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Nuclear magnetic resonance metabolomics and human liver diseases: The principles and evidence associated with protein and carbohydrate metabolism (Review)

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Abstract. During the last decade, metabolomics has become widely used in the field of human diseases. Numerous studies have demonstrated that this is a powerful technique for improving the understanding, diagnosis and management of various types of liver disease, such as acute and chronic liver diseases, and liver transplantation. Nuclear magnetic resonance (NMR) spectroscopy is one of the two most commonly applied methods for metabolomics. The aim of the present review was to investigate the results from recent key publications focusing on aspects of protein and carbohydrate metabolism. The review includes existing procedures, which are currently used for NMR data acquisition and statistical analysis. In addition, notable results obtained by these studies on protein and carbohydrate metabolism concerning human liver diseases are presented.

Contents

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Abbreviations: BCAA, branched-chain amino acids; AAA, aromatic amino acids; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; HR-MAS, high-resolution magic-angle spinning; MHE, minimal hepatic encephalopathy; NAFLD, non-alcoholic fatty liver disease; NMR, nuclear magnetic resonance; PCA, principal component analysis

Key words: metabolomics, chronic and acute liver disease, nuclear magnetic resonance

1. Introduction

Outcomes for patients suffering from liver diseases are particularly difficult to predict (1). Biochemical liver markers facilitate with disease diagnosis (2), however, there is a lack of useful biomarkers that allow physicians to identify patients at higher risk of developing these diseases or that enable physicians to predict treatment response. In order to accurately determine patient prognoses and to adapt therapeutic procedures to each patient's situation, three solutions are proposed as follows: i) Interpretation of novel data from clinical or biological tests; ii) identification of novel biomarkers; and iii) novel methods for processing of biochemical and clinical data. The identification and validation of biomarkers is currently a major focus of liver disease research, and aims to refine liver disease prognosis and to adapt therapeutic procedures to individual patient features. The characteristics defining these biomarkers are required to be: i) Applicable in routine practice, ii) discriminant, and iii) accurate, robust and universal. Proteomics and metabolomics are new approaches to the identification and characterization of novel biomarkers. High-throughput analysis of metabolism in a biological fluid now allows the evaluation of a large number of metabolites simultaneously [for example, in the case of liver cancer (3)]. It is widely accepted that the concept of identifying a single biomarker in routine clinical practice for the management of patients suffering from a liver disease is probably obsolete (4). Furthermore, it may be more informative to correlate biomarker levels with clinical events and to analyse its variation during follow-up than to have a single measurement of a given compound in a biological fluid. Metabolomic approaches necessitate a translational bridge between clinical and basic research. The aim

of the present review is to provide a state-of-the-art overview of nuclear magnetic resonance (NMR) metabolomic studies in the field of human liver diseases using the Pubmed database (from 1994 up until May 2016). The review briefly presents the different biofluids or tissues that have been investigated, the NMR experiments that are recorded and the multivariate analyses that were performed in the NMR metabolomic liver studies. In addition, the dysfunctions associated with protein and carbohydrate metabolism that have been illustrated in these studies are reviewed.

2. Data acquisition and statistical analysis

Data acquisition. Typical data acquisition has previously been reviewed (5). The majority of the studies investigated samples obtained from serum or plasma (Tables I and II) (6-25), which are usually the most pertinent biofluids for hepatological disease. Various studies sought to identify urine NMR fingerprints (22,26) and one study combined urine and plasma (18). Notably, using endoscopic retrograde cholangiopancreatography, Sharif *et al* (25) collected and evaluated bile samples by NMR. To limit inter-individual variability and to optimize metabolite concentration variations that correspond to intra-individual variability, previous studies collected sequential sera (19,27). The second type of sample investigated consisted of intact tissues that were evaluated by high-resolution magic-angle spinning (HR-MAS) spectroscopy, which is performed on whole tissue from liver biopsies and preserves the majority of the cellular architecture (6,12,14). The typical tissue mass required is between 15 and 25 mg, however, even 1 to 10 mg is sufficient when using smaller sample vessels. Concerning biopsies or tissues, a dual chloroform/methanol extraction may be performed to separate water-soluble and lipid-containing extracts (19). In all publications, classical NMR experiments (one-dimensional nuclear Overhauser effect spectroscopy and/or Car-Purcell-Meiboom-Gill) and 2D experiments (total correlation spectroscopy and J-resolved) were recorded. A study by Duarte *et al* (28) using heteronuclear multiple quantum coherence 1H-13C spectra proposed to easily identify metabolites from chemical shifts. Notably, this study also measured T1 and T2 relaxation times for CH_2-CH_2-CO lipid protons, which are correlated with lipid mobility. Another promising approach is to combine data from various-omics (metabolomics, proteomics, transcriptomics and genomics) techniques or from clinical or biological data. Andersson *et al* (20) combined NMR with mass spectrometry metabolomics and proteomics results. Cobbold *et al* (12) combined the results of *in vitro* 1H NMR spectroscopy with *in vivo* magnetic resonance spectroscopy to specifically determine lipid compositions.

Statistical analysis. The multivariate analysis methods, which are most commonly used in the studies discussed in the current review, are presented.

Unsupervised analysis. Analyzing spectroscopic data information with unsupervised Principal Component Analysis (PCA) is generally the first step of the statistical investigation (29). The aim of this technique is to reduce the multidimensional data space by preserving the majority of the variance (30). Usually, PCA is used for the detection of outliers or to observe clustering of the dataset according to important variations in metabolite profiles.

Supervised analysis. The second step in most of the statistical analyses for metabolomics is to use a regression-based method termed partial least squares (PLS) analysis. PLS analysis is the most widely used statistical method to interpret NMR multivariate data (31). PLS models attempt to establish a linear association between an X predictor matrix (e.g., spectrometric data of biological samples) and a Y response matrix (e.g., clinical results or treatments). Based on PLS analysis, PLS-discriminant analysis (DA) was introduced specifically for classification problems (32). Orthogonal projections to latent structures DA, which is an extension of PLS-DA, is based on applying an integrated orthogonal signal correction filter to remove variability not relevant to class separation (33). This statistical method has been increasingly used. The validation is essential, particularly from a clinical perspective. The only method of reliably estimating the ability of the model to predict Y values of novel individuals is to predict individuals from an independent dataset (i.e., those that were not used to build the model). The cross-validation method is predominantly used when no independent dataset is available. The principle of this technique consists of splitting the cohort into training and validation sets. The training set is used to build a model that is posteriorly tested with the validation set. The cross-validation process is completed when all samples in the cohort have been used at least once in the training and validation sets. Considering this principle, many cross-validation procedures have been proposed. These procedures may differ in the method by which the training and validation sets are constructed. A recent publication evaluated the impact of rows order on cross-validation quality parameters (34).

Further statistical methods. Additional methods will be cited, however a full explanation of the statistical basis of these methods is outside the scope of the present review. The significance analysis of microarrays and metabolites approach was used by Munshi *et al* (18). This method recognizes the chemical shifts (metabolites) with statistically significant differences between experimental groups by integrating data from a set of metabolite-specific t-tests wherein each chemical shift is given a score, calculated on the basis of changes in its level relative to the standard deviation of repeated measurements for those chemical shifts. The chemical shifts with scores above a defined threshold are considered as potentially significant.

Variable selection was used by Godoy *et al* (35). The n-1 components of the PCA (where n is the number of patients) were calculated. The components associated significantly with the question (discrimination between disease group and controls) were used to build a function predicting assignment to one group or the other.

Serkova *et al* (19) followed metabolite trajectories. Three blood samples (2, 24 and 48 h after liver transplantation) were used to measure and follow metabolite concentrations. An additional method used by Westerhuis *et al* (36) was paired

Table I. Protein and amino acid metabolism.

Metabolite	Reference	Model pathology	Variation	Sample	Author, year (Refs.)
Alanine	Adjacent tissue	HCC	+	Liver tissue	Yang, 2007 (6)
	Low-grade HCC	High-grade HCC	+	Liver tissue	Yang, 2007 (6)
	Survivors after transplantation	Non-survivors transplantation	+	Serum	Tripathi, 2009 (7)
	Controls	Cirrhosis (HBV/EtOH)	+	Serum	Qi, 2012 (8)
	Controls	NAFLD	+	Serum	Cheng, 2015 (9)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
		Acetaminophen toxicity	+	Urine	Winnike, 2010 (11)
	Cirrhosis	Mild or moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
		Cirrhosis gravity	-	Serum	Amathieu, 2011 (13)
Aspartate	Survivors after transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Cirrhosis	Fibrosis	-	Liver tissue	Martínez-Granados, 2011 (14)
Glutamate	Adjacent tissue	HCC	+	Liver tissue	Yang, 2007 (6)
	Low grade HCC	High grade HCC	+	Liver tissue	Yang, 2007 (6)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Controls	Cirrhosis (HBV/EtOH)	- and +	Serum	Qi, 2012 (8)
	Cirrhosis	HCC	+	Serum	Nahon, 2012 (16)
	Cirrhosis	Fibrosis	+	Liver tissue	Martínez-Granados, 2011 (14)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
	EtOH cirrhosis	HBV cirrhosis	-	Serum	Qi, 2012 (8)
Glutamine		Cirrhosis gravity	+	Serum	Amathieu, 2011 (13)
		Fulminant hepatic failure	+	Serum urine	Saxena, 2006 (17)
	Survivors	Non-survivors			
	HBV vs. HEV	HBV vs. HEV	+	Plasma or urine	Munshi, 2011 (18)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Adjacent tissue	HCC	+	Liver tissue	Yang, 2007 (6)
	Low-grade HCC	High-grade HCC	+	Liver tissue	Yang, 2007 (6)
	Functional liver transplantation	Non-functional liver transplantation	+	Blood (extraction)	Serkova, 2007 (19)
	Survivors transplantation	Non-survivors transplantation	+	Serum	Tripathi, 2009 (7)
	Cirrhosis	HCC	-	Serum	Nahon, 2012 (16)
	Cirrhosis	Fibrosis	-	Liver tissue	Martínez-Granados, 2011 (14)
	Cirrhosis	Mild or moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
	Controls	Cirrhosis (HBV/EtOH)	- and +	Serum	Qi, 2012 (8)
	EtOH cirrhosis	HBV cirrhosis	-	Serum	Qi, 2012 (8)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
		Ximelagatran	+	Plasma	Andersson, 2009 (20)
Glycine	Adjacent tissue	HCC	+	Liver tissue	Yang, 2007 (6)
	Low-grade HCC	High-grade HCC vs. low-grade HCC	+	Liver tissue	Yang, 2007 (6)
		Acetaminophen toxicity	+	Urine	Winnike, 2010 (11)
	Controls	NAFLD	-	Serum	Cheng, 2015 (9)
	Cirrhosis without MHE	Cirrhosis+MHE	-	Serum	Jimenez, 2010 (21)
	Cirrhosis	Mild vs. moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
	Control	HEV	-	Plasma	Munshi, 2011 (18)
	Control	HCC	-	Urine	Shariff, 2011 (22)

Table I. Continued.

Metabolite	Reference	Model pathology	Variation	Sample	Author, year (Refs.)
Histidine	Control	HEV+HBV	-	Urine	Munshi, 2011 (18)
	Survivors transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Controls	Cirrhosis (HBV/EtOH)	+	Serum	Qi, 2012 (8)
		Acetaminophen toxicity	+	Urine	Winnike, 2010 (11)
Isoleucine	Control	HEV	+	Plasma	Munshi, 2011 (18)
		Cirrhosis gravity	+	Serum	Amathieu, 2011 (13)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Cirrhosis-MHE	Cirrhosis+MHE	-	Serum	Jimenez, 2010 (21)
	Controls	Cirrhosis (HBV/EtOH)	-	Serum	Qi, 2012 (8)
Leucine	Adjacent tissue	HCC	+	Liver tissue	Yang, 2007 (6)
	Low-grade HCC	High-grade HCC	+	Liver tissue	Yang, 2007 (6)
		Cirrhosis gravity	+	Serum	Amathieu, 2011 (13)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Cirrhosis -MHE	Cirrhosis+MHE	-	Serum	Jimenez, 2010 (21)
	Controls	NAFLD	+	Serum	Cheng, 2015 (9)
	Control	Decompensated cirrhosis	-	Plasma	McPhail, 2016 (23)
	Controls	Cirrhosis (HBV/EtOH)	-	Serum	Qi, 2012 (8)
Lysine	Survivors transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
	Cirrhosis	Mild vs. moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
	Controls	Cirrhosis (HBV/EtOH)	-	Serum	Qi, 2012 (8)
Methionine		Cirrhosis gravity	+	Serum	Amathieu, 2011 (13)
	Functional liver transplantation	Non-functional liver transplantation	+	Blood (extraction)	Serkova, 2007 (19)
	Control	Decompensated cirrhosis	+	Plasma	McPhail, 2016 (23)
	Survivors transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Cirrhosis-MHE	Cirrhosis+MHE	+	Serum	Jimenez, 2010 (21)
Phenylalanine	Controls	Cirrhosis (HBV/EtOH)	+	Serum	Qi, 2012 (8)
	Survivors transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Controls	NAFLD	+	Serum	Cheng, 2015 (9)
	Control	Decompensated cirrhosis	+	Plasma	McPhail, 2016 (23)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
	Control	HEV+HBV	-	Urine	Munshi, 2011 (18)
	Non-injury	Injury	+	Serum	Ranjan, 2006 (24)
Proline	Control	HEV+HBV	+	Plasma/urine	Munshi, 2011 (18)
Threonine	Cirrhosis	Mild vs. moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
Tryptophan	Control	HEV+HBV	-	Plasma and urine	Munshi, 2011 (18)
Tyrosine	Survivors transplantation	Non-survivors	+	Serum	Tripathi, 2009 (7)
	Controls	Cirrhosis (HBV/EtOH)	+	Serum	Qi, 2012 (8)
	Control	Decompensated cirrhosis	+	Plasma	McPhail, 2016 (23)
	Controls	NAFLD	+	Serum	Cheng, 2015 (9)
	Control	HEV+HBV	-	Plasma	Munshi, 2011 (18)
	Non-injury	Injury	+	Serum	Ranjan, 2006 (24)

Table I. Continued.

Metabolite	Reference	Model pathology	Variation	Sample	Author, year (Refs.)
Valine	Control	HEV+HBV	-	Plasma and/or urine	Munshi, 2011 (18)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Cirrhosis-MHE	Cirrhosis+MHE	-	Serum	Jimenez, 2010 (21)
	Control	NAFLD	+	Serum	Cheng, 2015 (9)
	Control	Decompensated cirrhosis	-	Plasma	McPhail, 2016 (23)
	Controls	Cirrhosis (HBV/EtOH)	-	Serum	Qi, 2012 (8)
Ornithine	Control	HEV+HBV	-	Plasma and/or urine	Munshi, 2011 (18)
Glycine-conjugates	Non-cancer	Cholangiocarcinoma	+	Bile	Shariff, 2010 (25)
Taurine-conjugates	Non-cancer	Cholangiocarcinoma	+	Bile	Shariff, 2010 (25)
TMA	Controls	Cirrhosis (HBV/EtOH)	+	Serum	Qi, 2012 (8)
TMAO	Controls	HCC	-	Urine	Shariff, 2011 (22)
	Cirrhosis-MHE	Cirrhosis +MHE	+	Serum	Jimenez, 2010 (21)
	Cirrhosis	Mild vs. moderate fibrosis	-	Liver tissue	Cobbold, 2010 (12)
		Acetaminophen toxicity	-	Urine	Winnike, 2010 (11)
Uric acid	Functional liver transplantation	Non-functional liver transplantation	+	Blood (extraction)	Serkova, 2007 (19)
Hippurate	Controls	HCC vs. Cirrhosis	-	Urine	Shariff, 2011 (22)
Creatine	Low-grade HCC	High-grade HCC	+	Liver tissue	Yang, 2007 (6)
N-acetyl-asp	Control	HBV	-	Urine	Munshi, 2011 (18)
N-acetyl-glycoproteins		Cirrhosis gravity	-	Serum	Amathieu, 2011 (13)
	Control	Decompensated cirrhosis	-	Plasma	McPhail, 2016 (23)
	Control (HCV)	Cirrhosis (HCV)	-	Serum	Embade, 2016 (15)
	Cirrhosis	HCC	+	Serum	Nahon, 2012 (16)
	Cirrhosis-MHE	Cirrhosis+MHE	-	Serum	Jimenez, 2010 (21)

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; EtOH, ethanol; NAFLD, non-alcoholic fatty liver disease; HCV, hepatitis C virus; HEV, hepatitis E virus; MHE, minimal hepatic encephalopathy; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

analysis. Briefly, the between subject variation, which is described by the average of two observations from each subject (for example before and after treatment) is separated from the within subject variation, which is the difference between the two observations. This split provides the advantage of specifically discriminating the metabolic changes within individuals caused by the intervention and allows evaluation of the large variability between human subjects due to factors such as age, body mass index, genetics or environmental differences [for an example, refer to Goossens *et al* (27)].

Random forests were used by Liu *et al* (37). This algorithm is particularly efficient in cases of large datasets. It has been widely used in genomic and proteomic studies, and it may be suitable for metabolomic studies. In addition to the above-mentioned types of analyses, non-linear methods were used (38).

3. Metabolism in the liver

This section of the review presents the metabolic transformations described in the NMR studies that have attempted to characterize liver diseases. The liver is considered to be the major body organ for metabolism, storage and detoxification. The nutriments and xenobiotics that cross the intestinal barrier reach the liver before being delivered through the general circulation. In the liver, these compounds undergo metabolic transformations that may activate or deactivate the molecules. In the majority of cases, metabolic transformation produces metabolites that are directly usable by other organs.

In addition, the current review describes the major metabolic pathway impairments that are highlighted by NMR metabolomic studies of hepatic disease. The xenobiotic detoxification process that takes place in the liver will not be considered here. A recent review focused on hepatocellular carcinoma (HCC) (39) and lipid metabolism was recently investigated (40). Protein and carbohydrate metabolism modulations that are the result of liver pathologies are presented. Furthermore, a summary of these metabolic pathway modifications is provided in Tables I and II (6-25).

Protein metabolism. The liver is where the synthesis and degradation of proteins occur. Amino acids (AA) absorbed in the intestine

Table II. Glucose metabolism and the tricarboxylic acid cycle.

Metabolite	Reference	Model pathology	Variation	Sample	Author, year (Refs.)
Citrate	HBV	HEV	-	Urine	Munshi, 2011 (8)
	Control (HCV)	Cirrhosis (HCV)	+	Serum	Embade, 2016 (15)
	Healthy or cirrhosis	HCC	-	Urine	Shariff, 2011 (22)
	Functional liver transplantation	Non-functional liver transplantation	+	Blood (extraction)	Serkova, 2007 (19)
	Cirrhosis	Mild or moderate fibrosis gravity	+	Serum	Amathieu, 2011 (13)
Fumarate	Control	HEV+HBV	-	Plasma	Munshi, 2011 (8)
Succinate	Control	HBV	-	Urine	Munshi, 2011 (8)
	Control	Cirrhosis (HBV or EtOH)	+	Serum	Qi, 2012 (8)
	Control	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
Glucose	Adjacent tissues	HCC	-	Liver tissue	Yang, 2007 (6)
	Controls (HCV)	Cirrhosis (HCV)	+	Serum	Embade, 2016 (15)
	Cirrhosis	Fibrosis	-	Liver tissue	Martínez-Granados, 2011 (14)
	Low-grade HCC	High-grade HCC	-	Liver tissue	Yang, 2007 (6)
	Control	Cirrhosis (HBV or EtOH)	+	Serum	Qi, 2012 (8)
	Cirrhosis with low chronic liver failure severity	Cirrhosis with high chronic liver failure severity	+	Serum	Amathieu, 2011 (13)
	Cirrhosis-MHE	Cirrhosis+MHE	+	Serum	Jimenez, 2010 (21)
Glycogen	Adjacent tissue	HCC	-	Liver tissue	Yang, 2007 (6)
	Low grade HCC	High grade HCC	-	Liver tissue	Yang, 2007 (6)
	Mild Fibrosis (HCV)	Moderate fibrosis or cirrhosis (HCV)	-	Liver tissue	Cobbold, 2010 (12)
Lactate	Control	Cirrhosis (HBV or EtOH)	-	Serum	Qi, 2012 (8)
	HEV	HBV	-	Plasma	Munshi, 2011 (8)
	Functional liver transplantation	Non-functional liver transplantation	+	Blood (extraction)	Serkova, 2007 (19)
	Low-grade HCC	High-grade HCC	+	Liver tissue	Yang, 2007 (6)
	Cirrhosis-MHE	Cirrhosis+MHE	+	Serum	Jimenez, 2010 (21)
	Cirrhosis with low chronic liver failure severity	Cirrhosis with high chronic liver failure severity	+	Serum	Amathieu, 2011 (13)
Pyruvate	Control	Cirrhosis (HBV or EtOH)	+	Serum	Qi, 2012 (8)
	Control	Decompensated cirrhosis	+	Plasma	McPhail, 2016 (23)
	Control	NAFLD	+	Serum	Cheng, 2015 (9)
	Compensated cirrhosis	Decompensated cirrhosis	+	Serum	Qi, 2012 (10)
	Cirrhosis with low chronic liver failure severity	Cirrhosis with high chronic liver failure severity	+	Serum	Amathieu, 2011 (13)

HBV, hepatitis B virus; HEV, hepatitis E virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; EtOH, ethanol; MHE, minimal hepatic encephalopathy; NAFLD, non-alcoholic fatty liver disease.

are used in the liver for the synthesis of structure and transport proteins, as well as enzymes. AAs may also be deaminated for direct energy supply or gluconeogenesis. The degradation of proteins that takes place in the liver may also be used to supply energy needs. In the liver, various AAs enter the detoxification process by conjugation reactions. The result of AA catabolism is the production of ammonia, which is eliminated as urea and creatinine in the urine through the kidneys. All these metabolic pathways involve a large number of enzymatic activities, which are frequently impaired in hepatic diseases. Metabolomic studies of these liver impairments frequently report modifications of the AA content in serum, plasma, tissues and bile.

In serum and plasma NMR spectra, and in tissue spectra, alanine is the easiest AA to detect. Alanine participates in protein metabolism and may be considered as an anabolite and a catabolite of protein. In addition, alanine enters the energy metabolic pathways following deamination into lactate and this process occurs, for the most part, in the liver. The enzyme responsible for this deamination, alanine aminotransferase is routinely assayed as a marker of hepatic impairment.

Consequently, variations in alanine have been frequently mentioned in hepatic pathologies (13). According to trends observed in the variation of alanine concentrations in liver diseases, it appears that alanine levels increase in the presence of pathological samples when compared with non-pathological samples, and this increase is enhanced when the disease worsens. This is the case for tumours (6), cirrhosis (8,13) and for their complications, such as hepatic encephalopathy or cirrhosis decompensation (10,21). In the case of acetaminophen toxicity, elevated alanine levels are identified in urine (11).

Glutamine and glutamate are AAs involved in nitrogen metabolism. Glutamine is the most abundant AA and one of the entry points into the tricarboxylic acid cycle (TCA; citric acid or Kreb's cycle) following its deamination into ketoglutarate. Glutamate is a precursor of glutamine. In liver pathologies, the levels of glutamine are increased in plasma and serum. As observed for alanine, the levels of glutamine and glutamate increase in plasma and serum, and in liver tissue, in cases of HCC (6), cirrhosis [when compared with control subjects or with fibrosis in tissue (14) or serum (12)], hepatic transplantation failure (7,19), and in fulminant hepatic failure (17). The same increases may also be identified in urine samples in cases of fulminant hepatitis (17). The only cases where a decrease in serum glutamine levels were reported were during the discrimination between viral and ethanol aetiologies of cirrhosis (8) and when comparing the severity of cirrhosis (13).

Glycine is generated from serine, which is derived from 3-phosphoglycerate formed by glycolysis. Glycine participates directly in the urea cycle through its degradation into ammonia and CO_2. Consequently, variations in glycine levels follow those of glutamine and glutamate in hepatic diseases. However, hepatoencephalopathy is notable; when this neurologic side effect of cirrhosis is present, glycine levels in serum decrease (21). This may be associated with the role of this AA as an inhibitory neurotransmitter.

In NMR spectra, essential AAs are also detectable and frequently reported as branched-chain AAs (BCAAs) and aromatic AAs (AAAs). BCAAs include valine, leucine and isoleucine, which have resonances located in the same region of the NMR spectrum that frequently overlap. BCAAs are essential AAs that must be contained in the diet and they are involved in the regulation of protein synthesis, glucose metabolism and oxidation.

In liver tissue, only leucine has been found to be increased in HCC compared to adjacent non-tumour tissue and this increase is associated with a higher tumour grade (6). In plasma and serum, a BCAA decrease signals symptoms of encephalopathy in cirrhotic patients (21) and cirrhosis of viral or alcoholic aetiology when compared with non-cirrhotic patients. Conversely, an increase in leucine was reported in the hepatitis E infection when compared with controls, while the concentration of valine decreased. Similarly, with severity of alcoholic cirrhosis, the concentrations of leucine and isoleucine, but not valine are increased (13). Low levels of BCAA have been associated with neuropathological disorders associated with cirrhosis (21).

The AAA group of essential AAs produces signals in the region >6 ppm in NMR spectra. This region is generally less crowded in serum and urine spectra than the regions <5 ppm, thus, these AAs are easy to detect when increased in fluids and tissues. The primary AAs involved are tyrosine, phenylalanine and histidine. Phenylalanine and tyrosine are metabolically associated, as tyrosine is synthesized from phenylalanine by phenylalanine hydroxylase, and a genetic deficit in this enzyme is responsible for a metabolic disease termed phenylketonuria, which is characterized by abnormal excretion of phenylalanine in the urine. This disease causes neurologic disorders. The concentrations of these AAA have been reported to decrease in plasma (and urine) of patients with viral hepatitis infections (18). By contrast, AAA are reported to be increased in cirrhosis (8) and in cases of hepatic transplantation failure (7). The end point of catabolism for AAA is composed of fumarate and acetoacetate. Their increase may also be associated with energy supply modulation in the liver (8,18).

Downstream of AA metabolism, the aminated molecules are considered to be direct catabolites of AAs and proteins, as their role is to eliminate nitrogen in the form of ammonia produced by deamination of AAs. Ammonia is detoxified in the urea cycle, which occurs in the liver. However, to the best of our knowledge, urea levels were not referred to in the metabolomic studies of hepatic disease. Conversely, various nitrogen-containing compounds were found to be increased in urine, including trimethylamine N-oxide and creatine (22).

Glucose metabolism and the TCA cycle. The liver is directly involved in glucose metabolism and the consequent energy supply to the organism. The liver is the organ containing the highest levels of glycogen, the storage form of glucose, and this compound may be synthesized through hepatic gluconeogenesis. Other sugars may be transformed into glucose.

The major metabolites directly implicated in glucose metabolism and detected with NMR spectroscopy are glucose (detected in blood and tissues), glycogen (detected in liver tissue), lactate and pyruvate. Various other compounds participating in the TCA cycle are also present in biofluids and tissues in sufficient concentrations for detection by NMR spectroscopy. Those reported in the literature concerning hepatic diseases are citrate, fumarate and succinate (6,8). Their concentration may vary in opposite directions, depending on the pathology.

Glycaemia is a relatively stable biological value, as the majority of the hepatic pathways, including glucogenesis, glycolysis and gluconeogenesis attempt to maintain constant glucose levels. However, three different studies have shown an increase of glucose levels in serum in cases of cirrhosis. The first study compared viral and alcoholic cirrhosis to control subjects (8) and the second showed that levels of serum glucose increase with cirrhosis severity (13). The third study reported higher glycaemia for cirrhotic patients with encephalopathy than those without encephalopathy (21). Glycogen NMR signals are detected in liver tissues under resolution conditions offered by the HR-MAS technique. The glycogen content of liver tissues is decreased for high-grade HCC when compared with low-grade disease and to adjacent non-tumour tissue (6). Similarly, glycogen is decreased in cirrhotic tissues when compared with mild and moderate fibrosis (12). These lower glucose and liver glycogen levels appear to be associated with pathology grade for cirrhosis and carcinoma, and should be associated with lactate content, which is increased in high-grade HCC, when compared with low-grade HCC tissues (6), and in the sera of cirrhotic patients

with hepatoencephalopathy when compared with cirrhotic patients without encephalopathy (21). Subsequent to liver transplantation, lactate in the blood is also increased in cases of a non-functional transplant when compared with successful transplants (19) and in more severe cirrhosis (13). This pattern favours a deviation toward an anaerobic glycolysis pathway utilizing more glucose molecules for energy supply. However, lactate in the blood was found to be lower in cirrhotic patients than in control patients (8) and in cases of viral infection comparing the hepatitis B virus with the hepatitis E virus (18), indicating an indirect association between glucose, glycogen and lactate. It can be noted that pyruvate content is also modulated (8,10), as well as citrate (18,19) and the levels of other TCA compounds (including formate and succinate) (8,10,18).

It is apparent that impairments in liver glucose and energy metabolism are detected in liver tissue and have systemic consequences, which are detected in serum and plasma as the imbalanced profile of the metabolites participating in these pathways.

4. Conclusion

The overview of literature results presented in the current review indicates that NMR metabolomic studies provide important data for improving the diagnosis and prognosis of human liver diseases. This technique is non-invasive, simple, inexpensive and fast, and has high sensitivity and good specificity. The question that must be addressed now is how metabolomics will impact the healthcare of patients with liver diseases. It is tempting to speculate that intra-individual analysis of changes during follow-up of treated patients may provide essential information regarding therapeutic responses. Various conditions associated with a rigorous clinical methodological approach need to be addressed in this context. First, these research programs should be developed in large prospective cohorts of well-defined patients undergoing standardized therapeutic procedures. Second, in order to avoid biases that could influence metabolomic profiling of patients, attention should be paid with respect to the stage and cause of the underlying liver diseases. Third, repeated metabolomic profiling should be performed in samples drawn at different time points corresponding to specific clinical situations as follows: Before treatment, after treatment at the time of judgement of efficacy, and subsequently at interesting end-points (41,42). Fourth, combining datasets from different metabolomics (such as NMR and mass spectrometry) or omics (such as genomics and transcriptomics) techniques will improve the understanding of the different metabolic pathways. The development of statistical models for accurate interpretation of variations in metabolomic fingerprints may then be used to provide pivotal information for patient management. Integration of metabolomic-based diagnostic principles will likely be an essential tool for the development of personalized medicine for decision-making in liver disease management.

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