other clinical conditions, to date there is no specific biomarker that helps in the diagnosis or prognosis of acute lung injury (ALI), nor are there diagnostic tools to identify the appearance or progression of VILI.

The metabolomic approach is finding an increasing number of applications in critical illness,[9–11] and has been used for the diagnosis of sepsis in experimental models[12–14] and for the prediction of outcome in patients with trauma.[15,16] In a pioneer study in a model of ALI induced by intratracheal administration of tumor necrosis factor-α and interleukin 1-β in mice, it was demonstrated that ^{1}H-nuclear magnetic resonance spectroscopy (MRS) of lung tissue is useful as a biomarker of inflammation-induced ALI.[17] Liquid chromatography–mass spectrometry (MS) has also been used to define the metabolic profile in hyperoxic-[18] and

What We Already Know about This Topic

- There are no biomarkers for ventilator-induced lung injury

What This Article Tells Us That Is New

- Metabolomic studies document changes in the lungs of experimental animals with ventilator-induced lung injury; these pilot data suggest that it is possible to identify ventilator-induced lung injury with metabolic investigations

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The first three authors contributed equally to the article.

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γ-radiation–induced ALI.[19] More recently, ^{1}H-MRS has been used to identify the metabolome in patients with sepsis-induced ALI,[20] thus serving as a biomarker of this condition.

The discovery of biomarkers of VILI is useful for the identification of metabolic pathways involved in VILI and for a better understanding of the pathogenesis of this condition. The current pilot study was designed to identify metabolic biomarkers in an experimental model of VILI, a form of ALI not previously studied using MRS and MS through a data-driven metabolomics fingerprinting approach. Unlike preceding studies, which used samples from only one compartment (*e.g.*, lung tissue or serum), we studied different tissue compartments by analyzing lung tissue samples, bronchoalveolar lavage (BAL) fluid and serum. This allowed us to determine whether the inflammatory response is confined to the site of injury (the lung) or rather affects other organs. Our methods are likely to produce a solution that is very sample specific, and future ongoing studies will analyze how this solution replicates in an external sample. Our preliminary findings may enable sample size calculations for confirmatory studies and stimulate further research by applying metabolomics to improve the understanding of ALI.

Materials and Methods

Experiments were carried out following the Principles of Laboratory Animal Care (EU 609/86 CEE, Spanish Real Decreto 1201/05), and the research protocol was approved by our Institutional Review Board. Methods were slightly modified from those used in previous work.[3,5] Male Sprague–Dawley rats (Harlan Iberica, Barcelona, Spain) weighing 342.3 ± 5.4 g (mean ± SD) were housed, acclimatized to a 12-h light/dark cycle, and maintained on Purina rat chow and water ad libitum before the experiments. On the day of the experiment, rats were anesthetized with intraperitoneal ketamine (90 mg/kg) and diazepam (5 mg/kg). The femoral vein was cannulated for the continuous intravenous infusion of ketamine (50 mg kg^{-1} h^{-1}) and midazolam (8 mg kg^{-1} h^{-1}). A surgical tracheotomy was performed and a 14-gauge cannula was secured in place and connected to a mechanical ventilator (Babylog 8000 Plus; Dräger, Lübeck, Germany) setting ventilatory parameters as in the control group (*vide infra*).

A 20-gauge catheter was inserted into the left carotid artery for monitoring arterial blood pressure (Model 66S; Hewlett Packard, Meyrin, Switzerland). Body temperature was maintained during the experiment by placing the animals over a thermopad (Challoner Marketing Ltd., Amersham, England).

Study Protocol

As in previous studies,[3,5] once monitoring had finished, and after an equilibration period of 15 min on mechanical ventilation, animals received one of two ventilatory strategies for 2.5 h: low V_T ventilation (V_T, 9 ml/kg; positive end-expiratory pressure, 5 cm H_2O, [CONTROL], n = 14) and high V_T ventilation (V_T, 25 ml/kg; zero positive end-expiratory pressure [VILI], n = 10). In both groups, respiratory rate was 70 breaths/min, inspiratory time 0.3 s, expiratory time 0.56 s, and F_{IO_2} 0.45.

Physiological and Biochemical Measurements

Mean arterial pressure, peak inspiratory pressure (PIP), and V_T were registered. Arterial blood (t = 0 min and t = 150 min) was obtained for measurement of blood gases and lactate concentration (Gem Premier 3000; IL Instrumentation Laboratory, Bedford, MA).

Animals were sacrificed by exsanguination at t = 150 min, and serum was obtained and frozen at −80°C for metabolomic studies. Thereafter, a BAL was performed by instilling once 10 ml of saline.

Finally, the thoracic cage was opened and lungs were excised and extracted. The right lung was frozen and stored at −80°C for metabolomic analysis. The left lung was removed and expanded by intratracheal instillation of 10% formaldehyde for histological analysis under light microscopy (hematoxylin and eosin). A modified lung injury score was used as previously reported.[3,21]

Metabolic Spectra Acquisition

Lung tissue, BAL fluid, and serum spectra were obtained for high-resolution magic angle spinning and ^{1}H-MRS (Bruker AMX500 nuclear magnetic resonance [NMR] spectrometer; Rivas-Vaciamadrid, Spain).

Serum spectra were also acquired by high-performance liquid chromatography coupled to quadupole-time of flight MS[22] (see Supplemental Digital Content 1, http://links.lww.com/ALN/B10, which includes a detailed description of methods on NMR and MS data acquisition).

NMR Data Treatment

Principal components analysis was applied in order to extract the most discriminative spectral subset from the total pool of metabolites. Partial least square (PLS) analysis[23] was applied on NMR data to investigate significant differences between groups. Potential biomarkers were selected from the PLS correlation plots by Hotteling T^2 tests. The NMR statistical analysis was performed with the Metabonomic package (rel.3.3.1[24]; see Supplemental Digital Content 1, http://links.lww.com/ALN/B10, for a detailed description of NMR data treatment).

MS Data Treatment

Primary data treatment (filtering and alignment) was accomplished with Mass Profiler Professional 2.0 (Agilent, Santa Clara, CA) software. Features were filtered by choosing the data that were present in 100% of samples in any group. Filtering was performed for comparisons of the VILI and the control groups. In total, 575 features (of 7,796) were selected for further data treatment. A Student t test ($P < 0.05$) was performed on the filtered data and eight significant masses were found.

Partial least square-discriminant analysis (PLS-DA) and orthogonal PLS-DA for each comparison calculated for filtered data sets were conducted using SIMCA-P+ 12.0.1 (Umetrics, Malm, Sweden). One hundred seventy-six masses from S-plot with a cutoff point of ±0.05 (after orthogonal PLS-DA) were checked through jack-knifing, and 44 significant masses from jack-knifing were added up to the list with the Student t test. Accurate masses of features representing significant differences were searched against the METLIN, KEGG, LIPIDMAPS, and HMDB databases (see Supplemental Digital Content 1, http://links.lww.com/ALN/B10, for a more detailed description of MS serum preparation, data treatment, quality control, and compound identification).

Mass spectrometry multivariate statistical calculations and plotting were done using SIMCA-P+ 12.0.1 (Umetrics). According to the method used, the number of analyses in relation to the number of events prevents definitive conclusions about the utility of these predictors in future samples. The interpretation of our results has to take into consideration that this level of discrimination does not necessarily reflect some underlying solution that can be used in future studies.

Other Statistical Analysis

Hemodynamic and biochemical variables as well as differences between the control and the VILI groups for individual metabolites were compared by an unpaired unequal variance Student *t* test ($P \leq 0.05$).

The correlation between each metabolite and several physiological variables at t = 150 min was assessed by Spearman rank correlation analysis. A P value of 0.05 or less was considered statistically significant. Data are shown as mean ± SD. We used the statistical package SPSS 17.0 (Chicago, IL).

Results

Effects of Ventilation

Rats ventilated with high V_T showed a significant increase in PIP over time, as well as a significant decrease in Pa_{O_2} and mean arterial pressure (table 1).

Histological Findings

All lungs analyzed from the control group were normal at macroscopic inspection and at light microscopy, whereas lungs from the high V_T group showed capillary congestion, interstitial edema, type-I alveolar cell necrosis, and hyaline membrane formation covering the denuded epithelial surface (lung injury score 0.0 ± 0.0 *vs.* 23.4 ± 10.1, respectively; $P < 0.001$; fig. 1).

MRS Results

Partial least square analysis was applied on NMR data to investigate significant differences between groups. As compared with the control group, animals with VILI showed higher concentrations of lactate and lower concentration of glucose and glycine in lung tissue; and higher concentration of glucose, lactate, acetate, 3-hydroxybutyrate, and creatine in BAL fluid (see Supplemental Digital Content 2, http://links.lww.com/ALN/B11, tables 1and 2, which summarizes significant spectral regions of postulated metabolites).

Partial least square score plots of lung tissue and BAL fluid spectra (fig. 2) showed a perfect discrimination between the two groups.

MS Results

Partial least square-DA and orthogonal PLS-DA plots of serum samples showed a perfect discrimination between the control and the VILI groups (fig. 3).

Mass spectrometry analysis showed, among other changes, increased serum levels of phosphatidylcholine, oleamide, sphinganine, oxo-hexadecenal, and lysine, as well as decreased levels of lyso-phosphatidylcholine (lysophosphatidylcholine) and sphingosine (Supplemental Digital Content 2, http://links.lww.com/ALN/B11, table 2, which contains a lists of the identified compounds that are significantly different in serum samples from the control and the VILI groups by MS). Lyso-phosphatidylcholine, phosphatidylcholine, and fatty acid amides were confirmed with their characteristic fragments described in the literature.[25,26]

Table 1. Changes in Blood Gases, MAP, and Peak Inspiratory Pressure in Rats with VILI

		t = 0 min	t = 150 min	Delta	P Value
Pa_{O_2} (mmHg)	Control	175 ± 10	183 ± 8	8 ± 12	0.04
	VILI	164 ± 10	133 ± 9	–31 ± 24	
MAP (mmHg)	Control	120 ± 16	93 ± 11	–28 ± 15	0.01
	VILI	127 ± 15	81 ± 10	–46 ± 15	
PIP (cm H_2O)	Control	16.4 ± 2.0	17.4 ± 1.3	1.5 ± 1.0	0.001
	VILI	26.4 ± 1.9	33.5 ± 3.3	8.6 ± 1.2	
HCO_3^- (mM)	Control	16.4 ± 1.7	16.3 ± 1.9	–0.8 ± 1.1	0.5
	VILI	17.5 ± 1.5	16.6 ± 1.9	–0.4 ± 1.1	
Lactate (mM)	Control	2.6 ± 0.9	0.9 ± 0.3	–1.7 ± 0.9	0.07
	VILI	2.1 ± 0.9	1.1 ± 0.2	–1.1 ± 0.9	

Delta indicates the difference between t = 0 min and t = 150 min. P values refer to the comparison between delta in the control and the VILI groups for each variable. Values are mean ± SD.

MAP = mean arterial pressure; Pa_{O_2} = partial pressure of oxygen in arterial blood; PIP = peak inspiratory pressure; VILI = ventilator-induced lung injury.

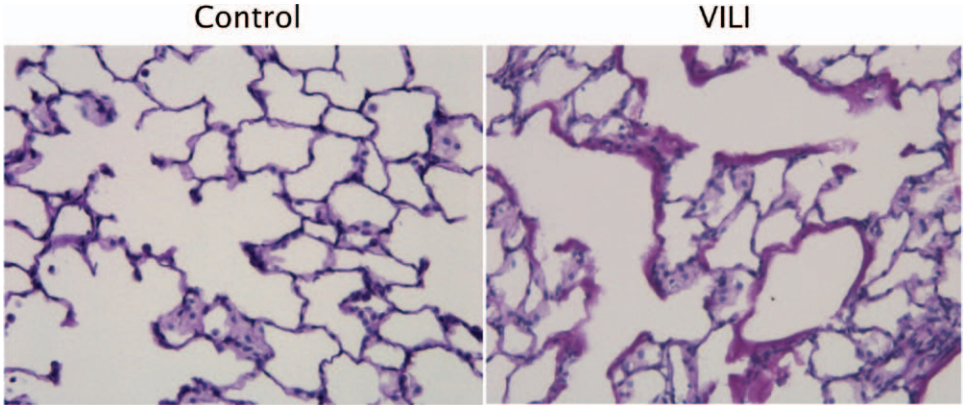

Fig. 1. Photomicrograph (light microscopy, hematoxylin and eosin) of representative lung slices (×20). Lungs from the control (low tidal volume) group were normal at macroscopic inspection and at light microscopy. Lungs from the ventilator-induced lung injury (VILI, high tidal volume) group showed capillary congestion, interstitial edema, alveolar epithelial necrosis, and thick hyaline membrane formation covering the denuded epithelial surface.

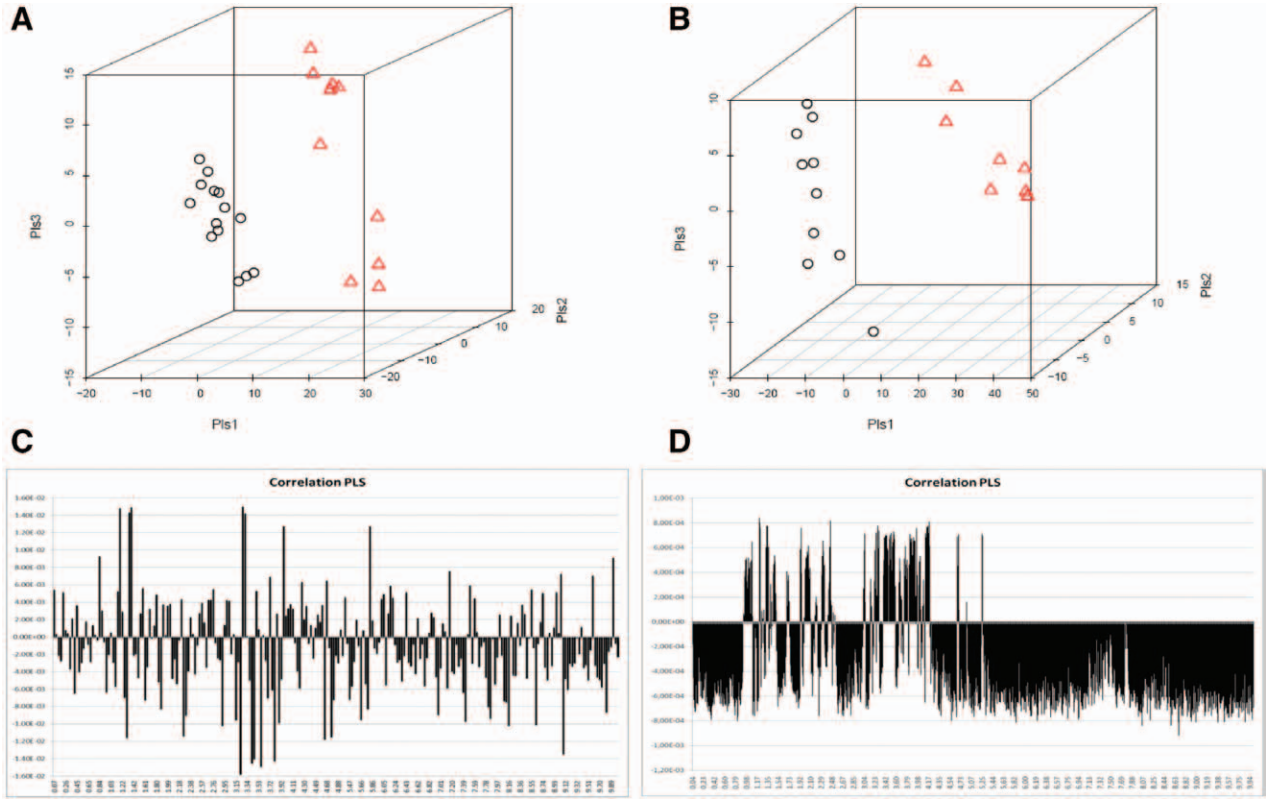

Fig. 2. Score and correlation plots of partial least squares (PLS) analyses performed on the ^{1}H-nuclear magnetic resonance spectra of lung tissue (*A* and *C*) and bronchoalveolar fluid (*B* and *D*) samples from the control (*circles*) and the ventilator-induced lung injury groups (*triangles*). The parameters for these models are (*A*) $R^2 = 0.998$, $Q^2 = 0.831$ and (*B*) $R^2 = 0.996$, $Q^2 = 0.812$. The most important bucket positions for the separation between ventilator-induced lung injury and control rats are selected with a significance level lower than 1.40e-02 and 7.00e-04, for lung tissue and bronchoalveolar fluid samples, respectively.

Correlation between Lung Injury and Metabolites

All metabolites identified by MRS whose concentration changed in VILI as compared with control animals, both in tissue and in BAL fluid samples (see Supplemental Digital Content 3, http://links.lww.com/ALN/B12, fig. 1, and Supplemental Digital Content 4, http://links.lww.com/ALN/B13, fig. 2, containing representative ^{1}H-NMR spectra of lung tissue and BAL fluid samples), showed a significant correlation with PIP, Pao_2, and lung injury score at $t = 150$ min (table 3). None of the compounds identified by MS in serum showed a significant correlation with PIP, Pao_2, or lung injury score.

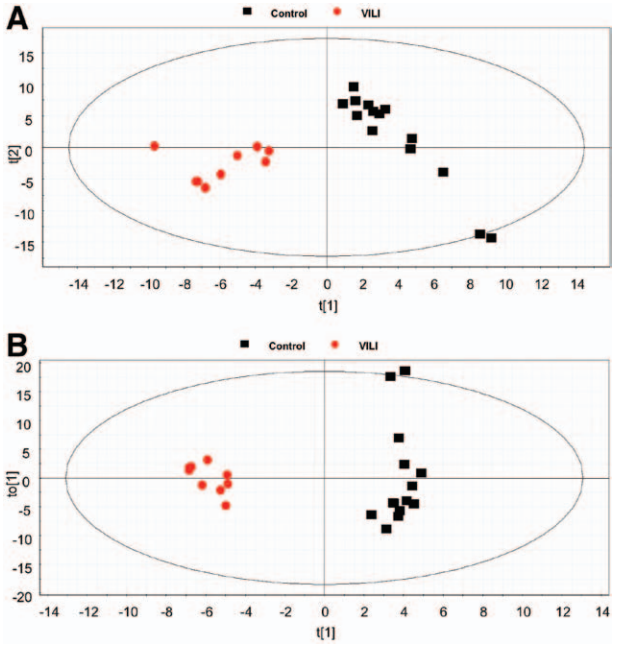

Fig. 3. Partial least squares analysis (*A*) and orthogonal partial least squares-discriminant analysis (*B*) plots of metabolite profiles in serum of control and ventilator-induced lung injury (VILI) (mass spectrometry). The parameters for these models are (*A*) $R^2 = 0.996$, $Q^2 = 0.781$ and (*B*) $R^2 = 0.979$, $Q^2 = 0.615$.

Table 2. Identified Compounds That Are Significantly Different in Serum Samples from the Control and the VILI Groups*

Sample	High	Low
Lung tissue†	Lactate	Glucose Glycine
BAL fluid†	Glucose Lactate Acetate 3-Hydroxybutyrate Creatine	
Serum‡	Phosphatidylcholine Oleamide Sphinganine Oxo-hexadecenal Hydroxy-oxo-cholenoic acid Deoxycortisol Lysine	Lyso-phosphatidylcholine Sphingosine Ethyl-dodecanoic acid Oxoisotretinoin Octadecadienol

* See also table 1, Supplemental Digital Content 2, http://links.lww.com/ALN/B11. † ^{1}H-nuclear magnetic resonance spectroscopy. ‡ Mass spectrometry.

BAL = bronchoalveolar lavage; VILI = ventilator-induced lung injury.

Discussion

The main findings of this pilot study are: (1) ^{1}H-MRS and MS are useful to define the metabolome characteristic of VILI in this experimental model; (2) the specific metabolites changing in animals with VILI suggest alterations in energy pathways and membrane lipids; (3) the change in the level of the different metabolites correlate with physiological variables.

Cell Energy Metabolism

Our findings of decreased glucose and increased lactate levels in lung tissue from rats with VILI are consistent with increased glucose use and altered aerobic metabolism. Similarly, the increased creatine levels in BAL fluid indicate impairment of normal cell energy production, as creatine-phosphate can be hydrolyzed to obtain adenosine triphosphate under conditions of decreased adenosine triphosphate production, and be transported along with lactate into the alveolar space. In addition, acetate (which cannot be used under conditions of anerobic metabolism) and glucose (from glycogen degradation) can also be transported into the alveolar space and be detected in the BAL fluid determinations.

These results are in line with the findings reported by Serkova *et al.*,[17] who found decreased high-energy phosphates, energy balance, and energy charge, as well as an increased lactate/glucose ratio in lung tissue from a model of ALI in mice. Further evidence pointing to altered energy metabolism in ALI has been reported in a model of silica-induced lung inflammation[27] and in patients with sepsis-induced ALI.[20]

Membrane Lipids

In the current study we found a particular lipid profile revealed by MS in serum from rats with VILI. Levels of lyso-phosphatidylcholine and sphingosine were decreased, whereas levels of phosphatidylcholine, oleamide, sphinganine, and hexadecenal were increased (see Supplemental Digital Content 5, http://links.lww.com/ALN/B14, fig. 3, for a proposed relationship among the different lipids found to be altered in the current study). Changes in levels of membrane phospholipids have been reported in serum from patients with sepsis-induced ALI[20] and in lung tissue from mice subjected to silica-induced lung inflammation.[27]

Lysophospholipids. Lysophospholipids are generated by hydrolysis of the fatty acid ester bond of membrane phospholipids by the action of phospholipase A1 or A2, and are precursors of a different class of lipid mediators including platelet-activating factor and endocannabinoids.28 Decreased lysophospholipids could be explained in the context of activation of the remodeling pathway for phosphatidylcholine synthesis.

Phosphatidylcholine. Phosphatidylcholine is a major component of cell membranes.[28] The mechanism for increased phosphatidylcholine in our model cannot be determined, and could involve cell membrane injury and subsequent release of its components. However, membrane components released from injured membranes would not be expected to enter the circulation but would rather remain in the alveolar space were they are endocytosed and degraded by alveolar macrophages,

Table 3. Correlation between Physiological and Morphological Variables and the Change in the Concentration of Different Metabolites by ^{1}H-NMR Spectroscopy

	PIP		PaO_2		LIS	
Metabolite	R^2	*P* Value	R^2	*P* Value	R^2	*P* Value
Lung tissue						
Glucose	0.73	0.001	0.34	0.010	0.65	0.001
Lactate	0.56	0.010	0.32	0.010	0.39	0.010
Glycine	0.48	0.010	0.33	0.040	0.52	0.010
BAL fluid						
Glucose	0.92	0.001	0.63	0.001	0.77	0.001
Lactate	0.81	0.001	0.55	0.001	0.60	0.001
Acetate	0.65	0.001	0.24	0.004	0.28	0.004
Hydroxybutytrate	0.64	0.001	0.31	0.04	0.35	0.01
Creatine	0.89	0.001	0.72	0.001	0.79	0.001

BAL = bronchoalveolar lavage; LIS = lung injury score; NMR = nuclear magnetic resonance; PaO_2 = partial pressure of oxygen in arterial blood; PIP = peak inspiratory pressure.

or used for surfactant synthesis by type II alveolar cells. Alternatively, lipid changes could set the scenario for the activation of the rate-limiting enzyme cytosine triphosphate:phosphocholine cytidylyltransferase, and increase *de novo* synthesis of phosphatidylcholine.

Oleamide. Oleamide is structurally related to anandamide,[29,30] another glycerolipid-derived regulatory molecule, and ligand for cannabinoid receptors. *Cannabinoids* have antiinflammatory and proapoptotic effects[31–33] and also play a role in the hypotension associated with various forms of shock.[31,34,35] Recent research has shown that the CB1 cannabinoid receptor is also coupled to the generation of the lipid second messenger *ceramide via* two different pathways. First, *via* sphingomyelin hydrolysis by *sphyngomyelinase*, yielding ceramide and phosphocholine (which is intermediate in the synthesis of phosphatidylcholine). The ceramide released has been linked to the regulation of metabolic functions, such as cannabinoid-mediated increased use of glucose and production of ketone bodies in primary astrocytes.[36] Second, the activation of palmitoyltransferase stimulating *de novo* synthesis of ceramide.[37–39] In the context of our findings, cannabinoid-mediated sphingomyelin hydrolysis yielding ceramide and phosphocholine could further provide substrate for phosphatidylcholine synthesis. In addition, cannabinoid-mediated activation of palmitoyltransferase could potentially explain the increased levels of *sphinganine* (one of the intermediate metabolites in *de novo* synthesis of ceramide).[40]

Sphingosine. Sphingosine can be released from ceramide, a process catalyzed by the enzyme ceramidase. Sphingosine can be phosphorylated *in vivo via* two kinases, sphingosine kinase type 1 and sphingosine kinase type 2, leading to the formation of sphingosine-1-phosphate, which is irreversibly transformed into hexadecenal and ethanolamine by sphingosine-1-phosphate lyase.[41,42] Thus, decreased levels of sphingosine in the current study could be explained by activation of ceramide synthase or by increased degradation to hexadecenal (the latter being supported by our findings of increased levels of hexadecenal). Sphingosine and sphingosine-1-phosphate, which are metabolites of ceramide, have antiapoptotic effects[41,43,44] and play a variety of roles in diverse cellular activities such as cell growth, cell motility, and immunity[45,46] and in the regulation of vascular permeability.[47] *Hexadecenal* has been associated with apoptosis and cytoskeletal reorganization, leading to cell rounding, detachment, and eventual cell death by apoptosis in multiple cell types, including HEK293T, NIH3T3, and HeLa cells.[42]

Collagen/Elastin Metabolism

Levels of glycine were decreased in lung tissue in our model whereas lysine serum levels were increased. Glycine and lysine account for 30 and 3%, respectively, of amino acid residues in collagen, glycine being the most abundant amino acid in this protein. Previous studies have shown altered collagen metabolism in ALI. In paraquat-induced ALI, type III procollagen mRNA was higher in rats with ALI than in controls.[48] In silica-induced ALI in mice,[27] increased levels of glycine, lysine, glutamate, proline, and 4-hydroxyproline were found, suggesting the activation of the collagen pathway. In addition, increased type-I procollagen in alveolar lining fluid from patients with adult respiratory distress syndrome or ALI subjects has been reported.[49]

Potential Role of Metabolomic Changes in the Pathogenesis of ALI

In the current study the physiological relevance of the findings was further substantiated by the significant correlation between the concentration of different altered metabolites and variables indicative of lung dysfunction (impaired gas exchange) or injury (increased PIP and greater histological injury).

Our findings may help our understanding of the pathogenesis of VILI. Specifically, increased formation of cannabanoids could be related to sphyngomyelinase activation and increased formation of the lipid second messenger ceramide, as potentially important events in the pathogenesis

of VILI (Supplemental Digital Content 3, http://links.lww.com/ALN/B12, fig. 3). In addition, increased hexadecenal could play a role in cytoskeletal abnormalities in the context of cell mechanical trauma. The results of the current study supporting a role for the involvement of the ceramide–sphingosine pathway in the pathogenesis of VILI are also in line with the recognized function of *ceramide* as a signaling molecule in the inflammatory response.[45,50,51] Indeed, a protective effect of sphingosine 1-P lyase inhibition (resulting in increased levels of sphingosine 1-P) has been reported in lipopolysaccharide-induced ALI in mice.[52]

Regardless of the interest of our results, this is not a definitive study. The issue of type-I error inflation has to be considered because the methodology did not include adjustment of the analyses for the many inferences that were generated to create the solution. If subsequently validated in larger sample sets, our results may improve the understanding and management of ALI, providing suitable biomarkers that can be used in clinical practice. The strengths of our study lie in its originality and the rigorous way in which the metabolomics experiments were conducted. It further provides some pilot data with which a sample size calculation can be conducted for a definitive study.

Limitations

The current study has several limitations. First, we studied only one time in point after the insult. Second, there was no intervention to inhibit one of the pathways proposed to have a pathogenic role in VILI. Third, the relevance of serum analysis to identify metabolic biomarkers is under discussion as the insult was primarily localized to the lungs. It is possible that changes detected in serum could reflect lipoprotein-associated lipid metabolism in serum rather than alterations in lung metabolic and signaling pathways. Fourth, this is an animal model in which an exaggerated form of VILI was produced, using V_T not used in humans. However, the V_T used in the current study is well in the range of the V_T used in other investigations, ranging from 20 to 42 ml/kg (see study by de Prost[53] for excellent review). It is possible that the specific metabolomic changes herein reported cannot be reproduced with different ventilatory patterns. Specifically, of particular relevance would be the study, not approached in the current investigation, of metabolomic changes induced by VILI in preinjured lung models. Fifth, it is acknowledged that mechanical ventilation using V_T 9 ml/kg in the control group may already cause some degree of lung injury, and that a lower V_T would have been more appropriate in the control group.

In summary, we report for the first time metabolomic changes associated with VILI. Future studies should confirm the value of MRS and MS in the identification of biomarkers for the diagnosis of VILI, particularly in preinjured lungs, or other forms of ALI in larger sample sets.

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Competing Interests

The authors declare no competing interests.

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Supplemental Digital Content 1

METHODS

Nuclear Magnetic Resonance data acquisition

Intact lung tissue samples (weighing approx. 10 mg) were examined using HR-MAS ^{1}H-NMR spectroscopy operating at 4 ºC to reduce metabolic degradation. ^{1}H-NMR spectroscopy was performed at 500.13 MHz using a Bruker AMX500 spectrometer 11.7 T (Bruker, Rivas-Vaciamadrid, Spain). The samples were placed into a 50 µl zirconium oxide rotor using a rinsed cylindrical insert, together with 15 µl of 0.1mM solution of Trimethylsilyl propanoic acid (TSP) in deuterium water (D_2O), and spun at 4000 Hz spinning rate to remove the effects of spinning side bands from the acquired spectra. Additionally, bronchoalveolar lavage (BAL) fluid lyophilized samples were diluted with 100 µl of D_2O and 100 µl of solution of TSP in D_2O and examined using a Bruker AV500 spectrometer operating at 4ºC.

Shimming and ^{1}H-NMR preparation time were reduced to a minimum, meanwhile the temperature for performing the ^{1}H-NMR analysis was chilled to 4 ºC to minimize metabolic changes. A number of bidimensional homonuclear experiments were performed to carry out the components assignments. Between consecutive 2D spectra, a control ^{1}H-NMR spectrum was always measured. No gross degradation was noted in the signals of multiple spectra acquired under the same conditions.

Standard solvent suppressed spectra were grouped into 16,000 data points for tissue samples analyses and into 32,000 data points for BAL analyses,

averaged over 256 acquisitions. The data acquisition lasted in total 13 min using a sequence based on the first increment of the Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence to effect suppression of the water resonance and limit the effect of B_0 and B_1 inhomogeneities in the spectra (relaxation delay-90º-t1-90º-tm-90º-acquire Free Induction Decay signal) in which a secondary radio frequency irradiation field was applied at the water resonance frequency during the relaxation delay of 2 s and during the mixing period (tm = 150 ms), with t1 fixed at 3 s. Tissue samples acquisitions were performed using a spectral width of 8333.33 Hz and BAL fluid acquisitions were acquired using a spectral width of 7507.5 Hz. Prior to Fourier transformation, the Free Induction Decay signals were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz. Spectra were referenced to the TSP singlet at 0 ppm chemical shift.

A standard gradient-enhanced Correlation Spectroscopy protocol was acquired under the following conditions: water presaturation during relaxation delay, spectral with of 5122.95 Hz in both dimensions, 2000 data points in f2 and 256 increments in f1. An unshifted sinusoidal window function was applied in both dimensions and zero filling in f1 dimension. ^{1}H-^{1}H Total Correlated Spectroscopy experiment was registered in the Time-Proportional Phase Incrementation phase sensitive mode, water pre-saturation during 1s relaxation delay, a spectral with of 5122.95 Hz in both dimensions, 60 ms mixing time, 2000 data points in f2 and 256 increments in f1. Zero filling in f1 and unshifted squared sinusoidal window function in both dimensions were applied before Fourier transformation.

Gradient-selected Heteronuclear Single Quantum Correlation experiments of serum and tissue samples were registered with the following parameters: 95 μs for Globally Optimized Alternating Phase Rectangular Pulse ^{13}C decoupling, 8333Hz and 21 kHz spectral widths in the ^{1}H and ^{13}C dimensions, respectively, 2000 data points in f_2 and 256 increments in f_1. Zero filling in f_1 and unshifted squared sinusoidal window function in both dimensions were applied before Fourier transformation.

In order to remove the random effects of variation in the water resonance suppression, the chemical shift regions between 4.90 and 5.30 ppm of tissue spectra, 5.00-5.20 ppm of serum spectra and 4.85-5.00 of BAL spectra, were excluded from the analysis. Similarly, the chemical shift region from 0 to 0.04 ppm containing the internal reference (TSP) was also excluded. The baseline correction (method by Rocke and Xi) (**1**) was automatically performed with the baseline correction tool of the Metabonomic R package (**2**). The Metabonomic R package is a graphical environment for the metabolomic analysis developed and maintained by our group in the public domain R framework (rel. 2.11.1) (**3**). ^{1}H-NMR spectra were automatically data-reduced to integral segments or buckets of equal length (δ0.01 ppm for BAL spectra and δ0.04 ppm for tissue and serum spectra) in order to compensate for variations in resonance positions (**4**) and they were normalized to the total sum of the spectral regions. 2D spectral processing and editing was performed with MestRenova v. 6.03 (Mestrelab Research S.L., Santiago de Compostela, Spain).

NMR data treatment

Principal Components Analysis (PCA) is the fundamental method in chemometrics (**5**). In PCA the data collected on a set of samples is resolved

into principal components. 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 on samples (concentrations) in the data set. Prior to PCA, the data were centered and Pareto scaled.

A Partial Least Squares (PLS) analysis (**6**) is a commonly used multivariate method for analyzing high-dimensional data. PLS analysis was applied to these data to investigate the significant differences between groups. The potential biomarkers selected from PCA loading plots were confirmed from PLS correlation plots by Hotteling's T^2 tests (**7**). Using the selected metabolites or chemical shifts, a PLS-Discriminant Analysis (PLS-DA) was developed as classificatory model. We have used the algorithm proposed by Ding and Gentleman* (tolerance for convergence=1e-03, maximum number of iteration allowed = 100). The number of PLS components used was chosen by the percentage of variance explained, the R^2 and the Mean Squared Error of Cross Validation graphics. PLS-DA models were trained with a number of random testing subjects and used afterwards to classify the rest of subjects as an internal validation. This process was repeated 200 times with random

* Ding B, Gentleman R: The gpls package: Classification using generalized partial least squares, version 1.3.1. Available at: ftp://ftp.auckland.ac.nz/pub/software/CRAN/doc/packages/gpls.pdf. Accessed October 7, 2013.

permutations of the data to reduce type I error. The percentages of correct classification were calculated as a measure of the model performance. The statistical computing and spectral processing described above were also performed with the Metabonomic package (rel.3.3.1).

Mass Spectrometry (MS) serum sample preparation

Protein precipitation and metabolite extraction was performed by adding 1 part of serum to 3 parts of cold (-20 °C) mixture of methanol and ethanol (1:1). Samples were then vortex-mixed and stored at -20 °C for 5 min. The supernatant was collected by centrifuging at 16 000× g for 10 min at 4 °C, and then the supernatant was filtered through a 0.22 μm nylon filter.

Quality control (QC) samples were prepared by pooling equal volumes of serum from each of the 23 samples. Five samples were independently prepared from this pooled serum following the same procedure as for the rest of samples. QC samples were analyzed throughout the run to provide a measurement not only of the system's stability and performance (**8**) but also of the reproducibility of the sample treatment procedure.

MS data acquisition

The High Performance Liquid Cromatography system consisted of a degasser, two binary pumps, and autosampler (1200 series, Agilent, Santa Clara, CA); 10 μL of extracted serum sample was applied to a reversed-phase column (Discovery HS C18 15 cm × 2.1 mm, 3 μm; Supelco, St. Louis, MO) with a guard column (Discovery HS C18 2 cm × 2.1 mm, 3 μm; Supelco). The system was operated in positive ion mode at the flow rate 0.6 mL/ min with solvent A composed of water with 0.1% formic acid, and solvent B composed of

acetonitrile with 0.1% formic acid. The gradient started from 25% B to 95% B in 35 min, and returned to starting conditions in 1 min, keeping the re-equilibration at 25% B for 9 min. Data were collected in positive electrospray ionization mode in separate runs on a quadrupole time-of-flight (QTOF) (Agilent 6520) operated in full scan mode from 50 to 1000 m/z. The capillary voltage was 3000 V with a scan rate of 1.02 scan per second; the nebulizer gas flow rate was 10.5 L/min.

The resulting data file was cleaned of extraneous background noise and unrelated ions by the molecular feature extraction tool in the MassHunter Qualitative Analysis Software U (Agilent). The molecular feature extraction then created a listing of all possible components as represented by the full QTOF mass spectral data. Exact mass databases quoted below were then searched for hits to identify the compounds.

MS data treatment

To avoid the risk of over-fitting for a PLS-DA model used for selection of statistically significant metabolites according to jack-knifed confidence intervals, the model was validated by use of a cross-validation tool **(9),** using a 1/3 out approach which has been described elsewhere **(10).** In brief, the dataset was divided into three parts, 1/3 of samples were excluded, and a model was built using the remaining 2/3 of samples. Excluded samples were then predicted by this new model, and the procedure was repeated until all samples had been predicted at least once. Each time the percentage of correctly classified samples was calculated.

QC of the MS methodology

QCs were checked along with the two other groups, VILI and control. For these three groups a PLS-DA model was built taking all variables (without any scaling) generated after molecular feature extraction in the mass spectrum (15016 variables in total). The robustness of the analytical procedure was evident by the tight clustering of QC samples obtained by mixing equal volumes of all samples. QCs were located in the center of the plot when sent to be classified by the model proving that separation between groups is not random, but due to real variability. The quality of the model built for three components was good (variance explained R2 = 0.907, and variance predicted Q2 = 0.383).

Compound identification

The nuclear magnetic resonance signals were identified according to the Human Metabolome Database (**11**), and characteristic cross-peaks from 2D spectra to help in the unequivocal assignation of these metabolites.

The identity of MS signals that were found to be significant in class separation was confirmed by liquid cromatography-MS/MS by using a QTOF (model 6520, Agilent). Experiments were repeated with identical chromatographic conditions as in the primary analysis. Ions were targeted for collision induced dissociation fragmentation on the fly based on the previously determined accurate mass and retention time. Comparison of the structure of the proposed compound with the fragments obtained can confirm the identity. Accurate mass data and isotopic distributions for the precursor and product ions can be studied and compared to spectral data of reference compounds, if available, obtained under identical conditions for final confirmation (HMDB, METLIN).

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Supplemental Digital Content 2

Table 1. Summary of significant spectral regions of postulated metabolites and their relative amounts from the control and the VILI groups (^{1}H-NMR Spectroscopy).

Sample	Bucket position (ppm)	Postulated metabolites	Relative intensity		P value
			Control	VILI	
Lung tissue	1.34	Lactate	42.6 ± 0.4	55.0 ± 0.5	<0.0001
	1.37	Lactate	36.5 ± 0.2	48.6 ± 0.5	<0.0001
	3.22	Glucose	32.2 ± 0.2	27.3 ± 0.2	0.0005
	3.26	Glucose	35.6 ± 0.2	28.5 ± 0.2	<0.0001
	3.30	Glucose	72.9 ± 0.5	59.1 ± 0.4	0.0006
	3.41	Glucose	47.2 ± 0.4	38.6 ± 0.3	<0.0001
	4.45	Glucose	71.8 ± 0.8	56.3 ± 0.5	<0.0001
	3.57	Glycine	93.6 ± 0.8	75.8 ± 0.8	<0.0001
	3.80	Glucose	22.7 ± 0.4	18.5 ± 0.3	0.0002
BAL fluid	1.20	Hydroxybutyrate	3.4 ± 0.1	11.8 ± 0.2	0.0002
	1.22	Hydroxybutyrate	3.1 ± 0.1	11.2 ± 0.2	0.0005
	1.23	Hydroxybutyrate	2.6 ± 0.1	8.5 ± 0.6	0.0023
	1.33	Lactate	14.1 ± 0.8	45.2 ± 0.9	0.0002
	1.35	Lactate	14.1 ± 0.8	45.2 ± 0.9	0.0002
	1.94	Acetate	11.5 ± 0.7	55.2 ± 0.9	0.0017
	2.45	Hydroxybutyrate	0.6 ± 0.0	1.8 ± 0.0	0.0017
	3.05	Creatine	3.2 ± 0.1	8.7 ± 0.2	0.0041
	3.25	Glucose	1.6 ± 0.3	4.6 ± 0.4	0.0050
	3.28	Glucose	1.5 ± 0.1	5.2 ± 0.3	0.0014
	3.30	Glucose	2.7 ± 0.3	9.0 ± 0.5	0.0028
	3.42	Glucose	1.7 ± 0.2	6.8 ± 0.3	0.0073
	3.49	Glucose	1.5 ± 0.1	8.1 ± 0.2	0.0076
	3.51	Glucose	2.4 ± 0.3	10.1 ± 0.4	0.0051
	3.55	Glucose	1.9 ± 0.2	4.0 ± 0.2	0.0032

	3.56	Glucose	2.3 ± 0.3	4.7 ± 0.3	0.0049
	3.84	Glucose	1.8 ± 0.2	5.2 ± 0.2	0.0061
	3.96	Glucose	2.6 ± 0.3	7.0 ± 0.3	0.0020
	4.12	Lactate	1.3 ± 0.1	3.0 ± 0.1	0.0030
	4.14	Lactate	2.4 ± 0.2	9.5 ± 0.3	0.0012
	4.15	Lactate	2.5 ± 0.2	9.7 ± 0.3	0.0010
	4.16	Lactate	1.0 ± 0.0	3.7 ± 0.2	0.0010
	4.17	Hydroxybutyrate	0.9 ± 0.0	3.6 ± 0.1	0.0027
	4.18	Hydroxybutyrate	0.6 ± 0.0	1.8 ± 0.1	0.0004
	4.69	Glucose	2.4 ± 0.6	7.4 ± 0.1	0.0056
	5.25	Glucose	1.3 ± 0.3	4.4 ± 0.2	0.0058

BAL, bronchoalveolar lavage. NMR, Nuclear Magnetic Resonance. VILI, ventilator induced lung injury.

Values are mean ± SD. Signal intensities were normalized to total sum of the spectral regions to calculate the relative intensity.

Table 2. Identified compounds that are significantly different in serum samples from the control and the VILI groups (mass spectrometry).

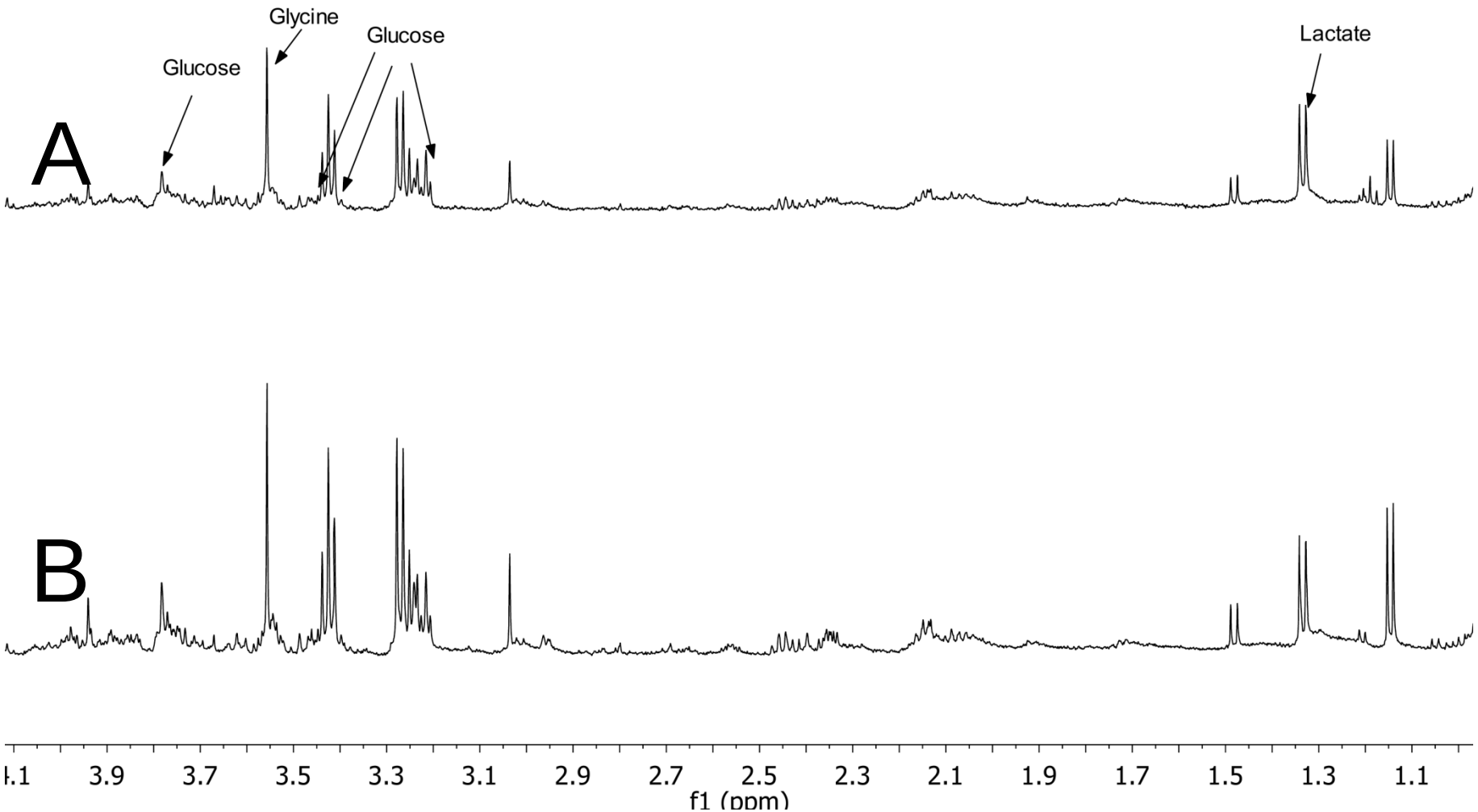
Compound	RT (min)	Measured mass (Da)	Mass error (ppm)	Identification	% change	CV for QCs (%)
Olemide (18:1)	28.35	281.2719	0.16	Standard	73.1	29.47
C16 Sphingosine (16:1)	11.57	271.2506	-1.95	272.1963, 254.2467, 114.0937, 100.0747	-40.5	24.14
C17 Sphinganine (17:1)	9.80	287.2821	-1.09	288.0801, 106.084, 88.0727, 57.0705	40.5	(†)
LysoPC (20:0)	28.30	551.3939	-2.16	552.4012, 184.0739, 104.1076, 86.0974	-26.6	18.75
PC (20:2/18:1)	34.47	795.6228	-15.69	796.5992, 309.2772, 184.0723	49.4	57.22
Lysine	0.57	146.1033	-15.25	Database isotopic distribution	50.0	17.36
Oxoisotretinoin	7.26	314.1879	-0.94	315.1943, 297.1007, 269.1889, 109.1007	-50.5	27.14
Hydroxy-oxo-cholenoic acid	8.75	388.2605	-2.17	389.2555, 371.2583, 355.2620, 149.0227, 71.06, 57.07	40.7	26.04
LysoPC (15:0)	16.86	481.3159	-1.88	482.3212, 184.0731, 104.1066, 86.0966	-25.6 *	29.54
LysoPC (18:0)	21.21	509.3476	-1.06	510.3538, 184.0725, 104.1068, 86.9063	-17.8 *	19.18
LysoPC (20:3)	18.94	545.3425	-10.34	546.3473, 184.0731, 104.1072, 86.0971	-22.9 *	26.41
Oxo-hexadecenal (16:1)	13.95	252.2083	-2.5	253.2127, 209.1879, 141.09, 71.0859, 43.05	10.5 *	6.15
Ethyl-dodecanoic acid (12:0)	26.38	228.209	0.35	229.1355, 89.0603, 87.0456, 43.0557	-16.6 *	22.08
Octadecadienol (18:2)	31.87	266.2631	8.03	267.269, 85.1008, 71.0855, 57.0705, 43.0551	-0.9 *	20.49
Deoxycortisol	6.54	346.2137	-1.99	Database isotopic distribution	21.9 *	19.37

* Compounds found from Jack-knifing.

† The CV for C17-sphinganine is not included, because dilution caused by the QCs preparation limited the detection range of less concentrated metabolites.

CV, coefficient of signal variation. Da: Dalton. PC, phosphatidylcholine. QCs, quality control samples. RT, retention time. VILI, ventilator induced lung injury.

Figure 1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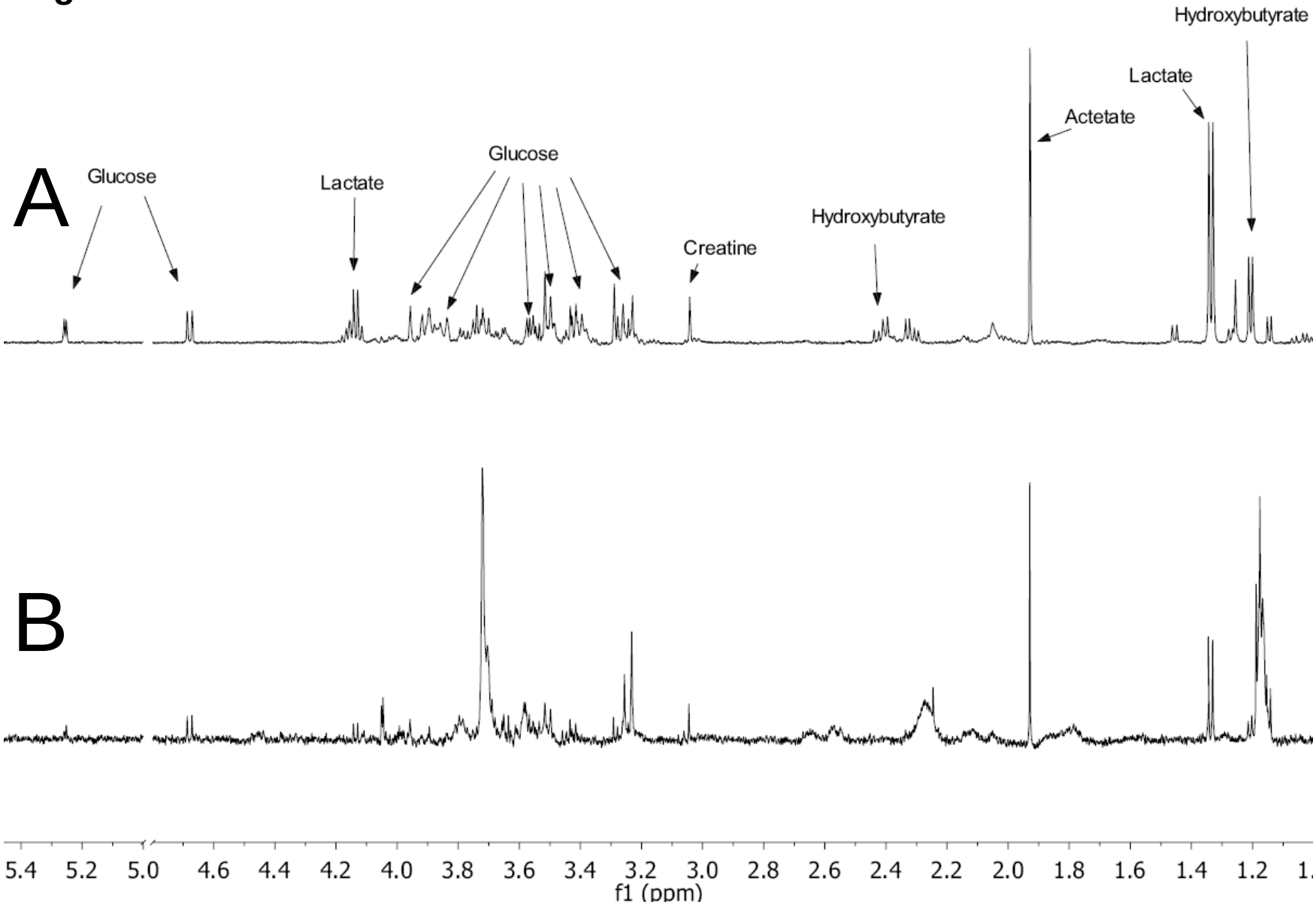
Glucose
Glucose
Lactate
A
B
4.1
3.9
3.7
3.5
3.3
3.1
2.9
2.7
2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
f1 (ppm)

Figure 2


A
Glucose
Lactate
Glucose
Creatine
Hydroxybutyrate
Actetate
Lactate
Hydroxybutyrate
B
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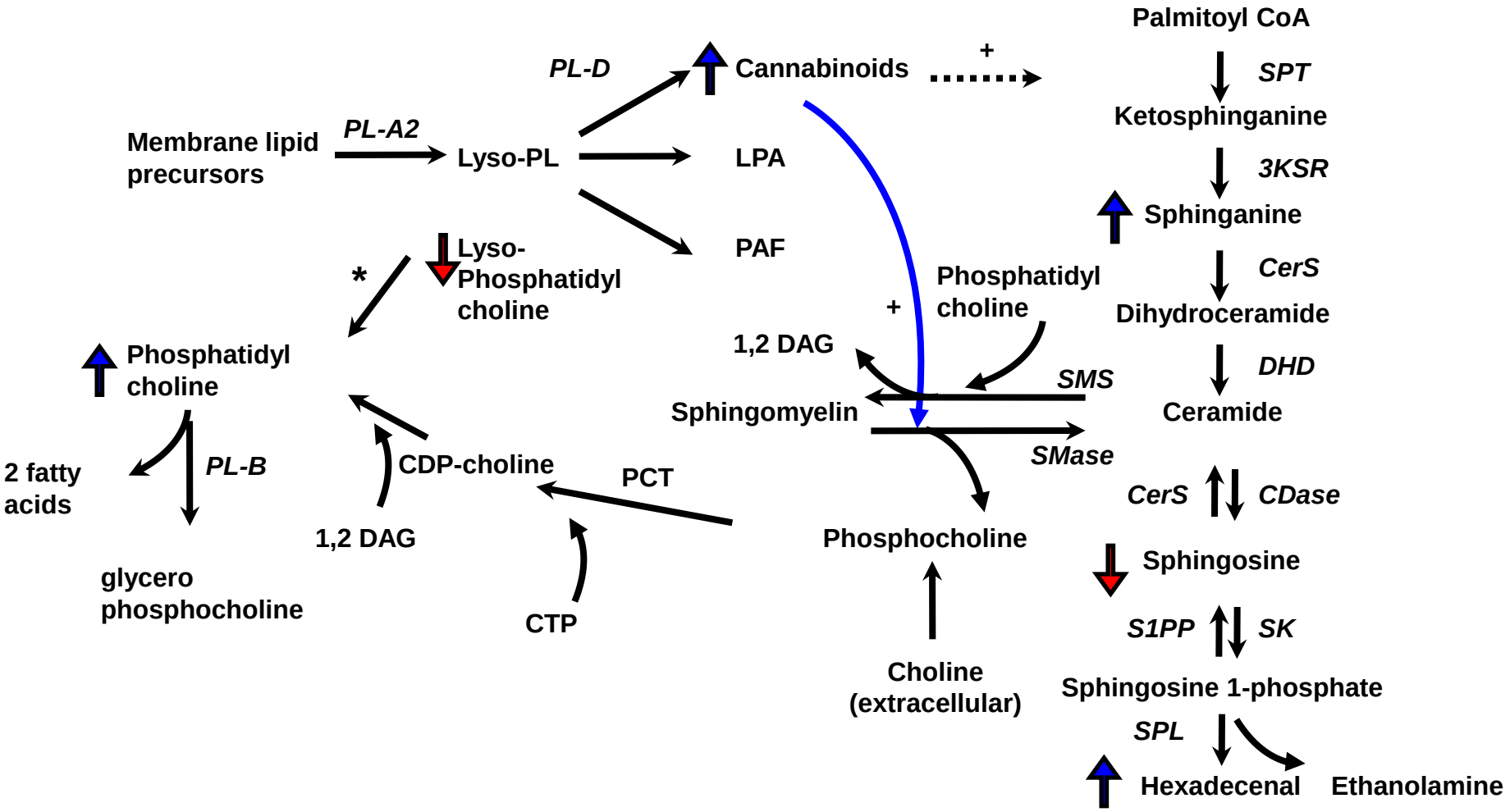
5.2
5.0
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
f1 (ppm)

Figure 3


Palmitoyl CoA
SPT
Ketosphinganine
3KSR
Sphinganine
CerS
Dihydroceramide
DHD
Ceramide
CerS
CDase
Sphingosine
S1PP
SK
Sphingosine 1-phosphate
SPL
Hexadecenal
Ethanolamine
PL-D
Cannabinoids
+
Membrane lipid precursors
PL-A2
Lyso-PL
LPA
PAF
Lyso-Phosphatidyl choline
*
Phosphatidyl choline
+
1,2 DAG
SMS
Sphingomyelin
SMase
Phosphatidyl choline
2 fatty acids
PL-B
glycero phosphocholine
CDP-choline
1,2 DAG
PCT
CTP
Phosphocholine
Choline (extracellular)

Chapter-3

Multiplatform analytical methodology for metabolic fingerprinting of lung tissue

Tissue metabolomics provides site specific information compared to specific bio-fluids measurements, hence the pathological condition of any disease state could be better explained. However the uses of tissue metabolomics are quite limited due to its invasive nature. Since decades clinicians are taking out biopsy sample from the diseased area for diagnostic purposes. Hence the application of tissue metabolomics on the available biopsy samples could provide fruitful information. Despite that another critical challenge for tissue analysis is the sample preparation due to its complex nature, especially its homogenization step. Generally in non-targeted metabolomics researchers focus on extraction procedure more than homogenization. In addition to tissue treatment, use of analytical techniques is also a major concern in non-targeted metabolomics. Compared to single, multiplatform approaches provide more metabolite information. The objective of this thesis is to apply the developed method in the quest for screening markers for lung injury. To achieve this goal, primarily an extensive review was accomplished on the recent MS based non-targeted metabolomics study of animal and human tissue.

A general review is accepted in *Bioanalysis* Journal and included in this Chapter-3.

Title: Analytical protocols based on LC-MS, GC-MS and CE-MS for non-targeted metabolomics of biological tissues.

Authors: Naz Shama, Moreira dos Santos Délia Chaves, Garcia Antonia, Barbas Coral

Bioanalysis, Accepted, April 2014.

The developed and validated methodology based on serum along with application, have been described in chapter-1 and in chapter-2, another methodology based on LC-MS on the rat models have been pronounced. Here in chapter-3, a methodology is optimized and validated for lung tissue applying multiplatform techniques. As MS provides much sensitivity compared to other techniques, so LC-MS, GC-MS and CE-MS were used in this study as the chosen analytical platforms. Previous studies have described lung tissue metabolomics using one or two, choosing from the available analytical platforms. This is the first time three different techniques were applied on lung tissue fingerprinting. Moreover a tissue pre-treatment was optimized focusing mainly on homogenization step, postulating the fact that homogenization is a very critical step in tissue metabolomics and it needs special consideration along with other

extraction steps. The primary objective was to obtain a single phase extraction and injecting in all instruments from same extraction procedure. To enhance the intensity in CE-MS it was necessary to increase the sample volume. To follow an easy protocol after homogenization for CE-MS the sample prepared following ultrafiltration. Whereas for LC-MS after extraction samples were directly injected and before GC-MS analysis sample extracts were derivatized. The developed method was then applied for a complete mouse lung profiling for the first time. Several compounds were selected from the profiled list, picking some from each, between and one among all the techniques. The particular compounds were of different classes and varying in their retention time/migration time. The methodology was validated in all platforms in terms of linearity, accuracy, precision, LOD and LOQ. Ultimately the developed and validated methods were then applied on lung samples from an animal model of sepsis and control.

The detailed description of the developed methodology and results of this study is published in *Analytical Chemistry*.

Title: Multiplatform analytical methodology for metabolic fingerprinting of lung tissue.

Authors: Naz Shama, Garcia Antonia, Barbas Coral

Analytical Chemistry, 2013, 85(22):10941-10948

DOI: 10.1021/ac402411n

The published manuscript has been attached with this Chapter-3.

Resumen

MÉTODOS ANALÍTICOS BASADOS EN LC-MS, GC-MS Y CE-MS PARA EL ESTUDIO METABOLÓMICO NO DIRIGIDO DE TEJIDOS BIOLÓGICOS

Autores: Shama Naz, Délia Chaves Moreira dos Santos, Antonia García, Coral Barbas

Revista: Bioanalysis (aceptado con *minor revision*, en marzo 2014 y enviada la revisión en abril).

La muestra de tejido, aunque su obtención es invasiva, proporciona la composición de metabolitos más fiel en una determinada zona del organismo. Por ello, la metabolómica aplicada a tejidos basada en técnicas de separación acopladas con espectrometría de masas, está cada vez más demandada en la investigación clínica. La aplicación de estas técnicas en metabolómica de tejidos, con enfoque no dirigido, permite la identificación de metabolitos muy diversos. Estos hallazgos podrían ayudarnos a entender las alteraciones que se producen a nivel molecular, que también pueden ser aplicadas en la práctica clínica como en el cribado de marcadores para el diagnóstico precoz de ciertas enfermedades. No obstante las muestras de tejidos, al tratarse de órganos heterogéneos, presentan una complicación adicional que debe ser tenida en cuenta para obtener perfiles analíticos amplios, representativos y reproducibles. Este manuscrito sobre estudios metabolómicos no dirigidos basados en MS, resume “el estado del arte” en el tratamiento de tejidos (humanos y animales) incluyendo quenching (inactivación de enzimas), homogeneización y extracción.

Title: Analytical protocols based on LC-MS, GC-MS and CE-MS for non-targeted metabolomics of biological tissues

Article type– Review article

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Abstract

Though invasive, site-specific metabolite information could be better obtained from tissues. Hence, highly sensitive mass spectrometry (MS) based metabolomics coupled to separation techniques are increasingly in demand in clinical research for tissue metabolomics application. Applying these techniques for non-targeted tissue metabolomics provides identification of distinct metabolites. These findings could help us to understand the alteration at the molecular level, which can also be applied in clinical practice as screening markers for early disease diagnosis. However, tissues as solid and heterogeneous samples pose an additional analytical challenge that should be considered in obtaining broad, reproducible and representative analytical profiles. This manuscript summarizes the state of art of tissue (human and animal) treatment (quenching, homogenization, extraction) for non-targeted metabolomics with MS.

Key terms

Metabolic fingerprinting: Metabolic fingerprinting can be defined as the global unbiased analysis of all small molecules that collectively constitute the entire metabolome.

Metabolic profiling: Is the targeted measurement of one/some known metabolites involved in a given biochemical pathway. Fingerprinting and profiling are frequently used indistinctively in literature.

Non-targeted analysis: Non-targeted metabolomics is the analysis of all possible small molecules present in a biological system for a particular physiological state in response to external or internal stimuli.

Multivariate data analysis: It involves the observation and analysis of more than one statistical outcome variable at a time, taking into account the effects of all variables on the responses of interest.

Quenching: Quenching is the inactivation of the metabolism. It should be rapid as compared to the metabolic reaction rates to have representative samples.

Extraction agent: An ideal extraction agent in metabolomics should extract as many intracellular metabolites as possible with minimal degradation and no enzymatic, chemical or physical modification of the metabolites.

Introduction

The field of metabolomics is an emerging and promising -omic science in systems biology which is aimed to depict the metabolic profile in complex systems through the combination of data-rich high throughput analytical techniques and multivariate data analysis. Metabolomics investigates single component effects on a biological system and offers a holistic approach in the exploration of the molecular details of multiple factors on an entire biological organism. Metabolomics techniques allows a high throughput analysis of small molecules in bio-fluids and tissues, giving metabolic profiles of the end products. Comparison of metabolic profiles from different phenotypes can be supportive in the identification of metabolic changes and as well as helping to understand the molecular mechanism, integrated biochemical pathways and disease progression [1-3]. There are many metabolites, in biological systems that change much faster than nucleic acids or proteins. Hence metabolites seem to depict more satisfactory changes in biochemical effects in any organism, representing a closer approach to determine biological end-point than genomics, transcriptomics and proteomics. Two different approaches have arisen in this field: targeted and non-targeted approach. The first approach can be defined as the targeted measurement of a selection of metabolites known to be involved in a given biochemical pathway that reflects the dynamic response to genetic as well as physiological modifications or the changes due to external stimuli in unicellular to multicellular biological systems [4-5]. On the other hand, the non-targeted approach is the global unbiased analysis of all small molecules that collectively constitute the entire metabolome and serves as a direct signature of biochemical activity in any sample of interest giving more information than targeted approach as it analyses all possible metabolites [6-7]. The non-targeted approach has been applied to different bio-fluids (urine, plasma/serum etc.) and tissues [8-13]. Though collection of tissue is invasive but tissue

metabolomics has many advantages over bio-fluids. The metabolomics modifications and the upstream regulations are first seen in tissue. Moreover, the pairwise comparison of tissue taken from diseased and non-diseased regions could reflect the interactions despite any individual differences. Global determination of metabolite concentrations in the tissues provides novel anatomical aspects of pathological conditions that cannot be obtained from target-specific fluid measurements. Providing more relevant information than systematic bio-fluids, tissue metabolomics has a greater importance in biomedical research. So far, many studies have already shown the applicability on a variety of animal tissues for metabolomics including liver, kidney, lung, brain and spleen from both rodents and other models [14-17]. Tissue metabolomics has been facilitated with the advances in nuclear magnetic resonance spectroscopy (NMR) and high resolution mass spectrometry (MS). Though NMR is characterized by a high technical reproducibility, fast analysis, and robust quantification of compounds, the intrinsic limitations of NMR are its poor sensitivity and signal overlap. The high resolution magic angle spinning NMR spectroscopy (HR-MAS NMR) is an ideal technique for the investigation of intact tissue specimens (10–50 mg) and permits spectra to be obtained with a resolution comparable to that observed in solution in a time that does not exceed a half of an hour for a routine analysis. However, poor sensitivity hampers the detection of low concentrations of metabolites, which could be important chemical biomarkers. In contrast, MS-based non-targeted metabolomics with separation techniques such as liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), provides higher sensitivity and molecular specificity [15]. For non-targeted tissue metabolomics analysis, separation with LC coupled to MS enables the most comprehensive metabolite coverage achievable to date but there are no single analytical technique covering the entire spectrum of the metabolome. Thus multiplatform approaches

including different separation techniques or even direct infusion and MS are all being employed in order to extend the wider metabolite coverage [10, 16]. Recent advances by coupling GC×GC and matrix assisted laser desorption ionization-time of flight (MALDI-TOF)-MS have also been applied in non-targeted analysis [18-19]. The use of GC×GC has shown to produce comprehensive enhanced metabolic coverage compared to conventional GC-MS and NMR [20-21]. One of the critical steps in non-targeted metabolomics studies is the structural characterization of identified metabolites especially when the compound of interest is of low concentration. A recent article has been published suggesting a workflow to overcome this limitation and increase the number of identified metabolites using LC-MS non-targeted metabolomics applied to brain tissue, liver, astrocytes, as well as nerve tissue [22]. The global metabolomics study involves differential comparison of healthy subject or treatment group without a prior knowledge of any metabolites. Therefore, possible applications of non-targeted tissue metabolomics approach to a variety of tissues was applied in order to discover clinically relevant biomarkers using different tissue types (lung, liver, brain, pancreatic, adipose, heart & kidney) to understanding disease and other biological processes, the effect of nutrition on health, the understanding the mechanisms of drug action, metabolism, or toxicity etc. [10, 23-29]. Researchers are also focusing on whole organ or animal profiling using non-targeted approach [30].

The aim of this review paper is to discuss the different challenges in the MS based non-targeted tissue metabolomics approach focusing sample preparation protocols, discussing different separation techniques and their application on different tissue types till date.

Major challenges in tissue sample preparation:

Tissue metabolites provide valuable insights into the biochemistry of disease, toxicity and response to drug administration and normal physiological characteristics because they contain an extraordinary amount of biological information, written in the language of cells, genes, proteins and metabolites [4, 31-32]. However, sample preparation remains a crucial variable in obtaining the most accurate information. The choice of sample pre-treatment methods which is an essential step, affects not only on the molecular features but also the biological interpretation of the obtained chromatographic data. The workflow describing the non-targeted tissue metabolomics is presented in Figure 1. The most common steps involved in all tissue fingerprinting comprise homogenization and metabolite extraction, in order to measure multiple small molecules to produce unique metabolic profiles.

Origin based tissue homogeneity:

Compared to bio-fluids, tissue collection and their homogeneity generate intrinsic challenges in non-targeted metabolomics. Muscle or fat samples may be expected to be quite homogenous but most other tissue types are not, liver has five different topographic lobes and they have different levels of enzymatic systems, kidney is another well-known heterogenic tissue type with medulla, cortex and multiple cell types with different structure and function throughout the nephron, and the brain is even more complicated [33]. Tissue from such organs could give rise to region specific results thus the region should be well defined before analysis is undertaken. Otherwise, the result may be erroneous and misleading. In cancer biology, tumors are being focused for non-targeted approach in order to understand the different metabolites between tumorous and non-tumorous region [34]. Though tumor tissue should be composed of the same cell type regional

differences are still present [35]. For example, in tumor itself there may be regions which are well oxygenated, where as other regions are not. Thus during sample collection care should be taken to collect sample from the same region for each sampling to avoid factors such as biological variability. Alternatively whole tissue or cross-sectional tissue analysis can help in overcoming this inconsistency.

Tissue collection and quenching metabolism:

Generally tissue samples are collected under anesthesia treatment in a randomized manner following ethics guideline. In order to avoid contamination from the anesthetic drug or blood, after collection the sample is usually thoroughly washed with deionized water or buffer [10, 22, 36]. Very interesting research has been published recently, where the effect of blood on liver analysis was evaluated by comparing perfused and non-perfused mice liver applying the non-targeted approach [37]. As blood circulates through different organs, it can carry other metabolites that are non-specific to liver and which could enhance the chances of overlapping or diluting the liver specific metabolites. The magnitude of metabolite contaminations from blood were seen with the elevated amounts of some amino acids, organic acids and sugars, with a clear overlap of blood and tissue metabolite profiles. Not only that, the study also confirmed the alterations in major blood related proteins through proteomics study.

One of the main limitations in tissue metabolomics analysis is the variation in metabolism within the tissue. The metabolism in tissue starts changing in seconds and metabolites are highly unstable at high temperature and easily degrade during ultra-sonication or homogenization. Thus quenching of metabolism to stop any metabolic reaction in the sample is mandatory. This quenching is usually obtained by using any one of the following, shock freezing with very low

temperature (usually liquid nitrogen -195.8 °C) or denaturing the enzymes with acid or solvents as fast as possible after tissue collection [38 - 41].

Homogenization:

The first need of tissue extraction is the physical disruption of the tissue sample in order to enable proper access of the extracting solvent to the tissue and as well obtaining a homogenous solution. Conventionally, breakdown of the frozen tissue achieved by grinding in liquid nitrogen cooled mortar and pestle or manual degradation of cold tissue with scissors [37] or by homogenizing the frozen tissue using an electric tissue homogenizer [41]. Mortar and pestle have been considered as a gold standard but this method requires considerable care to transfer the now ground tissue and in addition it is very labour intensive and time consuming. In addition, weighting the frozen powder is hardly reproducible due to water condensation, and after adding the solvent for extraction a clot of the frozen powdered tissue can be formed. This clot is very non-homogenously suspended and a further homogenization step is required. Moreover, there is a chance of sample carryover unless the mortar is thoroughly washed before the next sample. Although compared to mortar and pestle, homogenization with probe tip avoids many of the previous problems; it is also susceptible to sample carryover unless the probe is washed thoroughly between extractions. To avoid these problems, mechanical disruption using the Qiagen tissue lyser or Precellys 24-bead-based homogenizer is also used. With these systems multiple tissue samples can be homogenized simultaneously in a high-throughput manner, probably representing the most convenient and repeatable methodology but often not suitable for hard tissues [42-44]. Irrespective of the homogenization method used, tissue preparation is very labour intensive and represents a considerable bottleneck for metabolite profiling. The proper

disruption of tissue must be checked with the microscope. Moreover, these devices are effective for medium to high throughput extraction of metabolites. However, these devices focus on automating the homogenization process, whereas the addition of extraction solvents and sample filtration has not been fully streamlined. Apart from the techniques involved, the need of homogenization is closely linked with the objective of any research. The selection of solvent for homogenization depends on the category of metabolites to be analyzed and separation techniques to be applied. Usually homogenizations are done either with a separate solvent or along with the solvent used for extraction. Most of the methods published in the literature have used methanol in different proportions (100% /80% /50%) as homogenization solvent. Some of them have used deionized water and the rest used the extraction solvent in order to perform homogenization and extraction together. In any case, the homogenization solvent should contain an important proportion of polar solvent to promote the contact with the tissue whose composition is mainly water. A recent study on lung metabolomics applying the non-targeted approach has described the importance of the selection of homogenization solvent and how they affect metabolite coverage [10]. To avoid any metabolite loss during solvent extraction care should be taken during the homogenization and solvent selection.

Metabolite extraction:

The main goal of metabolite extraction in tissue metabolomics analysis is to obtain reproducible results with the broader possible range of metabolites. The primary step in metabolite extraction is the separation of unwanted compounds such as proteins. However the choice of metabolite extraction depends on the analytical tools and the metabolites of interest. Tissue extraction should be: i) as non-selective as possible, for wider metabolite coverage, ii) reproducible, iii) fast

and easy and iv) involving as few as steps as possible, enabling high-throughput analysis. Extraction of metabolites from tissues often is the most labour intensive and the rate limiting step and this is usually achieved either by single phase or biphasic extraction. The biphasic separation has been found to be popular because polar and non-polar small metabolites can be extracted simultaneously and each fraction can be analyzed separately. The biphasic method was first described by *Bligh and Dyer* using chloroform/methanol/water in a proportion to obtain two separate phases [45]. Further investigation on metabolite extraction strategies by *Le Belle and coworkers* also concluded that methanol/chloroform/water is the preferred method and concluded that this method may be mandatory for lipid-rich tissues [46]. Given the hazards associated with chloroform use, Matyash *et al.* proposed the usage of another organic solvent *methyl-ter-butyl-ether* and proved very useful in the extraction of polar and non-polar metabolites [47]. In another study, dichloromethane was used as an alternative to chloroform and proved to be superior [42]. Although multiphase extractions provide a higher number of extracted metabolites with higher concentrations, there are some metabolites which split in both phases creating problems during method validation. Moreover, metabolites which are very low in concentration can be lost due to dilution between phases and two analytical runs are necessary. Considering these facts a single extraction step would be ideal. Most of published works regarding non-targeted tissue metabolomics were focused on single phase separation typically using methanol as extraction solvent or monophasic solvent mixture of methanol/chloroform/water (Table 1). 24 solvent mixtures from six different solvents (methanol, ethanol, isopropanol, acetone, chloroform and water) were tested for a liver study applying the non-targeted approach. The researchers proved that the extraction yield could not be improved either with acetone or isopropanol or water, but that a single phase solvent mixture of methanol/ethanol/water (8.5/1/0.5, v/v/v/) showed better

extractions [37]. Our own study described that using one single phase and multiplatform analysis resulted in a wider metabolite coverage in mouse lung tissue. Not only that, this method was validated selecting metabolites of different physicochemical properties, covering the entire chromatogram in three different platforms in terms of linearity, accuracy, precision and the method proved to be very reproducible [10]. Using either single or biphasic separation, tissue extraction involves multiple steps that include solvent additions, mixing, and centrifugation. This is disadvantageous in terms of being time-consuming; furthermore, each step has the potential for introducing variation into the extraction protocol. The extraction yield and reproducibility does not only depend on the choice of solvent or phase separation. The addition of solvent during extraction also matters. The study of Huifeng *et al.* in developing a high-throughput methodology for the flatfish liver used three different solvent addition strategies. The original slow stepwise addition, the addition of solvents in a more rapid two-step protocol, and an all-in one addition of all solvents simultaneously and the quality of these extraction methods were evaluated based on metabolite yield, extraction reproducibility, and sample throughput. The results showed that the two-step method provided good quality data and more accurate snapshot of the liver metabolome [48]. The two step protocol was later used by Mason *et. al.*, in a non-targeted LC-MS based study of liver metabolite profiling and found optimal [42]. Even in our study a two-step solvent addition protocol has been followed [10]. In order to extract all possible metabolites (from polar to non-polar), it is quite usual to extract tissue sample using both aqueous and organic solvent. Adding them separately could enhance solvent specific metabolite extraction. Want *et al.* also provided detailed protocols for the preparation of animal and human tissue samples for obtaining non-targeted metabolic profiles based on the two steps method [49]. Another crucial point in non-targeted metabolomics is the large scale study sample preparation

and analysis. Usually researchers are attempting to reduce within-experiment analytical variation or any unavoidable sources of measurement error to be introduced which is very true for large-scale multi-batch experiments. In the quest to overcome this problem, the necessity for the development of robust workflows that minimize batch-to-batch variation have been well explained by Kirwan and co-workers [50].

MS based techniques involved in non-targeted tissue metabolomics:

This is a reliable analytical method that helps to make a primary trustworthy assumption from the identified discriminant metabolites. Several separation techniques are available to use along with MS detection such as LC, GC, CE, but also direct infusion can be used with FT-ICR.

LC-MS: LC coupled to MS is a powerful tool for metabolomics because it allows the separation and characterization of the majority of compounds. It can resolve different metabolite groups ranging from hydrophilic to hydrophobic. LC with MS detection has been used extensively in non-targeted metabolomics study rather than other detection method, because it has structural identification capabilities and detection with MS is more sensitive and accurate. However, ion suppression due to co-eluting compounds is the major limitation for LC-MS. The LC separation depends on the molecular properties of the analyte which determines what type of stationary phase (column type) and mobile phase are to be used for a better separation. In tissue metabolomic studies, reversed phase (RP) (C8/C18), normal phase (NP) and hydrophilic interaction chromatography (HILIC) are being used as common stationary phases depending on the specific class of compounds. Mostly published LC-MS based non-targeted tissue metabolomics studies used reversed phased silica based columns with various particle sizes

considering their sensitivity, lower detection limit and applicability to the majority of the compounds. The mixed use of RP with NP or HILIC has also been studied [51]. HILIC based separations are well suited for hydrophilic compounds as with classical RP columns many polar compounds have poor retention, eluting near the void volume. In a recent study, *Haitao Lv et al.* compared eight different categories of column and found that a RP pentafluorophenylpropyl showed better separation than RP C18, without the need for an ion pairing reagent [52]. Usually the gradient for RP based LC separation starts with high percentage of water and less organic solvent and HILIC based starts with mobile phase with high organic content with less aqueous modifiers. Almost all the RP, NP or HILIC based tissue extract separations were following this gradient criteria using water as aqueous and methanol, acetonitrile, isopropanol as organic solvents (see Table 1). The uses of different percentages of formic acid/ acetic acid/ ammonia have also been studied in order to increase metabolite ionization [10, 53-58]. In all cases electro spray ionization (ESI) has been chosen as the ionization mode using only positive or both positive and negative mode. While preparing tissue extract for LC-MS study using RP stationary phases, either using methanol alone or a mixture of organic compounds, a single phase has been preferred in most cases [10, 38, 51, 59-63]. For the combination of RP and HILIC or while focusing only on non-polar compounds, biphasic separations and separated injections of each phase were undertaken [64-65]. LC-MS based studies usually include an MS^n approach to characterize compounds from tissue extract [22]. The introduction of ultrahigh-pressure LC (UHPLC) with smaller column particle, operating at high pressure increases the efficiency by increasing both sensitivity and resolution with shorter analysis time. UHPLC-MS has also been applied on non-targeted tissue analysis resulting in wider metabolite coverage [38, 64]. Nicholson *et al*. have suggested a LC-MS-based workflow for the metabolic profiling of tissues

[49]. LC-MS based non-targeted approach has been used to identify the molecular mechanism or diagnosis of different disease states including cancer, acute kidney injury, Alzheimer's, depression, human immune deficiency virus infection, etc. [23, 54, 59, 60, 66].

GC-MS: GC-MS is the suitable comprehensive analytical tool for identification and quantification of volatile and semi-volatile organic compounds in complex mixtures, as it combines high separation efficiency with selective and sensitive mass detection. Moreover it can be used to identify unknown organic compounds both by matching spectra with reference spectra and by a priori spectral interpretation. Moreover, the compound identification is quite straight forward due to the extensive and reproducible fragmentation pattern obtained in full scan mode. Unlike other separation techniques GC has few limitations. Only thermally stable compounds with high vapor pressures can be analyzed by GC-MS, however, as the samples contain a complex mixture, sample preparation steps are quite long and complicated in order to properly vaporize the analyte in the mixture. Nearly all the published article about GC-MS based non-targeted tissue metabolomics used electron ionization source (EI) and up to now, very few have used exact mass analyzers. Fused silica capillary column with 5% phenyl group or 100% polydimethylsiloxane were used in most cases [36, 67-69]. Along with this a short guard column was also used in most of the cases in order to increase the sensitivity. In order to vaporize the polar metabolites with less thermal stability prior to GC analysis it is necessary to perform chemical derivatization which was usually done with an oximation reagent followed by silylation, or solely silylation with MSTFA or BSTFA with TMCS reagent for tissue metabolomics study [16, 20, 36, 68, 70-86]. Only one author used an alternative derivatization reagent with ethylchloroformate and the derivatization was done in two steps [87]. Many GC-MS

applications alone or in combination with LC-MS have been applied on almost all kinds of tissue type in order to find out the differing metabolites for cardiovascular disease, cancer, depression, age related disease, and as well developing databases (see table 1) [36, 72, 75, 78, 87-89]. Metabolite extractions were mainly focused on single phase using methanol or mixtures of other organic solvents with water. Separation of polar and non-polar metabolites has also been performed and analyzed separately. A two dimensional GC technique has also been applied in several studies [19, 37, 71-72]. The advantage of GC×GC-MS over GC-MS has been studied by *Mal M* in a non-targeted study of colorectal cancer. The study found broad significant metabolic space coverage compared to GC-MS. Moreover, the clustering of quality control samples was better with GC×GC-MS [71].

CE-MS: A major part of metabolites belong to the group of polar and ionic compounds. CE is able to separate a wide range of analytes from inorganic ions to large proteins. CE has an advantage over GC or LC for the resolution of these ionic compounds and even their isomers because CE separates metabolites according to their charge to mass ratio. On the other hand, GC and LC require the interaction with a stationary phase for metabolite separation. This separation criteria makes CE a complementary tool to the more established chromatographic separation technique. CE has more advantages over the other separation techniques, such as the analysis is fast, has high resolution, low cost, requires low sample volume (a few nanoliters) and requires very easy sample preparation steps. Though it has many advantages, CE has not been used extensively for metabolomics study due to several constrains. One of the main reasons behind this is the interface to combine with MS, which is not an easy task. Another factor is the low volume of sample injection. As the amount is very low, sensitivity is low too. The interphase ESI

(with or without sheath-flow) enables metabolites to change from liquid to gas phase and it is the most common interphase used for MS as it is easily adaptable with CE. However there are not many reports based on CE-MS non-targeted metabolomics and a very few related to tissue metabolomics. CE-ESI-MS based non-targeted approach has been applied on lung, adipose, heart, liver, aorta, muscle and on all rat organs [10, 57, 90-92]. Different homogenization and extraction steps were followed either using the monophasic mixture of methanol/ chloroform/water or only methanol. In all cases the extracts were dried and reconstituted before injection to avoid any interruption with the CE current. *Soga T.* has applied several CE-MS based non-targeted metabolomics approaches, following a similar extraction procedure for all tissue types and analytical conditions for analysis in positive and negative ionization modes [17]. Sample extracts were passed through a 5 kDa protein cutoff filter to get a clear solution for injection [57, 90-92]. In our own study we described a multiplatform method validation based on lung tissue using 30 kDa protein cut off filter to remove proteins or other tissue debris from the extract without any analysis problems. Moreover the homogenization and extraction was very simple only with 50 % methanol and later diluting it with equal volume of 0.1 M formic acid. Presently no ideal analytical platform exists, to cover the entire metabolome [10]. Different techniques have distinct advantages to investigate different groups of metabolites. So the use of multiplatform approaches could better characterize the entire metabolome, hence CE-MS can be a perfect complementary tool.

ICR-FT-MS: Ion cyclotron resonance–Fourier transform mass spectrometry (ICR-FT/MS) coupled to ESI source could provide fine resolved ions of small molecule in metabolomics analysis. It enables high throughput global analysis of compounds in a complex matrix with high

mass accuracy and fast identification solely based on the mass to charge ratio of each peak. However the current limitations of FT-ICR MS include lower technical reproducibility, less quantitative analysis, higher detection limit and less information as the identification is based only on the mass to charge ratio without additional information such as retention. ICR-FT-MS based non-targeted approach has been applied on liver, brain and heart tissue [25, 50, 93]. The extraction solvent was either methanol or a combination of methanol/ chloroform/ water to get one or two phases depending on the need of metabolite analysis.

Several studies including our own have described the utility of using multiplatform approaches [10, 23, 29, 39, 40, 57, 65, 88, 94]. We described a multiplatform non-targeted approach on lung tissue using LC-MS, GC-MS and CE-MS from method development, validation and successful application on sepsis and control [10]. The study provided high metabolite coverage and even the application described significant differences whereas sepsis is not related to lung. Moreover the amount of tissue required was minimum compared to other studies. The study showed the same homogenization process and very simple sample extraction protocol for all three platforms could be an idle for all tissue analysis.

Data treatment and pathway integration

Non-targeted metabolomics approach generates huge data sets which need to be handled with careful data handling and mining sense. Several statistical software (univariate or multivariate) are available for data analysis. Multivariate algorithm can often present these data sets as prediction of class separation and it is very important that this prediction is carried out in a relevant way. There are several strategies that are already being used in validating a statistical

model, which we are not going to describe as this is not the goal of this review paper. Generally, normalization of data from animal tissues is based on tissue weight. However, some organs are heterogeneous and may differ in water content among samples, levels of DNA, proteins or even one specific protein can be used for normalization. Data analysis always ends up with one or more statistically significant compounds which can give an insight in to the progress or pathogenesis of specific conditions. These metabolites need to be integrated to the particular pathway in order to find out its relation with specific disease, hence picturing a disease specific biomarker for clinical diagnosis and so on. Along with data mining software several databases are available online in order to identify the significantly different metabolites (such as METLIN, CEU mass mediator, LIPID MAPS etc.). It is necessary to validate biomarker with a target specific analytical method, validating analytical method, proper reference material and applying proficiency test.

Application on different biological tissues and findings

Compared to bio-fluid, tissue homeostasis is principally intracellular rather than extracellular. The physiological state of a complex tissue is reflected in the full complement of various metabolites by its constituent cells. Moreover, biomarkers derived from tumour tissues may provide higher sensitivity and specificity than those from bio-fluids, though obtaining tissue sample is always invasive (endoscopy or biopsy) [20]. Hence, not only bio-fluids but also tissue samples or biopsies are moving more and more into research focus including non-targeted metabolomics approach. The number of publications indexed by the terms non-targeted tissue metabolomics and tissue fingerprinting in PUBMED search is growing exponentially. Figure 2 demonstrates the percentage of publications based on different tissue types from the number of

publications returned with “un/non-targeted metabolomics, fingerprinting, human and animal tissue, mass spectrometry” keywords search of NCBI- PubMed. For clinical diagnosis purpose minimally invasive or non-invasive bio-fluids are preferred and in some cases biopsy samples. But in any condition the changes in metabolism are initiated at tissue level and later on the metabolites are excreted in bio-fluids. Thus non-targeted tissue metabolomics offers the opportunity to understand the site-specific molecular mechanism involved in any disease conditions. This also helps in the identification of potential biomarkers for the early diagnosis in bio-fluids and effective treatment. Till now most of the tissue metabolomics works have been applied mainly to understand the mechanism behind cancer. Application of non-targeted approaches on tumourous and non-tumourous tissue from liver, breast, prostate, colon, esophageal, bladder and gastric cancer revealed almost similar altered metabolic pathways related to glycolysis, amino acid metabolism, tricarboxylic acid cycle and fatty acid metabolism [61, 68-69, 95-98]. However, advanced steps on biomarker development based on the findings are still lacking. Some of the studies found very strong relationships for certain metabolites which proposed to be studied further in a targeted manner in order to obtain clinical markers. For example the ratio of betaine/ propionylcarnitine has been found significant to separate hepatocellular carcinoma patients from hepatitis and cirrhosis; palmitoleic acid has also been found in pancreatic cancer diagnosis and the metabolite ratio of cytidine-5-monophosphate/ pentadecanoic acid for breast cancer [52, 96]. Tissue metabolomics has also been applied on several other diseases. The purpose of several non-targeted tissue metabolomics applications with the sample preparation strategy have been described in Tables 1-8.

Executive summary

Targeted tissue for study

- MS based non-targeted metabolomics approach has been applied on almost all kind of tissue type though mostly on liver.

Homogenization and extraction

- Mechanical disruption of tissue was done using methanol and water as the homogenization solvent.
- 100% methanol and monophasic or bi-phasic mixture of methanol/ chloroform/ water was the most used extraction solvent.

Multiplatform study

- More analytical metabolite coverage was obtained using a combination of LC-MS, GC-MS and CE-MS rather than a single analytical tool.

Future perspectives

By the use of such highly sensitive and reliable MS based non-targeted metabolomics approaches, an improved ability to understand the site-specific mechanism of any disease state can be possible in complex biological specimens. However, the success depends on several factors, such as overcoming the challenges related to sample pre-treatment, spectral acquisition of metabolites, correlating the significant metabolites with biochemical pathways and validation of the identified metabolites in another set of samples applying both qualitative and quantitative approaches. Moreover, the identification of metabolites in non-targeted metabolomics studies is solely based on online databases which are not yet 100% complete. Reproducible spectra fragmentation is very useful for identifying compounds in GC-MS. Moreover with exact mass analyzers the potentiality could be exciting, but exact mass spectral libraries are not commercially available yet. As a result of all of this, many of the metabolites which could have

strong relationships with specific conditions remain unknown. Therefore, another challenge in non-targeted approach is to develop methodologies which will allow the fast identification of these ‘unknowns’. Though till now several applications have been published based on non-targeted tissue metabolomics, none of them have moved forward in order to provide a target specific marker, which requires analyzing a large set of samples as well as validating the strong differentiating metabolites to get reliable biomarkers.

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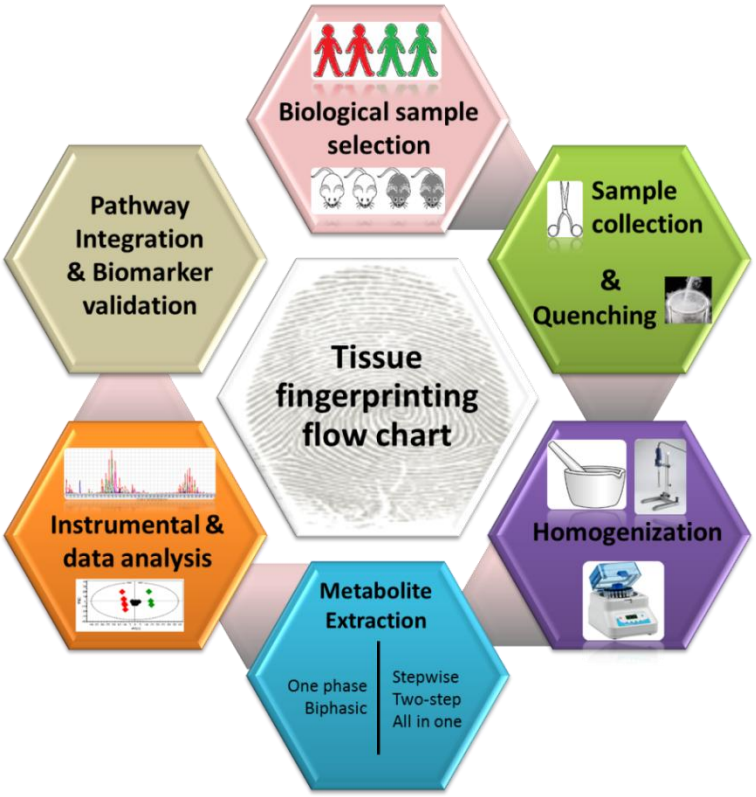

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836 **Figure 1:** General workflow involved in non-targeted tissue metabolomics.

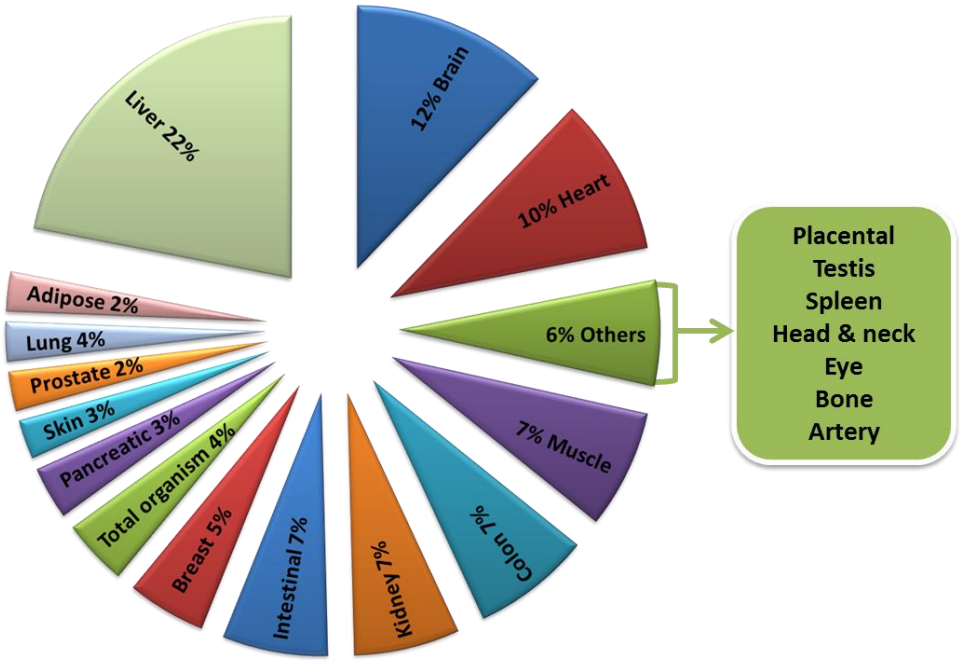

Figure 2: Percentage pie chart based on publications of different tissue types, searching keywords "un/non-targeted metabolomics, fingerprinting, human and animal tissue" in NCBI-PubMed.

Table 1: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**A-B**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Adipose	CE-MS	Understanding the mechanism of visceral fat accumulation and metabolic syndrome between and pre- menopause women.	Addition of IS containing methanol in frozen adipose tissue, homogenization, addition of water and chloroform (500,200,500), centrifugation, filtration by 5 kDa millipore filter, lyophilization, re-suspend in water before analysis.	[90]
Adipose	LC-MS	To identify pathways activated by feed restriction and to understand the contribution of insulin into chicken adipose physiology.	Tissue homogenizing in Liquid N_2 cooled mortar and pestle, metabolite extraction with chilled methanol containing IS, centrifugation, injection of supernatant.	[99]
Bladder	LC-MS	Identification of bladder cancer associated metabolic signature and disclosing the pre-carcinogenic metabolic process.	Tissue homogenization in ice cold 80% methanol containing IS, metabolite extraction with ice cold methanol: chloroform: water, deproteinization, drying the extract, re-suspending in solvent before injection.	[61]
Bone	GC-MS	Identification of metabolic pathways involved in the growth of bone metastases in order to improve cancer prognosis.	Tissue metabolite extraction with water: methanol: chloroform containing ISs using bead mill, extract drying and derivatization prior injection.	[73]
Brain	GC-MS	Evaluating therapeutic effects of antidepressants comprehensively in a depression rat model.	Tissue homogenization in water, addition of solvents, ISs & derivatizing reagent in the supernatant, ultra-sonication, addition of chloroform in the extract, adjusting the pH, repeating the derivatization process, drying chloroform layer with anhydrous sodium sulfate for subsequent GC-MS analysis.	[87]
Brain	UHPLC-MS	To assess the relative analytical power and potential usefulness of UHPLC- MS for studying the global polar metabolite changes in subjects with Alzheimer's disease.	Frozen tissue samples were lyophilized and milled to a fine powder, addition of 50% methanol, mixing, sonication, deproteinization by centrifugation, collection of supernatant for injection.	[60]
Brain	LC-MS	Investigating the link between histology to neural metabolites in rodent models of human immunodeficiency virus infection.	Tissue homogenization in methanol, addition of IS after serial dilution with water, deproteinization, centrifugation, supernatant collection, drying, reconstituting in 50 % acetonitrile, diluting 10000 times before injection.	[53]
Brain	LC-MS	Metabolomics study to see the biochemical mechanism of venlafaxine in brain tissues of rat model of depression.	Tissue homogenization in ice cold methanol, centrifugation, supernatant collection, drying reconstitution in water, addition of chloroform, centrifugation, injecting upper aqueous layer.	[54]
Brain	LC-MS	Global metabolomics analysis of GDE1 (-/-) mice.	Tissue homogenization with cold 80% methanol, sonication & centrifugation. Re-extracting the pellet, drying the combined extracts and reconstitution in water before analysis.	[100]

Table 2: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**B-C**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Brain	LC-MS	To identify the molecular mechanism and potential biomarker for intra uterine growth restriction in a rabbit model.	Addition of methanol: water mixture in tissue, ultra-sonication, centrifugation, drying the supernatant, reconstituting in 60% methanol with 0.1% formic acid and supernatant injection.	[55]
Breast	GC-MS	Investigating the metabolic alterations in breast cancer subtypes.	Tissue homogenization and metabolite extraction with degassed isopropanol: acetonitrile: water, drying and resuspending the extract in 50% acetonitrile, centrifugation, drying and derivatizing supernatant for analysis	[67]
Breast	GC-MS	Identifying the metabolic changes in the central pathways in invasive carcinoma and metabolic markers for breast cancer.	Tissue homogenization and metabolite extracting with isopropanol: acetonitrile: water, centrifugation, drying extracts, reconstituting in 50% acetonitrile, drying and derivatizing before analysis.	[96]
Breast	LC-MS & GC-MS	Elucidating tumor and stromal genomic characteristics that influences tumor metabolism through genomic and metabolomic analyses.	Sample preparation was done on robot system using 96 well plates. Briefly, Tissue homogenization in water, addition of ethanol acetate: ethanol (1:1) containing IS, centrifugation, repeated metabolite extraction from the supernatant with methanol: water (3:1) and dichloromethane: methanol (1:1), centrifugation, concentrating the supernatant before LC injection, and for GC drying and derivatizing.	[63]
Colon	GC×GC-MS	Investigating the global metabonomic profiling of colorectal cancer.	Addition of chloroform: methanol: water in tissue, ultra-sonication, drying & derivatizing supernatant before analysis. .	[71]
Colon	GC-MS	To investigate the metabolic changes in colorectal cancer.	Grinding frozen biopsy tissue, metabolite extraction with monophasic chloroform: methanol: water, centrifugation, drying and derivatizing supernatant before analysis.	[69]
Colon	GC-MS	To classify tumor and normal mucosae metabolic profile.	Tissue metabolite extraction with chloroform: methanol: water and ISs, ultra-sonication, centrifugation, drying the supernatant, addition of anhydrous toluene, drying and derivatizing before analysis.	[74]
Colon	GC-MS	Metabolic profiling of human colon tissue in terms of its sample stability, reproducibility, selectivity, linear response and sensitivity	Addition of monophasic mixture of chloroform: methanol: water in tissue, ultra-sonication, centrifugation, drying the supernatant, addition of toluene, drying and derivatizing before analysis.	[20]
Colon	UHPLC-MS & GC-MS	To determine a distinct metabolic profile during experimental colorectal carcinogenesis.	Tissue homogenization in water, metabolite extraction and deproteinization with methanol, centrifugation, direct injection of supernatant in UHPLC and for GC analysis drying and derivatizing.	[58]
Colon	MALDI – TOF-MS & LC×LC-MS	Focusing on the molecular mechanism of colorectal cancer.	Homogenization in water: methanol: chloroform, sonication, upper phase collection, repeating twice, mixing three upper phases, drying and reconstituting in 50% methanol before analysis.	[65]

Table 3: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**D-H**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Drosophila melanogaster	LC-MS	Establishing a baseline tissue map of Drosophila melanogaster, to show the interactions of different tissues within the whole organism.	Tissue dissection in Drosophila Schneider's medium, monophasic solvent of methanol: chloroform: water, homogenization, centrifugation, collection of supernatant for injection.	[41]
Eye	LC-MS	Characterizing the biochemical differences in vitreous of different animal species.	Addition of acetonitrile in the vitreous sample, deproteinizing by centrifugation, injection of supernatant in the system.	[62]
Gastric mucosa	GC-MS	To test the hypothesis that distinct metabolic profiles are reflected in gastric cancer tissue and exploring potential biomarker for gastric cancer.	Tissue lyophilization, metabolite extraction with mixture of methanol: chloroform, centrifugation, drying supernatant, addition of ethyl acetate, drying and derivatizing before analysis.	[75]
Gastric mucosae	GC-MS	To identify the difference of metabolomic profile between normal and malignant gastric tissue.	Metabolite extraction with monophasic mixture of chloroform: methanol: water and ISs in tissue, ultra-sonication, centrifugation, drying the supernatant, addition of anhydrous toluene, drying and derivatizing before analysis.	[98]
Head & neck	GC-MS	Metabolomics analysis of squamous cell carcinoma of the head and neck	Tissue homogenizing in methanol: water: chloroform, addition of IS and overnight shaking at room temperature, addition of water, centrifugation, drying the upper layer and derivatizing prior analysis.	[13]
Heart	CE-MS	To understand the chamber specific metabolism and pathophysiology in mouse heart.	Addition of IS containing methanol in frozen heart tissues (atria, right ventricle and left ventricle tissue), homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, re-suspend in water before analysis.	[91]
Heart	GCxGC-MS	To optimize the metabolite extraction from mouse heart tissue for GCxGC-TOFMS analysis.	Pulverized tissue in Liquid N_2 with mortar and pestle, re-homogenization with chloroform: methanol on ice, centrifugation after adding 50% methanol, drying and derivatizing the aqueous layer for analysis.	[19]
Heart	GC-MS	Investigating myocardial metabolic changes in depression rat model, to find the links between depression and cardiovascular disease.	Tissue homogenization in methanol containing IS, centrifugation, drying and derivatizing supernatants before GC-MS analysis.	[36]
Heart	LC-MS & CE-MS	To investigate the mechanistic basis of dilated cardiomyopathy in hamsters	Addition of IS containing methanol in tissue, homogenization, (for CE injecting by drying the supernatant and resuspending in water from the homogenate): for LC- addition of water and chloroform, (500,200,500) centrifugation, filtration, lyophilization, re-suspend in water before analysis.	[57]

Table 4: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**H-L**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Hippocampus	ICR-FT-MS	Metabolomics analysis of hippocampus tissues to understand the mechanism of Alzheimer's disease in transgenic mice.	Addition of 80% methanol in tissue, submerging in Liquid N_2, thawing, sonication, centrifugation, supernatant collection, drying, reconstituting in 50% acetonitrile with 0.1% formic acid, diluting 100 times before injection.	[25]
Hippocampus	LC-MS	Toxicological study of a neurotoxin (2,3,7,8-Tetrachlorodibenzo-p-dioxin) in a rat model	Tissue homogenization in methanol, centrifugation, drying and reconstituting the supernatant in 10% acetonitrile before injection.	[101]
Intestine	UHPLC-MS	Identifying metabolite markers of intestinal tissue injury after ionizing radiation exposure in a murine model.	Frozen tissue homogenization in 50% methanol containing IS, addition of acetonitrile, centrifugation, incubation on ice, drying and reconstituting the supernatant in water before analysis.	[102]
Intestine (ileum)	LC-MS	Monitoring the metabolic events associated with the gradual development of Crohn's disease -like ileitis in a mouse model.	Tissue homogenization in EDTA and BHT-buffer, addition of ISs, acidification by citric acid, deproteinization with methanol: ethanol (1:1), centrifugation, drying organic phase, reconstituting in 20% acetonitrile before analysis.	[56]
Kidney	LC-MS	To understand the metabolomic changes of ischemia/reperfusion -induced acute kidney injury and the protective effect of carnitine.	Tissue homogenization in ice cold PBS, centrifugation, supernatant collection for injection.	[59]
Kidney	LC-MS & GC-MS	To analyze the metabolic changes of three proximal tubule nephrotoxins.	Tissue homogenization in water, deproteinization with methanol containing ISs, centrifugation, supernatant injection in LC and for GC drying and derivatizing before injection.	[29]
Kidney	UHPLC–MS & GC–MS	Analyzing the systematic alterations of renal cortex metabolites to explore the related mechanisms of diabetic kidney disease.	Tissue homogenization in methanol containing IS, storing homogenates overnight, centrifugation, injection of supernatant in UHPLC and for GC derivatization before analysis.	[40]
Liver	GC×GC-MS	Evaluating the effect of blood on liver fingerprinting analysis by comparing perfused and non-perfused mice liver.	Tissue homogenization (24 different mixtures of 5 solvents), centrifugation, addition of internal standard (IS) and derivatization prior analysis.	[37]
Liver	GC×GC-MS	Assessing the feasibility of using commercially available software for non-target processing of GC×GC–MS data.	Addition of 80% methanol containing IS, ultra-sonication, centrifugation drying and derivatizing the supernatant before injection.	[72]
Liver	GC-MS	Evaluating metabolomics profile of hepatocellular carcinoma.	Tissue homonization in chloroform: methanol containing IS, diluting with chloroform: methanol, addition of sodium chloride solution, centrifuging supernatant, drying and derivatizing both phases before analysis.	[76]

Table 5: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**L-L**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Liver	GC-MS	Comparing the liver metabolome of specific pathogen-free and germ-free mice.	Tissue homogenization with methanol, addition of water and chloroform, deproteinizing upper layer by centrifugal filtering, addition of IS, lyophilization and derivatization before analysis.	[77]
Liver	GC-MS	To develop an optimized extraction method and comprehensive profiling technique for liver metabolites.	Grinding liver tissue using mortar and pestle, addition of solvent containing ISs, centrifugation, drying and derivatizing the supernatant before analysis.	[78]
Liver	GC–MS	Metabolomics evaluation of the altered biochemical composition after exhaustive and endurance exercises in rats.	Tissue homogenization in methanol, addition of water and chloroform, centrifugation, filtering aqueous layer, addition of IS, drying and derivatizing before analysis.	[84]
Liver	GC–MS	Investigating abnormal metabolic process in both serum and liver tissue of liver transplanted rats.	Tissue homogenization in sodium chloride, addition of acetone and ISs, incubation, centrifugation, drying supernatant and derivatizing for analysis.	[85]
Liver	ICR-FT-MS	To investigate the applicability of FT-ICR MS based metabolomics on biopsy sample to a small but well-defined cohort of patients undergoing liver transplantation.	Tissue homogenizing in 80% methanol, addition of chloroform and water, vortexing, incubation on ice, centrifugation, drying both the polar and nonpolar layers, and reconstituting before analysis.	[93]
Liver	ULPC-MS	To characterize the metabolic profile of steatosis in human tissue and to identify the potential disturbances in the hepatic metabolism of liver damage.	Homogenizing frozen tissue in 80% methanol containing ISs, metabolite extraction with chloroform and ethanol, centrifugation, separation of the layers and injection separately.	[51]
Liver	UHPLC−MS & GC−MS	Metabolomic investigation of the effect of paraoxonase-1 (PON1) deficiency on histological alterations and hepatic metabolism in mice after high-fat high-cholesterol diet.	Tissue homogenization, deproteinization with methanol, supernatant collection, drying, reconstitution in 0.1% formic acid & 6.5 mM ammonium bicarbonate for UHPLC. For GC-MS drying and derivatization before analysis.	[23]
Liver	LC-MS	Evaluating of the metabolic characteristics of tumor tissue and the impact of tumors on surrounding tissue.	Tissue homogenization in cold 80% methanol, ultra-sonication, deproteinization, centrifugation, supernatant collection, freeze drying and reconstitution in80% methanol before analysis.	[66]
Liver	LC-MS	Identification of differential endogenous metabolites and their molecular mechanism in hepatitis B related hepatocellular carcinoma	Tissue homogenization in deionized water, ultra-sonication, centrifugation, supernatant collection, deproteinization, centrifugation and filtering before injection.	[103]
Liver	LC-MS	Identifying the gene expression related to hepatocellular carcinoma and metabolite profiling of nonalcoholic fatty liver disease.	Liver tissue, homogenization in ice-cold methanol with 0.1 % formic acid, centrifugation, supernatant collection and injection.	[104]

Table 6: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**L-L**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Liver	LC-MS	Using data-driven procedures to improve metabolite extraction protocols for mammalian liver metabolomics analysis.	Tissue homogenization in methanol or PBS, dividing supernatant in two parts after centrifugation,, dilution with either 0.1 M formic acid or 0.1 N hydrochloric acid, re-centrifugation, filtering twice, applying solid-phase extraction before analysis.	[105]
Liver & Lung	LC-MS	Integration of in vivo sampling during liver and lung transplantation, sample preparation and global extraction of metabolites using solid phase microextraction.	Solid Phase Microextraction (SPME)	[106]
Liver & muscle	GC–MS	To test how artificial selection for high mass-independent maximal aerobic metabolic rate affects the metabolite profiles in seven generations rat.	Tissue pulverizing under dry ice and Liquid N_2, addition of cold methanol: chloroform, sonication, addition of chloroform: water & ISs, centrifugation, two separate phases processing for analysis.	[86]
Liver & muscle	UHPLC-MS	Developing single metabolite extraction protocol for simultaneously perform targeted and non-targeted metabolomics as well as lipidomics.	Crushing tissue using Qiagen tissue lyser, lyophilization, addition of methanol: *methyl-ter-butyl-ether*: water (two phase) for metabolite extraction, drying and reconstituting separately before injection.	[64]
Liver & muscle	LC-MS & GC-MS	To obtain an unbiased map and understand the metabolic decline during aging-related diseases in mammals.	Tissue homogenization, metabolite extraction with methanol containing ISs, supernatant injection in LC-MS, drying and derivatizing before analysis in GC-MS.	[88]
Liver, aorta, heart & muscle	CE-MS	Characterization of the metabolic imbalances of hypercholesterolaemia in Watanabe heritable hyperlipidaemic rabbits model.	Addition of IS containing methanol in tissue, homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, re-suspend in water before analysis.	[92]
Liver, kidney, heart, intestine & muscle	LC-MS	A broad profiling of hydrophilic metabolites from biological samples using a reversed-phase pentafluorophenylpropyl column.	Tissue homogenizing in 50% ice-cold methanol, addition of chloroform, vortex-mixing, centrifugation, analyze the supernatant.	[52]
Lung	LC-MS; GC-MS & CE-MS	Fingerprinting method validation and application on rat model of sepsis	Tissue homogenization in 50% methanol, metabolite extraction *methyl-ter-butyl ether*: methanol (one phase), centrifugation, injection of supernatant in LC, drying and derivatizing for GC. Deproteinizing the homogenate with 0.1 M formic acid by 30 kDa millipore protein cut-off filter, drying and reconstituting in 0.1 M formic for injection.	[10]

Table 7: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**L-P**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Lung	GC-MS	To identify clinical biomarker for lung cancer	Tissue homogenization with methanol: water:choloroform (2.5:1:1), addition of ISs, centrifugation, addition of water in the supernatant, centrifugation, freeze drying the supernatant and derivatizing before injection.	[16]
Lung & prostate	CE-MS	To understand the tissue-specific tumor microenvironments, in order to development of more effective and specific anticancer therapeutics.	Addition of IS containing methanol in frozen tissue, homogenization, addition of water and chloroform (500,200,500), centrifugation, filtration by 5 kDa millipore filter, lyophilization, re-suspend in water before analysis.	[70]
Muscle & liver	UHPLC-MS	To examine the metabolite alterations in liver and muscle tissues in mice after a high fat diet supplemented with betaine.	Grinding tissue samples with mortar and pestle, metabolite extraction with 90% methanol (100 mg of powdered tissue), centrifugation, and filtering supernatant before injection.	[107]
Pancreatic	GC-MS	Examining the metabolic changes for acute pancreatitis in cerulean- and arginine-induced pancreatitis mice model.	Tissue homogenization in methanol: water: chloroform, addition of IS; centrifugation, addition of more chloroform and water in supernatant, centrifugation, lyophilizing and derivatizing the supernatant before injection.	[79]
Pancreatic	LC & UHPLC-MS	Metabolic pathway identification in pancreatic ductal adenocarcinoma by integrating metabolomics and transcriptomics.	Tissue homogenization, deproteinization with methanol, supernatant collection, drying, reconstitution in 0.1% formic acid & 6.5 mM ammonium bicarbonate for UHPLC. For conventional LC in of 0.1% formic acid (in 10% methanol) before analysis.	[38]
Pancreatic	LC-MS & GC-MS	Identification of the metabolic pathways that are perturbed in pancreatic ductal adenocarcinoma.	Homogenization, addition of IS, protein precipitation, supernatant collection, vacuum drying, re-suspend in acidic solvent for LC-MS analysis and derivatization for GC-MS.	[94]
Placenta	GC-MS	To determine whether the altered O_2 tension affects the composition of the placental metabolome.	Tissue homogenizing with cold 50% methanol & PBS, centrifugation and supernatant collection, repeating process twice, addition of ISs, lyophilization and derivatization before analysis.	[80]
Placenta	UHPLC-MS & GC-MS	To describe methodologies used to interrogate data acquired from a wide range of complex metabolome.	Placental tissue cultured for 96 hours in a serum-based growth medium, suspending in the biomass pellets methanol, freezing in Liquid N_2 and thaw one ice, centrifugation, repeating 3 times. Repeating the extraction again with the pellet, lyophilize the combined supernatant and reconstitution in water for analysis.	[89]

Table 8: MS based application of non-targeted metabolite analysis on different tissue type along with the study purpose and sample pre-treatment: tissue types in alphabetical order (**P-W**).

Tissue Type	Techniques	Purpose of the study	Sample preparation	Reference
Prostate	LC-MS & GC-MS	Focusing on metabolomics signature of prostate cancer in tumor tissue.	Tissue homogenization in deionized water, metabolite extraction with 80% methanol, centrifugation, supernatant collection, direct injection in LC-MS, derivatizing before injecting in GC-MS.	[68]
Prostate	LC-MS & GC-MS	The initial focus was directed towards understanding the tissue metabolomic profiles as they exhibited more robust alterations.	Tissue extractions were performed in 4 steps using solvents (ethyl acetate: ethyl alcohol; methanol; methanol: water, and dichloromethane: methanol) containing IS. Combining all the supernatant, drying and reconstituting 10% methanol and 0.1% formic acid for LC and derivatization for GC analysis.	[108]
Rat (all organs)	CE-MS	Developing Mouse Multiple Tissue Metabolome Database	Addition of IS containing methanol in frozen adipose tissue, homogenization, addition of water and chloroform, centrifugation, filtration, lyophilization, re-suspension in water before analysis.	[17]
Renal	GC-MS	To characterize the key metabolic features of renal cell carcinoma.	Tissue homogenizing, centrifugation, drying the supernatant and derivatization prior analysis.	[81]
Sarcoma	LC-MS	To demonstrate the analysis of polar metabolites extracted directly from formalin-fixed, paraffin-embedded specimens.	Addition of methanol in tissue, incubation, centrifugation, drying the supernatant, re-suspending in HPLC grade water before analysis.	[109]
Skin	GC-MS	Explore the metabolic perturbation associated with ionizing radiation.	Tissues homogenization in ammonium bi-carbonate solution, addition of pre-chilled chloroform: methanol, centrifugation, drying and derivatizing the upper layer before analysis.	[82]
Skin	GC-MS	Analyzing the volatile metabolic signature of a malignant melanoma.	SPME	[83]
Spinal cord	UHPLC-MS & GC-MS	To examine a novel dietary strategy to provide significant anti-nociceptive benefits in rats of pain model.	Tissue homogenizing in water, protein precipitation with methanol containing ISs, centrifugation, drying and reconstituting the supernatant for UHPLC and derivatizing for GC analysis.	[39]
Whole Insect	LC-MS	Metabolomic analysis of the genus Metarhizium and Beauveria (biological pesticides).	Separation of medium from tissues, snap freezing in Liquid N_2, deproteinizing with acetonitrile, injecting the supernatant.	[110]
Whole Mussel	UHPLC–MS	To investigate the sex specific differences in the mussel metabolome to understand their reproductive physiology.	Tissue homogenization in methanol, centrifugation, addition of 5% methanol in sodium acetate buffer in supernatant, purifying, addition of IS, drying, reconstituting in 50% methanol and filtering prior analysis.	[102]

Resumen

METODOLOGÍA ANALÍTICA MULTIPLATAFORMA EN EL ESTUDIO DE HUELLA METABÓLICA DE TEJIDO PULMONAR

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El enfoque metabolómico basado en multiplataformas analíticas en las que se combinen cromatografía de líquidos-espectrometría de masas (LC-MS), cromatografía de gases-espectrometría de masas (GC-MS), electroforesis capilar-espectrometría de masas (CE-MS) y/o resonancia magnética nuclear (RMN), proporciona mayor información sobre el metaboloma, actualmente el principal punto de interés en el área de la metabolómica. No obstante, la realización del tratamiento de muestra, adquisición, preprocesamiento y análisis de los datos, de forma fiabilidad y adecuada resulta indispensable para la correcta interpretación biológica en estudios de metabolómica. Se deben conocer las características en cuanto a la eficacia de la metodología global aplicada a fin de poder encontrar diferencias significativas entre muestras. Esto lleva a la realización de la validación del método aplicado y así asegurar su eficacia y su adecuación al uso en la investigación metabolómica. El presente estudio se diseñó para desarrollar y optimizar un estudio global de huella metabólica (enfoque no dirigido), aplicado a tejido pulmonar, basado en técnicas analíticas múltiples y complementarias, siendo la primera investigación de esta índole en la literatura científica. Se ha realizado la optimización del método analítico (incluyendo tratamiento de muestra y método de análisis), caracterización y validación así como su aplicación a muestras reales. Se ha optimizado la composición del disolvente inicial utilizado en la homogeneización del tejido, algo que no se suele optimizar en los protocolos de obtención de extractos de tejido. Se aplicó esta metodología a un pool de muestra de tejido de pulmón con el fin de cubrir un amplio rango de metabolitos; se utilizaron los tres equipos basados en las tres técnicas reseñadas. Se han validado los tres métodos para todos los metabolitos en lo relativo a linealidad >0.99 y obteniendo una buena recuperación y precisión en todos los casos. Además se ha aplicado el método con éxito a muestras de tejido pulmonar procedente de ratas con sepsis y se han comparado con controles. Sólo son necesarios 20 mg de tejido y esa mínima cantidad es suficiente para todo el estudio incluyendo las tres técnicas; sólo se ha encontrado un metabolito estadísticamente significativo en común entre LC−MS y CE−MS. Ello prueba la importancia de la aplicación de una multiplataforma analítica en los estudios de metabolómica así como para el descubrimiento de biomarcadores.

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Multiplatform Analytical Methodology for Metabolic Fingerprinting of Lung Tissue

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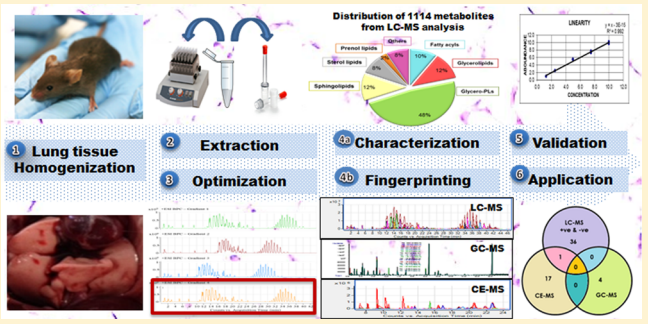
Supporting Information

ABSTRACT: Using multiplatform approaches providing wider information about the metabolome, is currently the main topic in the area of metabolomics, choosing from liquid chromatography−mass spectrometry (LC−MS), gas chromatography/mass spectrometry (GC/MS), capillary electrophoresis−mass spectrometry (CE−MS), and nuclear magnetic resonace (NMR). However, the reliability and suitability of sample treatment, data acquisition, data preprocessing, and data analysis are prerequisites for correct biological interpretation in metabolomics studies. The significance of differences between samples can only be determined when the performance characteristics of the entire method are known. This leads to performing method validation in order to assess the performance and the fitness-for-purpose of a method or analytical system for metabolomics research. The present study was designed for developing a nontargeted global fingerprinting approach of lung tissue, for the first time, applying multiple complementary analytical techniques (LC−MS, GC/MS, and CE−MS) with regards to analytical method optimization (sample treatment + analytical method), characterization, and validation as well as application to real samples. An initial solvent for homogenization has been optimized, which is usually overseen in the tissue homogenization protocol. A nontargeted fingerprinting approach was applied to a pooled sample of lung tissue using these three instruments to cover a wider range of metabolites. The linearity of the validated method for all metabolites was >0.99, with good recovery and precision in all techniques. The method has been successfully applied to lung samples from rats with sepsis compared to the control samples. Only 20 mg of tissue is required for the three analytical techniques, where only one metabolite was found in common between LC−MS and CE−MS analysis as statistically significant. This proves the importance of applying a multiplatform approach in a metabolomics study as well as for biomarker discovery.



Metabolomics is consolidating both as a theranostic, diagnostic therapy for individual patients and as an epidemiological tool to study populations. It utilizes the presence and concentration of small molecules in tissues and body fluids to construct a "fingerprint" which can be unique to the individual and environmental influences.[1] Global determination of metabolite concentrations in the tissues provide novel anatomical aspects of pathological conditions that cannot be obtained from target-specific fluid measurements. Providing more relevant information than systematic biofluids, tissue metabolomics has a great importance in biomedical research. So far, many studies have already shown the applicability of a variety of animal tissues for metabolomics, including liver, kidney, lung, brain, and spleen from both rodents and other models.[2−5] However, no single analytical technique covers the entire spectrum of the metabolome. Thus multiplatform approaches are necessary to extend the coverage, and mass spectrometric (MS) detection coupled to separation techniques such as gas chromatography (GC), capillary electrophoresis (CE), and liquid chromatography (LC) are becoming the most relevant tools due to their high sensitivity relative to nuclear magnetic resonance (NMR). Multiplatform metabolomics has been applied on different biological specimens either for quantitative or qualitative analysis.[6−9] Focusing on a single platform, certain metabolomics research has been applied to lung tissue, but to our knowledge very few have involved multiplatform fingerprinting of lung tissue involving two instrumental techniques chosen from LC−MS, GC/MS, and NMR.[4,10−12]

After instrumental attention, metabolites relevant to a specific phenotypic characteristic are identified, analyzing differences between metabolomes using biostatistics (univariate and multivariate data analysis; pattern recognition). However, the reliability and suitability of sample treatment, data acquisition, data preprocessing, and data analysis are prerequisites for correct biological interpretation in metabolomics studies. The significance of differences between samples can only be determined when the performance characteristics of the entire method, from sample preparation to data preprocessing, are known. Therefore, it is important to perform method validation to assess the performance and the fitness-for-purpose

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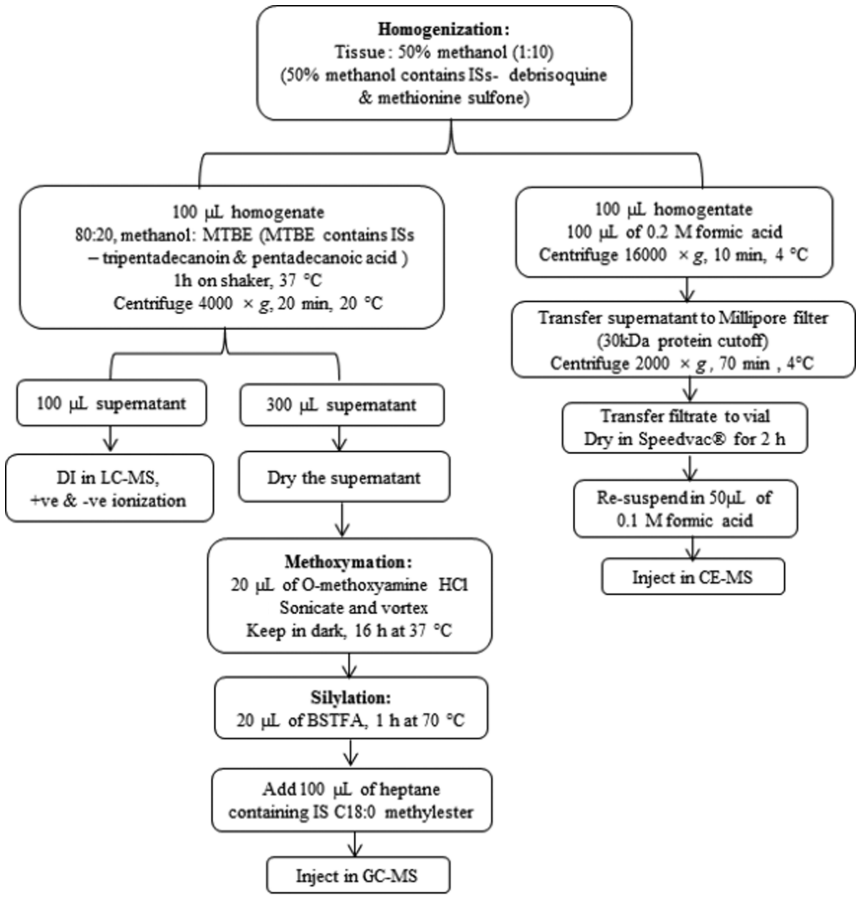

Figure 1. Final lung tissue preparation protocol flowchart for LC–MS, GC/MS, and CE–MS.

of a method or analytical system for metabolomics research. Several guidelines have described the parameters for validation of quantitative methods in terms of calibration (linearity and range), accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ), and limit of detection (LOD).[13,14] So far, there are no guidelines about analytical method validation for metabolomics fingerprinting. Common strategies used to validate the assays include the use of quality control samples (QCs) and the cross validation tool for validating the multivariate statistical model. Some researchers control the system performance, checking the signal-to-noise (S/N) ratio, plate number, retention factor, precision, and even linearity either in pooled samples or by diluting the pooled samples.[8,15,16] Pereira et al. described another way of method validation, in some way common to the quantitative method validation in nutritional plasma metabolomics by calculating the matrix effect and recovery after spiking six different xenobiotic standards. For large batch analysis, the method was validated in terms of the reproducibility of retention times and accurate masses in quality control samples (QCs).[17] Although the metabolic fingerprinting workflow includes the differential analysis of groups of samples in a semiquantitative way, applying the same validation parameters as mentioned above for quantitative analysis of a limited set of compounds with different physicochemical properties distributed along the profile is key for guaranteeing extraction recoveries and reproducibility.

The present study was designed for developing a nontargeted global fingerprinting approach of mouse lung tissue, applying for the first time multiple complementary analytical techniques (LC–MS, GC/MS, and CE–MS). The study was designed with regard to analytical method optimization (sample treatment + analytical method), validation, and characterization of mouse lung tissue through application to real samples. An initial solvent for homogenization has been optimized, which is usually overseen in tissue homogenization protocols, along with other steps of sample treatment using only 20 mg of lung tissue for three platforms. A very sensitive and robust method was optimized for lung tissue fingerprinting by three analytical platforms, validated it in terms of linearity, accuracy, precision, recovery, LOD and LOQ, and successfully applied to lung samples in an animal model with sepsis and control. The identified metabolites were in accordance with previous knowledge in the literature.

MATERIALS AND METHODS

Reagents. All the standards, reagents, and solvents used in this study are described in the Supporting Information (SI), and the compounds for validation are summarized in Table S1 of the Supporting Information.

Instrumentation and Conditions. The final instrumental conditions for lung tissue fingerprinting (detailed in the Supporting Information) were optimized from methods for LC–MS, GC/MS, and CE–MS previously developed in our laboratory.[18−20]

Samples. Information about mouse lung samples for fingerprinting and rat lung samples (control and sepsis) for application of the validated method are described in SI.

Sample Treatment Procedure for Metabolite Extractions. *Homogenization.* Intact lung samples were defrosted

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on the day of analysis from −80 °C and ground into small pieces using a mortar and pestle after dipping them in liquid nitrogen. Three different solvents were used for selecting the best solvent for homogenization, water with 5% formic acid (WF), water:methanol 50:50 (WM), and water:methanol 50:50 with 5% formic acid (WMF). The solvents were added before homogenization with a 1:10 ratio (1 mg of lung tissue: 10 μL of solvent) and then homogenized using homogenizer (HEIDOLPH DIAX 900, made in germany) for approximately 3 min. After performing homogenization, cells were checked under the microscope to ensure that cells are lysed. Finally, the global fingerprinting method validation and application were performed using WM (50% methanol). Internal standards (ISs), debrisoquine (for LC−MS positive ionization) and methionine sulfone (CE−MS positive ionization) were prepared along with the 50% methanol and added into the ground lung before accomplishing homogenization. A summary of the optimization assay is shown in Figure S1 of the Supporting Information.

Extraction procedure for LC−MS and GC/MS. For hydrophobic compounds, extraction methyl-tert-butyl ether (MTBE) was chosen as an organic solvent previously used by Matyash et al.[21] A total of 100 μL of lung homogenate was vortexed with 320 μL of methanol for about 2 min. Then 80 μL of MTBE-containing ISs, tripentadecanoin (for LC−MS negative ionization and pentadecanoic acid for GC/MS), was added, tightly capped as MTBE is very volatile, and placed on a shaker for 1 h at room temperature for metabolite extraction. The extracted samples were then centrifuged at 4000*g* at 20 °C for 20 min. After that, 100 μL of supernatant was transferred to a chromatography vial for LC−MS analysis and 300 μL to a separate vial for GC/MS analysis. For LC−MS analysis, the supernatant was injected directly into the system.

For GC/MS, 300 μL of supernatant was dried using a SpeedVac to eliminate water completely. Methoxymation was performed with 20 μL of *o*-methoxyamine hydrochloride (15 mg/mL in pyridine), sonicated and vortexed 3 times (2 min for each), and kept in the dark for 16 h. For silylation, 20 μL of BSTFA:TMCS (99:1) was added, vortexed for 5 min, and kept at 70 °C for 1 h. Prior to injection, 100 μL of heptane containing an IS for controlling the instrument performance C18:0 methyl ester (10 ppm) was added to each GC vial.

Extraction Procedure for CE−MS. One hundred microliters of lung homogenate was vortex mixed with 100 μL of 0.2 M formic acid and transferred to a centrifree ultracentrifugation device (Millipore Ireland Ltd., Ireland) with 30 kDa protein cutoff for deprotenization through centrifugation (2000*g*, 70 min, 4 °C). The filtrate was then transferred to the chromacol vial, dried using a SpeedVac, and resuspended in 50 μL of 0.1 M formic acid before CE−MS analysis.

Quality Control Samples. QC samples for method application were prepared by pooling equal volumes of lung homogenate from each of the 10 samples. Five samples were independently prepared from this pooled lung following the same procedure as for the rest of samples. QCs were analyzed throughout the run to provide a measurement not only of the system's stability and performance but also of the reproducibility of the sample treatment procedure.[22]

A flowchart for sample treatment is presented in Figure 1.

Nontargeted Fingerprinting of Mouse Lung. To characterize the mouse lung, nontargeted fingerprinting was performed. Six independent lung pools were prepared according to the optimized method and injected in LC−MS, GC/MS, and CE−MS, respectively, for global fingerprinting of mouse lung tissue.

Data Treatment. Precise software details and setting regarding the data processing are in the Supporting Information for all techniques.

Validation Study. The global fingerprinting method was validated in the mouse lung pool in terms of linearity, accuracy, instrumental precision, method precision (both with standards and samples), and LOQ and LOD in three analytical platforms (LC−MS, GC/MS, and CE−MS), according to the validation guide for quantitative metabolomics.

Linearity of the relative response for standards was studied by assaying in triplicate at least five concentrations, covering the expected values ranging from 25% to 200% of mean values found in a preliminary assay. Individual ranges are described in Tables S2−S4 of the Supporting Information for LC−MS, GC/MS, and CE−MS, respectively.

Recovery was estimated by comparing in triplicate the values obtained in spiked samples prepared in the linearity range, taking into account the endogenous concentrations, which had been previously measured in the samples.

Instrumental precision was tested to check the consistency of instrumental response for a given analyte in the midrange of the calibration curve. Instrumental precision was evaluated by multiple injections, $n = 10$, of a homogeneous standard solution.

Intraday ($n = 7$) precision of the method was checked by injecting individual preparations of standards and samples in the midrange of the calibration curve. Interday ($n = 14$) precision was tested in the same way, but on two different days, with the buffer and all the reagents freshly prepared.

The LOQ and LOD were calculated by using signal-to-noise ratio 3 and 10, respectively, according to the IUPAC method.[14]

Working Solutions and Standards. Individual 1000 ppm stock solutions of valine, proline, serine, carnitine, ornithine, citrulline, choline, threonine, histidine, spermidine, glutamic acid, malic acid, hypoxanthine, urea, stearic acid, arachidonic acid, oleic acid, cholesterol, triacylglycerol (18:2), cholesteryl-lester (20:4), disteroyl phosphatidylcholine, and dioleoyl phosphatidylcholine (presented in the validation Table S2−S4 of the Supporting Information) were prepared in their corresponding solvent and stored at −20 °C. From these solutions, an intermediate 10 ppm solution of compound was prepared and stored at 4 °C during the working week and these solutions were appropriately diluted on the day of the analysis.

RESULTS AND DISCUSSION

Metabolomics profiling of biological tissues has become an important area of research in biomarker discovery. However, efficient extraction of metabolites from tissue samples through customized sample preparation procedures is a critical parameter in accurate metabolomics analysis. Thus, a new approach to ensure the detection of a wider metabolite range present in biological tissue using three different analytical platforms with the same sample treatment is more challenging. A critical step in tissue sample preparation is the selection of a homogenization solvent, as this is the first step for extraction which is being overseen most of the time. Three different combinations of solvent for homogenization were tested: WF, WM, and WMF for all platforms in three replicates. Water is necessary for a good contact with the tissue; formic acid could help to break metabolite protein interactions, and methanol is a good cosolvent to act as a bridge for further extraction. The aim

Table 1. Summarizing the comparison of MFE data sets from LC−MS positive, LC−MS negative, and CE−MS positive analyses with three homogenization solvents

filtering	LC−MS positive			LC−MS negative			CE−MS positive		
	WF	WM	WMF	WF	WM	WMF	WF	WM	WMF
100% filter by frequency	2589	3056	2787	569	642	652	483	303	304
RSD < 30%	2263	2780	2555	536	600	607	408	252	263
RSD < 25%	2159	2685	2466	526	585	592	395	240	250
RSD < 20%	2014	2522	2342	508	557	569	370	228	232

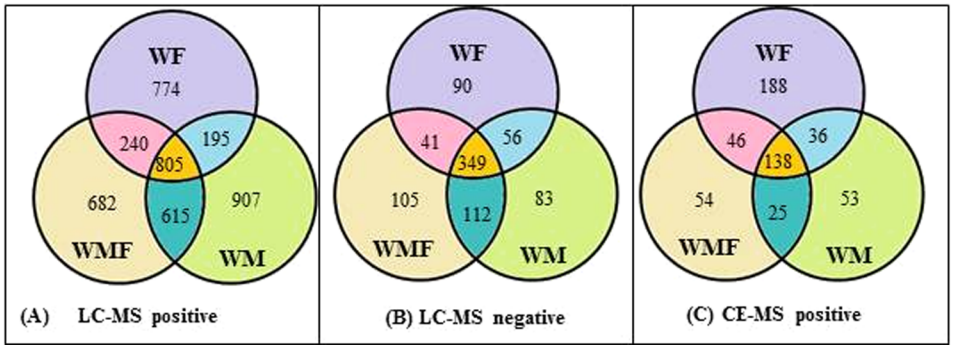

Figure 2. Venn diagrams presenting data for identified features detected in all three replicates of lung pool in three homogenization solvents WF, WM, and WMF [WF−water with 5% formic acid, WM−water:methanol, 50:50, WMF−water:methanol, 50:50, with 5% formic acid and direct injection (DI)]. (A) Comparison in LC−MS positive data after filtering (less than 20% RSD in QCs and 100% frequency in one group). (B) Comparison in LC−MS negative data after filtering (less than 30% RSD in QCs and 100% frequency in one group). (C) Comparison in CE−MS positive data after filtering (less than 30% RSD in QCs and 100% frequency in one group).

of this study was to develop a robust, sensitive, and easy-to-apply protocol for the global fingerprinting of lung sample using three different analytical platforms to cover as much as possible the metabolite range, as well as to validate it as a method to characterize mouse lung metabolic fingerprinting so it could be applied to study a rat model of control and sepsis samples.

Optimization of Sample Treatment and Analytical Conditions. *LC−MS.* Three independent lung pools were prepared for each homogenization solvent to select the optimal one. Accordingly, for each analytical platform, the criteria for choosing a solvent were set up. For LC−MS along with sensitivity and reproducibility, the number of molecular features and categories of compounds were considered. As listed in Table 1 and Figure 2, higher numbers of molecular features were obtained using WM and WMF and at higher rates of reproducibility, represented by more features with a lower % relative standard deviation (% RSD) in both ionization modes. In addition, all three preparations were compared in terms of the number of metabolites found and identified in databases, as well as their biochemical classes. It was observed that broader categories of lipids regarding polarity were identified using WM. More specifically, along with other lipids, cardiolipin and sphingoid bases were only found with WM.

The LC−MS method was optimized, focusing mainly on reducing the analysis time and better separation of polar to nonpolar compounds. Four different chromatographic gradients were tested, and finally a gradient starting with 82% organic solvent was selected, considering shorter analysis time. A comparison of these gradients is presented in Figure S2 of the Supporting Information.

GC/MS. The criteria for choosing an optimum homogenization solvent for GC/MS was concerned with the higher sensitivity and reproducibility of eluted compounds. The idea of adding formic acid instead of adding only water or methanol was to help in the separation of amino acids, short chain amino acids, and all other weak acid compounds. On the other hand, the presence of formic acid in the homogenization solvent could convert the amino acids to free acid promoting the loss of volatile compounds during the drying step as they are in free acidic form (HA). To minimize this loss, the acidic pH in the supernatant for GC/MS analysis was neutralized (pH 6−7) by adding ammonium hydroxide. A comparison of pH neutralization results are summarized by calculating their average total response in triplicate in Figure 3. WM and WMF with neutral pH showed reproducible results compared to others. To select the best option for GC/MS, the identified compounds were divided in several groups, as presented in Table 2, and their better elution and sensitivity were calculated based on percentage. With WM, around 78% of the compounds were

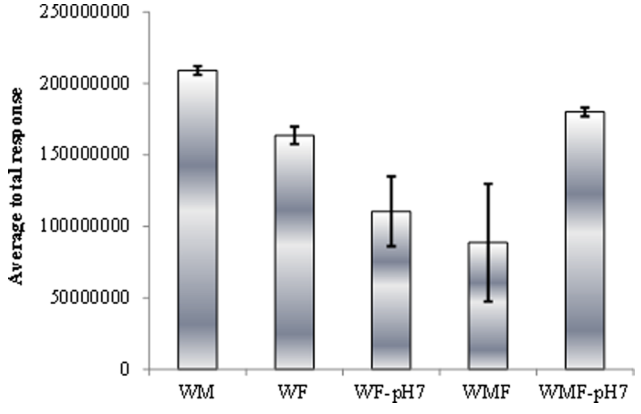

Figure 3. Bar chart showing the relationship between five homogenization solvent used in GC/MS with standard error of mean bar.

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Table 2. Comparison of WM and WMF-pH7 Solvents According to Elution of Six Categories of Compounds in GC/MS

category (no.) of compounds	WM	WMF, pH 7	common
amino acids (16)	9	1	5
free organic acid (11)	8	3	0
free fatty acids (8)	0	6	2
N-containing compounds (10)	9	0	1
sugars (15)	9	4	2
others (6)	5	0	1
% of compounds	78.5	38.5	

eluted with better reproducibility and higher sensitivity as compared to the other.

GC/MS analytical conditions were previously published from our lab and used in this experiment without any modification (described in the Supporting Information).[19]

CE–MS. Developing a sample treatment protocol was more challenging for CE–MS analysis. The primary idea for this research was one time sample preparation and analyzing in three analytical platforms, as described in Figure S1 of the Supporting Information. After the extraction pronounced in the extraction procedure for the LC–MS & GC/MS section, three aliquots were made: 100 μL for LC–MS, 150 μL for CE–MS, and 150 μL for GC/MS. The aliquot for CE–MS was dried under SpeedVac and resuspended in 0.1 M formic acid. The number of molecular features (167) and sensitivity were very poor. Moreover, the retention times were shifting and there were problems with capillary blockage. Thus, another sample treatment protocol using ultrafiltration for CE–MS was tested. The protocol previously described for serum analysis was optimized for lung pool analysis.[20] The sensitivity, reproducibility, and number of molecular features (691) were increased more than three times without any interruption or capillary blocking. The optimized instrumental conditions were then used for selecting the homogenization solvent, taking into account the similar criteria described for LC–MS and summarized in Table 1 and Figure 2. The sensitivity and reproducibility were the same in all cases, but the numbers of features were higher in WF. Similar database hits were observed for WM, WMF, and WF, except some phospholipids that were found with WF, which are in common with LC–MS findings.

However, LC–MS and GC/MS optimization results clearly reflect that WM was the best choice for these two platforms, and in order to have an identical initial sample protocol (homogenization step) for lung tissue, as a compromise WM was also chosen for CE–MS as the homogenization solvent. Since the uncommon features (phospholipids) were in common with LC–MS analysis. The solvent proportion for extraction, methanol:MTBE (80:20) was chosen without further optimization, as it is documented that 80% methanol combined with organic solvents provides better extraction while studying both polar and nonpolar compounds.[8] Another criterion for establishing a robust method is the sample volume. The published literature on lung tissue metabolomics has reported a use of more than 30 mg of tissue either for single or multiplatform metabolomics analysis.[10,11] But with this optimized method, we have identified a broader range of metabolites (covering polar to nonpolar and different biochemical classes), with only 20 mg of tissue for the three techniques. Thus this optimized method is very much applicable to the biopsy sample in biomedical research.

Global Fingerprinting of Mouse Lung. Six independent preparations were injected in three instruments for understanding the global metabolite content (fingerprinting) in mouse lung tissue.

Putatively identified compounds from LC–MS positive and negative ionizations are presented as a percentage pie chart in Figure 4. The tentatively identified compounds are also presented in Table S5 of the Supporting Information, according to their category, subcategory, and numbers presented in both

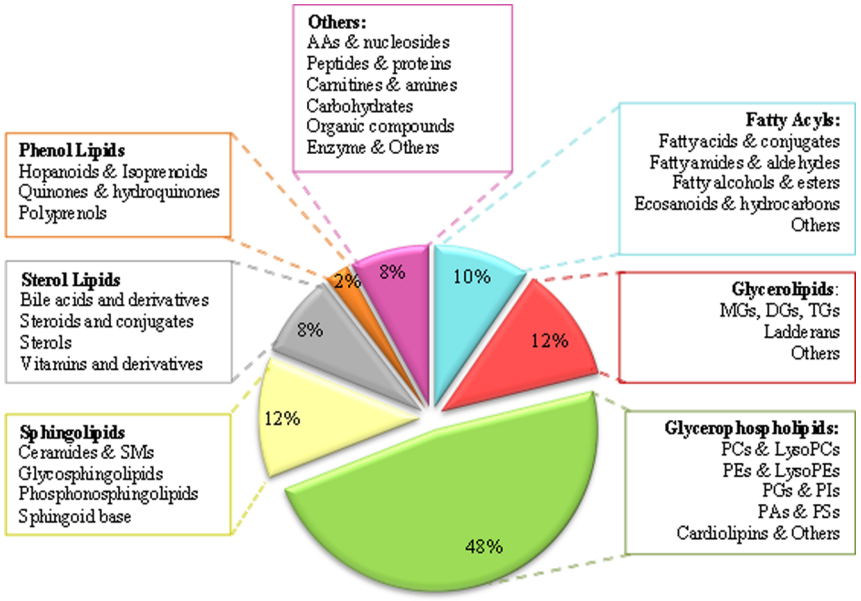

Figure 4. Pie chart representing percentages of different classes of compounds found after nontargeted fingerprinting of mouse lung pool in LC–MS (positive and negative ionization). SPhingomyelins (SMs), monoacylglycerols (MGs), diacylglycerols (DGs), triacylsglycerols (TGs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphoglycerol (PGs), phosphoinositols (PIs), phosphatidicacids (Pas), and phosphatidylserines (PSs).

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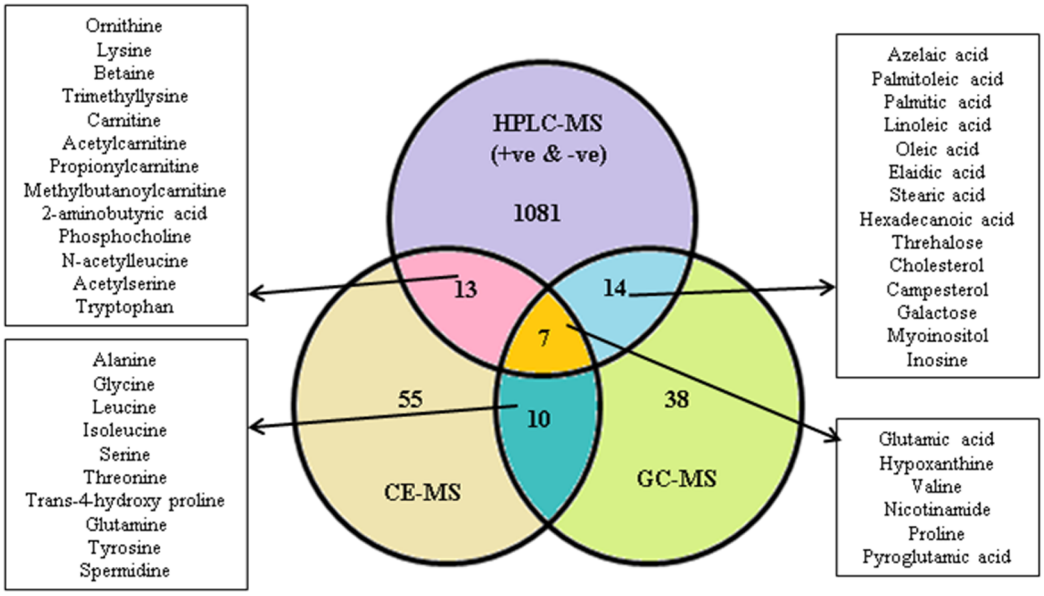

Figure 5. Venn diagram presenting data for identified features detected in all 6 replicates for mouse lung pool compared between the three analytical platforms (LC−MS, GC/MS, and CE−MS).

ionization modes. In positive ionization, 954 compounds were identified, whereas 292 compounds were identified in negative ionization, with 132 compounds identified in common. Among the identified compounds, 48% were glycerophospholipids and 34% were other lipids such as sphingolipids, glycerolipids, sterol lipids, and prenol lipids. Remaining were fatty acyls (12%) and others (8%), such as amino acids, peptides, proteins, neucleoside bases, carnitines, amines, and carboxylic and organic compounds. Compounds were eluted within a specific gradient region in the chromatogram and the marked window in the chromatogram according to their elution region is presented in Figure S3 of the Supporting Information. The compounds eluted according to their polarity. Almost 1114 compounds were identified putatively from LC−MS analysis in positive and negative ionization.

After filtering by 100% of the frequency in the 6 replicates of at least one group and applying a filter of 30% RSD on QCs, out of 210 features, 85 compounds were tentatively identified in CE−MS. The list of profiled compounds along with their migration time, formula, and compound category are presented in Table 6S of the Supporting Information. The identified compounds are mostly amino acids and their derivatives, carboxylic acid and organic compounds, neucleobases, amines, carnitines, and fatty acids. Some dipeptides and tripeptides were also putatively identified. An electropherogram of a lung pool fingerprinting has been presented in Figure S4 of the Supporting Information.

The identified compounds from GC/MS have been marked in the chromatogram for lung fingerprinting with the ISs in Figure S5 of the Supporting Information. The compounds were identified according to the Fiehn and NIST libraries. Mostly amino acids, free fatty acids, and sugars were found among the 69 identified compounds.

Very few studies have been performed on lung tissue using single or multiplatform approaches. In recent times, with the use of NMR high-resolution magic angle spectroscopy, a comparison study has been performed on lung biopsy samples from humans, pigs, rats, and mice, where 39 metabolites were identified from mouse lung biopsy samples.[23] Another metabolomics study on different human organ biopsy samples was carried out using LC−MS and GC/MS, resulting in the identification of aroung 299 compounds from a single lung biopsy.[11] On the other hand, in a CE−MS-based study, 114 metabolites have been identified in lung tissue.[24] Whereas, in our multiplatform study, 1114, 69, and 85 metabolites of different biochemical classes were identified from only 20 mg of lung tissue using LC−MS, GC/MS, and CE−MS, respectively. The relations of identified compounds from these three different analytical platforms are presented in a Venn diagram (Figure 5). Only seven compounds were found in common among these three techniques, including glutamic acids, hypoxanthine, valine, nicotinamide, proline, and pyroglutamic acid. The common compounds between LC−MS and GC/MS were mostly free fatty acids and their derivatives (14 compounds). On the other hand, between LC−MS and CE−MS, the common compounds were amino acids and their derivatives and some carnitines (13 compounds). Ten amino acids were found in common with CE−MS and GC/MS analysis. The fact that very few compounds identified were common between the three techniques proves the usefulness of using multiplatform metabolomics when exploring and covering a wider range of metabolic pathways. As we know, no single technique is enough to cover the entire metabolome. Even from the category of compounds, it can be seen that each technique analyzes a unique category of compounds. Though LC−MS is a very well-known technique to cover a wide range of compounds from polar to nonpolar, it is the combination with CE−MS and GC/MS that adds more information about charged compounds and free and volatile metabolites, respectively.

Validation. A complete validation was performed with 22 different metabolites presented in Table S1 of the Supporting Information for LC−MS (9), GC/MS (10), and CE−MS (10), and considering the retention time or migration time covering the entire chromatogram or electropherogram, different functional groups, polarities, and molecular mass. Several compounds were observed in common among the instruments. In consideration of this fact, some validation compounds were

chosen in common between and among analytical techniques. Valine was chosen in common for three platforms, oleic acid and cholesterol were chosen for LC−MS and GC/MS, proline and serine for CE−MS and GC/MS, and carnitine was chosen for LC−MS and CE−MS. By performing the validation for these representative metabolites a good insight into method performance and reliability of the analytical data for different compound classes of the method could be obtained. The summary of the validation parameters for the selected metabolites is shown in Table S2−S4 of the Supporting Information accordingly for LC−MS, GC/MS, and CE−MS, including both standards and samples and will be briefly discussed. With regard to the validation parameters obtained from mouse lung pool for all techniques, the standards fit the linear model ($r > 0.99$) for all metabolites. No bias was found for GC/MS and CE−MS platform because the confidence limits of the intercept included the zero value but there was little bias in the LC−MS regression parameter.

Recoveries ranged from 87.5% to 107.8% for LC−MS, 90.0% to 98.9% for CE−MS and 89.6% to 107.7% for GC/MS, except for hypoxanthine which was 116.5% and taking into account their RSDs, it did not statistically differ from 100 ($p < 0.05$).

Instrumental precision for LC−MS ranged from 0.5% to 2.4% for standards ($n = 10$). Intra-assay precision ranged from 0.7% to 3.3% ($n = 7$) and interassay precision from 0.7% to 3.7% ($n = 14$). When seven samples prepared from the same pool were treated and run in the same assay, the daily RSDs ranged from 0.6% to 2.9% and from 3.9% to 11.2% in different days.

The GC/MS instrumental precision ranged from 1.4% to 4.5% for standards ($n = 10$). Intra-assay precision ranged from 0.7% to 3.3% ($n = 7$) and interassay precision from 1.8% to 5.0% ($n = 14$). When seven samples prepared from the same pool were treated and run in the same assay, the daily RSDs ranged from 1.2% to 2.5% and from 2.0% to 7.0% in different days.

On the other hand, instrumental precision for CE−MS ranged from 2.2% to 4.7% for standards ($n = 10$). Intra-assay precision ranged from 0.9% to 3.8% ($n = 7$) and interassay precision from 1.7% to 4.9% ($n = 14$). When seven samples prepared from the same pool were treated and run in the same assay, the daily RSDs ranged from 0.6% to 4.9% and from 2.7% to 7.2% in different days.

The theoretical LOD[14] calculated by the signal-to-noise method for these metabolites ranged from 0.0006 to 20.8, 0.04 to 0.006, and 0.06 to 0.5 ppm for LC−MS, GC/MS, and CE−MS, respectively. The LOQ was in the range of 0.002 to 69.4, 1.60 to 0.05, and 0.2 to 1.9 ppm for LC−MS, GC/MS, and CE−MS, accordingly.

Application on Sepsis and Control Lung Tissue Samples. As a proof of concept, the final multiplatform method for global lung fingerprinting was applied on sepsis and control samples from rats. It should be pointed out that in a previous experiment involving murine intra-abdominal sepsis, pulmonary injury was discarded as the etiology of death based on broncheoalveolar lavage levels of pro- and anti-inflammatory cytokines and neutrophils, pulmonary vascular permeability, or histological evidence of damage.[25] Therefore, lung damage was minimum and the methodology should be able to detect slight differences.

Quality Control of the Methodology. QCs were made from homogenized samples before sample treatment and prepared along with the sepsis and control samples. Samples were randomized, and QCs were run at the beginning, in between, and at the end to check the performance of the instrument and also the quality of the method. For quality checking, chromatograms, electropherograms, and identified compounds raw data (data preprocessing as described in the supporting data treatment section) from lung samples and QCs were aligned together giving 1350, 352, 180, and 71 features, respectively, from LC−MS positive, LC−MS negative, CE−MS, and GC/MS analysis. A principle component analysis (PCA) model was built for each technique, considering the two groups and taking into account all variables generated from the mass spectra. The robustness of the analytical procedure was tested by the clustering of the QC samples in the PCA model at the center of the plot (Figure 6, panels a−d of the Supporting Information). The quality of the mode built for two components was acceptable for LC−MS positive with the variance explained, $R^2 - 0.609$, and variance predicted, $Q^2 - 0.0829$; for LC−MS negative with the variance explained, $R^2 - 0.451$, and variance predicted, $Q^2 - 0.292$; for CE−MS with the variance explained, $R^2 - 0.551$, and variance predicted, $Q - 0.059$; and for GC/MS with the variance explained, $R^2 - 0.533$, and variance predicted, $Q^2 - 0.289$.

Sample Classification and Statistical Analysis. To perform sample classification, chromatograms and electropherograms obtained from the 10 investigated lung samples were aligned in Mass Profiler Professional, version B.12.01. Data filtering was performed by choosing the data present in 100% of the samples in any one of the groups and selecting features with RSD less than 35% present in QCs. To discriminate between the groups, both PCA and partial least-squares-discriminant analysis (PLS-DA) models were built for these filtered features. The quality of the model built for two components was very good, considering the variance explained and the variance predicted for LC−MS and CE−MS. For GC/MS, only the PCA model was obtained with reasonable variance classified and predicted; however, a PLS-DA model could not be built.

Statistically significant metabolites were selected by univariate and multivariate statistics. From this, 37, 4, and 8 significant metabolites were putatively identified from univariate and multivariate statistics, respectively, from LC (positive and negative), GC, and CE−MS analysis. Only one compound was in common between LC−MS positive and CE−MS platform, which is propionyl carnitine. The results are presented in a Venn diagram showing the comparison between analyses (Figure S7 of the Supporting Information). The identified significant compounds are in accordance with literature for sepsis in animal models.[26−28] The final identification and interpretation for these compounds is in progress with larger sets of samples.

CONCLUSION

In conclusion, a simple method has been optimized and validated for global lung metabolic fingerprinting using LC−MS, GC/MS, and CE−MS. Compounds eluting in the different profiles have been tentatively identified and are complementary, permitting the detection of a wide range of compounds. This proves the importance of the multiplatform approach of this study in the field of lung fingerprinting. Validation parameters are adequate for bioanalysis. Moreover, the validated method was applied as a proof of concept on sepsis samples caused by cecal ligation and puncture, and the results showed the potential for sample classification even though there are preliminary evidence of no physiological changes in the lung.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.G. and C.B. participated in the research design. S.N. performed sample and data analysis. S.N., A.G., and C.B. contributed to the writing of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information (SI)

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Title: Multiplatform Analytical Methodology For Metabolic Fingerprinting Of Lung Tissue.

Table of content:

Table S6. List of putatively identified compounds from CE-MS lung fingerprinting.

Figure S1. Schematic flow chart planned for mouse lung tissue fingerprinting in three analytical platforms. WF-water with 5% formic acid, WM – water: methanol, 50:50, WMF - water: methanol, 50:50 with 5% formic acid & DI- direct injection.

Figure S2. LC-MS base peak chromatograms from four different gradient conditions.

Figure S3. LC–MS extracted compound chromatogram acquired from the lung pool in - (A) positive ionization & (B) negative ionization. The marked windows are according to the compound class and their elution time.

Figure S4. CE–MS extracted compound electropherogram acquired in positive ionization.

Figure S5. GC–MS total ion chromatogram marking the identified compounds peaks. Panel A and B. (1) Pyruvic acid, (2) L-(+)lactic acid, (3) Glycolic acid, (4) L-alanine 1, (5) Glycine, (6) Oxalic acid, (7) 3-hydroxybutyric acid, (8) L-mimosine 1, (9) DL-3-aminoisobutyric acid, (10) L-valine 2, (11) Urea, (12) Phosphoric acid, (13) L-leucine-2, (14) DL-isoleucine 2, (15) L-proline 2, (16) Glycine, (17) Succinic acid, (18) Catechol, (19) Glyceric acid, (20) Uracil, (21) Fumaric acid, (22) L-serine 2, (23) L-threonine 2, (24) Aminomalonic acid, (25) Nicotinamide, (26) D-malic acid, (27) Aspartic acid 2, (28) trans-4-hydroxy-L-proline 2, (29) L-pyroglutamic acid, (30) L-glutamic acid 1, (31) Creatinine, (32) L-glutamic acid 2, (33) N,O-Bis-phenylalanine, (34) Lauric acid, (35) L-asparagine 2, (36) D-lyxose, (37) Xylitol, (38) Arabitol, (39) Ribitol, (40) Glycerol 1-phosphate, (41) L-glutamine 3, (42) o-phosphocholamine, (43) Azelaic acid. (44) Hypoxanthine-1, (45) 3-phosphoglycerate, (46) Cirtic acid/ isocitric acid, (47)

Dehydroascorbic acid 1, (48) Myristic acid, (49) Fructose 2, (50) D-allose 1/tallose, (51) D-Mannose/ glucose/ galactose/ allose/ altrose, (52) D-allose 2/ mannose 2/ glucose 2/ gluconic acid, (53) Dehydroascorbic acid 4, (54) L-tyrosine 2, (55) Xanthine, (56) Palmitoleic acid, (57) Palmitic acid, (58) Myo-inositol/ all-inositol, (59) Linoleic acid, (60) oleic acid, (61) Elidic acid, (62) Stearic acid, (63) Xanthotoxin, (64) Spermidine 2, (65) D-glucose 6-phosphate 1, (66) D-glucose 6-phosphate 2, (67) Inosine, (68) Hexadecanoic acid, (69) Lactulose/ sucrose, (70) Trehalose, (71) Maltose 1, (72) Cholesterol, (73) Campesterol, IS15:0-pentadecanoic acid, IS18:0-methyl stearate.

Figure S6. PCA models built with the whole data sets for checking the QCs. (A) Scores plot from LC-MS positive analysis. Quality of the model: R2=0.609, Q2=0.0829, (B) Scores plot from LC-MS negative analysis. Quality of the model R2=0.451 Q2=-0.292, (C) Scores plot from GC-MS analysis. Quality of the model R2=0.533, Q2=0.289, (D) Scores plot from CE-MS positive analysis. Quality of the model R2=0.551, Q2=0.059. Empty triangles, sepsis; empty squares, control; filled circles, quality control.

Figure S7. Venn diagrams presenting the comparison of putatively identified significant compounds form rat lung (sepsis and control) among the three analytical platforms (LC-MS, GC-MS and CE-MS).

Materials and methods

Reagents

All 22 standards for validation and five internal standards (IS) present in supplementary information (table S1), were purchased from Sigma (Steinheim, Germany). HPLC grade methanol and analytical grade methyl-tert-butyl ether (MTBE) were from Sigma–Aldrich (Steinheim, Germany); formic acid for MS was purchased from Sigma-Aldrich (St Louis, USA) and ammonia 30 % and sodium hydroxide from Panreac (Barcelona, Spain). Reverse-osmosed purified water from Milli-Q Plus 185 (Millipore, Bedford, USA) was used in the preparation of buffers, standard solutions and dilutions. Reagents for derivatization O-methoxyamine hydrochloride and BSTFA: TMCS, 99:1 (Sylon BFT) were purchased from Sigma-Aldrich (St Louis, USA) and Supelco (Bellefonte, PA, USA) respectively. Chromacol 03-FIV HPLC Vials with fixed 0.3 mL glass inserts (Chromacol, Welwyn Garden City, UK) were chosen and used in all instrumental platforms.

Instrumentations and conditions

LC-MS: The analysis was performed using an high pressure liquid chromatography (LC) system (1200 series, Agilent Technologies, Waldbronn, Germany) coupled to an Agilent quadruple time-of-flight (QTOF) (6520) MS with electrospray ionization source. The HPLC system consisted of a degasser, two binary pumps, temperature controlled auto sampler and column oven. During all analyses, two reference masses were used: *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive ionization mode and *m/z* 112.9856 ($C_2O_2F_3(NH_4)$) and

m/z 1033.9881 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for negative ionization mode. These masses were continuously infused into the system to allow constant mass correction.

An Agilent Poroshell 120 EC-C8 column (150 mm × 2.1 mm, 2.7 µm) with a guard column Ascentis® Express C8 (5 mm × 2.1 mm, 2.7 µm) was used. The chromatographic and mass spectrometric conditions were optimized following a method developed in our laboratory for plasma. [18] In brief, the employed gradient consisted of mobile phase A (5 mM ammonium formate in Milli-Q water) and mobile phase B (5 mM ammonium formate in methanol) pumped at 0.5 mL/min. Initial conditions at time 0 were 82% B, increasing to 96% B in 30 min. This was then held until 38 min. The gradient then increased to 100% B by 38.5 min and held until 40.5 min. The starting condition was returned to by 42 min, followed by an 8 min re-equilibration time, taking the total run time to 50 min. The column temperature was maintained at 60 °C.

Mass spectrometry detection was performed in both positive and negative ESI mode in full scan from 100 to 1200 *m/z*. The mass spectrometer source conditions consisted of a capillary voltage of 3500 V (positive mode) or 4500 V (negative mode), while both ionization modes used a scan rate of 1.02 scans/s: a nebulizer gas flow rate of 10.0 L/min, a source temperature of 350 °C, and a source pressure of 40 psig. The sample tray temperature was maintained at 15 °C and the injection volume was 5 µL for both ionization modes. Data were acquired in MassHunter Work-Station B.05.00 (Agilent Technologies).

GC-MS: The derivatized extracts were analyzed with an Agilent GC instrument (7890A) interfaced to an inert mass spectrometer with triple-Axis detector (5975C, Agilent Technologies). Derivatized samples were injectied in 2 µL volumes in split mode using an Agilent autosampler (7693). The split ratio was 1:5 to 1:10 with 3−10 mL/min helium split flow

into a Restek 20782 deactivated glass wool split liner. Separation was achieved using a 10 m J&W precolumn (Agilent Technologies) integrated with a 122−5332G column: DB5-MS 30m length, 0.25 mm i.d. and 0.25 μm film consisted of 95% dimethyl/5% diphenyl polysiloxane (Agilent Technologies). The helium carrier gas was used at a constant flow rate of 1 mL/min through the column. The column was initially maintained at 60 °C for 1 min which was then increased at the rate of 10 °C/min to reach a final temperature of 325 °C, and held for 10 min before cool-down.

Temperatures of the injector, transfer line, filament source and quadruple were maintained at 250 °C, 280 °C, 230 °C and 150 °C, respectively. The electron ionization source was operated at −70 eV. MS detection was achieved in scan mode over a mass range *m/z* 50−600 at a rate of 1 spectra/s. A mixture of n-alkanes (C8−C28) dissolved in n-hexane was run prior to the experimental samples for retention index determination. Data were acquired using the Agilent MSD ChemStation Software (Agilent Technologies). All these conditions were optimized previously. [19]

CE-MS: The experiments were performed using an Agilent 7100 CE system coupled to an Agilent 6224 Accurate-Mass TOF-MS system (Agilent Technologies, Wilmington, USA). The coupling was equipped with an electrospray ionization source. A 1200 series ISO Pump from Agilent Technologies was used to supply sheath liquid. The capillary for separation was from Agilent Technologies (Germany) with a diameter of 50 μm and length 96 cm. The experiment was carried out in positive ionization mode. Data were acquired using 3 DE-CE ChemStation B.04.02 for CE and MassHunter Work- Station B.05.00 (Agilent Technologies)for MS. The conditions for the separation CE and detection in MS were optimized from a previously developed method in our laboratory. [20] The new capillary was conditioned for 30 min with 1 M

sodium hydroxide, followed by 30 min with 2 M ammonium hydroxide and finally 30 min with background electrolyte (BGE) (0.8 M formic acid in 10% Methanol). Before each analysis the capillary was flushed for 5 min (950 mbars pressure) with BGE. Sample injections were performed over 50 s with 50 mbar pressure. After each sample injection the BGE was injected for 20 s at 100 mbar pressure to improve the reproducibility. The separation conditions included 25 mbar of pressure and 30 kV of voltage. The current observed under these conditions was 20 μA. Before each injection the instrument automatically replaced the BGE.

The MS was operated in positive ionization mode and the other conditions were, drying gas 10 L/min, nebulizer 10 psi, voltage 3500 V, fragmentor 100 V, gas temperature 200 °C and skimmer 65 V. For MS, the total separation time (35 min) was divided into two segments; the first segment was for 1 min without applying nebulization and the remaining 34 min with nebulization.[29] The sheath liquid used for detection in the positive ionization mode consisted of 50 % methanol, 50 % water, 4 μL of formic acid and reference standards (10 μL of 5 mM $C_5H_4N_4$, *m/z* - 121.0509 + 30 μL of 2.5 mM $C_{18}H_{18}O_6N_3P_3F_{24}$, I-922.0098), using a flow rate of 0.6 mL/min (1/100 split). BGE and sheath liquid were freshly prepared and degassed by sonication for 5 min prior to use in order to ensure proper and reproducible ionization.

Samples

Lung pool for non-targeted fingerprinting and method validation:

For method optimization, non-targeted fingerprinting, and validation a pool of lung homogenate was prepared by homogenizing 10 mouse lungs (weighing approximately 1.6 gm), aliquoted in small volumes, kept at – 20 °C and thawed on the day of analysis to avoid freeze thaw effects.

Rat lung samples for method application

Experiments were carried out following the Principles of Laboratory Animal Care (EU 609/86 CEE, Real Decreto 1201/05 BOE 252, Spain) and the research protocol was approved by the Getafe Hospital, Madrid, Spain. Cecal ligation and puncture (sepsis) were performed on six male Sprague-Dawley rats (Harlan Iberica, Spain) and four SHAM rats were used as control. The intact lung was collected from each rats separately, quickly dipped in liquid nitrogen to quench metabolism and kept at -80 ºC for metabolomics studies.

Data treatment:

LC-MS and CE-MS: For global profiling the raw data collected by the LC-MS and CE-MS was cleaned of background noises, and unrelated ions by the Molecular Feature Extraction (MFE) tool in MassHunter Qualitative Analysis Software (B.05.00, Agilent Technologies). The MFE then creates a list of all possible components as represented by the full TOF mass spectral data. Each compound is described by mass, retention time and abundance. Parameters selected for data extraction by MFE were as follows: the limit for the background noise was set to 200 counts, to find co-eluting adducts of the same feature, the following adduct settings were applied: +H, +Na, +NH4 in LC-MS positive ionization, +H, +Na for CE-MS positive ionization and –H, +HCOO for LC-MS negative ionization. Dehydration neutral losses were allowed in both platforms and salt domination for positive ions in LC-MS.

Primary data treatment (alignment and filtering) was performed with the Mass Profiler Professional v.B.12.01 (Agilent) Software. Data were filtered by choosing the data that were present in 100% of all samples under any condition.

For the method application in control and sepsis samples the same MFE algorithm, alignment and filtering were used. Differences between control and sepsis were evaluated for individual metabolites using a *t*-test or jack-knifed confidence interval. The jack-knifed algorithm was used for a partial least square discriminant analysis (PLS-DA) model. Before any statistical calculations, logarithmic transformation of data was performed in order to approximate a normal distribution. Univariate statistical analyses (*t*-tests) were performed using Microsoft excel, where unpaired unequal variance was assumed. Multivariate statistical analysis (jackknifed confidence intervals) as well as other multivariate calculations and plotting were performed using SIMCA-P+ 12.0.1 (Umetrics).

Accurate masses of features representing significant differences were searched against METLIN, KEGG, LIPID MAPS and HMDB databases. The matched compounds were identified not only using the accurate mass but also checking their isotopic pattern. Only those features were kept which were giving higher match. Many of the compounds were identified by matching the retention time from our in-house library. *GC−MS data treatment and identification:* The acquired data represented by Total Ion Current Chromatogram (TIC) were carefully examined by visual inspection of the quality of chromatograms and signal of internal standards. Peak detection and de-convolution were performed automatically using Automated Mass Spectrometry Deconvolution and Identification System (AMDIS) software. Metabolites were identified by comparing their mass fragmentation patterns with those available in the NIST mass spectral library and Fiehn RTL library.

Table S1. List of validation compounds and internal standards (ISs) for LC-MS, GC-MS and CE-MS.

No	Compounds	LC-MS	GC-MS	CE-MS
1	Valine	+*	+	+
2	Proline	-**	+	+
3	Serine	-	+	+
4	Carnitine	+	-	+
5	Ornithine	-	-	+
6	Citrulline	-	-	+
7	Choline	-	-	+
8	Threonine	-	-	+
9	Histidine	-	-	+
10	Spermidine	-	-	+
11	Glutamic acid	-	+	-
12	Malic acid	-	+	-
13	Hypoxanthine	-	+	-
14	Urea	-	+	-
15	Stearic acid	-	+	-
16	Arachidonic acid	+	-	-
17	Oleic acid	+	+	-
18	Cholesterol	+	+	-
19	Triacylglycerol (18:2)	+	-	-
20	Cholesteryl ester (20:4)	+	-	-
21	Disteroyl PC	+	-	-
22	Dioleoyl PC	+	-	-
23	Debrisoquine (IS)	+ (positive mode)	-	-
24	Tripentadecanoin (IS)	+ (negative mode)	-	-
25	Pentadecanoic acid (IS)	-	+	-
26	Methyl stearate (IS)	-	+	-

27	Methionine sulfone (IS)	-	-	+

* + sign denotes compound validated using the corresponding instrumentation

** - sign denotes compound was not validated using the corresponding instrumentation

Table S2. Validation data for selected metabolites in mouse lung tissue with the optimized LC-MS method

Validation criteria	Disteroyl PC	Dioleoyl PC	CE 20:4	TG 18:2	Arachidonic acid	Valine	Carnitine	Oleic acid	Cholesterol
Linearity									
Slope	80.3×10^{7} $\pm7.4\times10^{7}$	$1.5\times10^{7}\pm$ 1.0×10^{6}	$1.3\times10^{6}\pm$ 1.0×10^{5}	$9.6\times10^{6}\pm$ 7.0×10^{5}	$6.0\times10^{5}\pm$ 4.0×10^{4}	$2.0\times10^{4}\pm$ 1.0×10^{2}	$1.3\times10^{6}\pm$ 7.0×10^{5}	$2.0\times10^{4}\pm$ 2.0×10^{3}	$1.7\times10^{5}\pm$ 9.0×10^{3}
Intercept	$-7.0\times10^{7}\pm$ 2.7×10^{7}	$1.2\times10^{7}\pm$ 2.0×10^{7}	$8.0\times10^{4}\pm$ 6.0×10^{4}	$41.9\times10^{6}\pm$ 4.2×10^{6}	$2.0\times10^{5}\pm$ 2.0×10^{4}	$18.1\times10^{4}\pm$ 5.3×10^{4}	$1.0\times10^{6}\pm$ 4.0×10^{5}	$2.0\times10^{4}\pm$ 1.0×10^{4}	$32.0\times10^{5}\pm$ 6.4×10^{5}
r	0.990	0.992	0.992	0.993	0.993	0.995	0.996	0.992	0.996
Range (ppm)	0.1-0.6	3.5-30	0.125-1	1.8-10	0.1-0.6	100-800	1.25-10	0.75-6	15-120
Accuracy (%)	107.8	87.5	104.5	99.9	93.8	98.5	91.8	98.2	97.8
RSD (%)	1.5	3.9	4.8	2.6	1.7	1.9	1.1	2.1	3.9
Instrumental precision (n=10), % RSD	2.4	1.1	1.7	1.2	1.5	1.9	2.1	0.8	0.5
Method precision with standard									
Intra-day (n=7), %RSD	3.3	0.8	1.1	3.0	1.4	0.7	1.8	0.7	0.8
Inter-day (n=14), %RSD	3.7	1.3	1.8	3.3	1.4	0.8	2.0	0.7	0.8
Method precision with sample									
Intra-day (n=7), %RSD	2.9	2.2	2.1	4.1	2.5	0.8	1.8	1.9	0.6
Inter-day (n=14), %RSD	5.2	8.9	5.0	5.6	3.9	11.2	6.7	5.8	3.4
LOD (ppm)	0.006	0.005	0.01	0.0006	0.02	20.8	0.007	0.2	0.4
LOQ (ppm)	0.02	0.015	0.04	0.002	0.08	69.4	0.02	0.6	1.2

Validation criteria	Valine	Urea	Proline	Serine	Malic acid	Glutamic acid	Oleic acid	Stearic acid	Cholesterol	Hypoxanthine
Linearity										
Slope	27.8×10^5 $\pm 2.0\times10^5$	33.4×10^5 $\pm 2.8\times10^5$	31.4×10^5 $\pm 2.6\times10^5$	28.2×10^5 $\pm1.6\times10^5$	33.2×10^5 $\pm2.8\times10^5$	14.0×10^5 $\pm1.3\times10^5$	3.8×10^5 $\pm 0.2\times10^5$	4.9×10^5 $\pm0.4\times10^5$	4.0×10^5 $\pm0.2\times10^5$	2.7×10^5 $\pm0.2\times10^5$
Intercept	-1.5×10^5 $\pm1.2\times10^5$	-29.3×10^5 $\pm17.4\times10^5$	-1.5×10^5 $\pm1.6\times10^5$	-1.8×10^5 $\pm1.0\times10^5$	-3.4×10^5 $\pm2.6\times10^5$	-12.7×10^5 $\pm6.5\times10^5$	-12.3×10^5 $\pm4.2\times10^5$	-8.2×10^5 $\pm4.6\times10^5$	-47.9×10^5 $\pm21.8\times10^5$	-0.2×10^5 $\pm0.3\times10^5$

Table S3: Validation data for selected metabolites in mouse lung tissue with the optimized GC-MS method

r	0.993	0.990	0.991	0.996	0.99	0.991	0.997	0.992	0.995	0.993
Range (ppm)	0.13-1.0	1.25-10.0	0.13-1.0	0.125-1.0	0.188-1.50	1.0-8.0	5.0-40.0	2.5-20.0	18.75-150.0	0.25-2.0
Accuracy (%)	96.3	107.7	98.5	98.9	89.6	94.0	98.8	97.8	91.8	116.5
RSD (%)	4.7	2.6	4.7	4.2	2.8	5.7	3.8	5.6	7.2	3.6
Instrumental precision (n=10), % RSD	3.7	2.1	3.0	3.2	1.4	3.8	1.5	2.3	1.9	4.5
Method precision with standard										
Intra-day (n=7), % RSD	2.5	2.9	3.3	3.2	0.7	4.2	2.4	2.1	1.6	2.7
Inter-day (n=14), % RSD	2.7	3.0	3.3	3.9	1.8	4.3	2.8	2.3	2.0	5.0
Method precision with sample										
Intra-day (n=7), % RSD	1.2	1.4	1.5	1.2	2.0	1.4	2.0	1.6	1.9	2.5
Inter-day (n=14), % RSD	3.4	3.1	4.1	3.0	2.0	6.1	2.3	2.5	3.0	7.0
LOD (ppm)	0.003	0.016	0.003	0.006	0.033	0.01	0.37	0.48	0.33	0.07
LOQ (ppm)	0.01	0.05	0.01	0.02	0.11	0.03	1.22	1.60	1.09	0.21

Table S4. Validation data for selected metabolites in mouse lung tissue with the optimized CE-MS method

Validation criteria	Carnitine	Ornithine	Citrulline	Choline	Threonine	Serine	Histidine	Spermidine	Valine	Proline
Linearity										
Slope	$4.1\times10^{5}\pm 2.0\times10^{4}$	$2.2\times10^{5}\pm 1.0\times10^{4}$	$3.0\times10^{5}\pm 1.0\times10^{4}$	$3.7\times10^{5}\pm 1.0\times10^{4}$	$3.3\times10^{5}\pm 1.0\times10^{4}$	$2.4\times10^{5}\pm 1.0\times10^{4}$	$2.8\times10^{5}\pm 1.0\times10^{4}$	$3.1\times10^{5}\pm 2.0\times10^{4}$	$5.6\times10^{5}\pm 2.0\times10^{4}$	$5.3\times10^{5}\pm 3.0\times10^{4}$
Intercept	$-1.7\times10^{5}\pm 3.4\times10^{5}$	$-5.0\times10^{4}\pm 5.3\times10^{5}$	$4.0\times10^{3}\pm 1.7\times10^{4}$	$2.6\times10^{5}\pm 7.9\times10^{5}$	$-1.3\times10^{5}\pm 1.1\times10^{5}$	$-1.0\times10^{5}\pm 1.2\times10^{5}$	$-7.0\times10^{4}\pm 5.0\times10^{4}$	$-26.2\times10^{5}\pm 17.1\times10^{5}$	$-1.7\times10^{5}\pm 1.7\times10^{7}$	$-1.5\times10^{5}\pm 1.8\times10^{5}$
r	0.997	0.994	0.997	0.998	0.997	0.997	0.996	0.995	0.998	0.996
Range (ppm)	3.75-30	0.75-6	0.25-2	11.25-90	1.5-12	2.25-18	0.75-6	1.875-15	1.5-12	1.25-10
Accuracy (%)	92.0	96.4	98.3	98.9	95.3	94.7	93.8	97.7	90.0	90.4
RSD (%)	3.5	4.3	2.2	2.8	1.8	3.6	3.7	2.2	7.9	2.3
Instrumental precision (n=10), % RSD	3.9	3.6	4.0	2.2	2.2	3.9	3.0	3.6	3.3	4.7
Method precision with standard										
Intra-day (n=7), % RSD	1.4	3.8	1.3	0.9	2.7	3.1	1.3	2.0	1.4	3.8
Inter-day (n=14), % RSD	4.3	4.2	3.9	1.7	3.3	4.1	3.8	3.0	3.4	4.9
Method precision with sample										
Intra-day (n=7), % RSD	4.4	4.5	5.0	0.6	4.1	4.2	3.1	2.8	3.9	4.9
Inter-day (n=14), % RSD	5.0	4.7	5.9	2.7	5.1	4.9	4.2	4.1	3.9	7.2
LOD (ppm)	0.07	0.5	0.06	0.6	0.25	0.2	0.06	0.3	0.16	0.15
LOQ (ppm)	0.25	1.6	0.2	1.9	0.8	0.6	0.2	1.0	0.5	0.5

Table S5. List of putatively identified compounds in mouse lung fingerprinting from LC-MS positive and negative analyses.

Category	Subcategory	LC-MS Positive	LC-MS negative	Common
Fatty Acyls	Fatty acids and conjugates	29	27	6
	Fatty amides	20	5	2
	Fatty Aldehydes	7	0	0
	Fatty alcohols	4	0	0
	Ecosanoids	3	3	0
	Fatty Esters	2	0	0
	Hydrocarbons	9	1	0
	Others	2	3	0
	Total	**76**	**39**	**8**
Glycerolipids	Monoacylglycerols	9	1	0
	Diacylglycerols	38	2	0
	Triacylglycerols	61	0	0
	Ladderans	9	0	0
	Glyceroacyldiacylglycerols	4	6	3
	Others	1	1	0
	Total	**122**	**10**	**3**
Glycerophospholipids	Phosphatidylcholines	167	43	36
	Phosphatidylethanolamines	126	46	39
	Phosphoglycerols	16	31	7
	Phophoinositols	20	17	11
	Phosphatidylserines	59	41	18
	Phosphatidicacids	65	7	3
	Ladderans	6	0	0
	Cardiolipins	1	0	0
	Others	2	1	1
	Total	**462**	**186**	**115**
Sphingolipids	Ceramides	59	2	1
	Sphingomyelins	32	1	1
	Neutral glycosphingolipids	30	4	2
	Phosphonosphingolipids	5	0	0
	Acidic glycosphingolipids	1	1	1
	Sphingoid base	5	0	0
	Total	**132**	**8**	**5**
Sterol Lipids	Bile acids and derivatives	15	0	0
	Steroids and conjugates	15	2	0
	Sterols	34	3	0
	Vitamins and derivatives	22	3	0

	Total	**86**	**8**	**0**
Prenol Lipids	Hopanoids	1	0	0
	Isoprenoids	11	2	0
	Polyprenols	1	0	0
	Quinons and hydroquinons	10	2	0
	Total	**23**	**4**	**0**
Others	Amino acids and derivatives	6	11	0
	Carnitines	11	1	0
	Carbohydrates	3	1	1
	Alkaloids	4	0	0
	Amines	1	0	0
	Dipeptides	2	1	0
	Tripeptides	14	8	0
	Enzyme	1	0	0
	Organic compounds	2	4	0
	Polyketides	5	1	0
	Saccharolipids	4	0	0
	Protein	0	1	0
	Monosaccharides and derivatives	0	5	0
	Nucleosides and derivatives	0	4	0
	Total	**53**	**37**	**1**

Table S6. List of putatively identified compounds from CE-MS lung fingerprinting.

No	Category	Name	Formula	Migration time (min)
1	Amino acids	Alanine	$C_3H_7NO_2$	14.7
2		Serine	$C_3H_7NO_3$	16.1
3		Cytosine	$C_4H_5N_3O$	15.5
4		Proline	$C_5H_9NO_2$	17.4
5		Valine	$C_5H_{11}NO_2$	15.7
6		Threonine	$C_4H_9NO_3$	16.8
7		Leucine	$C_6H_{13}NO_2$	16.5
8		Isoleucine	$C_6H_{13}NO_2$	16.4
9		Aspartic acid	$C_4H_7NO_4$	18.2
10		Glutamine	$C_5H_{10}N_2O_3$	11.2
11		Lysine	$C_6H_{14}N_2O_2$	11.6
12		Glutamic acid	$C_5H_9NO_4$	17.4
13		Methionine	$C_5H_{11}NO_2S$	17.1
14		Histidine	$C_6H_9N_3O_2$	12.2
15		Phenylalanine	$C_9H_{11}NO_2$	18.6
16		Arginine	$C_6H_{14}N_4O_2$	11.9
17		Tyrosine	$C_9H_{11}NO_3$	18.8
18		Citrulline	$C_6H_{13}N_3O_3$	17.5
19		Tryptophan	$C_{11}H_{12}N_2O_2$	17.4
20		Cystine	$C_6H_{12}N_2O_4S_2$	17.7
21	Amino acid	*N,N*-dimethylglycine	$C_4H_9NO_2$	13.2
22	derivatives	Sacrosine	$C_3H_7NO_2$	14.7
23		Betaine	$C_5H_{11}NO_2$	18.2
24		3-hydroxy-pyrroline-carboxylic acid	$C_5H_7NO_3$	16.6
25		Pyroglutamic acid	$C_5H_7NO_3$	16.6
26		4-hydroxy-proline	$C_5H_9NO_3$	18.6
27		Ornithine	$C_5H_{12}N_2O_2$	11.5
28		Methylthreonine/ *N*-hydroxyvaline	$C_5H_{11}NO_3$	16.7
29		2-aminoadipic acid	$C_6H_{11}NO_4$	17.3
30		Carnitine	$C_7H_{15}NO_3$	13.6
31		*N*-methylhistidine	$C_7H_{11}N_3O_2$	12.5
32		*N*-acetylleucine	$C_8H_{15}NO_3$	17.7
33		N^6-acetyllysine	$C_8H_{16}N_2O_3$	14.7
34		N^6,N^6,N^6-trimethyllysine	$C_9H_{20}N_2O_2$	12.0
35		N^G,N^G-dimethylarginine	$C_8H_{18}N_4O_2$	12.8
36		Acetyl carnitine	$C_9H_{17}NO_4$	14.5
37		N^6-acetyl- N^6-hydroxylysine	$C_8H_{16}N_2O_4$	15.2

38		Propionylcarnitine	$C_{10}H_{19}NO_4$	14.9
39		Lysopine	$C_9H_{18}N_2O_4$	15.8
40		Butanoylcarnitine	$C_{11}H_21NO_4$	15.2
41		Methylbutyroyl carnitine	$C_{12}H_{23}NO_4$	15.5
42		Hexanoyl carnitine	$C_{13}H_{25}NO_4$	15.8
43		Hydroxyisovaleryl carnitine	$C_{12}H_{23}NO_5$	15.9
44		Argininosuccinic acid	$C_{10}H_{18}N_4O_6$	15.4
45	Fatty Acyls	2-aminobutyric acid	$C_4H_9NO_2$	12.2
46		8-amino caprylic acid	$C_8H_{17}NO_2$	13.5
47	Amines and	Nicotinamide	C_6H_6N2O	12.3
48	derivatives	Phenylacetamide	C_8H_9NO	17.9
49		Nicotinamide-*N*-oxide	$C_6H_6N_2O_2$	13.5
50		Spermidine	$C_7H_{19}N_3$	8.9
51		Fucosamine	$C_6H_{13}NO_4$	20.6
52		Spermine	$C_{10}H_{26}N_4$	8.5
53		Choline	$C_5H_{13}NO$	11.2
54		Acetylcholine	$C_7H_15NO_2$	13.1
55		Phosphocholine	$C_5H_{14}NO_4P$	17.4
56	Carboxylic acids	Pipecolic acid	$C_6H_{11}NO_2$	16.8
57		Dihydroorotic acid	$C_5H_6N_2O_4$	9.5
58	Organic compounds	Creatine/ Guanidinopropionic acid	$C_4H_9N_3O_2$	14.4
59	and derivatives	Allopurinol	$C_5H_4N_4O$	18.9
60		Hypoxanthine	$C_5H_4N_4O$	18.9
61		Pyrimidine acetic acid	$C_6H_6N_2O_2$	13.5
62	Nucleaobases	Dimethyluracil	$C_6H_8N_2O_2$	13.4
63		Cytidine	$C_9H_{13}N_3O_5$	15.5
64		Thiamine	$C_{12}H_{16}N_4OS$	10.9
65		Adenosine	$C_{10}H_{13}N_5O_4$	13.3
66		Guanosine	$C_{10}H_{13}N_5O_5$	20.3
67		*S*-Adenosyl-homocysteine	$C_{14}H_{20}N_6O_5S$	14.3
68		*S*-Adenosyl-methionine	$C_{15}H_{22}N_6O_5S$	11.9
69	Peptides	Ala-Gly	$C_5H_{10}N_2O_3$	11.2
70		Gly-leu	$C_8H_{16}N_2O_3$	14.7
71		Glu-Gly	$C_7H_{12}N_2O_5$	18.8
72		Val-Ser	$C_8H_{16}N_2O_4$	15.2
73		Ile-Ser	$C_9H_{18}N_2O_4$	15.8

74	Ile-Val	$C_{11}H_{22}N_2O_3$	16.5
75	Lys-Val	$C_{11}H_{23}N_2O_3$	11.4
76	Cys-Homocysteine disulfide	$C_7H_{14}N_2O_4S_2$	16.5
77	Glu-Leu	$C_{11}H_20N_2O_5$	19.9
78	Glu-Glu	$C_{10}H_{16}N_2O_7$	20.2
79	Ser-Ala-Asn	$C_{10}H_{18}N_4O_6$	15.4
80	Ala-Trp-OH	$C_{19}H_{17}N_3O_6$	22.5
81	Tyr-Ser-Lys	$C_{18}H_{28}N_4O_6$	13.3
82	Ile-His-Gln	$C_{17}H_{28}N_6O_5$	14.8
83	*S*-Glutathionyl-cysteine	$C_{13}H_{22}N_4O_8S_2$	18.5
84	Arg-Trp-His	$C_{23}H_{31}N_9O_4$	14.1
85	Glutathione disulfide	$C_{20}H_{32}N_6O_{12}S_2$	19.1

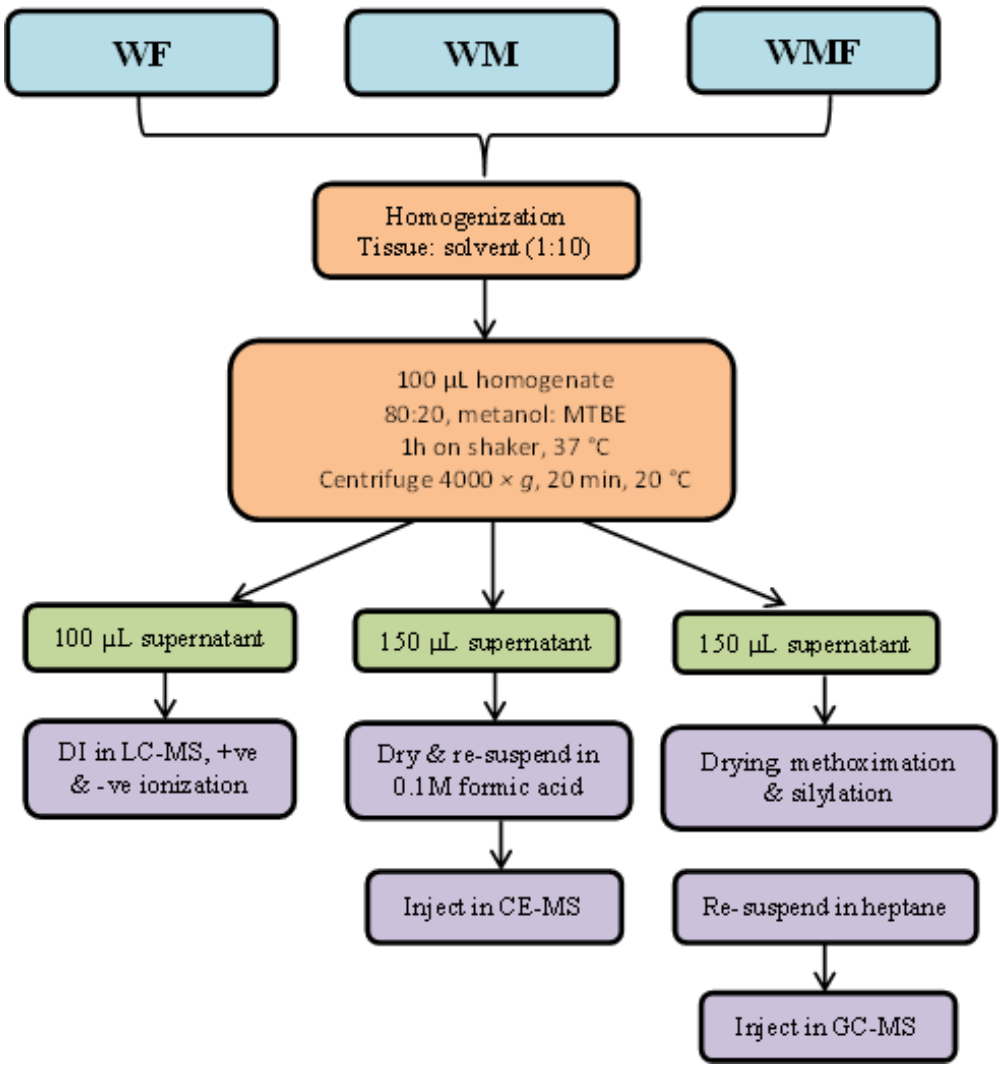

Figure S1. Schematic flow chart planned for mouse lung tissue fingerprinting in three analytical platforms. WF-water with 5% formic acid, WM – water: methanol, 50:50, WMF - water: methanol, 50:50 with 5% formic acid & DI- direct injection.

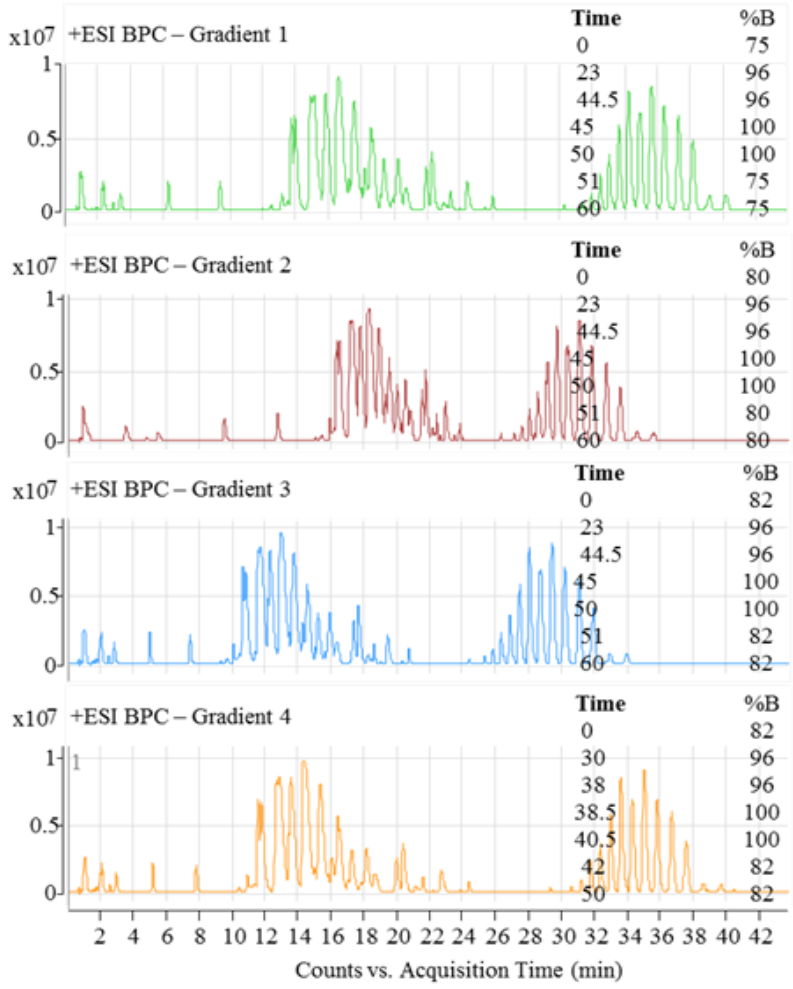

Figure S2. LC-MS base peak chromatograms from four different gradient conditions.

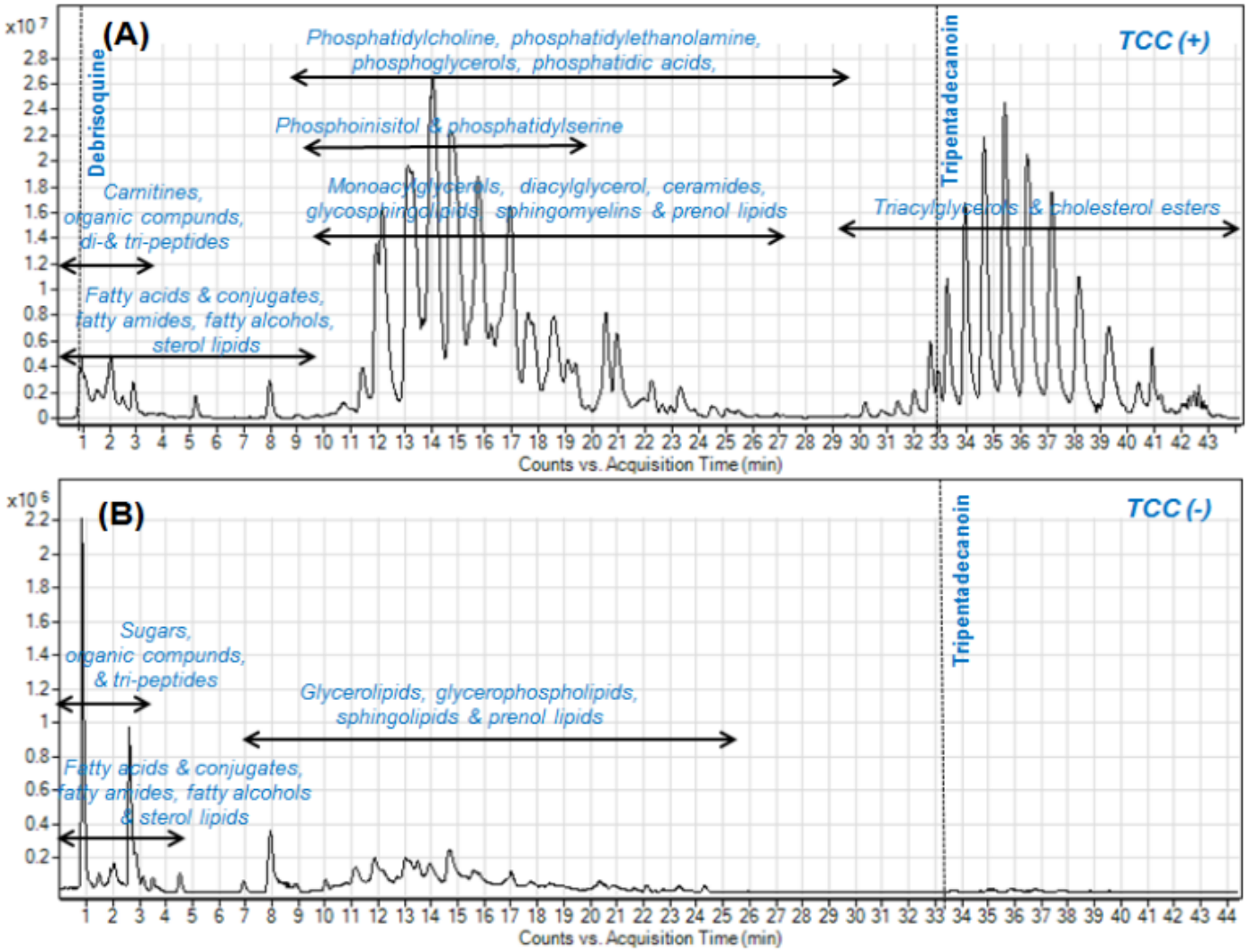

Figure S3. LC–MS extracted compound chromatogram acquired from the lung pool - (A) positive ionization & (B) negative ionization. The marked windows are according to the compound class and their elution time.

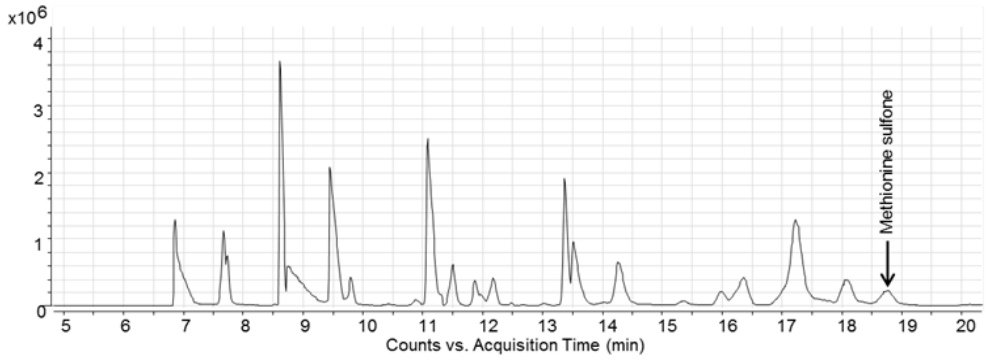

Figure S4. CE–MS extracted compound electropherogram acquired in positive ionization.

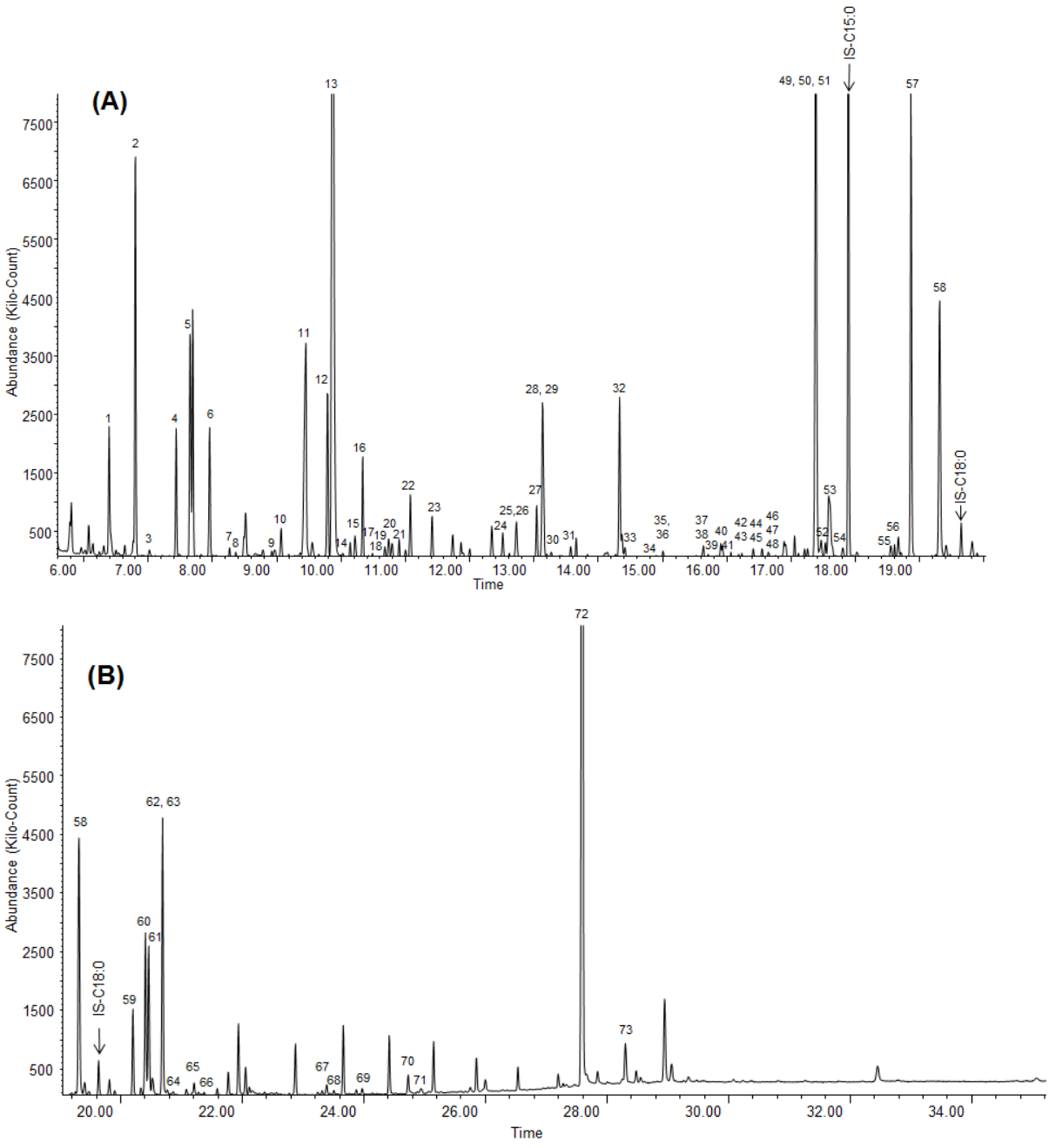

Figure S5. GC–MS total ion chromatogram marking the identified compounds peaks. Panel A and B. (1) Pyruvic acid, (2) L-(+)lactic acid, (3) Glycolic acid, (4) L-alanine 1, (5) Glycine, (6) Oxalic acid, (7) 3-hydroxybutyric acid, (8) L-mimosine 1, (9) DL-3-aminoisobutyric acid, (10) L-valine 2, (11) Urea, (12) Phosphoric acid, (13) L-leucine-2, (14) DL-isoleucine 2, (15) L-proline 2, (16) Glycine, (17) Succinic acid, (18) Catechol, (19) Glyceric acid, (20) Uracil, (21) Fumaric acid, (22) L-serine 2, (23) L-threonine 2, (24) Aminomalonic acid, (25) Nicotinamide, (26) D-malic acid, (27) Aspartic acid 2, (28) trans-4-hydroxy-L-proline 2, (29) L-pyroglutamic

acid, (30) L-glutamic acid 1, (31) Creatinine, (32) L-glutamic acid 2, (33) N,O-Bis-phenylalanine, (34) Lauric acid, (35) L-asparagine 2, (36) D-lyxose, (37) Xylitol, (38) Arabitol, (39) Ribitol, (40) Glycerol 1-phosphate, (41) L-glutamine 3, (42) o-phosphocholamine, (43) Azelaic acid. (44) Hypoxanthine-1, (45) 3-phosphoglycerate, (46) Cirtic acid/ isocitric acid, (47) Dehydroascorbic acid 1, (48) Myristic acid, (49) Fructose 2, (50) D-allose 1/tallose, (51) D-Mannose/ glucose/ galactose/ allose/ altrose, (52) D-allose 2/ mannose 2/ glucose 2/ gluconic acid, (53) Dehydroascorbic acid 4, (54) L-tyrosine 2, (55) Xanthine, (56) Palmitoleic acid, (57) Palmitic acid, (58) Myo-inositol/ all-inositol, (59) Linoleic acid, (60) oleic acid, (61) Elidic acid, (62) Stearic acid, (63) Xanthotoxin, (64) Spermidine 2, (65) D-glucose 6-phosphate 1, (66) D-glucose 6-phosphate 2, (67) Inosine, (68) Hexadecanoic acid, (69) Lactulose/ sucrose, (70) Trehalose, (71) Maltose 1, (72) Cholesterol, (73) Campesterol, IS15:0-pentadecanoic acid, IS18:0-methyl stearate.

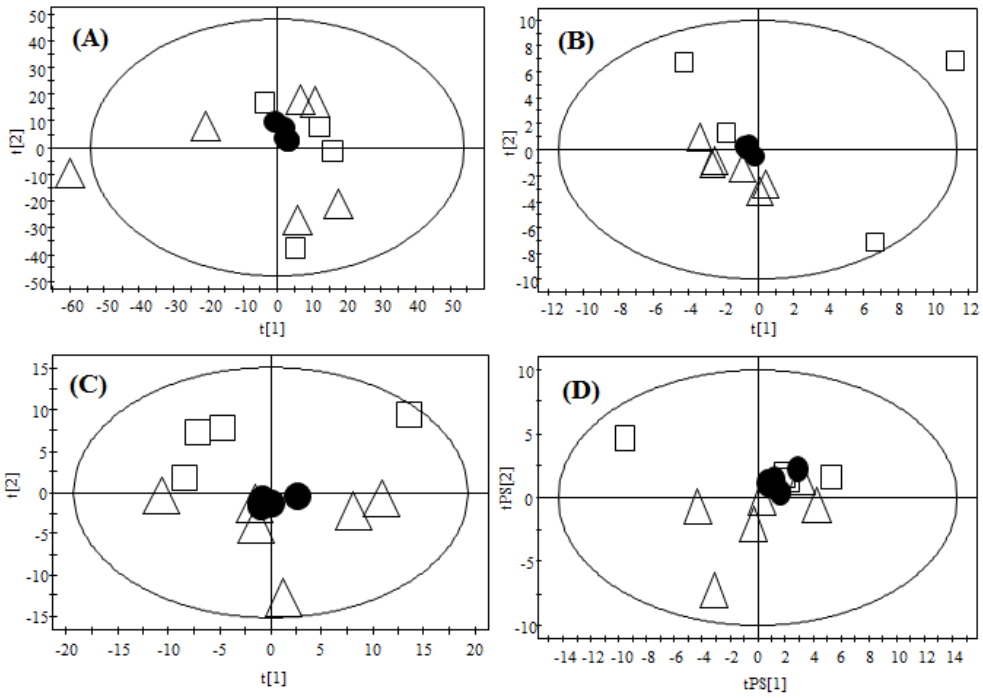

Figure S6. PCA models built with the whole data sets for checking the QCs. (A) Scores plot from LC-MS positive analysis. Quality of the model: R^2=0.609, Q^2=0.0829, (B) Scores plot from LC-MS negative analysis. Quality of the model R^2=0.451 Q^2= - 0.292, (C) Scores plot from GC-MS analysis. Quality of the model R^2=0.533, Q^2=0.289, (D) Scores plot from CE-MS positive analysis. Quality of the model R^2=0.551, Q^2=0.059. Empty triangles, sepsis; empty squares, control; filled circles, quality control.

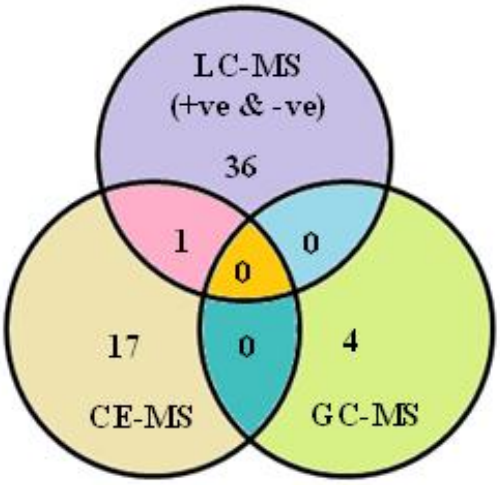

Figure S7. Venn diagram presenting the comparison of putatively identified significant compounds form rat lung (sepsis and control) among the three analytical platforms (LC-MS, GC-MS and CE-MS).

Reference:

(29) Domínguez-Alvarez, J.; Rodríguez-Gonzalo, E.; Hernandez-Mendez, J.; Carabias-Martínez, Rita. *Anal. Chem.* **2011**, 83, 2834–2839.

Chapter- 4

Unveiling the effect of ventilation in an animal model of sepsis through a multiplatform lung fingerprinting approach

Being extremely heterogeneous, the accurate progression of ALI in patients whether induced by mechanical ventilation or sepsis is still not known. Several cell specific biomarkers are available; however the degree of sensitivity and specificity is very low. The actual molecular mechanism could be better obtained from the metabolomics study. Numerous serum/plasma metabolomics study has been performed including our won, on ALI induced by mechanical ventilation or by sepsis separately, postulating the involvement of oxidative stress, tissue damage, altered lipid and collagen metabolism. All the published research was undertaken using one or two instrumental techniques. However, it is evident that tissue metabolites provide more inherent site specific information compared to bio-fluids. Moreover, combination of several analytical techniques enables sufficient metabolite coverage. Chapter-1 and Chapter-2 describes the serum metabolite information of the ALI model. On the other hand, Chapter-3 designates the multiplatform (LC-MS, GC-MS and CE-MS) fingerprinting approach for lung tissue. Here in Chapter-4 the application of the multiplatform lung tissue method is going to be implemented. Moreover instead of applying only one single model, here the method is going to be applied on three different type animal models, sepsis, ALI induced by mechanical ventilation (VILI) and ALI induced by sepsis (SI-ALI). The purpose of this study is to find out the metabolomics changes in lung tissue associated with lung injury induced by mechanical ventilation in animals with sepsis.

A manuscript has been is submitted in *American Journal of Respiratory and Critical Care Medicine* and included in this chapter-.

Title: Unveiling the effect of ventilation in an animal model of sepsis through a multiplatform lung fingerprinting approach

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American Journal of Respiratory and Critical Care Medicine, Submitted, in April 2014.

Resumen

APLICACIÓN DE UN ESTUDIO DE HUELLA METABÓLICA BASADO EN MULTIPLES TÉCNICAS ANALÍTICAS EN EL DESCUBRIMIENTO DEL EFECTO DE LA VENTILACIÓN MECÁNICA EN UN MODELO ANIMAL DE SEPSIS

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Revista: American Journal of Respiratory and Critical Care Medicine (enviado abril 2014)

Objetivo: Realización por primera vez del estudio global de huella metabólica en muestras de tejido de pulmón de rata para identificación de cambios en metabolitos asociados con daño pulmonar agudo inducido por ventilación mecánica y sepsis.

Métodos: Ratas que habiendo sido sometidas a CLP (perforación y ligadura cecal) o ratas SHAM (sus controles tras cirugía pero sin perforación ni ligadura cecal), 24 horas después de la intervención recibieron ventilación mecánica durante 2.5 h con Volumen tidal=9 ml/kg, PEEP=0 cm H2O (n=9 y n=12, sin y con CLP); o Volumen tidal =25 ml/kg, PEEP=5 cm H2O (n=13 y n=12, sin y con CLP, respectivamente). Tras obtener las muestras de tejido de pulmón, se procedió al análisis de huella metabólica utilizando un multiplataforma analítica basada en las técnicas LC-MS, GC-MS Y CE-MS.

Resultados: Se identificaron cambios en metabolitos característicos de sepsis y de VILI. Las muestras de tejido pulmonar de ratas con sepsis y VILI presentaban un perfil metabolómico característico comparado con las muestras de ratas con sepsis y sin VILI. Los cambios encontrados indicaron un aumento del estrés oxidativo, así como cambios en el metabolismo energético, metabolismo de la purina, carnitina, aminoácidos, ciclo de la urea, vitaminas, colágeno, ceramidas-esfingomielinas y fosfolípidos.

Conclusión: Se puede identificar un perfil metabolómico diferenciado en muestras de tejido de pulmón de rata con daño pulmonar inducido por ventilación mecánica.

Summary

Non-targeted metabolomics approach offers the complete representation of a complex biological system through the combination of data-rich high throughput analytical techniques and MVDA. In comparison to other complex molecules such as proteins or nucleic acids, many metabolites changes faster, thus metabolites reflect the more adequate changes of any biochemical effects in an organism. Non-targeted metabolomics permits the analysis of all the small molecules (metabolites) that collectively constitutes the entire metabolome in a given biological fluids and tissues. Numerous analytical methods have been developed for non-targeted metabolomics based on NMR and MS coupled with different separation techniques such as LC, GC and CE. Working with these highly sensitive techniques requires development of robust and reliable method, which will be utilized for its intended use. The crucial points in any analytical method development are the validation of every step to get reliable and reproducible result and the challenges are completely new for non-targeted approach and different to target methods. Beside method validation, sample pre-treatment also requires careful consideration. Different sample types are being employed in metabolomics. However most commonly used sample types are bio-fluids such as urine and serum/plasma due to their minimally invasive nature. Although invasive, tissue analysis provides site specific information because in any altered conditions the changes are first seen at tissue site.

ALI is an important cause of pulmonary and non-pulmonary morbidity in patients who survive hospitalization. Patients with severe breathing problem due to various factors/reasons admitted to ICU, where they are provided with mechanical ventilation to acquire proper respiration. The mechanical ventilation with high tidal volume could affect the lung through the process of continuous stretching and de-stretching, this condition is termed as VILI. Laterally due to other reasons, 50% severe septic patients admitted to ICU develop lung injury which is termed as SI-ALI. Upon injury the normal epithelial fluids transport disrupted and contribute to lung epithelial flooding. Interleukin-1β and tumor necrosis factor-α have been evaluated to improve the understanding of ALI pathophysiology. However, the diagnostic or prognostic capability of these factors as a biomarker are very much non-specific. Thus there is a need for specific screening markers which can be used for easy diagnosis of ALI and also help in understanding the pathophysiology, irrespective of its heterogeneous characteristics. The study of metabolomics is of great clinical interest because of the urgent need to: (i) identify metabolic profile to extend the understanding of ALI pathophysiology and mechanism and (ii) identify new screening markers for disease diagnosis and helping the clinicians' in advance to follow a proper therapeutic treatment procedure, which is lacking in ICU.

Considering the above mentioned facts the main goal of this dissertation is to develop and validate methodologies both in single and multiplatform aspects, suitable for serum and tissue non-targeted metabolomics approaches, providing reliable and effective data sets. Upon developing and validating methodologies for non-targeted metabolomics analysis, the following purpose of this project is to apply them on serum and lung samples from animal models of lung injury which will help in identifying markers

associated to ALI as well its pathophysiology and molecular mechanisms. To attain these objectives the present work was divided in four chapters.

Chapter-1:

First of all an intensive review was done on the available validation strategies for MS-based non-targeted metabolomics approach. There are many methods existing for serum non-targeted analysis centered on LC-MS, GC-MS and NMR. Among the little published CE-MS serum metabolomics methods, none of them are adequately validated, involves high sample volume and time consuming sample preparation steps. In this section, a CE-MS method for non-targeted serum analysis was developed by using the high mass accuracy capabilities of TOF mass analyzer in combination with automated feature extraction and database search. A very easy and less time consuming serum sample pre-treatment, suitable for CE-MS analysis was also established. Traditional SPE extraction protocol which is usually time-consuming and also one step ultrafiltration protocol were tested. However, one step ultrafiltration with high protein cut-off, minimal sample volume, and lowest dilution, provided more accurate, precise with high recovery, better reproducibility and wider metabolite information compared to traditional SPE protocol. The initial sample volume is always a very crucial point specially while studying animal model. Different serum volumes were tried and to keep it as less with better reproducibility 100 µL was selected, which is much lower than previously published CE-MS based serum treatment protocols. Generally water is a very common solvent for CE-MS sample pre-treatment, however extraction with water provides identification of only free metabolites. While performing deproteinization, to remove proteins from serum, some metabolites could be retained by the protein. In order to improve the compound categories, an organic solvent, 5% acetonitrile was added and the signal instability generated from the addition of organic solvent was minimized by adding electrolyte (0.2 M formic acid). Several dilutions were tested, 1:1 was chosen for final sample treatment. Along with other instrumental parameter the injection time was also optimized to 35 seconds, to lessen the amount of sample for injection. A complete fingerprinting was performed in order to characterize rat serum using CE-MS, giving 35 identified compounds of different classes including organic acids, amino acid derivatives and carnitines. Many other metabolites were found but could not be identified due to the un-availability of authentic standards. The metabolite types prove the usefulness of CE-MS and its complementariness to other techniques when exploring altered metabolic pathways. The robustness of the developed methodology was tested by validating the method in terms of linearity, accuracy, precision, LOD and LOQ, choosing some identified compounds form the fingerprinting list. Different compound categories with various retention times covering the entire electropherogram were considered. All the metabolites used for validation fit the linear model ($r > 0.99$) without any biasness and with high accuracy and precision, demonstrating the reliability and reproducibility of the developed method. Eventually the validated method was then applied on serum samples of rat model of VILI, a mode of ALI that was not studied before using CE-MS. A set of distinctive metabolites were seen

separating VILI from control groups. A decrease in arginine and an increase in asymmetric-di-methyl arginine and ornithine postulated that the condition ALI provoke lung airway remodeling, inflammation and oxidative stress. On the contrary, during mechanical ventilation lung also experiments a protective mechanism through cholinergic anti-inflammatory reflex. An increased level of choline was also found in the VILI animal model in this study, confirming that this protective mechanism is active in this pathology. Alongside this application, this developed method has been applied to several other application successfully and being routinely used in the laboratory for serum/plasma analysis.

Chapter-2:

LC-MS represents an important analytical platform for non-targeted metabolomics, advancing with high sensitivity and potentiality for biomarker identification. Thus the broad applicability of LC-MS to metabolites of all classes leads to accept it as a first choice of consideration. Previous studies have described the application of non-targeted metabolomics approach on lung injury induced by hyperoxic, gamma-irradiation and sepsis using NMR and LC-MS. However there are no publications available for non-targeted metabolomics application on VILI models. Thus an already developed method in our laboratory for serum analysis based on LC-QTOF-MS was applied on the VILI and control samples of rat to discover distinctive metabolite information than CE-MS. The repeatability of the methodology was tested by the clustering of QC samples, which were prepared by the aliquot of same sample volume of VILI and controls. This type of method validation is the most commonly used by researchers as an alternative validation approach. To find representative differentiating markers for ALI, obtained features were then filtered by choosing the data that had “present” calls in 100% of samples in any group. Applying univariate and MVDA, 44 significant masses were found, 15 of them were then confirmed by MS/MS analysis. The identified metabolites were belonging to the class of phospholipids, steroids, vitamin, amino acids, fatty amide and bile acids. The PLS-DA and OPLS-DA models showed the distinguishing separation of VILI from control group. The observed metabolites were then correlated with the biological pathways to find their relation with ALI. Sphingosine and sphingosine-1-phosphate are the metabolites of ceramide pathway which has anti-apoptotic activity, and it has been proposed that the balance of ceramide and sphingosine-1-phosphate determines the fate of the cell. Hexadecenal is also associated with cell apoptosis. The decreased sphingosine level could be involved in the cell apoptosis by the activation of ceramide synthase or by the increased degradation of hexadecenal. Decreased lyso-phospholipids and increased oleamide and phospholipids could be explained by the interruption in the lands cycle, which regulates the phospholipid metabolism. The changes in lysine, vitamin and bile acids proved the known facts about ALI which are altered collagen metabolisms and multi-organ failure. According to the results, the damage that occurs in the surfactant as a result of oxidative stress, promotes the generation of lysophosphatidylcholines that are degraded by lysophospholypase di-esterase on endocannabinoids that promote de novo synthesis of ceramides. This contributes to ALI by activating the

metabolic pathway in which ceramide is converted into sphingosine-1-phosphate that inhibits apoptosis of neutrophils. One of its metabolites detected by LC-QTOF-MS is hexadecenal, which causes alterations in cytoskeletal reorganization and apoptosis. Lastly observations of this present study including the changes in certain metabolites and lipids shed light into the pathogenesis of ALI.

Chapter-3:

Before developing non-targeted metabolomics method for lung tissue analysis a critical review was performed to evaluate the available research based on animal/human tissue using MS detection approaches. Tissue provides more innovative information than bio-fluids; however the sample preparation is its main challenge. A LC-MS, GC-MS and CE-MS based multiplatform method has been developed for the non-targeted analysis of lung tissue, in combination with automated feature extraction and database search. An initial solvent for homogenization was optimized choosing from: water with 5% formic acid, water: methanol 50:50 and water: methanol 50:50 with 5% formic acid. Except CE-MS, LC-MS and GC-MS optimization results clearly reflected that water: methanol 50:50, was the best choice for these two platforms. In order to have an identical initial sample protocol (homogenization step) for lung tissue, as a compromise water: methanol 50:50, was also chosen for CE-MS as the homogenization solvent. Along with homogenization solvent, the sample volume was also optimized. Using this optimized method and with only 20 mg of tissue for the three techniques, a broader range of metabolites were putatively identified, covering polar to non-polar and different biochemical classes. This sample volume is very much applicable to biopsy sample in biomedical research. An extraction protocol was optimized targeting single phase extraction, using 80:20 methanol: MTBE, and injecting the same extract in all platforms. However the high quantity of organic solvent employed for non-polar metabolite extraction exerts less signal intensity in CE-MS. Hence to obtain better signal and reproducible analysis, the developed one step ultrafiltration protocol for CE-MS analysis was applied for lung tissue. Keeping other instrumental conditions similar, only the injection time was optimized to 50 seconds to increase the metabolite numbers. The 80:20 methanol: MTBE extract was split in two parts, 100 µL injected directly in LC-MS and 300 µL dried and derivatized for GC-MS analysis. A previously optimized instrumental condition for non-polar compounds was applied for LC-MS analysis; however the gradient was optimized to decrease the analysis time. Concomitant analyses with these multiplatform techniques a complete mouse lung fingerprinting, 1254 metabolites (1114, 69 and 85 metabolites from LC-MS, GC-MS and CE-MS respectively) of different biochemical classes were identified from only 20 mg of lung tissue. The importance of multiplatform approach was proved by the fact that only seven compounds were found in common. A particular technique is not sufficient to cover the entire metabolome which has also been proved by the fact that each technique analyses a unique category/ class of compounds. With the developed method, LC-MS provided a wide range of compounds from polar to non-polar, however the combination of CE-MS and GC-MS added more information about charged compounds and, free and

volatile metabolites respectively. A complete validation was performed in all three platforms selecting ten different compounds from each technique from mouse lung profiling, considering the retention/ migration time, covering the entire chromatogram/ electropherogram and various functional groups, polarities and molecular masses. Compounds were chosen independent and common between and among analytical techniques. All the metabolites used for validation fit the linear model ($r > 0.99$) with high accuracy and precision. The optimized and validated method was then applied on a small set of lung samples of animal model of sepsis and control successfully. After chemometrics and statistical analysis 48 significant compounds were detected from three platforms, only propionyl-carnitine was common in LC-MS and CE-MS. Though sepsis was implemented through CLP but the significant compound from lung analysis proved the literature findings that sepsis ultimately results in multi-organ failure. The obtained result support the utility of multiplatform application in the rapid and simple screening for alterations in any type of biopsy tissue analysis.

Chapter-4:

In this section the developed non-targeted multiplatform method for lung tissue was applied on rat lung tissue, to find out the exact reason behind SI-ALI, whether it is the effect of mechanical ventilation or sepsis. To accomplish this study animal models of sepsis (induced by CLP), VILI (injury by mechanical ventilation and SI-ALI (injury due to sepsis as explained) along with their corresponding control groups were used. The developed method was successfully applied to the lung tissue of these models. The obtained features were filtered applying 80% filter by frequency and 30% CV on quality control for all platforms. The GC-MS metabolites were identified by comparing their mass fragmentation patterns with those available in the NIST mass spectral library and Fiehn Retention Time Library. In CE-MS, compounds were identified by matching the retention time from the in-house library and rest of them were kept as putative. However in LC-MS, metabolites were putatively identified not only using the accurate mass but also checking their isotopic pattern (higher score pattern) and retention time. Both univariate and MV statistical analyses are applied, 112, 35, 15 and 39 significant metabolites were obtained respectively from LC-MS (+), LC-MS (-), GC and CE-MS analysis, in total for all comparisons. The quality of the entire run was validated by the clustering of QCs in PCA models respective to the instrumental analysis. Both the PCA and PLS-DA models for the six groups distinctively showed that the separation is due to ventilation rather than sepsis. Although effect of sepsis, ventilation, and ventilation along with sepsis were checked separately, provided a well separation in all platforms. The identified significant metabolites were then correlated with the corresponding biological pathways. Metabolic changes indicated increased oxidative stress, changes in purine, energy, carnitine, amino acid, urea cycle, vitamins, collagen, ceramide-sphingomyelin and phospholipid metabolism.

In summary, with the proposed research objectives rapid and simple methodologies have been developed and validated both in single and multiplatform aspects for non-targeted metabolomics. Validation parameters are adequate for bio-analysis and sample treatments are very simple permitting the detection of a wide range of compounds. A complete fingerprinting is obtained for rat serum and mouse lung. Finally the application of the validated methods was able to answer questions based on the need to solve a problem of great social and economic impact with high morbidity and mortality of ALI in ICU.

Conclusions

General Conclusions

Along with particular, this dissertation has proved that the combination of different techniques is required for the comprehensive analysis of all the small molecules in a biological system, termed as metabolome. However before applying any metabolomics screening approach it is quite necessary to develop and validate a sensitive and robust, 'fit-for-purpose' method. Here, in this dissertation two different strategies have been presented towards this final goal (i) development and validation of non-targeted metabolomics approach based on both particular and combinatorial platforms and (ii) application of these developed methodologies to an animal model of lung injury.

- A CE-MS based metabolomics study based on rat serum samples was capable of obtaining relevant information: 35 compounds were identified including organic amines, amino acids, amino acid derivatives and carnitines. The class variety of compounds proves the usefulness of CE-MS and its complementariness to other techniques when exploring altered metabolic pathways. Making use of the high mass accuracy capabilities of this analyzer in combination with automated feature extraction and database search has demonstrated to be a valuable tool for metabolomics.

- A very simple serum pretreatment consisting of one step ultrafiltration (30 kDa protein cut-off filter) and dilution has been included in this metabolomics study based on CE-MS. Moreover, required sample volume is minimal, only 100 µL. This procedure has been validated in terms of linearity, accuracy and reproducibility for seven different metabolites covering the entire electropherogram and different metabolite classes.

- The application of this CE-MS metabolomics approach to an animal model of ALI induced by ventilation was able to find out distinctive metabolites that changed markedly in serum of rats with VILI as compared to control in search for screening markers. A decrease in arginine and an increase in asymmetric-di-methyl arginine and ornithine postulated that the condition acute lung injury provoke lung airway remodeling, inflammation and oxidative stress. On the contrary during mechanical ventilation lung also exerts a protective mechanism through cholinergic anti-inflammatory reflex. An increased level of choline was found in the VILI animal model in this study, confirming that this protective mechanism is active in this pathology.

- A multiplatform method based on MS was optimized for the non-targeted analysis of lung tissue using LC-MS, GC-MS and CE-MS in combination with automated feature extraction and

database search. Sample treatment for a one step procedure using water: methanol (50:50) as homogenization solvent, and MTBE: methanol (80:20) as extraction solvent for LC-MS and GC-MS resulted to be the best choice. Only 20 mg of tissue was required for the global analysis with these three techniques. A broader range of metabolites was putatively identified, covering polar to non-polar and different biochemical classes. This sample volume is very adequate to biopsy sample in biomedical research.

- Only seven compounds were found in common after analysis of lung samples with the three techniques. This proved the importance of the multiplatform approach. Moreover, a particular technique is not sufficient to cover the entire metabolome. LC-MS provided a wide range of compounds from polar to non-polar. However the combination of CE-MS and GC-MS added more information about charged compounds and, free and volatile metabolites respectively. Concomitant analyses with these multiplatform techniques showed a complete mouse lung fingerprinting, 1254 metabolites (1114, 69 and 85 metabolites from LC-MS, GC-MS and CE-MS respectively) of different biochemical classes were identified.

- This procedure based on three platforms applied to mouse lung metabolomics has been validated in terms of linearity, accuracy and reproducibility selecting ten different metabolites for each platform covering the entire profiles, different metabolite classes and molecular masses.

- 48 significant compounds were found after application of the optimized and validated multiplatform method for a metabolomics study in an animal model of sepsis and control. Only propionyl-carnitine was common in LC-MS and CE-MS. Sepsis was produced through CLP, not related to lung. However the significant compounds found from lung analysis proved the literature findings that sepsis ultimately results in multi-organ failure.

- The obtained results support the utility of this multiplatform metabolomics approach in the rapid and simple screening of lung tissue.

- An already developed method for non-targeted serum metabolomics based on LC-MS was applied successfully on the VILI and control samples of rat to discover distinctive metabolite information than CE-MS. The multivariate models showed a characteristic separation of VILI from control group. Fifteen compounds were confirmed by MS/MS analysis. The identified metabolites

were phospholipids, steroids, vitamin, amino acids, fatty amide and bile acids. A decrease in sphingosine and an increase in hexadecenal could be involved in the cell apoptosis by activating the ceramide pathway. On the other hand, a decrease in lysophospholipids and elevated oleamide and phospholipids could be explained by the interruption of lands cycle, which regulates the phospholipid metabolism. The changes in lysine and other vitamin and bile acids proved the known fact about ALI, which is altered collagen metabolism and multi-organ failure. Lastly observations of this present study including the changes in certain metabolites and lipids shed light into the pathogenesis of ALI.

- The developed and validated method for multiplatform lung fingerprinting method was successfully applied to the lung tissue samples from sepsis, VILI and SI-VILI. The purpose of the study was to identify metabolic changes in lung tissue associated with lung injury induced by mechanical ventilation in animals with sepsis, using for the first time a fingerprinting approach. Lung tissue samples from septic rats with VILI were characterized by a specific metabolic profile as compared to samples from septic rats without VILI. Metabolic changes indicated increased oxidative stress and changes in purine, energy, carnitine, amino acid, urea cycle, vitamins, collagen, ceramide-sphingomyelin and phospholipid metabolism. In summary, the present study expanded the previous information on changes induced by sepsis and VILI in experimental animal models. And also showed that VILI in the context of sepsis, a more clinically relevant scenario than VILI induced in normal lungs.

Conclusiones Generales

Además de las conclusiones particulares, estos trabajos han probado que para el análisis global de moléculas pequeñas en un sistema biológico, lo que se denomina metabolómica, se necesita la combinación de técnicas diferentes. No obstante, antes de aplicar cualquier metodología de cribado en metabolómica resulta muy necesario el desarrollo y validación de un método sensible y robusto adecuado para el fin propuesto. Aquí, en esta tesis, se han presentado dos estrategias diferentes con este fin (i) desarrollo y validación de un método para el estudio metabolómico no dirigido basado en plataformas analíticas únicas o múltiples y (ii) aplicación de las metodologías desarrolladas al estudio de un modelo animal de ALI (daño pulmonar agudo).

- El estudio metabolómico basado en CE-MS aplicado a muestras de suero de rata fue capaz de obtener información relevante: se identificaron 35 compuestos incluyendo aminas, aminoácidos, derivados de aminoácidos y carnitinas. Los diferentes tipos de compuestos pruebaron la utilidad del CE-MS en el estudio de rutas metabólicas alteradas así como su complementariedad con otras técnicas. La utilización de las prestaciones del analizador de masa exacta en combinación con la extracción automática de los posibles compuestos y la búsqueda en bases de datos han demostrado ser una muy valiosa herramienta en metabolómica.

- En el estudio anterior se ha incluido un pretratamiento de muestra de suero muy simple, basado en una única etapa de ultrafiltración (membrana de corte de 30 kDa) seguido de dilución. Además, el volumen de muestra necesario es muy pequeño, sólo 100 µL. Este procedimiento se ha validado en lo relativo a linealidad, exactitud y reproducibilidad para el análisis de siete metabolitos diferentes cubriendo el electroferograma completo así como tipos de metabolitos muy diferentes.

- La aplicación de la metodología desarrollada y validada, basada en CE-MS, a un modelo animal de daño pulmonar agudo inducido por ventilación mecánica (VILI) con el fin de encontrar marcadores de cribado, fue capaz de encontrar metabolitos diferentes que cambiaban notablemente en suero de ratas con VILI comparadas con los controles. A partir de la disminución de arginina y el aumento de dimetil arginina asimétrica y ornitina se propone que la condición de ALI provoca un remodelado de los canales de ventilación en los pulmones, en la inflamación y en el estrés oxidativo. Por el contrario, durante la ventilación mecánica, los pulmones también experimentan un mecanismo de protección a través de un reflejo

antiinflamatorio colinérgico. Un aumento de colina en los animales con VILI, confirma que este mecanismo protector está activo en dicha patología.

- Se ha optimizado un método basado en una multiplataforma analítica para estudios metabolómicos no dirigidos aplicado a muestras de pulmón en el que se utilizan LC-MS; GC-MS y CE-MS en combinación con extracción automática de *features* o posibles compuestos y búsqueda en bases de datos. El tratamiento de muestra en un solo paso basado en la mezcla H_2O: metanol (50:50), como disolvente en la homogeneización y metil terbutil éter (MTBE): metanol (80:20), como mezcla en la extracción para LC-MS y GC-MS, ha resultado ser la mejor opción y únicamente 20 mg de muestra de tejido son necesarios para el análisis global con las tres técnicas. De esta forma, se identificaron putativamente una mayor variedad de metabolitos, cubriendo diferentes polaridades y tipos de compuestos bioquímicos. En relación con la cantidad de muestra que se obtiene en las biopsias, este volumen de muestra resulta muy adecuado en la investigación biomédica.

- Tras aplicar la metodología multiplataforma anterior a muestras de tejido pulmonar sólo se han encontrado siete compuestos en común entre las tres técnicas. Ello prueba la importancia del enfoque multiplaforma; además una técnica en particular no es suficiente para cubrir el metaboloma completo. La técnica LC-MS proporcionó un amplio rango de compuestos desde polares a no polares, aunque la combinación de CE-MS y GC-MS añadió más información sobre compuestos cargados así como compuestos en su forma libres y volátiles respectivamente. El análisis completo con esta metodología analítica multiplataforma aplicada a muestras de pulmón de ratón permitió una huella metabolómica más completa, que incluía la identificación de 1254 metabolitos: 1114, 69 y 85 metabolitos por LC-MS, GC-MS y CE-MS respectivamente) de diferentes clases bioquímicas.

- Se ha validado la metodología multiplataforma para el estudio de huella metabólica en muestras de pulmón de ratón en términos de linealidad, exactitud y reproducibilidad, para lo cual se han seleccionado diez metabolitos diferentes, de clases diferentes, de masas diferentes y cubriendo el perfil cromatográfico en su totalidad.

- Se han encontrado 48 compuestos con diferencias estadísticamente significativas tras la aplicación en un modelo animal de sepsis y sus controles, del método metabolómico multiplataforma una vez optimizado y validado. Únicamente se encontró un metabolito en común entre LC-MS y CE-MS, la propionil carnitina. Aunque se indujo la sepsis por perforación y ligadura cecal, los compuestos significativamente diferentes encontrados en el análisis de pulmón estaban en consonancia con la literatura relativos a que finalmente la sepsis acaba en fallo multiorgánico.

- Finalmente se puede concluir que los resultados confirman la utilidad de la metodología multiplataforma analítica para el estudio de huella metabólica de tejido de pulmón consiguiendo un cribado rápido y sencillo.

- El método basado en LC-MS previamente desarrollado para el análisis metabolómico no dirigido en muestras de suero fue aplicado con éxito en muestras de rata control y VILI a fin de encontrar información complementaria a la obtenida por CE-MS. Los modelos obtenidos por análisis multivariante mostraron una separación clara entre las muestras pertenecientes a ambos grupos. Se identificaron quince compuestos mediante análisis por MS/MS, resultando ser fosfolípidos, esteroides, vitaminas, aminoácidos, amidas de ácidos grasos y ácidos biliares. Se ha encontrado una disminución en los niveles de esfingosina y un aumento en los de hexadecenal que pudiera estar relacionado con la apoptosis celular por activación de la ruta de las ceramidas. Además, una disminución en los niveles de lisofosfolípidos y elevación de oleamida y fosfolípidos pudiera explicarse por alguna interrupción en el ciclo de Lands en su regulación del metabolismo fosfolipídico. Los cambios observados en lisina, ácidos biliares y vitaminas han probado, como ya era conocido, que la enfermedad ALI viene acompañada de alteraciones en el metabolismo del colágeno y fallo multiorgánico. Además de ello, el resto de los hallazgos facilitarán el estudio de la patogénesis del ALI.

- El método basado en LC-MS previamente desarrollado para el análisis metabolómico no dirigido en muestras de tejido pulmonar fue aplicado con éxito en muestras de pulmón de rata con sepsis, VILI y SI-VILI. El objetivo del estudio fue la identificación de los cambios metabólicos en tejido pulmonar asociados a VILI en animales con sepsis, siendo pioneros en esta aplicación basada en el estudio de huella metabólica. Las muestras de tejido pulmonar de ratas con sepsis y VILI presentaban un perfil metabolómico característico comparado con las muestras de ratas con sepsis y sin VILI. Los cambios encontrados indicaron un aumento del estrés oxidativo, así como cambios en el metabolismo energético, metabolismo de la purina, carnitina, aminoácidos, ciclo de la urea, vitaminas, colágeno, ceramidas-esfingomielinas y fosfolípidos. Por último, estos trabajos amplían la información previa existente hasta ahora sobre los cambios inducidos por sepsis y VILI en modelos animales de experimentación. Además muestran que VILI comparado con sepsis, se constituye como un daño más grave clínicamente que el VILI inducido en pulmones normales.