

## Lipidomics in non-alcoholic fatty liver disease

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### Abstract

Non-alcoholic fatty liver disease (NAFLD), the most common chronic liver disorder in Western countries, comprises steatosis to nonalcoholic steatohepatitis (NASH), with the latter having the potential to progress to cirrhosis. The transition from isolated steatosis to NASH is still poorly understood, but lipidomics approach revealed that the hepatic lipidome is extensively altered in the setting of steatosis and steatohepatitis and these alterations correlate with disease progression. Recent data suggest that both quantity and quality of the accumulated lipids are involved in pathogenesis of NAFLD. Changes in glycerophospholipid, sphingolipid, and fatty acid composition have been described in both liver biopsies and plasma of patients with NAFLD, implicating that specific lipid species are involved in oxidative stress, inflammation, and cell death. In this article, we summarize the findings of main human lipidomics studies in NAFLD and delineate the currently available information on the pathogenetic role of each lipid class in lipotoxicity and disease progression.

**Key words:** Lipidomics; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Lipotoxicity; Fatty acids; Ceramides

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**Core tip:** Lipidomics is a new rapidly growing field that allows the overall and detailed investigation of the whole lipid composition in a given biology matrix. Lipid profiling of liver biopsies of patients with non-alcoholic fatty liver disease (NAFLD) has previously

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revealed several changes in glycerophospholipids and sphingolipids concentrations and alterations in fatty acid pattern compared to healthy control. However, findings from lipidomics studies in plasma samples are inconsistent. We review the main findings of lipidomics studies and the important pathophysiological role of specific lipid species in lipotoxicity and development of NAFLD.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver diseases in the Western countries, affecting approximately 25% of the general population<sup>[1]</sup>. NAFLD encompasses a wide spectrum of liver histological features, ranging from mild hepatic steatosis (non-alcoholic fatty liver, NAFL) to nonalcoholic steatohepatitis (NASH)<sup>[2]</sup>. The hallmark of NAFLD is the hepatic intracellular accumulation of lipids and the subsequent formation of lipid droplets in hepatocytes<sup>[3]</sup>. NASH, the more progressive form of the disease, is characterized by the presence of hepatic steatosis accompanied by lobular inflammation, hepatocellular damage, and fibrosis and associated with an increased risk of developing cirrhosis and hepatocellular carcinoma<sup>[4]</sup>. In fact, NASH-related cirrhosis is believed to become the leading cause of liver transplantation in the future<sup>[5]</sup>.

NAFLD is commonly associated with insulin resistance and type 2 diabetes mellitus and is considered an independent risk factor for cardiovascular disease<sup>[6]</sup>. Obesity, physical inactivity, consumption of nutritionally imbalanced food, and unhealthy dietary and other lifestyle habits are also associated with NAFLD, and lifestyle modifications involving physical activity and diet have been shown to improve hepatic steatosis and liver fibrosis<sup>[6-8]</sup>. Although there has been remarkable progress in the elucidation of NAFLD pathogenesis, the pathophysiological pathways underlying lipotoxicity and transition of simple steatosis to NASH are still incompletely understood<sup>[9]</sup>. Recent lipidomic studies revealed marked changes in the fatty acid pattern and phospholipid composition in liver samples of NAFLD patients, suggesting that perturbations in lipid metabolism are a key factor in the pathogenesis and progression of NAFLD<sup>[10,11]</sup>. Furthermore, liver biopsy remains the only reliable but invasive method to diagnose NAFLD and differentiates NASH from simple steatosis. Thus, the non-invasive diagnosis of NASH is still an unmet need. Alterations occurring in plasma lipid molecules identified by lipidomic techniques which cannot be determined in every day clinical practice, may have utility as non-invasive biomarkers of disease progression<sup>[12]</sup>.

The present review article focuses on the main findings of the alterations occurring in lipidome in NAFLD patients and the interpretation of pathophysiological role of several identified lipid classes in the development and progression of NAFLD.

## PATHOGENESIS OF NAFLD AND ROLE OF LIPIDS

The pathogenesis of NAFLD is considered to be a multifactorial process and the underlying mechanisms involved in the progression of the disease are complex. Intrahepatic fat accumulation, the hallmark of the disease, is the result of increased uptake of fatty acids, increased *de novo* lipogenesis, and impairment in export and oxidation of fatty acids<sup>[3]</sup>. Obesity through expansion and dysfunction of adipose tissue and insulin resistance through subsequent reduction of adipose tissue lipolysis lead to increased efflux of free fatty acids<sup>[13]</sup>. Moreover, the hyperinsulinemia associated with insulin resistance promotes *de novo* fatty acid synthesis in the liver by activating the sterol regulatory element binding protein-1c (SREBP-1c), a transcriptional regulator of lipogenic genes<sup>[14]</sup>. These free fatty acids as well as those from dietary sources either undergo  $\beta$ -oxidation or are esterified with glycerol to form triglycerides. Then, triglycerides are stored in hepatocytes and form lipid droplets or are packaged and exported as very-low-density lipoprotein (VLDL)<sup>[3]</sup>. Thus, a dietary overload and

insulin resistance promote the hepatic fat accumulation, as observed in NAFLD<sup>[15]</sup>.

Intracellular deposition of lipids in NAFLD and the subsequent increased demand for metabolism of excess fatty acids lead to production of reactive oxygen species (ROS), elevation of oxidative or endoplasmic reticulum (ER) stress, and activation of Jun N-terminal kinase, all of which result in mitochondrial dysfunction and cell death<sup>[16]</sup>. Cell injury, in the setting of steatosis, is also largely attributed to activation of inflammatory pathways. Adipose tissue dysfunction leads to secretion of pro-inflammatory cytokines and alters the production and secretion of adipokines, such as leptin and adiponectin that are involved in the modulation of inflammation and insulin resistance<sup>[15]</sup>. Hepatic inflammation in fatty liver is considered to be triggered by a variety of compounds, such as damage-associated molecular patterns (DAMPs) released from hepatocytes, gut-derived bacterial endotoxin, free fatty acids, and free cholesterol<sup>[17]</sup>. Cytokine-induced liver inflammation, the subsequent activation of Kupffer and hepatic stellate cells, and lipotoxicity induced by free fatty acids and other lipotoxic bioactive lipids are involved in chronic liver injury and are thought to be responsible for progression from NAFL to NASH and development of fibrosis<sup>[18]</sup>.

Over the past decade, our knowledge regarding lipotoxicity has been greatly expanded and recent progress in lipidomics analyses has given new insights into lipid profiling and pathophysiological mechanisms involved in chronic inflammation and cell injury. Investigation of liver and serum lipidome in patients with NAFLD has disclosed that perturbations in lipid metabolism are a key factor for the development of NAFLD and that several complex lipid species, including sphingolipids and glycerophospholipids, are involved in lipotoxicity and the pathogenesis of NASH.

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## LIPIDOMICS STUDIES IN NAFLD

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Lipidomics is defined as the detailed characterization of lipid molecular species and of their structure and biological role in a given matrix including cell, tissue, and biological fluid<sup>[19]</sup>. This relatively new research field is a subset of metabolomics and represents a powerful approach to obtain a comprehensive overview of whole lipid metabolism in a biological system or even in specific disease state<sup>[20]</sup>. Lipidomics includes the identification and characterization as well as the quantification of thousands of lipid molecular species in a biological matrix<sup>[21]</sup>. This rapidly growing advanced field incorporates analytical techniques that are utilized for lipid separation and detection, such as high-performance liquid chromatography (HPLC), electrospray ionization mass spectroscopy (ESI MS), and nuclear magnetic spectroscopy (NMR)<sup>[19,22]</sup>.

The first lipidomics studies in NAFLD patients, as seen in **Table 1**, were conducted in liver biopsies and focused mainly on the analysis of fatty acid composition. Araya *et al.*<sup>[10]</sup> was the first to report an increased n-6:n-3 ratio in liver lipids of NAFLD patients accompanied by a decrease of the long chain polyunsaturated fatty acid (PUFA) of n-3 and n-6 series in liver TAG, such as arachidonic, eicosapentaenoic, and docosahexanoic acid. A depletion of long chain n-3 and n-6 PUFA in NASH patients has also been reported by a later study, regardless of the dietary FA intake, suggesting that the biosynthetic pathways of these lipids are impaired<sup>[23]</sup>. Indeed, later studies on enzymatic activities confirmed the decreased activity of  $\Delta 5$  desaturase, a key enzyme in essential n-3 and n-6 PUFA synthesis<sup>[24]</sup>. However, the first most comprehensive lipidomic study in liver biopsies, which included quantification of major lipid classes, was carried by Puri *et al.*<sup>[11]</sup>. In this study, lipidomic analyses identified marked changes not only in the fatty acid composition but also in the total phospholipid content<sup>[11]</sup>. Alterations of phospholipid content in liver biopsies of NASH patients have also been reported by other studies, implicating that phospholipid synthesis is impaired in NASH and is associated with disease progression<sup>[24]</sup>.

The research later focused on the study of the alterations occurring in plasma and serum samples of patients with NAFLD. In view of the fact that the liver is the key organ of metabolism and that plasma lipids under fasting conditions reflect mainly the lipids exported from the liver, changes in the circulating lipidome could be correlated with those in the liver during NAFLD progression. Interestingly, the changes observed in plasma fatty acid and phospholipid composition were discrepant from those reported in liver samples<sup>[25,26]</sup>. Moreover, as seen in **Table 2**, the findings of lipidomic studies conducted on plasma samples are inconsistent. According to Puri *et al.*<sup>[26]</sup>, no significant differences were observed in the plasma phospholipid subclasses of patients with NAFLD compared to healthy controls. However, recent studies report statistically significant changes in plasma phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC),

**Table 1 Summary of main liver lipidomics studies in non-alcoholic fatty liver disease**

Ref.	Tissue	Main findings in NAFLD patients compared to healthy controls	Main findings in NASH patients compared to NAFL patients
Puri <i>et al</i> <sup>[11]</sup> , 2007	Liver	<b>Increased:</b> DAG, TAG, total SFA, total PUFA; stepwise increase in the mean TAG/DAG ratio, FC/PC ratio and hepatic FC from normal livers to NAFL to NASH. <b>Decreased:</b> Total PC in both NAFL and NASH; AA in FFA, TAG, and PC in NASH; EPA and DHA in TAG in NASH.	The n-6:n-3 FFA ratio increased in NASH
Araya <i>et al</i> <sup>[10]</sup> , 2004	Liver, adipose tissue (fatty acid composition)	<b>Increased:</b> n-6:n-3 ratio, n-6 LCPUFA in liver phospholipids, total MUFA. <b>Decreased:</b> Long-chain PUFA of the n-6 and n-3 series in liver TAG, AA/LA ratio, EPA + DHA)/ALA in liver TAG, n-3 LCPUFA in phospholipids, total PUFA, n-3 PUFA, n-6 PUFA, AA, EPA, DHA.	The n-6:n-3 ratio increased in NASH
Allard <i>et al</i> <sup>[23]</sup> , 2008	Liver, red blood cells (fatty acid composition)	<b>Increased:</b> MUFAs, palmitoleic acid (16:1 n9), and oleic acid (18:1 n9) in NASH compared to control group. <b>Decreased:</b> Total n-3 PUFA, long-chain n-3 (EPA + DHA) and long-chain n - 6 (AA) PUFA in NASH compared to control; RBC-FA composition similar among the three groups.	<b>Decreased:</b> Total n- 6-PUFA in NASH compared to NAFL
Chiappini <i>et al</i> <sup>[24]</sup> , 2017	Liver	<b>Increased:</b> C14:0, C16:0, C16:1n-7, C18:1n-7, C18:1n-9, and C18:2n-6 in NASH. <b>Decreased:</b> Total SM, PI, PS, PE, PC in NASH.	Lipid signature of NASH (32 lipids). <b>Decreased:</b> AA, EPA, and DHA; total Cer.

NAFLD: Non-alcoholic fatty liver disease; NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; DAG: Diacylglycerol; TAG: Triacylglycerol; SFA: Saturated Fatty acids; PUFA: Polyunsaturated fatty acids; FC: Free cholesterol; PC: Phosphatidylcholine; FFA : Free fatty acids; LCPUFA: Long chain polyunsaturated fatty acid; MUFA: Monounsaturated fatty acid; RBC-FA: Red blood cell-fatty acids; SM: Sphingomyelin; PI: Phosphatidylinositol; PS: Phosphatidylserine; PE: Phosphatidylethanolamine; EPA: Eicosapentaenoic acid (C20:5n-3); DHA: Docosahexanoic acid (C22:6n-3); AA: Arachidonic acid (C20:4n-6); LA: Linoleic acid (C18:2n-6); ALA:  $\alpha$ -linolenic acid (C18:3n-3); Cer: Ceramides.

and sphingomyelin contents among healthy subjects and NAFL and NASH patients<sup>[25,27]</sup>.

Due to discrepancy between the findings in plasma lipidomic analyses and the need to discover novel non-invasive biomarkers to distinguish NASH from NAFL, several studies for lipidomics analysis were performed in both plasma and liver biopsy samples<sup>[28,29]</sup>. A total of 48 common analytes with an overlap in both tissues were identified in a comprehensive lipidomic study conducted both in liver and plasma samples of patients with NAFLD. These analytes were mainly sphingolipid species, such as dihydroceramides, 1-deoxydihydroceramides, and longer chain ceramides, suggesting that perturbation of sphingolipid metabolism is involved in the pathogenesis of NAFLD<sup>[28]</sup>.

The alterations occurring in each lipid class as well as the possible mechanisms underlying these changes in NAFLD will be discussed below.

## GLYCEROPHOSPHOLIPIDS

Glycerophospholipids are major components of cellular membranes and a source of physiologically active compounds. They serve as signaling molecules and as anchors for proteins in cell membranes.

Phosphatidylcholine (PC) is one of the most abundant phospholipids in mammals and a major component of cellular membrane lipids. PC levels were reported to be decreased in the liver samples of patients with NAFLD<sup>[11,24]</sup>. However, there are conflicting data concerning the changes occurring in serum PC<sup>[25-27]</sup>.

From a metabolic point of view, in most mammalian cells, PC is produced *de novo* from dietary choline *via* the cytidine 5'-diphosphate CDP-choline pathway<sup>[30]</sup>. In hepatocytes, up to 30% of PC comes from the conversion of phosphatidylethanolamine (PE) to PC, a reaction which is catalyzed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT)<sup>[31]</sup>. The synthesis of PE occurs *via* a CDP-ethanolamine pathway and *via* decarboxylation of phosphatidylserine (PS). Up to now, a few number of lipidomic studies mentioned alterations in PE in NAFLD patients. Liver PE content was found to be decreased among subjects with NASH, but in another study

**Table 2 Summary of main lipidomics studies in plasma and serum in non-alcoholic fatty liver disease**

Ref.	Tissue	Main findings in NAFLD patients compared to healthy control	Main findings in NASH patients compared to NAFL patients
Puri <i>et al</i> <sup>[26]</sup> , 2009	Plasma	<b>Increased:</b> DAG, TAG, MUFA, dihomo-gamma-linolenic acid, palmitoleic acid, oleic acid, palmitoleic acid to palmitic acid ratio in NAFLD; stepwise increase in lipoxygenase (LOX) metabolites 5-HETE, 8-HETE, and 15-HETE from healthy controls to NAFL to NASH; 11-HETE in NASH compared with controls. <b>Decreased:</b> LA; total plasmalogen levels in NASH compared with controls.	
Zheng <i>et al</i> <sup>[81]</sup> , 2012	Plasma phospholipids fatty acid composition	<b>Increased:</b> Dihomo-gamma-linolenic acid (C20: 3n-6), total SFA in phospholipids. <b>Decreased:</b> Eicosanoic acid (C20: 0), cis-11-octadecenoic acid (C18: 1n-7), DHA in PL.	
Loomba <i>et al</i> <sup>[89]</sup> , 2015	plasma eicosanoid lipidomic profile	<b>Increased:</b> 15-HETE, 5,6-diHETrE. <b>Decreased:</b> 12,13-diHOME.	<b>Increased:</b> 11,12-diHETrE, dhk PGD2, and 20-COOH AA. <b>Decreased:</b>
Walle <i>et al</i> <sup>[80]</sup> , 2016	Serum (fatty acid composition)	<b>Increased:</b> Palmitoleic acid in CE in individuals with NAFLD. <b>Decreased:</b> LA and total n-6 fatty acids in TAG in individuals with NASH.	<b>Increased:</b> SFA in TAG were higher in subjects with NASH, myristic acid in CE and TAG, Stearic acid in TAG. <b>Decreased:</b>
Tiwari-Heckler <i>et al</i> <sup>[27]</sup> , 2018	Serum	<b>Increased:</b> PC and SM in NAFL and NASH. <b>Decreased:</b> Lysophosphatidylethanolamine in NAFL and NASH individuals.	<b>Increased:</b> PE in patients with NASH.
Ma <i>et al</i> <sup>[27]</sup> , 2016	Plasma	<b>Increased:</b> PS and PI in NAFL and NASH, DHA and AA in PS in NAFL and NASH.	

NAFLD: Non-alcoholic fatty liver disease; NAFL: nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; DAG: Diacylglycerol; TAG: Triacylglycerol; SFA: Saturated Fatty acids; MUFA: Monounsaturated fatty acids; PC: Phosphatidylcholine; HETE: Hydroxyeicosatetraenoic acid; 5,6-diHETrE : 5,6 dihydroxy- eicosatrienoic acid; 12,13-diHOME: 12,13-dihydroxy-9- octadecenoic acid; CE: Cholesteryl ester; PE: Phosphatidylethanolamine; LA: Linoleic acid (C18:2n-6); DHA: 11,12-diHETrE: 11,12-dihydroxy- eicosatrienoic acid; dhk PGD2: 13,14-dihydro-15-keto prostaglandin D2; 20-COOH AA: 20-carboxy arachidonic acid; SM: Sphingomyelin; PE: Phosphatidylethanolamine; PS: Phosphatidylserine; PI: Phosphatidylinositol.

serum PE levels were increased in these patients<sup>[24,27]</sup>.

The ratio of PC/PE in the liver reflects the activity of PEMT<sup>[32]</sup>. In a shotgun MS-based targeted lipidomic analysis, researchers observed a statistically significant decrease of the hepatic PC/PE ratio in NAFLD patients<sup>[32]</sup>. Similarly, a low PC/PE ratio was also reported in red blood cell membrane of NAFLD patients and is considered as a biomarker of NAFLD. Additionally, a loss-of-function polymorphism in the *PEMT* gene seems to be associated with susceptibility in NAFLD<sup>[33]</sup>. However, when this parameter was calculated in plasma of NAFLD patients, no significant differences were observed among the healthy controls and NAFL and NASH patients, suggesting that compensatory mechanisms are activated in an attempt to maintain the plasma PC/PE ratio<sup>[25]</sup>.

The low hepatic PC levels and the altered hepatic PC/PE ratio seem to have major implications in the development of NAFLD, but the pathophysiology of the lipid-induced processes is not fully understood. PC is the only phospholipid molecule that is known to regulate the assembly and secretion of lipoproteins<sup>[34]</sup>. Low hepatic levels



of PC, due to its synthesis impairment, have been found to impair the VLDL secretion and reduce significantly the levels of circulating VLDL lipoproteins. A dysfunction of VLDL secretion results in hepatic accumulation of TGs, as observed in many animal model studies<sup>[35,36]</sup>. Moreover, low PC levels have been previously described to activate sterol regulatory element-binding protein 1 (SREBP1)<sup>[37]</sup>. The activation of SREBP1, as mentioned above, leads to upregulation of lipogenic gene expression, thus resulting in increased *de novo* lipogenesis and formation of lipid droplets in hepatocytes.

From a structural point of view, disturbances in the proportion of PC and PE possibly affect the structure of the phospholipid bilayer of cell membrane. PC has a cylindrical shape and is distributed mainly in the outer monolayer of plasma membrane. On the contrary, PE is described as conical, and is located mostly in the inner monolayer<sup>[38]</sup>. A low PC/PE ratio possibly leads to rearrangement of PE in the outer monolayer, resulting in a loss of membrane integrity and increased permeability to pro-inflammatory molecules such as cytokines. Thus, the release of cellular contents, such as calcium, accompanied by an increase in influx of cytokines, initiates the inflammation in NAFLD<sup>[39]</sup>.

As far as the rest of the glycerophospholipids is concerned, only a small number of lipidomics studies have previously reported statistically significant changes of their abundance in NAFLD<sup>[11,24,25]</sup>. Likewise, the findings from lipidomics studies conducted on liver samples were inconsistent with those from plasma samples of NAFLD patients.

Chiappini *et al.*<sup>[24]</sup> found that the levels of PS and PI were decreased in liver biopsy samples of patients with NASH compared with control individuals, whereas in a recent lipidomic study, no statistically significant differences were found in hepatic PS and PI among the control group, patients with NAFL, and those with NASH<sup>[24,29]</sup>. On the contrary, plasma PS and PI were found to be increased in NAFL and NASH compared with the control, while another study reported only an increase of serum PI in NASH patients compared to patients with simple steatosis<sup>[25,40]</sup>. Tiwari-Heckler *et al.*<sup>[27]</sup>, on the other hand, reported no significant changes in the amount of circulating PI among controls, NAFL patients, and NASH patients, but it is worth noting that in this study liver biopsy was not performed in all included subjects. These glycerophospholipids are also components of cellular membrane and are associated with cellular signaling and cellular apoptosis<sup>[41,42]</sup>. Given the important role of these lipids, differences observed in their hepatic or plasma levels may be involved in the development and progression of NAFLD.

Lysophosphatidylcholine (LPC) is a biologically active lipid and is considered an important mediator of hepatic lipotoxicity<sup>[43]</sup>. In liver biopsies from patients with NASH, LPC was found to be increased and this elevation seems to follow the disease severity<sup>[11,44]</sup>. However, several plasma and serum lipidomic studies failed to detect any statistically significant changes in the LPC content in patients with NAFL or NASH<sup>[25-27]</sup>. Interestingly, a recent study in biopsy proven patients with NAFLD found that plasma LPC species were decreased in patients with NASH<sup>[45]</sup>. Furthermore, another study reported that LPC diminished in patients with NAFLD<sup>[46]</sup>. This finding combined with an increase of TGs with low carbon number and double-bond content and a decrease of ether phospholipids has been proposed as a useful biomarker capable of estimating the percentage of liver fat in patients with NAFLD.

LPC is generated from PC by the action of secretory or lipoprotein-bound phospholipase A2 (PLA2). Also, LPC in plasma originates by the activity of lecithin-cholesterol acyltransferase (LCAT) as well as the activity of endothelial lipase. Hepatic secretion is also considered as a source of plasma LPC<sup>[47]</sup>. The increased hepatic LPC content could be attributable to an increase in hepatic biosynthesis or to an increase of total LPCs transported back to the liver by albumin or alpha 1-acid glycoprotein (AGP)<sup>[48]</sup>. As concerns the LPC levels in plasma, an impairment either on LCAT activity or PLA2 activity, as well as an increased turnover of LPC to PC or lysophosphatidic acid and sphingosine-1-phosphate are probable causes of diminished LPC levels in plasma. In fact, lipoprotein associated phospholipase A2 levels were found to be decreased in patients with NAFLD, whereas LCAT activity was higher in subjects with NAFLD, as inferred from a Fatty Liver Index > 60<sup>[49,50]</sup>. Moreover, a study in mice reported lower levels of palmitoyl-, stearoyl-, and oleoyl-LPCs in NASH compared to animals with NAFL, suggesting that the activity of lyso-PC acyltransferase, that catalyzes the recycle of LPCs to PC, is elevated in NASH<sup>[51]</sup>.

LPC as a bioactive molecule, seems to be involved in the pathogenesis of NAFLD and the transition from simple steatosis to NASH. LPC affects the whole liver lipid metabolism and has been found to downregulate genes involved in fatty acid oxidation and upregulate genes involved in cholesterol biosynthesis<sup>[52]</sup>. Furthermore, LPC has been demonstrated *in vitro* to trigger apoptosis of hepatocytes, probably

through disruption of mitochondrial integrity, whereas inhibitors of phospholipase A2 were shown to decrease palmitate-induced lipotoxicity and cell apoptosis<sup>[52,53]</sup>. Lastly, lipotoxicity induced by LPC could be mediated by release of proinflammatory and pro-fibrogenic molecules from hepatocytes or the enhanced turnover of LPC to profibrogenic lysophosphatidic acid<sup>[54]</sup>.

Plasmalogens are a class of glycerophospholipids carrying a vinyl ether bond in sn-1 and an ester bond in sn-2 position of their glycerol backbone. The biosynthesis of plasmalogens is a complex multistep process that takes place in peroxisomes and the endoplasmic reticulum<sup>[55]</sup>. Circulating plasma plasmalogens levels have been previously found to be decreased in patients with NASH and were negatively associated with obesity<sup>[26,56]</sup>. Furthermore, a depletion of total ether phospholipids has also been found in patients with NAFLD<sup>[46]</sup>. Lipidomic studies in liver biopsies of patients with NAFLD, however, failed to detect any changes in plasmalogen levels, probably due to their significantly lower liver concentrations compared to the rest of glycerophospholipids<sup>[57]</sup>. The liver contains low amounts of plasmalogens, although the enzymes involved in their synthesis are active in this tissue. This reduction might be attributable to their synthesis in the liver, and subsequent transport by lipoproteins to other tissues<sup>[57]</sup>. More interestingly, lipidomic analyses in NAFLD patients carrying the GG-genotype of *PNPLA3*, who are at a higher risk for more advanced disease and fibrosis, revealed lower levels of total plasma plasmalogens compared to subjects with CC- and CG-allele<sup>[27]</sup>.

Plasmalogens represent a key structural component of the cell membrane and may be involved in ion transport and cholesterol efflux. They have been described as signaling molecules and may also serve as precursors for eicosanoid biosynthesis<sup>[58]</sup>. Several studies have shown that plasmalogens, by virtue of their vinyl ether, function as endogenous antioxidants<sup>[59]</sup>. The deficiency in plasmalogens, which has been reported in plasma of NASH patients, could be attributed to oxidative stress-induced peroxisome damage and subsequent impairment of plasmalogen biosynthesis<sup>[55]</sup>. In fact, a recent study reported that endogenous hepatic plasmalogens, through a PPARα-dependent mechanism, prevent the development of hepatic steatosis and NASH in mice<sup>[60]</sup>.

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## SPHINGOLIPIDS

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Sphingolipids are a special group of phospholipids which contain a sphingosine backbone. Even though sphingolipids are very low in abundance compared with glycerophospholipids, they are considered important structural components of cell membrane<sup>[61,62]</sup>. They are involved in the arrangement of membrane lipid domains and cell signaling of major biological processes, such as cell survival and immune responses<sup>[62]</sup>. Lipidomic studies revealed changes in levels of sphingomyelin (SM), ceramides, and dihydroceramides in plasma and liver biopsies of patients with NAFL and NASH, implicating that alterations in sphingolipid metabolism are associated with the development and severity of NAFLD<sup>[24,28,45]</sup>.

SM is the most abundant sphingolipid and its plasma levels have been previously reported to correlate with body mass index (BMI)<sup>[56,61]</sup>. In NAFLD, the results from lipid profiling of liver and plasma are inconsistent. SM was found to be decreased in liver biopsies of patients with biopsy proven NASH<sup>[24]</sup>, but Puri *et al*<sup>[11]</sup> reported a non-statistically significant increase of this sphingolipid in patients with NASH. In other lipidomic studies, in which the control group was also morbidly obese, no significant differences were observed in the total sphingomyelin levels among the control, NAFL, and NASH groups<sup>[25,29,40]</sup>. Tiwari-Heckler *et al*<sup>[27]</sup>, however, reported an increase of total serum SM in NAFL and NASH patients compared to healthy controls. Moreover, individual sphingomyelin species, specifically SM (36:3), (d18:2/16:0), (d18:2/14:0), (d18:1/18:0), (d18:1/16:0), (d18:1/12:0), and (d18:0/16:0), were found to be increased in serum of patients with NAFLD compared to healthy subjects<sup>[63]</sup>, whereas Zhou *et al*<sup>[45]</sup> reported that circulating sphingomyelin cluster with representatives SM (d18:1/24:1), SM (d18:1/16:0), SM (d18:1/22:0), SM (d18:1/24:0), SM (d18:1/18:0), SM (d18:1/20:0), SM (d18:1/23:0), SM (d18:0/16:0), and SM (d18:0/20:4) was decreased in NASH patients compared to non-NASH subjects. Although there is no consensus on whether SM increases or decreases along with disease severity, studies in transgenic mice lacking the sphingomyelin synthase gene, revealed a strong association between liver SM levels and insulin resistance<sup>[64]</sup>. Further studies are needed to assess the relationship between SM metabolism and progression of NAFLD.

Numerous studies suggest that ceramide is a major contributing factor to insulin

resistance<sup>[65]</sup>. Ceramides and ceramide-derived sphingolipids are structural constituents of cell membranes, which also possess cell-signaling properties. Even though ceramide synthesis occurs in many organs, the liver is a key site for ceramide synthesis and in fact data from several studies suggest that sphingolipids, such as SM and ceramides, are found in higher quantity in the liver compared to other tissues<sup>[65,66]</sup>. Moreover, ceramide levels have been reported to be increased in the plasma of patients with prediabetes and ceramides were also increased in plasma and liver biopsies of patients with NAFLD<sup>[28,40,67]</sup>.

Ceramide synthesis can occur through three different pathways: (1) *A de novo* pathway that includes four sequential reactions with serine palmitoyl-CoA transferase (SPT) representing the rate-limiting enzyme of this pathway; (2) Through hydrolysis of SM catalyzed by sphingomyelinase (SMase); and (3) A salvage pathway<sup>[68]</sup>. *De novo* synthesis has been described to be stimulated by a diet rich in saturated fat<sup>[69]</sup>. Furthermore, increased hepatic free fatty acid influx, inflammation induced by TNF $\alpha$  and IL1, and oxidative stress can all increase the activity of SPT and activate *de novo* synthesis of ceramides<sup>[68,70]</sup>. All these three conditions are involved in the etiopathogenesis of NAFLD and represent important regulators of *de novo* ceramide synthesis<sup>[3]</sup>. Aside from the activation of *de novo* synthesis, inflammation increases ceramides by up-regulating the activity of sphingomyelinase<sup>[71]</sup>. Adiponectin, an adipokine involved in NAFLD pathophysiology, affects also the ceramide production. Adiponectin *via* receptors appears to upregulate the expression of ceramidase, the enzyme that converts ceramides to sphingosine-1-phosphate (S1P). Patients with NAFLD exhibit lower adiponectin levels than healthy subjects and this seems to contribute to the already increased concentration of ceramides<sup>[72]</sup>.

Ceramides, through their function as signaling molecules, have several physiological effects that contribute to the pathogenesis of steatosis and steatohepatitis. In particular, ceramides have been previously reported to decrease insulin sensitivity in skeletal muscle and hepatocytes<sup>[65]</sup>. In fact, a previous animal study reported that administration of inhibitors of ceramide biosynthesis resulted in a significant improvement of insulin resistance<sup>[70]</sup>. While increase of inflammatory cytokines leads to increased ceramide production, it is likely that ceramides through feedback mechanisms lead to increased production of cytokines and induce further processes of inflammation<sup>[65]</sup>. In addition, ceramides are involved in increased oxidative stress, mitochondrial dysfunction, and cell apoptosis<sup>[65,73]</sup>. Finally, there is evidence that ceramides may regulate the synthesis of HDL lipoproteins and thereby affect the reverse cholesterol transport. In a study in Western diet rat models, administration of myriocin - an inhibitor of ceramide biosynthesis - not only improved insulin resistance and steatosis, but also increased ApoAI production rate and consequently the production rate of HDL lipoprotein<sup>[74]</sup>.

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## NEUTRAL LIPIDS

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As far as neutral lipid classes are concerned, a limited number of studies have been conducted to investigate whether quantitative changes in their content are observed in patients with NAFLD. Triacylglycerols (TG), as expected, were found to be increased in liver biopsies of patients with NAFLD, whereas no statistically significant differences were observed in free fatty acid (FFA) hepatic content<sup>[11,29]</sup>. Diacylglycerols (DG) were also increased in the liver and interestingly, the ratio of TG/DG was increased in a stepwise manner from NAFL to NASH, suggesting that diacylglycerol acyl transferase (DGAT) is possibly involved in the pathogenesis of NAFLD<sup>[11]</sup>. In fact, inhibitors of DGAT-2 decreased hepatic steatosis, ballooning, and fibrosis in mice<sup>[75]</sup>. Moreover, recently this study was extended in phase 1 clinical trial in humans and steatosis and clinical markers of liver function were improved<sup>[76]</sup>.

Several studies have demonstrated that cholesterol homeostasis is disturbed in NAFLD<sup>[77,78]</sup>. Hepatic free cholesterol accumulation has been correlated with disease progression from simple steatosis to NASH without an increase in cholesterol esters<sup>[11]</sup>, whereas the findings about esterified cholesterol are contradictory<sup>[11,29]</sup>. Free cholesterol is considered a cytotoxic lipid that is involved in hepatotoxicity by disrupting membrane integrity and inducing oxidative stress, mitochondrial dysfunction, and apoptosis<sup>[79]</sup>. Thus, the observed increase of free cholesterol might contribute to liver injury and disease progression.



## FATTY ACIDS

Numerous studies have demonstrated that the fatty acid composition of lipids is altered in patients with simple steatosis and NASH. Total saturated fatty acids were found to be increased in liver biopsies of patients with NAFLD<sup>[11]</sup>. Especially, an increase in individual saturated fatty acids such as myristic acid and palmitic acid was found in liver samples of patients with NASH<sup>[24]</sup>. Walle *et al*<sup>[80]</sup> conducted a comprehensive study in serum fatty acid composition and reported an increase in total saturated fatty acids in triacylglycerols in NASH patients compared to patients with simple steatosis. Furthermore, serum levels of myristic acid in cholesterol esters and triacylglycerols and those of stearic acid in triacylglycerols were found to be increased in patients with NASH<sup>[80]</sup>. Total saturated fatty acids were reported also to be increased in plasma phospholipids in patients with NAFLD<sup>[81]</sup>. The increased *de novo* lipogenesis occurring in NAFLD as well a diet enriched in those types of fatty acids might be the main cause for the increase of saturated fatty acids in the liver and serum of patients with NAFLD<sup>[82]</sup>. In addition, saturated fatty acids exhibit pro-apoptotic properties and also, are involved in the pathogenesis of steatosis. The increase of saturated fatty acids in hepatocytes results in endoplasmatic reticulum stress, increased caspase activation, and hepatocellular apoptosis<sup>[83]</sup>.

Total monounsaturated fatty acids were also found to be increased in the liver and plasma of NAFLD patients<sup>[10,23,26,29]</sup>. In some cases, this increase was driven by palmitoleic acid and oleic acid<sup>[23,26]</sup>. These individual fatty acids are generated by the enzyme stearoyl-Coa desaturase (SCD1) from saturated fatty acids. The increase of monounsaturated fatty acids could be attributable to increased *de novo* lipogenesis activity and increased activity of SCD1<sup>[84]</sup>. In fact, Chiappini *et al*<sup>[24]</sup> demonstrated that the gene expression of *SCD1* was significantly increased in NASH patients in accordance with the increase of oleic and palmitoleic acid. Monounsaturated fatty acids are considered to contribute to the development of steatosis, but are more efficient in incorporating into hepatocyte triglycerides, thus they are less lipotoxic than saturated fatty acids. A potential protective role of monounsaturated fatty acids against lipotoxicity has also been suggested through the promotion of triglycerides accumulation in hepatocytes<sup>[85]</sup>.

The most common finding in lipidomic studies is the decrease of long chain PUFA. Specifically, a decrease in eicosapentaenoic acid, docosahexanoic acid, and arachidonic acid was reported in several lipidomic studies performed in the liver and plasma of patients with NAFLD<sup>[10,11,23,25]</sup>. The depletion of these n-3 and n-6 PUFA may be attributed to either a dietary deficiency or impaired biosynthesis. The generation of these PUFA is a multistep process in which several elongase and desaturases enzyme are involved. In NASH patients, the activities of fatty acid desaturase 1 (FADS1) and fatty acid elongase 6 (ELOVL6) were decreased<sup>[24]</sup>. Furthermore, the decreased activity of FADS1 is considered a key pathogenetic factor in the progression of simple steatosis to NASH. Another interesting finding is the increased n-6/n-3 ratio observed in liver biopsies of patients with NASH<sup>[10,11]</sup>. PUFA, especially n-3, are involved several biological processes and exhibit a protective role against lipotoxicity and insulin resistance<sup>[86]</sup>. Restoration of hepatic n-3 content improved steatosis and insulin resistance and decreased lipid peroxidation and necroinflammation in a mouse model of steatohepatitis<sup>[86]</sup>. Moreover, PUFA interact with transcription factors and modulate the expression of genes involved in lipid metabolism and fibrogenesis<sup>[87,88]</sup>.

PUFA serve also as precursors for the synthesis of proinflammatory eicosanoids and specialized pro-resolving mediators (SPMs). The biosynthesis of these lipid species involves several enzymes such as cyclooxygenases and lipoxygenases. Puri *et al*<sup>[26]</sup> reported a stepwise increase of lipoxygenase metabolites of arachidonic acid in plasma from control to NAFL and NASH, whereas no significant differences were observed in the plasma cyclooxygenase products of arachidonic acid among the study groups. Specifically, the lipoxygenase metabolites 5-HETE, 8-HETE, 11-HETE, and 15-HETE were found to be increased in plasma of patients with NASH<sup>[26]</sup>. Later, Loomba *et al*<sup>[89]</sup> investigated the plasma lipidomic profile of eicosanoid in patients with NAFLD and reported a significant increase of arachidonic acid-derived metabolites 11,12-diHETrE, dhk PGD2, and 20-COOH AA in plasma of patients with NASH compared to subjects with NAFL.

## LIMITATIONS OF PLASMA LIPIDOMICS STUDIES IN NAFLD

The findings of lipidomics studies conducted in plasma or serum of patients with

NAFLD, as mentioned before, are inconsistent. Lack of consistency is observed also between findings from plasma and liver studies. Interestingly, the discrepancies between liver and plasma findings regard mainly glycerophospholipid composition rather than fatty acid composition. In general, liver lipidomics studies revealed a decrease in glycerophospholipid species, such as PC, PE, PS, and PI, in NAFL patients and in some cases this alteration was profound only in the setting of NASH. On the contrary, most plasma lipidomic studies failed to detect depletion of these lipids and in some cases plasma glycerophospholipids were found to be increased in patients with NAFLD compared to the control group. Plasma glycerophospholipids are carried and distributed in lipoprotein classes. Plasma PC and PE are mainly distributed in HDL lipoprotein and 50% of hepatic PC is derived from circulation probably through hepatic uptake of HDL-PC<sup>[90,91]</sup>. Hence, low hepatic glycerophospholipid content, in an attempt to maintain adequate levels of these lipids, could lead to activation of unknown compensatory processes resulting in increased delivery of HDL-associated phospholipids and subsequent increase in plasma levels.

Moreover, findings regarding SM content in the liver and plasma are also inconsistent. Approximately 50% of plasma SM is found in LDL and 40% in HDL, and it is worth noticing that plasma SM levels correlate with BMI<sup>[56,90]</sup>. Differences in lipidomics study design including the selection of obese study population as a control group could explain the discordant findings. Furthermore, alteration in SM content in lipoprotein particles due to dietary factors, obesity, and unknown compensatory mechanism could be responsible for the differences observed in liver and plasma studies regarding sphingolipid species.

Further lipidomic studies focused on phospholipid content of lipoproteins in NAFLD patients should address this issue and delineate the changes observed in the setting of NAFLD.

## NONINVASIVE DIAGNOSIS OF NASH THROUGH LIPIDOMICS

At present, the diagnosis of NAFLD and the distinction of NASH from simple steatosis require liver biopsy and histological assessment. Nevertheless, liver biopsy is an invasive, costly, and time-consuming procedure. Hence, there is a growing interest in developing noninvasive methods for differential diagnosis of NASH and evaluation of treatment outcomes. Lipidomic studies carried out in liver biopsies of patients with NAFL and NASH patients reported alterations of hepatic lipid profile and several studies investigated if these changes were also observed in plasma or serum. Plasma lipidomic studies reported changes in the concentration of several lipids between patients with NASH and NAFL, but as highlighted above the results are inconsistent. As seen in [Table 2](#), saturated fatty acids in TGs, such as myristic acid and stearic acid, were found to be increased in patients with NASH compared to subjects with NAFL<sup>[80]</sup>. Moreover, plasma eicosanoid lipidomics analyses revealed a significant increase of arachidonic acid-derived metabolites (11,12-diHETrE, dhk PGD2, and 20-COOH AA) in patients with NASH compared to subjects with NAFL and researchers suggested that these eicosanoids may have a utility as biomarkers for the noninvasive diagnosis of NASH<sup>[89]</sup>. Lipoxygenase metabolites 5-HETE, 8-HETE, 11-HETE, and 15-HETE were also found to be increased in plasma of patients with NASH and these metabolites seem promising predictive biomarkers of NASH<sup>[26]</sup>.

Gorden *et al*<sup>[28]</sup> investigated the alterations of liver and plasma lipidomic profiles in patients with NAFLD categorized in three subgroups of disease progression. The study population included healthy subjects, patients with simple steatosis, patients with NASH, and subjects with cirrhosis. Lipidomic analyses in combination with aqueous metabolites analyses led to identification of 48 common analytes, which presented variation across disease stage and an overlap in both tissues. These analytes were sphingolipid species, such as dihydroceramides, 1-deoxydihydroceramides, and longer chain ceramides, implicating that sphingolipid metabolism is impaired and additionally involved in disease progression and transition of simple steatosis to NASH. Furthermore, Gorden *et al*<sup>[28]</sup> identified a panel of 20 plasma lipids that can be used to distinguish NASH from simple steatosis. This panel included dihydrosphingolipids, ether phosphatidylcholines, and other individual species. However, the number of patients that participated in this study is relatively small and validation of these findings in larger cohort of patients is needed<sup>[28]</sup>. Later, Zhou and his team developed an MS-based model and diagnostic score for NASH with an area under the receiver operating characteristic of 0.86. The NASH ClinLipMet score included AST, fasting glucose, glutamate, isoleucine, glycine, lysophosphatidylcholine

16:0, and phosphoethanolamine 40:6 along with *PNPLA3* genotype. This score needs also external validation<sup>[45]</sup>.

## CONCLUSION

Recent advances in lipidomics technology have made it possible to profile lipidome of liver tissues and plasma in NAFLD and compare the findings among the different stages of disease. Lipidomic profiling accompanied by experimental studies using pharmacological reagents to alter synthesis or metabolism of certain lipids, has given additional insights into mechanisms governing lipotoxicity and disease progression. In this review, the most interesting findings of lipidomics analyses are summarized and the interpretation of these findings in the pathogenesis of NAFLD is discussed. The inconsistencies observed between the findings of plasma and liver lipidomics studies in NAFLD have also been underlined and future studies will need to address this issue. Moreover, even if a small number of studies identified specific lipids or a panel of lipids as biomarkers of disease progression, these findings need further external validation from a large cohort of patients.

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