Liver Transplantation Biomarkers in the Metabolomics Era

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Abstract

The term biomarker usually refers to the biochemical molecules used in basic and clinical research, and also in the clinical practice, as surrogate markers that offer the advantage of being an objective, quantifiable, and reproducible measure. The

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most common applications of biomarkers include diagnosis, screening and monitoring of disease, assessment of response during therapy, risk assessment, and prognosis. Metabolomics or metabonomics enables the determination of hundreds of small molecules at the same time, which provides more comprehensive information than the determination of a single biomarker. Using metabolomics as an approach for searching biomarkers is supported by its capabilities to detect subtle metabolic changes triggered by external stimuli or perturbation. Metabolome changes are quite dynamic compared to genomics and transcriptomics, or even proteomics. Therefore, such metabolite alterations are found early in different samples, like tissues, cell lysates, blood, serum, plasma, feces, urine, etc. Application of metabolomics in liver transplantation is still in its early stages and has focused mainly on studying three aspects: post-reperfusion damage and rejection and dysfunction of the organ. In the current era when lack of organs suitable for transplantation is the most important limiting factor, the existence of an accepted functional assessment of grafts before transplantation would help to not only recover initially discarded organs but to also assess the therapies used to improve the quality of these organs. Different metabolic approaches have been used to search for objective markers of graft function and quality, but further analytical and clinical validation in multicentre studies is mandatory before they are incorporated into clinical routines.

Keywords

Omics • Biomarkers • Metabonomics • Metabolomics • Mass spectrometry • Bile acids • Phospholipids • Liver transplant • Ischemia reperfusion injury • Graft dysfunction

List of Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ADMA	Asymmetric dimethylarginine
BA	Bile acids
DBD	Donation after brain dead
DCD	Donation after circulatory dead
C18	Aliphatic chain of length 18
CEAD	Colorimetric electrochemical array detection
CIT	Cold ischemia time
CS	Cold storage
EAD	Early allograft dysfunction
ECD	Extended criteria donor
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
FXR	Farnesoid X receptor
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography

HR-MAS H-NMR	High-resolution magic angle spinning
H-NMR	Proton nuclear magnetic resonance spectroscopy
IGF	Initial good function
IP-LC	Ion-pairing liquid chromatography
IRI	Ischemia reperfusion injury
JNK	c-Jun N-terminal kinase
LC	Liquid chromatography
LT	Liver transplant
MRM	Multiple reaction monitoring
NIH	National Institute of Health
NO	Nitric oxide
NOS	Nitric oxide synthase
NMP	Normothermic machine perfusion
OPLS-DA	Orthogonal projection to latent structures-discriminant analysis
PCA	Principal component analysis
PLS	Partial least square
PLS-DA	Partial least square-discriminant analysis
PNF	Primary non-function
QC	Quality control
Q-ToF	Quadrupole time of flight
ROS	Reactive oxygen species
Rt	Retention time
RP	Reversed phase
TQ	Triple quadrupole
UPLC	Ultraperformance liquid chromatography
WHO	World Health Organization

Key Facts of Metabolomics

- Metabolomics/metabonomics can be defined as the holistic determination of low-molecular-weight (<1.5 kDa) molecules present in a biological system (cell, tissue, or organism).
- According to the Human Metabolome Database, human metabolome size is estimated to be composed of around 42,000 endogenous and exogenous metabolites, including lipids, small peptides, carbohydrates, cofactors, amino acids, etc.
- As a result of being downstream of the activity of genes and proteins, the metabolome constitutes a closer approach to the phenotype than genes and transcripts, or even proteins.
- The most common analytical techniques used to study the metabolome are nuclear magnetic resonance spectroscopy and mass spectrometry. The latter is usually coupled to previous separation techniques, such as liquid chromatography, gas chromatography, and capillary electrophoresis.
- MS-based metabolomics can be performed by two different approaches: (i) untargeted metabolomics, which aims to determine the global metabolomic

profile, and (ii) targeted metabolomics, where only a subset of the metabolome is determined.
- From a human perspective, metabolomics can be applied to body tissues or fluids, and selection strongly conditions the metabolites that are expected to be detected and the meaning of the altered metabolomic patterns.
- Metabolomic studies in liver organ transplants have focused on three aspects: (i) ischemia reperfusion injury, (ii) graft dysfunction, and (iii) assessment of donor liver quality before transplantation.

Definitions of Words and Terms

Biomarker	The characteristic that is objectively evaluated as an indicator of normal biological and pathogenic processes or pharmacological responses to a therapeutic intervention.
Cold ischemia time	The period from the time the organ is perfused in the donor with preservation solution until organ implantation starts in the recipient.
Donor after circulatory death	Organ donors in which the death is certified after cardiac arrest.
Donors after brain death	Organ donors after the diagnosis of brain stem death.
Early allograft dysfunction	It is the dysfunction of the organ after being transplanted not related to other causes such as vascular complications, infection, or rejection.
Extended criteria donors	Organ donors with characteristics beyond standard limits that may compromise the outcome in the recipient after using his/her organs.
Ischemia reperfusion injury	The damage that occurs within the transplanted graft when it is reperfused in the recipient after an ischemia period in cold storage.
Metabolite	Low-molecular-weight molecules ($<$1.5 kDa) that are the intermediates of biochemical reactions.
Metabolome	Collection of all the metabolites present in a given biological system (i.e., cell, biofluid, tissue, organism, etc.).
Metabolomics	The comprehensive and quantitative analysis of all the metabolites present in a specific cellular, tissue, or biological sample.
Metabonomics	The quantitative measurement of the dynamic multiparametric metabolic response of living

	systems to pathophysiological stimuli or genetic modification.
Targeted metabolomics	Guided (quantitative) determination of a predefined set of metabolites of interest.
Untargeted metabolomics	Holistic/global determination of the metabolites present in a given biological specimen.
Warm ischemia time	The period from when organ implantation starts until it is reperfused in the recipient.

Introduction

The term "biomarker" results from the combination of the terms "biological marker" and has been defined by the National Institute of Health (NIH) as "the characteristic that is objectively evaluated as an indicator of normal biological and pathogenic processes or pharmacological responses to a therapeutic intervention" (Biomarkers Definition Working Group 2001). A broader definition by the World Health Organization (WHO) has defined biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (WHO International Programme on Chemical Safety 1993). The utilization of small molecule measurement has been a common clinical practice in the field of medicine for more than 100 years (Wishart 2005); biochemical measurements of urine glucose, serum creatinine, or urea are still a very useful tool in the clinical practice. Nowadays, the term biomarker mostly refers to molecular or biochemical molecules which have become surrogate markers in basic and clinical research and also in clinical practice and have the advantage of being an objective, quantifiable, and reproducible measure (Strimbu and Tavel 2010). Biomarkers offer different applications in the field of human health, not only once the disease is present but also to predict the future. The intended uses of biomarkers comprise: (i) diagnosis of symptomatic patients; (ii) detection or screening of disease by enabling intervention in an earlier and potentially more curable stage than under usual clinical diagnostic conditions; (iii) monitoring disease, following the response during therapy, with the potential for adjusting the level of intervention (e.g., dose) on a dynamic and personal basis; (iv) risk assessment, which leads to preventive interventions for those at sufficient risk; (v) prognosis, which allows to adjust therapy (more or less aggressive) for patients according to the expected prognosis; (vi) prediction, which provides guidance in selecting specific therapy for patients or tailoring its dose for safety and efficacy purposes.

The term "omics" refers to a field of science defined as "the molecular or biochemical characterisation of pools of biological molecules, such as genes and genomes, transcripts and transcriptomes, proteins and proteomes, and small molecules, metabolites and metabolomes, which together encode the structure and

function of an organism or organisms, and can be used to explore their dynamics and flexibilities" (Gilbert et al. 2014). "Omics" technologies (i.e., genomics, transcriptomics, proteomics, and metabolomics) are characterized by the simultaneous determination of multiple parameters in a single biological sample (Gomez-Lechon et al. 2010). Metabolomics measures the downstream products of the "omics cascade" and thus provides information that is not accessible through other alternative "omics," such as genomics, transcriptomics, or proteomics (Dettmer et al. 2007; Leon et al. 2013). Metabolites are the intermediates of biochemical reactions. Hence their levels are defined by both their concentration and the functional properties of the enzymes, where the latter are the result of the integration of transcription, translation, posttranslational modifications, and allosteric effects, which results in an integrative effect between the capabilities of the system under study and its interaction with the environment (Villas-Boas et al. 2005). As a result of being downstream of the activity of genes and proteins, the metabolome constitutes a closer approach to the phenotype than genes, transcripts, or proteins. So metabolomics is more informative of the functional status of cells than other "omics" agents (Kaddurah-Daouk et al. 2008; Fig. 1). While common biochemical analyses also assess levels of metabolites, the main difference compared to metabolomics is that the latter measures hundreds of small molecules at the same

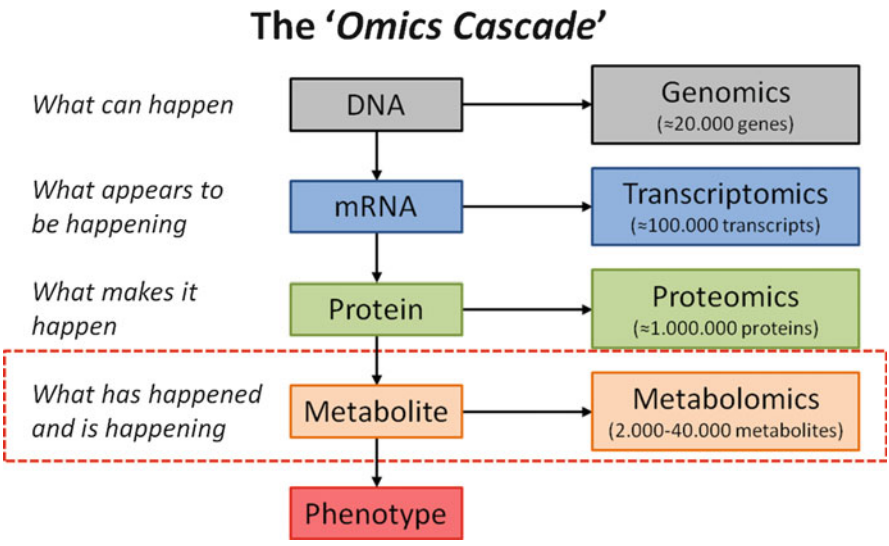

Fig. 1 The "omics cascade" in systems biology. Omics sciences provide a complete overview of the system under study. Biological information goes from genes (genomics) that provide information about what can happen through mRNA (transcriptomics) and proteins (proteomics), which provide information about what appears to happen and what makes it happen, respectively, and finally to metabolites (metabolomics), which represent the perturbations of the genome, transcriptome, and proteome and, therefore, provide information of what has happened and what is happening

time, which provides much more information than the simple determination of a single marker (Wishart 2005).

Metabolomics

The main idea behind metabolomics is that diseases produce changes in body fluids and tissues, which was already known in ancient Greece, and is represented in the diagnostic "urine charts" used since the Middle Ages, where the colors, smells, and tastes of urine were related to various medical conditions. Indeed gas chromatography (GC) and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) have been employed to perform the metabolic profiling of biological samples for decades (Nicholson and Lindon 2008). Despite the formal definitions of metabolome, which refer to the quantitative complement of all the low-molecular-weight molecules present in a cell in a particular physiological or developmental state (Oliver et al. 1998), metabonomics, defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999), or metabolomics, defined as "the comprehensive and quantitative analysis of all the metabolites present in a specific cellular, tissue, or biological sample," are much more recent. In generic terms, metabonomics/metabolomics can be defined as the holistic determination of the low-molecular-weight (<1.5 kDa) molecules present in a biological system (cell, tissue, or organism), such as lipids, small peptides, carbohydrates, cofactors, amino acids, etc. Human metabolome size is estimated as being composed of around 42,000 endogenous and exogenous metabolites according to the Human Metabolome Database (http://www.hmdb.ca/) (Wishart et al. 2013). The number of major metabolites, that is, those at higher concentrations and with the most relevant functions, is estimated to be around 2,000 (Beecher 2003). In order to provide a couple of examples, the human urine metabolome is estimated to be composed of 3,100 compounds, with the predominance of highly polar compounds (Bouatra et al. 2013), while the human serum metabolome is estimated to be composed of around 4,600 metabolites, half of which are phospholipids, and over a 1,000 glycerolipids (Psychogios et al. 2011).

Metabolomics Workflow

The basic working approach with any "-omic" science, including metabolomics, is shown in Fig. 2. Given their relevance, certain aspects of this approach must be highlighted. The experimental design is key in any metabolomic study because of the vast experimental variability that an inadequate design can imply. Some noteworthy aspects are defining the cohort study, selecting the type of biological samples of interest to perform the analysis, deciding sample treatment/processing, choosing the analytical method, etc.

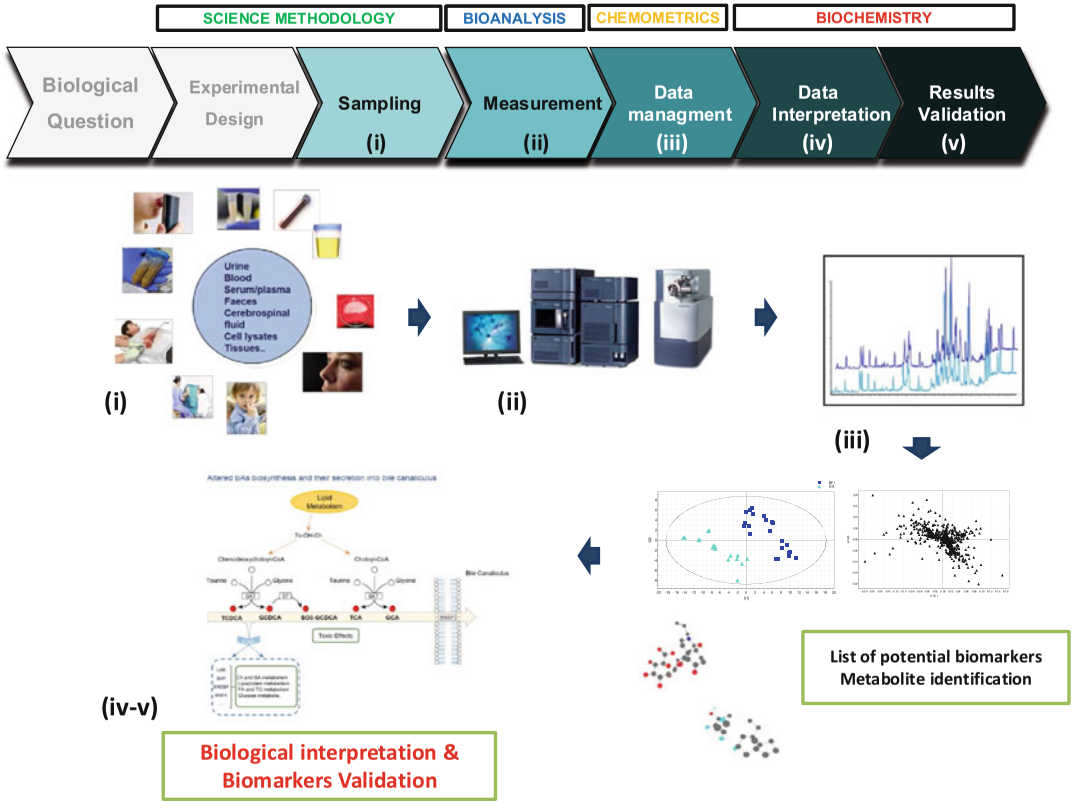

Fig. 2 A general metabolomic workflow. The figure represents the different steps involved in the sample metabolomics analysis, from sample collection and preparation *i*) to validating the results (*v*) through metabolite determination (*ii*) and data analyses (*iii-iv*)

Samples Most Widely Used in Metabolomics

Metabolomics, when referring to human samples, can be applied to body tissues or fluids. While the metabolic profiling of tissues is expected to more closely reflect their function, most of the current data available in the literature correspond to metabolomic analyses in fluids, probably because they can be obtained without resorting to invasive procedures (Dunn et al. 2005). For biofluids, metabolite levels not only reflect the status of the organ of biosynthesis but are dependent on other several factors at an organism's level. Sample preparation is a key step in a metabolomic study as it strongly conditions the results, and several factors have to be evaluated at both the time of sampling and during sample processing as they can affect their quality, such as type of sample, the containers used to store them, storage time, the necessity to add any preservatives, and the time taken to process them (Holland et al. 2003). While the most common procedure for body fluids is to perform protein precipitation using an organic solvent, with tissues, metabolites have to be efficiently extracted. In any case, sample processing should avoid potential interferences, ensure minimal loss of metabolites, and be compatible with subsequent analytical procedures (Leon et al. 2013).

Platforms Used to Measure the Metabolome

The metabolome presents a high diversity of components (amino acids, carbohydrates, lipids, organic acids, etc.) with very different chemical structures and properties (from ionic or very polar to highly hydrophobic compounds). It is almost impossible to determine the complete metabolome using a single analytical platform, thus the combination of complementary techniques (covering both sample preparation and analysis) is required to achieve comprehensive metabolome coverage (Villas-Boas et al. 2005; Dettmer et al. 2007). The most frequent analytical techniques used to study the metabolome are NMR spectroscopy and mass spectrometry (MS), the latter is usually hyphenated to previous separation techniques (Robertson 2005). NMR and MS, in their different configurations, are complementary rather than opposite platforms (Table 1). Thus the use of different analytical techniques has a positive impact on widening metabolome coverage (Leon et al. 2013).

^{1}H-NMR spectroscopy is based on the detection of all the proton signals present in a given sample. The main advantages of NMR spectroscopy are: (i) it is a nondestructive technique; therefore, samples may be used in further analyses; (ii) it requires no or little sample preparation; (iii) it is possible to perform analyses with solid samples; (iv) it is an intrinsically quantitative technique; (v) it is possible to perform structural analyses; (vi) its high robustness allows easy lab-to-lab comparisons. However, the NMR application is hampered by its low resolution and sensitivity, difficulty in interpreting the obtained spectra, and presence of analytes deficient in protons or which possess protons that can be readily interchanged with the solvent (Clarke and Haselden 2008; Villas-Boas et al. 2005).

In MS, the analytes present in the sample are ionized and characterized by their mass-to-charge ratio (m/z). MS detection is usually preceded by a separation technique that aims to resolve the individual components present in complex biological

Table 1 Summary of advantages and limitations of MS and NMR techniques

Platform	Advantages	Disadvantages
NMR	Quantitative Nondestructive High throughput Requires minimal sample preparation Robust, highly reproducible technology Compatible with liquids and solids	Low sensitivity (μM range) Low resolution Complex data processing Requires large sample volume/quantity Limited to protonated compounds
MS	High versatility, has the potential of covering a wide part of the metabolome Can be hyphenated to previous separation techniques (i.e., GC, LC, and CE) High sensitivity (especially in the case of LC-MS, nM-pM range) High resolution and selectivity (when needed/desired) Possibility of targeted and untargeted metabolic profiling modes Usually requires low sample volume/quantity	Destructive Limited reproducibility (mainly when hyphenated to LC and CE) Usually requires extensive sample preparation Long analysis times when hyphenated to previous separation techniques Quantitation highly dependent on calibration curves and appropriate internal standards and chemical reference compounds

matrices. The most widely used separation techniques coupled to MS are GC, liquid chromatography (LC), and capillary electrophoresis (CE). GC is used to separate volatile (and nonvolatile, after derivatisation) metabolites (Dunn 2008; Lenz and Wilson 2007). CE separates polar ionisable compounds based on their *m/z* (Ibanez et al. 2012). LC is by far the most widely used separation technique in metabolomics and allows the separation of metabolites based on their chemical properties according to the stationary phase of the selected chromatographic column (Lenz and Wilson 2007; Dettmer et al. 2007). Traditional LC separations have been performed by reversed phase (RP) chromatography (Lenz and Wilson 2007). RP-LC is usually performed with C18-bonded silicas as stationary phases and a water to methanol or acetonitrile gradient. RP-LC is suitable for retaining and separating medium-polar and nonpolar metabolites and is a good option as a starting point in metabolomic studies. However, very polar compounds elute in the void volume or with minimal retention. Hydrophilic interaction chromatography (HILIC) and ion-pairing liquid chromatography (IP-LC) are two alternative strategies for the separation of metabolites that are poorly retained in RP. IP-LC is based on the use of ion-pair modifiers, large ionic molecules that have both a hydrophobic region that interacts with the stationary phase and a charged region that interacts with the analyte, while maintaining the typical water to methanol/acetonitrile gradient of RP-LC (Cajka and Fiehn 2016). The stationary phases used in HILIC chromatography include amine, amide, or free silanol groups, and, unlike RP, separation starts with a high proportion of organic solvent, while water is considered a highly eluotropic solvent. Metabolite retention is a combination of liquid-liquid

partitioning, adsorption, ionic interactions, and hydrophobic retention and heavily depends on the nature of the analyte and the composition of the mobile phase (Buszewski and Noga 2012). No single separation technique is able to resolve and detect the complete range of metabolites that may be present in a complex biological sample. Therefore, achieving the most comprehensive metabolome coverage may require the use of several column chemistries, or even complementary separation techniques (Dettmer et al. 2007; Leon et al. 2013). The main advantages of MS hyphenated to separation techniques are: (i) its high sensitivity, several orders of magnitude lower than NMR; (ii) its high resolution and selectivity; (iii) the possibility of performing fragmentation analyses to thus confirm the identity of the detected metabolites and the identification of unknown and unexpected compounds; and (iv) information is easier to handle than in NMR spectroscopy (Leon et al. 2013; Villas-Boas et al. 2005). In addition, MS allows analyses not only in a holistic or global way (untargeted or non-directed analyses), where the aim is to analyze the largest possible number of compounds, but it also enables guided analyses (targeted), in which detection focuses on a specific group of metabolites of interest, which enables work to be done with increased sensitivity and quantitatively (Robertson 2005). Although the latter may not be considered a true "omics" approach because it basically consists in a biased analysis, which is usually driven by a hypothesis or by a priori knowledge of the system under study, targeted analyses may be considered an important part of untargeted metabolomics. Once potential biomarkers have been deciphered by untargeted metabolomics analyses, they should be validated by performing targeted quantitative analyses (Fig. 3; Leon et al. 2013). Regarding MS detection, untargeted metabolomics requires instruments with a sensitive full-scan mode and accurate mass measurement. Quadrupole time of flight (Q-TOF) mass spectrometer meets such requirements as it combines the stability of a quadrupole with the high efficiency, sensitivity, and accuracy (<5 ppm) of a TOF. It also offers mass fragmentation capabilities for metabolite identification (Lahoz et al. 2006). Thanks to its capabilities, the Q-TOF mass spectrometer has become the instrument of choice for untargeted analysis approaches, although other instruments, such as Orbitrap or Fourier Transform Ion Cyclotron Resonance (FT-ICR), also meet high mass resolution capabilities (Leon et al. 2013). Targeted metabolomics requires the unambiguous identification and quantification of metabolites of interest. Triple quadrupole (TQ) mass spectrometers, which mostly work in the multiple reaction monitoring (MRM) mode, are the most common platform to perform targeted analyses. TQ mass spectrometers have a lower mass resolution than Q-TOF but offer greater sensitivity, specificity, robustness, and a wider dynamic range (Leon et al. 2013). Given that LC-MS is the most commonly used platform for holistic metabolome analysis, the following subsections of the present section are detailed for this particular case, although some steps can be extended to other analytical platforms.

Quality Control Analysis

The main premise in a metabolomics study is that the levels of detected metabolites reflect the biological status of the system under study. So besides the careful selection and definition of the abovementioned factors (i.e., type of sample, sampling

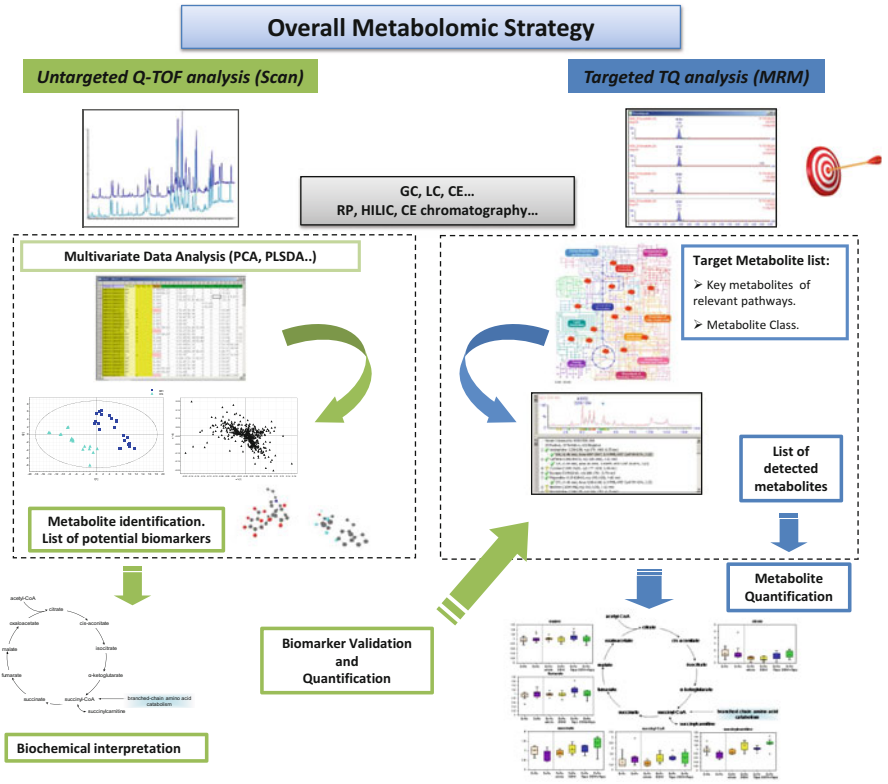

Fig. 3 Global MS-based metabolomics strategy. An initial assessment is made by using unbiased untargeted analyses to obtain a broad view of the metabolome to search for potential biomarkers. In a second step, a targeted approach is performed to provide an unambiguous quantitative assessment of the metabolites of interest

conditions, sample processing, analytical conditions to perform the metabolome analysis, etc.), it is mandatory to implement a quality assurance strategy during sample preparation and analyses to ensure the quality of the results and to also minimize and detect any sources of variation unrelated to the biological nature of the samples (Garcia-Cañaveras et al. 2011; Quintás et al. 2012). As real method validation is hardly achievable in untargeted metabolomics (Naz et al. 2014), quality assurance is usually based on the addition of internal standards to samples, the inclusion of quality control (QC) samples (i.e., blanks, pooled samples, commercially available pools, etc.), and the careful design of the sample acquisition process (Garcia-Cañaveras et al. 2011; Naz et al. 2014).

Metabolomic Data Analysis

Holistic (untargeted) LC-MS-based metabolomics analyses generate huge amounts of data. The information obtained for each sample is arranged into a great three-

dimensional matrix (retention time (rt), *m/z*, and intensity), in which each feature is informative of a metabolite, fragment, or adduct present in the sample. Before performing statistical analyses, it is necessary to process metabolomics data. Among other steps, processing includes the alignment of chromatographic peaks among samples and the standardization of variables to enable inter-sample comparisons. These processes can be performed by vendor's software (e.g., Markerlynx, Markerview, Masshunter, ProGenesis), by open access software (e.g., XCMS, MZmine), or by in-house scripts (e.g., R, Matlab) (Leon et al. 2013). Processed information can then be subjected to both uni- and multivariate analyses, although the latter is preferred given the nature of the data (Fig. 4). Regarding multivariate data analyses, two types of methods can be adopted: non-supervised (e.g., Principal Components Analysis (PCA)) and supervised methods (e.g., Partial Least Squares Discriminant Analysis (PLS-DA) or orthogonal projection to latent structures-discriminant analysis (OPLS-DA). In both analyses, the vast quantity of the experimental variables obtained (mega matrix) is reduced to a small number of latent variables (principal components) that explain in a much simpler way the similarities or differences between the samples (observations) and variables (metabolites) responsible for these differences or similarities. In a PCA, the first principal component describes the main difference or variance between samples, the second principal component (independent and orthogonal) describes the residual variance of samples after that explained by the first component, and so on. The main difference between supervised and non-supervised methods is that the algorithm in the latter relies on previous knowledge to, for example, classify samples (observations). So it finds the latent variable that best describes the differences between samples by taking into account which group they belong to (Robertson 2005; Kaddurah-Daouk et al. 2008). As in data preprocessing, data analyses can be performed by specific software (i.e., MassProfiler Professional, SIMCA, etc.), with freely available packages or in-house built scripts that use the R software (R Core Team 2014), or even with user-friendly guided tools, as is the case of the MetaboAnalyst (Xia et al. 2015), which is a set of online tools for metabolomic data analysis and interpretation.

Metabolite Identification

The last step in a metabolomic analysis is to identify the potential biomarkers selected in the multivariate analysis (Fig. 4). According to Metabolomics Standards Initiative criteria (Sumner et al. 2007), four levels of metabolite identifications can be found in the published metabolomics literature: (1) identified compounds: identity has been corroborated by the analysis of the authentic standard under the same analytical conditions and parameters, such as retention time (rt), and MS and mass fragmentation (MS/MS) spectra can be matched; (2) putatively annotated compounds (e.g., without chemical reference standards, based on physicochemical properties and/or spectral similarity with public/commercial spectral libraries); (3) putatively characterized compound classes (e.g., based on the characteristic physicochemical properties of a chemical class of compounds or by spectral similarity to known compounds of a chemical class); (4) unknown compounds: although

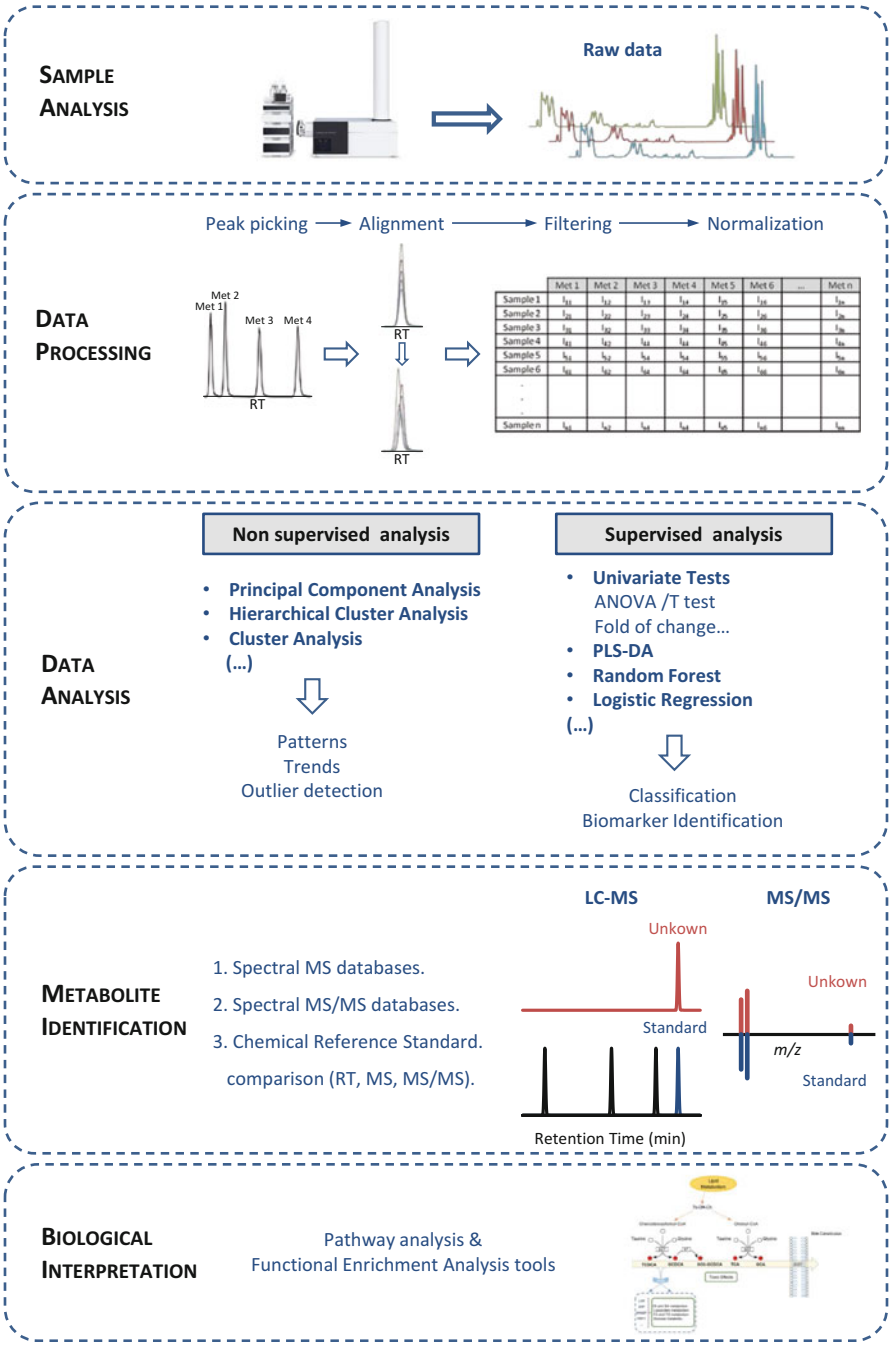

Fig. 4 The data workflow in MS-based metabolomics analyses. Several steps are needed before raw data can be interpreted and placed in a biological context. The first data workflow step is termed data processing and may include different steps, such as peak peaking, peak alignment, filtering, and data normalization or transformation. In the end, the raw data generated during sample analyses

unidentified or unclassified, these metabolites can still be differentiated and quantified according to spectral data (Sumner et al. 2007). While the definitive assignment of a feature (characterized by rt and *m/z*) to a known identity requires the use of chemical reference standards, initial metabolite identification stages are usually performed based on the use of publicly or commercially available databases. Some useful available online metabolite databases that allow to search both MS and MS/MS data are the Human Metabolome Database (Wishart et al. 2013), the LIPID MAPS-Nature Lipidomics Gateway (Fahy et al. 2007), the Metlin Database (Smith et al. 2005), and MassBank (Horai et al. 2010).

Biological Interpretation

After identifying the metabolites that are significantly altered as a result of a given pathophysiological situation of interest, it is of special relevance to place them in a context to extract useful and meaningful information (Fig. 4). Online pathway analysis tools, such as MBRole 2.0 (López-Ibáñez et al. 2016), metabolite set enrichment analysis, and metabolic pathway analysis, for metabolomic data interpretation integrated into MetaboAnalyst (Xia et al. 2015), may be helpful for this purpose.

Metabolomics in Organ Transplantation

Metabolomics/metabolic profiling, either MS or NMR based, has been used in many human health (or biomedicine) areas and covers a wide spectra of matrices from cells in culture (IPS, hepatocytes, cell lines, etc.) (Garcia-Cañaveras et al. 2016) to body fluids (urine, serum, bile, cerebrospinal fluid, etc.) (Soga et al. 2006; Trushina et al. 2013; Garcia-Cañaveras et al. 2012) and even tissues (liver, tumors, heart, adipose tissue, etc.) (Garcia-Cañaveras et al. 2011; Chan et al. 2009) and in a high diversity of study fields (cancer, cardiovascular disease, nonalcoholic fatty liver disease, diabetes, toxicity, etc.) (Brindle et al. 2002; Puri et al. 2007; Rhee et al. 2011; Cortes et al. 2014; Dang et al. 2009; García-Cañaveras et al. 2015). The success in such a diversity of biological samples and fields of application reflects the potential that metabolomics has to be really and fully incorporated into the clinical field.

Metabolomics has become an extremely useful tool to characterize the metabolic changes that can take place in an organ. Application of metabolomics in transplantation is still in its early stages, but metabolomic studies in solid organ transplants

Fig. 4 (continued) is arranged in a mega matrix that contains all the information. Then data analysis procedures are applied, which include both supervised and non-supervised methods and also uni- and multivariate techniques. Those features were found to be relevant according to the aim of the data analysis and are then subjected to metabolite identification procedures (various grades of identification confidence can be achieved based on the criteria established by the Metabolomics Standards Initiative). Finally, biological interpretation can be simplified by using freely available web-based tools to perform pathways analyses and/or functional enrichment analyses

have generally focused on monitoring three situations: (i) post-reperfusion damage, (ii) rejection, and (iii) organ dysfunction (Wishart 2008). Of all the material published on the matter, 60% is about renal transplants, followed by the liver (21%), heart (10%), pancreas (5%), and lungs (6%). Most metabolite measurements for organ transplantation have been performed ex vivo using body fluids like urine, serum, or bile (Sinclair et al. 1974; Saude et al. 2004; Hauet et al. 2000; Serkova et al. 2005; Silva et al. 2005; Gibelin et al. 2000; Martin-Sanz et al. 2003). Examples in organ transplantation include the diagnosis of acute cardiac rejection by analyzing plasma by ^{1}H-NMR spectroscopy (Mouly-Bandini et al. 2000), profiling acute renal rejection by GC-MS (Mao et al. 2008), and monitoring kidney transplant patients' immune responses and drug effects in early recovery by means of urine samples analyzed by ^{1}H-NMR (Stenlund et al. 2009). Most measured molecules are related to metabolic processes that generally exist in any living being, such as glycolysis, gluconeogenesis, lipid metabolism, etc. Changes in universal metabolites, such as glucose, citrate, lactate, ATP, and AFP reflect changes in cell viability, like apoptosis, levels of oxygenation (anoxia, blood flow), local pH, and homeostasis in general (Saude et al. 2004). These molecules provide information about cell function or cell stress and, therefore, about organ function. Other less used metabolites, like thromboxane, histamine, or chlorotyrosine, could reflect the immune function of inflammatory response (Sinclair et al. 1974; Saude et al. 2004).

Liver Transplantation

The use of metabolomics in liver transplantation (LT) is a challenge as it deals with an organ involved in many metabolic processes. Such a complex scenario hinders the possibility of finding a single biochemical test to generally assess liver function (Sakka 2007). The therapeutic success of LT has meant that more patients are susceptible to taking advantage of it. This increased demand has generated longer waiting lists, plus an increase in morbidity and mortality. In view of this situation, and in order to increase the number of donors, the criteria which define whether an organ is suitable or not have gradually changed, which means that "marginal" or "extended criteria" livers are being used with the subsequent risk of postoperative complications appearing, such as severe graft dysfunction and primary liver failure (Vilca Melendez et al. 2000). The open questions are: To what extent can we expand donor criteria? Which criteria should we apply to make the decision as to whether a liver can be used or not? And, in this context, can metabolomics provide valuable information? Despite the fact that organs from extended criteria donors (ECD) are not optimal, they are a good alternative to dying while being on a transplant waiting list (Busuttil and Tanaka 2003). Many factors play an important role in the onset of graft dysfunction or primary failure (Chen et al. 2007). Damage caused by ischemia/reperfusion injury (IRI) could be responsible for graft dysfunction in many cases. Hypothermia lowers the metabolism and helps maintain essential metabolic functions but induces cell damage (alterations in calcium homeostasis, cytoskeleton modifications, and local tissue destruction by proteases). Reperfusion implies the

production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, all of which are involved in IRI (Silva et al. 2007). Factors like pH, inflammatory response, and microcirculation changes aggravate cell damage. Despite their importance, all the mechanisms that cause graft dysfunction are not yet completely known.

During the first week after transplantation, most grafts show some sign of liver dysfunction, 20% of which are attributed to a defect in the liver's metabolic capacity. Some of the factors responsible for this should be considered *a priori* as potentially controllable, and they include the metabolic and functional quality of the graft. The availability of an objective criterion to assess graft quality before implant would be extremely useful for making decisions that would minimize the risk of severe metabolic dysfunction and/or primary liver failure. If this type of information could be made available together with the usual provided information, such as an anatomopathological study, it could avoid discarding an organ simply because of its macroscopic appearance (surgeon-related subjective criterion that does not always coincide), or elusive histology, and would therefore increase the number of useable organs based on objective criteria selection. Several studies have been carried out about LT using NMR and MS to quantify the graft injury secondary to cold preservation (Silva et al. 2007; Gibelin et al. 2000), graft recovery following transplant (Silva et al. 2007), and to also identify diagnostic and prognostic biomarkers of graft rejection and dysfunction (Martin-Sanz et al. 2003; Singh et al. 2003; Melendez et al. 2001; Cortes et al. 2014; Table 2). More detailed information about the data published on metabolomics, IRI, and graft function in LT is provided.

Ischemia Reperfusion Injury

During LT, a donor graft initially undergoes a period of ischemia from the time it is retrieved from the donor until blood supply is restored on reperfusion in the recipient, which enhances any damage produced during the ischemic period. This situation is termed IRI, which can result in poor graft function after transplantation (Serracino-Inglott et al. 2001). In 2003, *Martin-Sanz et al.* demonstrated decreased nitric oxide (NO) synthesis as a result of higher rates of nitric oxide synthase (NOS) inhibition in blood samples before reperfusion in recipients who presented graft dysfunction after LT. They indicated that lower NO levels can cause ischemia related to vasoconstriction and can participate in IRI (Martin-Sanz et al. 2003). A potent NOS synthase named asymmetric dimethylarginine (ADMA) was found in these patients, which showed increased levels in parallel to cold ischemia time (CIT) duration.

High lactate levels are usually found in ischemic processes where cells start anaerobic glycolysis, which results in raised lactate and lower pyruvate levels (Sommer and Larsen 2004). *Silva et al*. observed high levels of lactate and pyruvate upon reperfusion, which slowly normalized during the following 12 h if the liver recovered from initial ischemic insult. These authors also described that the levels of four amino acids (alanine, GABA, glutamate, and taurine) lowered during the monitoring period, but at different rates and time points (Silva et al. 2007).

Table 2 Metabolic markers associated with ischemia reperfusion injury and graft dysfunction in humans

Author	Condition	Increased metabolites	Platform	Decreased metabolites	Samples
Vilca-Melendez 2001	Posttransplant	Phosphatidylcholine	^{1}H-NMR		Bile
Martin-Sanz et al. 2003	Ischemia reperfusion injury	Methylarginine dimethylarginine	HPLC-MS		Perfusate Blood
Singh et al. 2003	Graft dysfunction	Glutamine	^{1}H-NMR	Urea (urine)	Blood Urine
Silva 2005	Ischemia reperfusion injury	Lactate, pyruvate, glycerol, alanine, glutamate, GABA, taurine, arginine (<19 h)	HPLC-MS CEAD	Arginine (<19 h) Synthesis of NO	Dialysate
Serkova 2007	Graft dysfunction	Lactate, uric acid, and citrate	^{1}H-NMR		Blood
Tripathi et al. 2009	Graft dysfunction	Lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine	^{1}H-NMR		Blood (serum)
Hrydziuszko et al. 2010	Ischemia reperfusion injury	Urea and urea cycle intermediate levels (e.g., N4-acetylaminobutanal, 5'-methylthioadenosine) Increased bile acid levels (e.g., chenodeoxyglycocholate, glycodeoxycholate, glycochenodeoxycholate, and glycholate) Disturbance of energy metabolism (formate, orthophosphate, ADP, fumarate, succinate)	FT-ICR MS CEAD		Liver tissue Dialysate
Cortes et al. 2014	Graft dysfunction	Lysophosphatidylcholines and lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, bile acids, and products of histamine metabolism	UPLC-MS		Liver tissue

In 2009, *Hrydziuszko et al*. compared the metabolic profile of liver biopsies after both procurement and reperfusion and used a FT-ICR MS-based metabolomics approach with colorimetric electrochemical array detection (CEAD) in microdialysates for the first time. The main metabolic changes observed upon reperfusion were increased urea production, higher urea cycle intermediate levels (e.g., *N*4-acetylaminobutanal, 5-methylthioadenosine), and raised bile acid levels (e.g., chenodeoxyglycocholate, glycodeoxycholate, glycochenodeoxycholate, and glycholate). Further molecular changes included the anticipated disturbance of energy metabolism, with consistent increases in several metabolites (e.g., formate, orthophosphate, ADP), particularly those involved in oxidative phosphorylation (e.g., fumarate, succinate) in post-reperfusion biopsies. One of these authors' major findings was that the metabolite profile in a donor after circulatory death (DCD) in the cold phase was similar to the metabolic profile after reperfusion in those livers obtained from donors after brain dead (DBD) (Hrydziuszko et al. 2009).

Graft Function Assessments

Extended criteria donors in LT (elderly donors, DCD, etc.) are increasingly becoming a source of organs, which are more susceptible to ischemic insult. IRI plays a central role in posttransplant complications, especially in graft function. Therefore, the expansion of donor criteria requires an objective quality graft assessment to predict or avoid complications (Vogel et al. 2012). Many tests have been evaluated to assess the pretransplant graft function. To date, however, none has found its place in the clinical practice (Vilca Melendez et al. 2000). Bile secretion has been generally accepted as an early posttransplantation sign of liver recovery (Ericzon et al. 1990). However, bile secretion was not studied in donor livers until 1998, when *Vilca-Melendez et al*. focused on analyzing its profiling (bile acid composition) in donors upon organ retrieval, and in recipients immediately after reperfusion, after developing a standardized bile collection technique (Vilca-Melendez et al. 1998). This study showed no difference in bile flow to differentiate between "suboptimal" and normal grafts. However, these "suboptimal" grafts showed a higher concentration of bile acids, which indicated that bile flow did not increase appropriately with the higher concentration of bile acids. The same authors postulated that this finding could be related to water secretion impairment at a canalicular level or due to a reduction in the bile acid-independent promoters of bile flow, such as glutathione, bicarbonate, calcium, sodium, potassium, glucose, amino acids, and organic acids. The donor bile from suboptimal grafts had a higher proportion of cholic acid than normal grafts. It is well known that the canalicular bile flow depends not only on the amount of bile acids secreted but also on bile acid composition (Howard and Murphy 1990).

An impairment in the urea cycle during acute liver failure results in abnormally high levels of blood ammonia, which triggers glutamine synthesis (Suarez et al. 2002) and decreased urea levels. *Sing et al*. observed higher glutamine levels in blood and urine, and lower urea levels in urine, by ^{1}H-NMR spectroscopy in a patient who presented liver failure related to vascular complications after transplantation. Thus monitoring glutamine levels in blood and urine, along with urea levels in urine, has been proposed as a predictor of graft function (Singh et al. 2003).

Increased circulating amino acids (e.g., tyrosine, glutamine, leucine) have been correlated with decreased catabolism by the liver, which reflects hepatocyte injury and death (Saxena et al. 2006). These results were confirmed by *Tripathi et al*., who analyzed serum specimens by ^{1}H-NMR in liver transplant patients preoperatively and at various time points following transplantation. These authors observed high levels of lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine in the patient who died after LT due to graft dysfunction (Tripathi et al. 2009). This finding agrees with the earlier analyses performed both on patients with experimental models of chronic liver failure (Tietge et al. 2002).

Duarte et al. analyzed liver biopsies, which were collected at three different time points during LT: before organ retrieval, during cold storage, and after implantation. The purpose was to find metabolic signatures that reflected graft success. The metabolomic platform used was high-resolution magic angle spinning (HR-MAS) ^{1}H-NMR, a variation of conventional ^{1}H-NMR that can be performed on solid samples, and it was the first metabolomic application of such spectroscopy used with human liver tissues. A larger amount of triglycerides and unsaturated lipids, and lower levels of phospholipids, were found on the grafts with fatty infiltration (Duarte et al. 2005). Recently, *Xu et al*. described for the first time different lipid profiles between two types of donors as DBD and DCD. For this purpose, they initially performed an untargeted approach, followed by a targeted analysis using UPLC-MS. DCD livers showed higher concentrations of LysoPCs, which is a known precursor of the platelet-activating factor, a potent phospholipid inflammatory associated with IRI (Xu et al. 2015).

Despite all the potential biomarkers described in the literature, transplant surgeons still have to rely on subjective donor data interpretations, evaluations of the macroscopic appearance of the graft (shape, color, appearance, and feel), and occasionally on the histological analysis of a liver biopsy, in order to assess the graft's suitability for use. Liver biopsies assess the degree of steatosis, fibrosis, sepsis, and ischemia. Yet some controversy still exists when evaluating organs that present mild to moderate steatosis. What this reflects is both the difficulty to predict graft functionality based on changes in morphology (Angele et al. 2008) and lack of a functional assessment that really helps rule out grafts with a high risk of primary non-function, or to accept organs that, based on subjective assessments, would have been ruled out for transplant. Recently, a metabolomics attempt to predict donor liver function after transplantation has proven to be a useful tool to assess organs before transplantation. In this work, a metabolomic pattern, which allows donor quality assessment, has been deciphered by using a MS-based metabolomic approach to analyze the liver biopsies collected after organ retrieval (Cortes et al. 2014). The novelty of this approach lies in the use of two chromatographic techniques (i.e., RP and HILIC), which allow the coverage of the metabolites identified in the previous LC-MS-based analysis to be extended (Cortes et al. 2010). Using multivariate data analysis (i.e., PCA and PLS-DA), the relationship between the metabolomic profile present in liver biopsies and their subsequent function in the

recipient according to the Olthoff classification (Olthoff et al. 2010) was investigated. A set of 93 metabolites was used, which are significantly involved in the metabolic processes related to early allograft dysfunction (EAD) and initial good function (IGF) distinctions, and are made up of amino acids and peptides, carbohydrates, vitamins and cofactors, bile acids, carnitines, fatty acids, products of the glycerolipid metabolism, lysophospholipids, phospholipids, and sphingomyelins. The patients who presented EAD showed significantly higher levels of lysophosphatidylcholines and lysophosphatidylethanolamines, lysophospholipids, phosphatidylcholines, phosphatidylethanolamine, phospholipids, sphingomyelins, bile acids, and products of histamine metabolism (Fig. 5). The lipidomic pattern found in this study could prove to be an interesting diagnostic instrument in clinical practice as phospholipid homeostasis alteration may indicate cellular membrane disruption, which would thus trigger different mechanisms of hepatocellular death (Arora et al. 1997). It has been recently demonstrated that lysophosphatidylcholines are toxic metabolites generated by the hydrolysis of the phospholipids catalyzed by phospholipase A2, which acts as a promoter of cell death (Kakisaka et al. 2012; Han et al. 2008). The significant accumulation of lysophosphatidylcholines observed in the EAD group suggests a greater predisposition of these grafts to lipid-dependent apoptosis, which can dramatically affect posttransplant graft functioning. Bile salts are considered key signal molecules as physiological ligands of the farnesoid X receptor, an intracellular sensor that controls the expression of the genes involved in the metabolism of lipids, lipoproteins, and glucose (Hylemon et al. 2009). Previous publications have suggested that bile salts are powerful function markers used to monitor LT and rejection (Vilca Melendez H et al. 2004). In agreement with the findings described by *Vilca-Melendez et al*., the excessive accumulation of bile salts and phospholipids observed in poor-quality liver grafts could make the initial bile excretion in the liver graft difficult, once it has been implanted, which is considered an early sign of graft function (Vilca Melendez et al. 2004; Hedaya et al. 2009). The key role of homeostasis in bile salts during progression after LT is supported by previous studies that have reported the altered expression of numerous genes related to the synthesis of bile acids and their transport (e.g., BAAT, CYP7A1, SULTA2, MDR2, BSEP), as well as the nuclear factors involved in the regulation of these genes (e.g.,: HNFα, FXR, SREBF1) associated with early graft dysfunction (Fouassier et al. 2007; Defamie et al. 2008). In grafts that present EAD, alterations have been found in other metabolic pathways that are not directly related to lipid metabolisms, such as histidine metabolism. High histamine levels could be indicative of the adaptive response to liver damage as a result of CIT or graft preservation for the purpose of reducing the release of cytokines thorough histamine H4 receptor activation (Motoki et al. 2008). It has also been proven that histamine acts via receptors H1-H4 to trigger the signal for metabolic pathways by proliferating and differentiating from cholangiocytes. These regulating processes have to be forced under cholestatic conditions, like those previously described in grafts with EAD (Francis et al. 2012).

Pathway	In bckgnd.	hits	*p-value*	*Adj. p-value*
Phospholipid Biosynthesis	19	6	2.70E-05	5.64E-04
Bile Acid Biosynthesis	49	5	1.85E-02	6.78E-02
Histidine Metabolism	11	4	4.90E-04	3.04E-03
Ammonia Recycling	18	4	4.46E-04	3.16E-03
Aminoacyl-tRNA biosynthesis	28	3	2.06E-02	6.78E-02
Urea Cycle	20	2	1.37E-02	1.14E-02
Glycerolipid Metabolism	13	2	3.08E-02	8.85E-02
Glycerol Phosphate Shuttle	8	2	1.18E-02	6.78E-02
Glutathione Metabolism	10	2	1.85E-02	6.78E-02

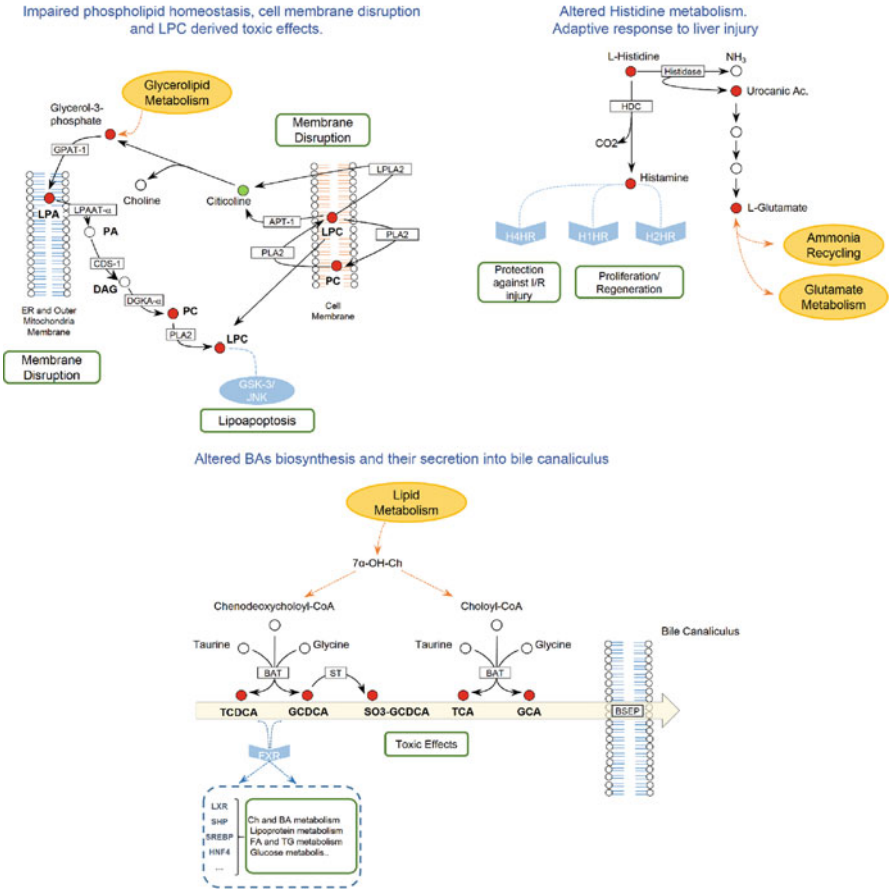

Fig. 5 Biological interpretation of the EAD metabolomic biosignature. (**a**) Summary panel for the metabolite set enrichment analysis where metabolic pathways are ranked according to their impact on EAD. (**b**) Impaired phospholipid homeostasis derives in cell membrane disruption and provokes toxic effects, triggered by lysophosphatidylcholine (LPC) accumulation. (**c**) Altered lipid metabolism provokes downstream alterations of bile acid biosynthesis and accumulation derives into toxic effects and FXR receptor activation. (**d**) Alteration in histidine metabolism as a response to ischemia. The metabolites depicted in green and red are, respectively, down- or upregulated

Future Applications of Metabolomics in Liver Transplantation

The increasing number of patients on waiting lists for LT has driven transplant centers to use ECD, which has raised the posttransplantation incidence of EAD up to 27%. This condition is associated with increased rates of suffering acute cellular early rejection, sepsis, and with longer intensive care unit and hospital stays, which can result in higher rates of graft loss, recipient morbidity, and mortality (Briceño and Ciria 2010; Salvalaggio et al. 2013). As previously mentioned, many efforts have been made to assess graft function before transplant by evaluating different liver metabolism aspects: bile secretion, hepatic protein synthesis, drug-metabolizing capabilities, organ morphology, etc. (Vilca-Melendez et al. 2000). However, a well-accepted functional assessment of an organ before its transplantation is still lacking. Therefore, the discovery of objective biomarkers that can assess graft quality and anticipate its later function would be greatly appreciated and be of much interest, especially when evaluating ECD. In addition, the need to increase the donors' pool has led to an increased utilization of DCD grafts, which itself is considered an extended criteria that has been associated with higher early graft failure and cholangiopathy rates (Garcia-Valdecasas et al. 1999; Kukan and Haddad 2001). While such grafts are being assessed, it is difficult to evaluate the liver damage caused, particularly, by warm ischemia time, which cannot be observed macroscopically. Therefore, markers that could predict the posttransplant graft function would constitute an invaluable tool to help decide whether to accept it or not.

Normothermic machine perfusion (NMP) has become an emerging preservation modality designed to maintain the liver metabolically active during storage and has the potential to prevent injury associated with low temperature and to promote physiological organ repair following ischemic cell damage. Several animal studies have demonstrated the feasibility and superiority of normothermic liver perfusion during cold storage (CS) with a lower inflammatory response after reperfusion and longer survival rates (Fondevila et al. 2011). The benefits of this technique are that it allows the thorough analysis of its quality by measuring parameters, e.g., bile production, lactate, glucose, and oxygen composition. In this context, the metabolomic analysis of bile and/or the perfusate obtained from the graft would add not only the liver quality assessment but also the effect of normothermic preservation on the graft. More importantly, this metabolomic analysis would become a priceless tool during organ assessment to recover livers that have been previously discarded when preserved in cold.

Although the use of metabolomics in organ transplantation is in its early stages, the above-described applications indicate its huge potential in this field. Assessing donor liver quality before LT in order to make the most of limited resources available, and to anticipate possible clinical complications related to graft function, remains a challenge. Furthermore, a rigorous validation of the metabolomic procedures in multicentre studies is mandatory to ensure their usefulness in a clinical environment.

Summary Points

- The term biomarker refers mostly to macromolecules or metabolites which have become surrogate markers in basic and clinical research and in clinical practice and offer the advantage of being an objective, quantifiable, and reproducible measure.
- Biomarkers offer different applications in the human health field, not only once the disease is present but also to predict its potential appearance.
- Metabolomics, through the simultaneous measurement of hundreds of small molecules, can provide a more comprehensive "snapshot" than the simple determination of a single marker.
- Metabolomic studies in solid organ transplants have focused especially on monitoring three situations: post-reperfusion damage, rejection, and dysfunction of the organ.
- Markers of graft dysfunction and ischemia reperfusion damage indicate the following metabolic pathways/function: amino acids, urea cycle, bile acids, and energy metabolism.
- The most novel use of metabolomics in liver transplantation is the assessment of donor liver quality before transplantation.
- The following pathways/groups of metabolites have been found to be markers of organ quality, determined based on the function of the graft once transplanted: bile acids, histamine metabolism, phospholipids, and lysophospholipids.
- Metabolomics has the potential to be a crucial tool in assessments of graft quality before liver transplantation, in anticipating clinical complications related to graft function and in assessing the parameters that affect graft quality during its manipulation before implantation.

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