Metabolomics in nonalcoholic fatty liver disease - a new technique for an open horizon

Dana A. Crișan, Corina I. Radu, Alina M. Suciu, Florina Romanciuc, Carmen Socaciu, Mircea Grigorescu

Abstract. The diagnosis of nonalcoholic fatty liver disease (NAFLD), the detection of non-alcoholic steatohepatitis (NASH) and the assessment of prognosis of this widespread disease is a permanent lately preoccupation of researches from different medical fields: hepatology, metabolic disease, diabetes, cardiology. Some biological techniques tried to have an insight over the pathophysiology of the steatosis, revealing important but insufficient data regarding this disease. Profiling the metabolomics products is a new emergent technique that promises to reveal some unknown aspects in NAFLD field. Data from mass spectrometry and nuclear magnetic resonance spectroscopy combined with statistical modeling have permitted the authors to analyze and interpret metabolic signatures in terms of metabolic pathways and protein interaction networks. We review here the metabolomics data found for different stages of NAFLD: first for simple steatosis, than for NASH which associates inflammation, and finally, for fibrosis and cirrhosis that brings about complications with high morbidity and mortality, hoping that this new technique will increase the knowledge in diagnosing and treating NAFLD.

Key Words: steatosis, NASH, metabolomics, fibrosis.

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Introduction

The spectrum of fatty liver disease comprises different aspect of the disease starting with simple steatosis passing to nonalcoholic steatohepatitis (NASH), which can add different stages of fibrosis, up to cirrhosis (Michielotti et al 2013). Nonalcoholic fatty liver disease (NAFLD) is assessed morphologically, if simple steatosis without hepatocyte injury (ballooning) in present, while NASH is defined by the presence of steatosis associating inflammation and ballooning, with or without fibrosis (Chalasani et al 2012). The prevalence of NAFLD can reach 46% in developed nations (Farrell et al 2012) and affects both children and adults (Torres et al 2012). In Romania, a study conducted in 2008 on 3005 patients, revealed a prevalence of NAFLD of 20% (Radu et al 2008).

A diagnosis of NAFLD implies an increased risk of related diseases as cardiovascular disease, diabetes, colonic adenomas, hypothyroidism, and polycystic ovary syndrome (Torres et al 2012). The impact of morbidity and economic burden is significant as NAFLD has a high prevalence among liver diseases in the Western countries. As a consequence, it has been reported an increasing proportion of patients with fatty liver who undergo liver transplantation (15%–20%) (Newsome et al 2012). Diagnosis, staging and prognosis of NAFLD and NASH remain the most important challenges in this field (Jepsen et al 2011). Imaging techniques (ultrasound and magnetic resonance) are the most reliable methods for the diagnosis of NAFLD, but liver biopsy stays as gold standard of diagnosis (Oh et al 2008; Kleiner et al 2005). Un undesirable face of the imaging techniques is the procedure cost and the lack of specificity especially when the assessment of steatohepatitis and fibrosis staging are the main goals. Neither liver biopsy does not lack drawbacks, as it is expensive, subjective and invasive procedure, encumbered by different complications and sampling error (Ratziu et al 2005). The detection of fibrosis can be assessed using (transient) elastography (Castera et al 2006) or serum biomarkers combined in different panels (FibroTest) (Poynard et al 2004; Imbert-Bismut et al 2004).

The issue becomes mode difficult when the target is the detection and staging of steatosis. The biological non-invasive methods are lacking accuracy in diagnosing steatohepatitis and fibrosis in patients with fatty liver, even if there have been some attempts (ELF test, NAFLD fibrosis score) (Morra et al 2003; Imbert-Bismut et al 2004). Offering a holistic and noninvasive approach, the emergent method called metabolomics gives a new perspective of diagnosis and staging in NAFLD. This new technique brings the advantage of identifying hundreds of metabolites characteristic for different diseases. Metabolomics is particularly suitable for the assessment of liver injury, given that the most widely used samples for testing are serum or urine, compared to other diseases like cancer, where tissue is available for different types of analysis as transcriptomics and proteomics (Barr et al 2010). The metabolic changes revealed through targeted analysis will be speculated for the detection of new potential biomarkers for fatty liver diagnosis and treatment.
Metabolomics seems to offer the advantage of being less dependent on species than gene or protein markers, allowing a direct comparison with animal models and short-circuiting the way of applying in practice what the lab research shows (Heijne et al 2005).

**Aim**

We aimed to review the importance of metabolomics in diagnosing and staging NAFLD and its progressive form – NASH. In addition, we also aimed to analyze the metabolomics value in staging liver fibrosis associated to NAFLD.

**NAFLD**

Considering that this technique is relatively new described, we found few data regarding the accuracy of metabolomics in diagnosing and staging NAFLD in humans. There are, however, more studies on animal model that might create an image of the metabolomic profile modified in fatty liver. A brief presentation of the results found in international databases for metabolomics aspects in NAFLD is summarized below (adapted after Beyoğlu&Idlee 2013, Dumas et al 2014):

- **Barr et al 2010** - Subjects and tissue: human serum; Platform: UPLC; Up-regulated: Arachidonic acid, PC, LPC (24:0); NASH vs. NAFLD; PC (14:0/20:4), LPC (18:1); Down-regulated: FAs: 20:2n6, 18:3n3/18:3n6, LPCs, SMs, TDA, TCDCAs, DG (18:0/22:6) NASH vs. NAFLD; LPC (24:0)
- **Puri et al 2009** - Subjects and tissue: human plasma; Platform: HPTLC – LCMS; Up-regulated metabolites: Triacylglycerol, FAs, 15-HETE Myristic acid, palmitic acid, myristoleic acid, palmitoleic acid, vaccenic acid, oleic acid, g-linolenic acid, steardonic acid, docosapentanoic acid. Progression from NAFLD to NASH: lipoxigenase metabolites, arachidonic acid; 5-HETE, 15-HETE.
- **Beyoğlu&Idle 2013, Dumas et al 2014**: NASH: lipoxygenase metabolites, arachidonic acid, 5-HETE, 15-HETE, Myristic acid, palmitic acid, stearic acid, oleic acid, g-linolenic acid, 15-lipoxygenase products (HETE), 15-HETE products (HEPE).
- **Dumas et al 2006** - Subjects and tissue: mice serum; Down-regulated: choline, TMAO, dimethylamine, methylamine; Up-regulated: creatine, glycercate, isovalerate, pyruvate.
- **Puri et al 2009** - Subjects and tissue: human plasma; Platform: HPLC; Up-regulated: homocysteine, cysteine, cysteinylglycine; Down-regulated: glutathione. NASH specific: 11-HETE.
- **Sunny et al 2015** - Subjects and tissue: human and mice plasma; Platform: NMR based metabolic flux analysis; Up-regulated: BCAA, long chain acylcarnitines.

The lipidomic profile, briefly presented above, points some important aspects. There are metabolites that are increased in NAFLD, like phospho-choline, choline, betaine (Toye 2007, Li 2011). This observation indicates that there is an increased turnover of phosphatidylcholine and phosphatidylethanolamine species in the liver, free fatty acids being released through the action of phospholipases A1 and A2. There is a variety of types of fatty acids depending on chain length, saturation pattern and lipid oxidation in NAFLD. The increased ratio of fatty acids indicates increased activity of different enzymes from desaturase type and peroxisome dysfunction. These results suggest that de novo lipogenesis is implied in NAFLD development. Furthermore, there are studies that showed an inflammatory status in steatosis, especially linked to the progression from NAFLD to NASH, indicating that lipogenesis associated with NAFLD and NASH is directly involved in the production of free fatty acids with lipotoxic properties which goes hand in hand with synthesis of inflammatory lipids (eicosanoids) (Puri et al 2009; Meikle et al 2011).

Fatty acids, if not catabolized by b-oxidation, will be stored in the liver as triacylglycerols (Vinaixa et al 2010, van Ginneken et al 2007, Hyde et al 2010). Therefore, the observation that fatty liver is not just storage of fat in the liver but rather a rearrangement of lipids in the liver, might be true (van Ginneken et al 2005).
2007). Some types of triacylglycerols TG(44:2) and TG(48:3) are massively increased in the liver (2427% and 1198%, respectively) as it has been described in an animal model. These types of triacylglycerols are frequently found in adipose tissue and these observations suggest that adipose tissue may be a source of triacylglycerols deposited in the liver in NAFLD (van Ginneken et al 2007). Secondly, elevated hepatic concentrations of various lysophosphatidylcholine (LPC), lysophosphatidyl-ethanolamine (LPE), and phosphatidylethanolamine (PC) species have been reported for human steatotic vs. non-steatotic livers (Garcia-Canaveras et al 2011). These molecules are obvious candidates for the elevated choline and choline metabolites discussed above. One common finding is the increased level of lipid species in serum/plasma, such as triacylglycerols (Puri et al 2009; Vinaixa et al 2010; van Ginneken et al 2007; Hyde et al 2010), diacylglycerols (Vinaixa et al 2010), cholesterol esters (van Ginneken et al 2007; Hyde et al 2010), sphingomyelins (Vinaixa et al 2010), various bile salts (Barr et al 2010, Kalhan et al 2011, Garcia-Canaveras et al 2011), together with lactate (Toye et al 2007; Li et al 2011) and glutamate (Li et al 2011; Noguchi et al 2011). In addition, cysteine-glutathione disulfide and both oxidized and reduced glutathione (GSH) were all reported to be depressed in the liver and serum/plasma (Kalhan et al 2011, Garcia-Canaveras et al 2011). The level of glucose has been reported to be low in subjects with food deprivation used to induce liver steatosis, but not all the studies are in agreement in this respect (Toye et al 2007; Li et al 2011; Griffin et al 2007). These observations together with increased level of lactate, pyruvate and alanine might suggest that NAFLD engages in cytosolic glycolysis and oxidative stress (Toye et al 2007; Li et al 2011). Soga et al revealed another group of serum markers for detecting NAFLD: g-glutamyl dipeptides. The presence of g-glutamyl dipeptides indicates increased GSH synthesis as a mark of oxidative stress response (Soga et al 2011). Under oxidizing conditions, the pool of GSH decreases; as a consequence, the activity of g-glutamyl-cysteine-synthetase increases, leading to g-glutamyl-cysteine formation. Excess of g-glutamyl-cysteine is metabolized by g-glutamyl-transpeptidase, which catalyzes the substitution of cysteine with amino acids, and the synthesis of different g-glutamyl dipeptides, released afterwards into the bloodstream. These findings are important as serum levels of g-glutamyl-transpeptidase together with aminotransferases are routinely assessed in patients with liver diseases, especially in alcoholic and nonalcoholic steatohepatitis (Soga et al 2011; Griffin et al 2007).

The metabolism of BCAA is also modified in NAFLD. A study including human and animal model demonstrated a link between BCAA and tri-carboxylic acid (TCA) cycle, reflecting hepatic insulin resistance. Insulin stimulation revealed a strong relationship between human plasma BCAA and insulin resistance. Further more, human plasma BCAA are positively correlated with long chain acylcarnitines, indicating the modulation of mitochondrial metabolism by BCAA. The authors concluded that BCAA are essential to mediate mitochondrial TCA cycle, the impairment of BCAA metabolism, contributing to mitochondrial dysfunction in NAFLD (Sunny et al 2015). A very important and constant observation in different studies conducted on NAFLD is the increased level of bile acids in serum/plasma (Barr et al 2010; Kalhan et al 2011). Bile acids act as signaling molecules in the liver implied in lipid and glucose homeostasis (Wei et al 2009). Certain bile acids, in particular chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA), are endogenous ligands that activate the farnesoid X receptor (FXR) (Wang et al 1999). The nuclear receptor FXR interferes with the conversion of cholesterol to bile acids (Torres et al 2012). The observation that NAFLD exists in the presence of increased serum/plasma concentrations of glycocholate, taurocholate, glycochenodeoxycholate (Kalhan et al 2011) and deoxycholate (Barr et al 2010) is at least interesting if not surprising.

**NASH**

NASH is a special form of NAFLD, with progressive capacity due to a major inflammatory component (Farrell et al 2012). NAFLD may switch to NASH, but more than 80% of cases will remain at simple steatosis with no or minimal progression to cirrhosis and a very low risk of death (Torres et al 2012). It has been estimated that >11% of NASH cases will develop cirrhosis in 15 years. The inflammation involved in steatohepatitis is still a good subject for research, even though there are some authors suggesting that visceral adipose tissue has a pro-inflammatory compound (Farrell et al 2012). Puri et al showed that eicosanoids and products of oxidation of the inflammatory arachidonic acid by lipoxygenase are particularly affected during the progression from NAFLD to NASH (Puri et al 2009). The investigators showed that, among eicosanoids, an increase of lipoxygenase products (hydroxyeicosatetraenoic acids (HETE)) contribute to inflammation and that 11-HETE is specific to NASH (Puri et al 2009).

There are relatively few metabolomic studies targeting the NASH pathophysiology and the aspects that segregate NASH from NAFLD. As with NAFLD, triacylglycerol sand several fatty acids were elevated in plasma (Cobbold et al 2009) and several other fatty acids and LPC were decreased in plasma (Kalhan et al 2011). Other authors reported modifications in only three phospholipids (Toye et al 2007). Glucose, glutamate and taurine were found to be raised in NASH in a study developed using NMR, which does not have the power to detect a large range of molecules (Toye et al 2007; Li et al 2011). An important point of view over the NASH pathogenesis was brought by a study combining metabolomics with genetics (Tanaka et al 2012). In this study, NASH was generated in mice fed a methionine- and choline-deficient (MCD) diet. UPLC-ESI-TOFMS metabolomics revealed a statistically significant decrease of LPC (16:0), LPC (18:0) and LPC(18:1) in serum with a significant increase in tauro-b-muricholate, taurocholate, and 12-HETE for MCD fed mice compared to mice on a normal diet. When comparing the subjects with a positive control formed genetically obese ob/ob mice with severe steatosis and severe induced inflammation, the authors observed the same changes in LPCs and bile acids as the MCD fed mice. Thus, the decline in serum LPC and rise in serum bile acids is a signature of the inflammatory component of NASH, rather than the steatotic component. To further investigate the mechanisms involved in the changes of LPC and bile acid homeostasis mentioned above in the NASH model, hepatic mRNA levels were determined by qPCR for genes involved in the metabolism and transport of LPC, bile acids and 12-HETE. It was observed an up-regulation of lysophosphatidylcholine
Table 1. Metabolomics for the assessment of fibrosis in NALFD (adapted after Beyoğlu et al 2013)

<table>
<thead>
<tr>
<th>Subjects and tissue</th>
<th>Platform</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat urine (Gou et al 2013)</td>
<td>GCMS</td>
<td>Propionate and leucine</td>
<td>2-oxoglutarate, taurine, glyceroles, tyrosine, 1-methylhistidine, phenylalanine</td>
</tr>
<tr>
<td>Human serum (Gao et al 2009)</td>
<td>NMR</td>
<td>Acetate, pyruvate, glutamine, “N-acetylglycoproteins”,</td>
<td>LDL, VLDL, leucine, isoleucine, valine, acetooacetate, choline, unsaturated lipid</td>
</tr>
<tr>
<td>Human serum (Waldhier et al 2011)</td>
<td>GCxGCTOFMS</td>
<td>D-alanine, D-proline</td>
<td>L-alanine, L-valine, L-isoleucine, L-tyrosine, L-serine, L-asparagine</td>
</tr>
<tr>
<td>Human serum (Lian et al 2011)</td>
<td>UPLC-ESIQTOFMS</td>
<td>GCDDA, GCA, L-acetylcarnitine, myristamide, oleamide</td>
<td>LPCs, myristamide, oleamide</td>
</tr>
<tr>
<td>Human serum (Huang et al 2013)</td>
<td>UPLC-ESIQTOFMS</td>
<td>LPC (16:0), LPC (18:0), LPC (18:1), LPC (18:2)</td>
<td>CDCA, 7-ketolithocholic acid, urobilin, urobilinogen</td>
</tr>
<tr>
<td>Human serum (Du et al 2011)</td>
<td>LCMS</td>
<td>GCDDA</td>
<td>LPCs</td>
</tr>
<tr>
<td>Human (pediatric) plasma (Pastore et al 2014)</td>
<td>HPLC</td>
<td>Homocysteine, cysteine</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PA, palmitic acid; OA, oleic acid; NMR, nuclear magnetic resonance spectroscopy; HPTLC, high performance thin-layer chromatography; LCMS, liquid chromatography–mass spectrometry; GC/MS, gas chromatography with flame ionization detection; UPLC, ultra-performance liquid chromatography; ESI, electrospray ionization; TQMS, triple quadruple mass spectrometer; QTOFMS, quadruple time-of-flight mass spectrometer; TMAO, trimethylamine-N-oxide; TG, triacylglycerol (triglyceride); FA, fatty acid; 15-HETE, (±)-15-hydroxy5Z,8Z,11Z,13E-eicosatetraenoic acid (non-enzyme oxidation product of arachidonic acid); DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GCDCA, glycochenodeoxycholic acid; TCDDA, taurochenodeoxycholic acid; LPC, lysophosphocholine; SCD1, branched chain amino acids; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein. LC, liver cirrhosis; CHB, chronic hepatitis B; TMA, trimethylamine; CCl4, carbon tetrachloride; FTA, unsaturated fatty acid units (-CH = CH-CH2-). GCxGC-TOFMS, twodimensional gas chromatography time-of-flight mass spectrometry. OPLS-DA, orthogonal partial least squares projection to latent structures-discriminant analysis, PE, phosphatidylethanolamine.

Fibrosis

The consequence of any inflammatory process in the liver is the occurrence of fibrosis. When this connective tissue determines septa and hepatic nodules, it can be considered a cirrhotic stage. The oxidative stress is the one who initiates and entertains the inflammatory reactions involved in the occurrence of fibrosis (Yeh et al 2007). Liver steatosis and especially NASH are considered lately an important cause of chronic liver disease that generates cirrhosis, among other well known etiologies like alcohol, hepatic B and C viruses (Yeh et al 2007). The interest of researches in finding noninvasive techniques for the diagnosis of fibrotic stage and for detecting early cirrhosis is increasing lately. A lot of imagery techniques and serum markers were studies, most of them lacking a clear value to distinguish between each stage of fibrosis, (Wiegand et al 2013) other being prohibitive because of the cost. In this case, the new metabolomic technique needs to be tested for the accuracy of detecting fibrosis. There are few studies published on this subject, some of them found to be representative are presented in table 1.

Some studies conducted on animal model used different types of inducing cirrhosis and finally concluded that there are no major changes in urinary metabolome in cases of cirrhosis determined in healthy rats compared to cirrhosis occurred on fatty liver (Constantinou et al 2007; Gou et al 2013; Zhang et al 2013). In rats treated with thyoacetamide, liver extract showed higher...
levels of lactate, suggesting anaerobic metabolism (Constantinou et al 2007), while in rats treated with carbon tetrachloride and also a hepatotoxic medicinal plant (scoparone) it has been observed a decrease in urinary amino acids and gut flora metabolites (Gou et al 2013; Zhang et al 2013).

Metabolomic studies performed on human subjects analyzing serum or feces din not show very clear and congruent data. Still, an increased serum concentration of non-essential amino acids (Gao et al 2009) and some D-amino acids (Waldhier et al 2011) has been reported, while other essential amino acids had decreased serum concentration. This observation is in accordance with the already reported impairment in metabolizing proteins and amino acids of cirrhotic patients (Gao et al 2009; Waldhier et al 2011; Lian et al 2011). Other studies observed a decrease of LPCs in the serum of cirrhotic subjects with virus B or alcohol etiology (Qi et al 2012). The data can be extrapolated to NASH, as the pattern is similar. As far as biliary acids are concerned, a high concentration of glycochenodeoxycholic acid and glycocholic acid were observed in the serum of cirrhotic patients (Qi et al 2012).

As the impairment of ammonium detoxification in cirrhosis is well known, the metabolomics confirms this modification through the shift from hepatic glutamine to glucose and glutamine (Martinez-Granados et al 2011). Another metabolic chain found to be modified in cirrhosis was the reduced serum carnitine and increased serum palmitoleoylcarnitine and oleoylcarnitine concentrations (Lin et al 2011). An interesting observation regarding metabolomic changes in cirrhotic patients was reported related to a modification in patients’ feces (Huang et al 2013). The authors reported an increased concentration of major LPCs in cirrhosis and a decreased excretion of chenodeoxycholicacid and 7-ketolithocholicacid, similar metabolic changes being reported also in NALFD. Concurrently, similar metabolomics changes have been reported in cirrhotic patients, detected in serum this time, with an increased level of biliary acids and decreased level of LPCs (Du et al 2011). In a study on human pediatric population analyzing plasma thiols, increased levels of Hcy and Cys were found in patients with NAFLD. A positive correlation was observed between Hcy and Cys and the presence of fibrosis in children with NAFLD. High levels of Hcy and Cys probably correlates with a pattern of more severe histological liver damage, due to mechanisms that require further studies (Pastore et al 2014).

Conclusion

Metabolomic technique seems to be appropriate and useful for the diagnosis and assessment of different forms of NAFLD. A very important aspect is the detection of the progressive form of fatty liver (NASH) and the assessment of prognosis especially related to the stage of fibrosis. In a non-expensive and holistic manner, the emergent technique of metabolomics is a promising method to analyze this disease.

Acknowledgement

This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/138776.


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Citation


Editor

Ştefan C. Vesa

Received

4 August 2015

Accepted

23 August 2015

Published Online

24 August 2015

Funding

European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/138776

Conflicts/Competing Interests

None reported