**Name of Journal:** *World Journal of Gastroenterology*

**Manuscript NO:** 63390

**Manuscript Type:** MINIREVIEWS

**Advances in paediatric nonalcoholic fatty liver disease: Role of lipidomics**

Di Sessa A *et al*. Lipidomics and fatty liver in children

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**Received:** January 27, 2021

**Revised:** April 6, 2021

**Accepted:** June 4, 2021

**Published online:**

**Abstract**

Due its close relationship with obesity, nonalcoholic fatty liver disease (NAFLD) has become a major worldwide health issue even in childhood. The most accepted pathophysiological hypothesis is represented by the “multiple hits” theory, in which both hepatic intracellular lipid accumulation and insulin resistance mainly contribute to liver injury through several factors. Among these, lipotoxicity has gained particular attention. In this view, the pathogenic role of different lipid classes in NAFLD (*e.g.,* sphingolipids, fatty acids, ceramides, *etc.*) has been highlighted in recent lipidomics studies. Although there is some contrast between plasma and liver findings, lipidomic profile in the NAFLD context provides novel insights by expanding knowledge in the intricate field of NAFLD pathophysiology as well as by suggesting innovative therapeutic approaches in order to improve both NAFLD prevention and treatment strategies. Selective changes of distinct lipid species might be an attractive therapeutic target for treating NAFLD. Herein the most recent evidence in this attractive field has been summarized to provide a comprehensive overview of the lipidomic scenario in paediatric NAFLD.

**Key Words:** Fatty; Liver; Lipidomics; Children; Nonalcoholic fatty liver disease

Di Sessa A, Riccio S, Pirozzi E, Verde M, Passaro AP, Umano GR, Guarino S, Miraglia del Giudice E, Marzuillo P. Advances in paediatric nonalcoholic fatty liver disease: Role of lipidomics. *World J Gastroenterol* 2021; In press

**Core Tip:** Insightful data from lipidomics studies have recently expanded knowledge about nonalcoholic fatty liver disease pathophysiology. In fact, different lipids have been found to exert specific pathogenic roles in liver injury through several pathways (in particular by impairing insulin signalling). Given the cardiometabolic burden of nonalcoholic fatty liver disease even in childhood, lipidomics findings might improve strategies for nonalcoholic fatty liver disease treatment by providing novel therapeutic targets.

**INTRODUCTION**

An increasing prevalence of nonalcoholic fatty liver disease (NAFLD) up to 25% has been found in adults, but alarming paediatric data have also been reported[1-3]. Due to the obesity epidemic, NAFLD has become the most prevalent liver chronic disease in childhood, affecting 3%-10% of the general paediatric population and up to 50% of obese children with a relevant cardiometabolic burden[2,4]. It includes different degree of hepatic steatosis ranging from simple fat accumulation to steatohepatitis and fibrosis, but its pathogenesis remains to be fully elucidated[1].

To date, “multiple hits” have been recognized in the NAFLD pathophysiology, with a pivotal role of the hepatic intracellular lipid accumulation and insulin resistance favouring liver damage through several factors such as lipotoxicity, oxidative stress, inflammation, genetics, gut axis, metabolic, and dietary factors[3,5]. Among these pathogenic factors, lipotoxicity has gained particular remarkable attention[6]. Lipid induced oxidative stress, inflammation, and cell death have been largely studied as major players of this process, and their interplay represent a critical step in both NAFLD development and progression[6-8]. Of note, it must be considered that the chronic inflammation closely related to lipotoxicity represents one of the most important features of several metabolic diseases such as obesity and type 2 diabetes, resulting in a dangerous “vicious circle” with dramatic clinical implications[6]. In fact, lipotoxicity affects a broad range of tissues such as adipose tissue, heart, brain, pancreatic islets, and skeletal muscle with a complex interrelation favouring the development of metabolic syndrome[6].

Although intrahepatic fat accumulation has been widely accepted as the hallmark of NAFLD, overwhelming evidence showed that both quantity and quality of accumulated hepatic lipids play a pathogenic role in NAFLD[7,9-12]. In particular, specific lipid classes such as sphingosine, diacylglycerols, and ceramides act as liver damaging agents through multiple pathways[6,7,9,13,14].

To date, the growing interest in lipidomic studies has provided meaningful data regarding lipid profiles involved in the pathogenesis of liver injury and its modulation as a potential therapeutic target[8,11,12-14]. In view of the clinical relevance of these findings in the NAFLD treatment scenario, evidence is currently available in adults and in children[15-19] (Tables 1 and 2).

We aimed to summarize the most recent findings regarding lipidomic studies in paediatric NAFLD by providing an overview of the different lipid class and their potential therapeutic implications.

**LIPIDS IN NAFLD**

Triacylglycerols (TAG) are the most representative lipidic class accumulated in the liver of NAFLD patients. Nevertheless, they would seem to be protective against lipotoxicity due to lipid overload. Lipotoxicity is mainly caused by increasing levels of saturated fatty acids (SFAs), free cholesterol, glycerophospholipids, sphingolipids, and deficient levels of phospholipids, u-3 polyunsaturated fatty acids (PUFAs), or PUFA-derived specialized proresolving mediators[20,21]. Monounsaturated fatty acids (MUFAs), lysophosphatidylcholine (LPC), and ceramide are also increased while phosphatidylcholine (PC) is reduced in nonalcoholic steatohepatitis (NASH)[22,23].

Of interest, lipidomic studies conducted in both plasma and serum of NAFLD patients reported overlapping results with those found in the liver. In addition, increased levels of total SFA in phospholipids[24], metabolites of lipoxygenase, 5- hydroxyeicosatetraenoic acid (HETE), 8-HETE[25], 15-HETE, 5,6 dihydroxy- eicosatrienoic acid[26], palmitoleic acid in cholesteryl ester[27], PC and sphingomyelin[28], phosphatidylserine, and phosphatidylinositol[29] were found in plasma and serum of NAFLD patients. On the other hand, decreased levels of eicosanoic acid (C20: 0), cis-11-octadecenoic acid (C18: 1n-7), docosahexaenoic acid in phospholipids[24], 12,13-dihydroxy-9-octadecenoic acid[26], and lysophospatidylethanolamine[28] have been described.

***Fatty acids***

The increased share of free fatty acids reaching the liver in NAFLD has been related to three main mechanisms: lipolysis of adipose tissue, *de novo* lipogenesis, and diet[30].

Both hepatic[31] and plasma[24] findings from adults and children[32] with NAFLD showed an increased content of SFAs.Of concern, SFAs seem to be one of the major players involved in lipotoxicity. In fact, evidence linked inhibition (genetic or pharmacological) of the enzyme converting SFAs to MUFAs, namely stearoyl-CoA desaturase-1, to different processes such as hepatocyte apoptosis, lipotoxicity, and development of steatohepatitis[33]. Consequently, balancing between MUFAs and SFAs might represent a central player in the progression from isolated hepatic steatosis to progressive steatohepatitis and fibrosis[34].

MUFAs were also found to be increased in liver and plasma of both adult and paediatric patients with NAFLD[25,32,35-37]. This lipid class has been considered as less lipotoxic than SFAs because channelling free fatty acids into MUFAs allow their incorporation into triglycerides and storage in lipid droplets[34].

The long chain PUFAs such as eicosapentaenoic, docosahexanoic, and arachidonic acids were decreasedin liver and plasma of patients with NAFLD[29,31,35,36].This reduction could be due to impairments in dietary intake or the biosynthesis process. A pivotal pathogenic role in NAFLD progression (from simple steatosis to NASH) has been attributed to decreased activity of fatty acid desaturase 1, an enzyme involved in the PUFAs metabolism[6].

Through the activity of hepatic cytochrome P450 enzymes, PUFAs derived from monoepoxides are collectively termed epoxyeicosanoids[38-40]. Those deriving from arachidonic acid (epoxyeicosatrienoic acid), eicosapentaenoic acid (epoxyeicosatetraenoic acid), and docosahexaenoic acid (epoxyeicosapentaenoic acid) have anti-inflammatory, antisteatotic, and antifibrotic properties[41]. In a recent paediatric study[32], 40 youths with biopsy-proven NAFLD underwent lipidomic evaluations by analysing liver tissue and blood samples. Upregulated hepatic epoxyeicosatrienoic acid, epoxyeicosatetraenoic acid, and epoxyeicosapentaenoic acid levels were found in children with steatosis. This might be due to reduced activity and protein expression of soluble epoxide hydrolase, metabolizing epoxyeicosanoids to vicinal diols. On the contrary, at the stage of fibrosis the aforementioned epoxyeicosanoids were found to be decreased in liver and plasma because of a potential reduction of cytochrome P450 epoxygenase expression. Therefore, the cytochrome P450 epoxygenase/soluble epoxide hydrolase pathway seem to represent a potential innovative pharmacologic target for NAFLD treatment.

Proinflammatory molecules are also derived from PUFAs. Puri *et al*[25] found increased plasma levels of arachidonic acid (5-HETE, 8-HETE, 11-HETE, and 15-HETE) lipoxygenase metabolites in NAFLD and NASH patients compared to lean normal controls. Moreover, plasma arachidonic acid-derived metabolites 11,12-dihydroxy- eicosatrienoic acid, 13,14-dihydro-15-keto prostaglandin D2, and 20-carboxy arachidonic acid levels were found to be significantly increased by Loomba *et al*[26] in subjects with NASH than those with NAFLD.

With respect to the wide cardiometabolic burden of NAFLD, changes in FA metabolism have also been linked to its related comorbidities including obesity, diabetes, and cardiovascular risk[20,28,29].

***Neutral lipids***

The hallmark of NAFLD is the accumulation of lipid droplets in the hepatocytes containing TAG[7]. TAGs were found to be increased in both plasma and liver of patients with NAFLD compared to healthy controls[25,31]. They represent a less toxic form of storing lipids. The inhibition of diacylglycerol acyltransferase 2, the enzyme catalysing the final step in the assembly of TAG, reduced steatosis but at the same time increased hepatic free fatty acids, lipid peroxidation, oxidative stress, necroinflammation, and fibrosis[42]. In mice defective for perilipin-5, a protein binding lipid droplet and regulating TAG storage, the reduction in the size of lipid droplets caused increased lipolysis and lipotoxicity[43].

Diacylglycerols (DAG) were also increased in plasma and liver of patients with NAFLD[25,31], and the ratio of TAG/diacylglycerols seemed to increase in the evolution from NAFLD to NASH[31].

In a paediatric study, Draijer *et al*[8] performed lipidomic analyses in plasma samples of 21 children with obesity and proton magnetic resonance spectroscopy-detected hepatic steatosis compared to the lipidome of 21 samples of nonsteatotic subjects with obesity. The authors found an overall significant increase in NAFLD patients of serum alkyl-diacylglycerols [TG(O)], in particular 8 TG(O) species (TG(O-52:0), (TG(O-52:1) TG(O-52:2), TG(O-54:1), TG(O-54:2), TG(O-52:3), TG(O-56:1) and TG(O-56:2)].

Finally, it should also be noted that the amount of hepatic free cholesterol increases with the progression to NASH, without an increase in cholesterol esters[31]. It is considered a cytotoxic lipid disrupting membrane integrity and inducing oxidative stress, mitochondrial dysfunction, and apoptosis[44].

***Glycerophospholipids***

Glycerophospholipids represent a significant lipidic fraction of the cell membrane. Reduced hepatic PC levels were observed in both NAFLD and NASH subjects[22,31]. However, conflicting data about alterations in serum were reported[6]. Low hepatic levels of PC influenced circulating very low-density lipoproteins, which are therefore reduced with consequent hepatic accumulation of TAG[45,46]. Low levelsalsoincreased de novo lipogenesis and formation of lipid droplets in hepatocytesby activation of sterol regulatory element-binding protein 1[47].

Liver phosphatidylethanolamine (PE) content was found to be decreased among subjects with NASH[22], while serum PE increased[28]. The enzyme phosphatidylethanolamine n-methyltransferase catalyses the reaction converting PE to PC. Studies reported that a loss-of-function polymorphism in the phosphatidylethanolamine n-methyltransferase gene predisposed to NAFLD susceptibility[48]. Lower hepatic PC/PE ratio was also reported in NAFLD individuals. Interestingly, a reduced PC/PE ratio in red blood cell membranes has been found to enhance predisposition to NAFLD[49]. As a consequence, loss of membrane integrity and higher permeability to proinflammatory factors were observed[50].

Paediatric data reported significantly increased PE serum levels and reduced specific etherphospholipid classes such as alkyl/alkenyl-phosphatidylethanolamine, alkyl/alkenyl-lysophosphatidylethanolamine [LPE(O)], and alkyl/alkenyl-phosphatidylcholine in subjects with NAFLD. When looking at individual lipid species, two LPE species such as LPE (20:3) and LPE (22:5) were found to be increased[19].

Phosphatidylserine and phosphatidylinositol were decreased in the liver[22] but increased in the plasma[29] of NAFLD patients. However, these results are conflicting in other studies[25,28,51].

LPC was increased in the liver of NASH patients[6], while no statistically significant changes in plasma and serum of LPC content were reported in patients with NAFLD or NASH[28,29]. LPC derived from PC by the action of lipoprotein associated phospholipase A2 at the intracellular level, whereas in the extracellular milieu by lecithincholesterol acyltransferase activity. In patients with NAFLD, phospholipase A2 levels were found to be decreased, while those of lecithincholesterol acyltransferase increased[52,53]. LPC downregulates genes involved in fatty acid oxidation, upregulates genes involved in cholesterol biosynthesis, and promotes apoptosis of hepatocytes[54]. Inhibitors of phospholipase A2 decreased palmitate-induced lipotoxicity and cell apoptosis[54,55].

In the liver of NAFLD patients no change in plasmalogen content was reported[56], while this class was decreased in the plasma of NASH patients[25,57]. Animal data demonstrated that a specific mechanism (involving peroxisome proliferator-activated receptor-alpha) sustained by endogenous hepatic plasmalogens may prevent liver steatosis and NASH[58].

Circulating plasmalogen levels, particularly 16:0 and 18:1, were found to be reduced in NAFLD individuals with the GG-genotype of patatin-like phospholipase domain-containing 3 (*PNPLA3*) compared to those with the C or CG allele[28]. The *PNPLA3* gene is highly expressed in hepatic stellate cells of the liver and adipose tissue and encodes adiponutrin, a protein exerting both lipase and acyltransferase activity[59]. Adiponutrin variant p.I148M [rs738409 (G)] enhanced PUFA content of TAGs and diacylglycerols and negatively affected both PC synthesis and lipid droplet hydrolysis[60].

An elegant paediatric study examining NAFLD genetic factors demonstrated that *PNPLA3* rs738409 (G) represented the strongest determinant of the presence of NAFLD as compared to healthy controls and conferred the highest risk of severity of steatosis. Interestingly, a specific steatosis pattern (including an increased percentage of portal inflammation) was reported in homozygous *PNPLA3* rs738409 (G) patients[61].

In addition to NAFLD, a significant association of these compounds has been found in a larger cardiometabolic-related context such as obesity and cardiovascular disease[51,57].

***Sphingolipids***

Sphingolipids are structural components of cellular membranes and signalling molecules in mammalian cells. Conflicting resultswere found about sphingomyelin (SM) trends in NAFLD and NASH patients[22,28,29,37,51]. Barr *et al*[62] found an increase in serum levels of certain sphingomyelin species, such as 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) in NAFLD individuals compared to controls. Instead, reduced circulating levels of 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) were observed by Zhou *et al*[63] in NASH adult subjects compared to controls. Moreover, increased serum levels of two SM species such as SM(d39:0) and SM (d41:0) were found in the serum of NAFLD paediatric patients[19].

Higher ceramide levels were found in plasma and liver biopsies of NAFLD subjects[51,64,65]. These lipids decreased insulin sensitivity in skeletal muscle and hepatocytes[66] and enhanced several unfavourable biological processes such as oxidative stress, mitochondrial dysfunction, and cell apoptosis[66,67]. Moreover, they seem to regulate the synthesis of high-density lipoproteins. Animal data reported that myriocin, acting through ceramide biosynthesis inhibition–promoted insulin receptor and steatosis and enhanced apolipoprotein AI production rate, resulting in an increased high-density lipoprotein production rate[68].

In a prospective study[20] including 80 obese children, total ceramide concentration was significantly increased in the serum of obese and NAFLD patients than in the reference group. In addition, increased levels of distinct fatty acid ceramides, such as myristic, palmitic, palmitoleic, stearic, oleic, behenic, and lignoceric were observed in children with NAFLD compared to controls. Furthermore, a significant positive association of total ceramide levels with homeostasis model assessment and insulin levels was reported[20].

Taken together, these findings might pave the way for a wider risk assessment for these patients, as suggested by paediatric evidence indicating a significant association of distinct sphingolipids with NAFLD and with its cardiometabolic burden including obesity, cardiovascular disease, and metabolic derangements[20,69-71].

**CONCLUSION**

Lipidomic studies have added novelty by allowing an accurate characterization of lipidomic profile of both plasma and liver tissues in NAFLD[7,12,17]. Besides experimental data providing additional insights about the pathophysiology of NAFLD and its progression, there is a growing body of evidence from human studies[8,14,20]. In particular, a clear effect for specific ceramides in impairing insulin signalling pathways has been found[10,13,15].

Interestingly, different lipid classes have been demonstrated to exert pathogenic distinct roles in NAFLD and in other metabolic diseases such as obesity, metabolic syndrome, and type 2 diabetes[14,15]. Thus, manipulation of the expression of certain lipids (*e.g.,* selective lowering of specific ceramides) might represent a novel target for both prevention and treatment of these diseases. In fact, this attractive therapeutic approach might pave the way for novel strategies to counteract the increasing NAFLD-related cardiometabolic burden even in childhood.

Further research is needed to validate these findings and to provide a more comprehensive assessment of the exact pathogenic role of specific lipids in the NAFLD context.

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**Footnotes**

**Conflict-of-interest statement:** No conflict of interest.

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**Manuscript source:** Invited manuscript

**Peer-review started:** January 27, 2021

**First decision:** March 29, 2021

**Article in press:**

**Specialty type:** Gastroenterology and hepatology

**Country/Territory of origin:** Italy

**Peer-review report’s scientific quality classification**

Grade A (Excellent): 0

Grade B (Very good): 0

Grade C (Good): C

Grade D (Fair): 0

Grade E (Poor): 0

**P-Reviewer:** Hyun J **S-Editor:** Fan JR **L-Editor:** Filipodia **P-Editor:**

**Table 1** **Comparison between adult and paediatric lipidomic findings in nonalcoholic fatty liver disease**

|  |  |  |
| --- | --- | --- |
| **Lipid class** | **Changes in adult NAFLD patients** | **Changes in paediatric NAFLD patients** |
| SFAs | Increased in liver[31] and in plasma[24] | Increased in liver and in plasma[32] |
| MUFAs | Increased in liver[35,36] and in plasma[25] | Increased in liver and in plasma[32] |
| PUFAs | Increased total PUFAs in liver[31] and n-6 LCPUFA in liver phospholipids[35]. Decreased LCPUFA of the n-6 and n-3 series in liver TAG, n-3 LCPUFA in phospholipids, total PUFA[35,36], n-3 PUFA[35], n-6 PUFA[32] | Increased in liver and in plasma[32] |
| PUFAs derived | Increased 5-HETE, 8-HETE, 11-HETE, and 15-HETE in NAFLD and NASH patients[27].Increased 11,12-diHETrE, dhk PGD2, 20-COOH AA in NASH patients[26] | Increased EDPs, EEQs, EETs with progression of steatosis; reduced with progression of fibrosis[32] |
| TAG | Increased in liver[31] and in plasma[25] | Increased (TG[O]); TG (O-52:0), TG (O-52:1), TG (O-52:2), TG (O-52:3), TG (O-54:1), TG (O-54:2), TG (O-56:1) and TG (O-56:2) in serum[19] |
| DAG | Increased in liver[31] and in plasma[25] | No available data |
| FC | Increased in liver[31] | No available data |
| PC | Reduced in the liver[31], conflicting data for changes in serum[28,29] | Reduced serum alkyl/alkenyl-phosphatidylcholine (PC[O]) levels[19] |
| LPC | No statistically significant changes in plasma and serum[28,29] | No available data |
| PE | Decreased in liver[22] and increased in serum of NASH patients[28] | Increased PE in serum[19] |
| LPE | Decreased in serum of patients with NAFLD and NASH[28] | Increased LPE (20:3) and LPE (22:5); decreased [LPE(O)]) in serum[19] |
| PS | Reduced in liver[22], increased in plasma[29] | No available data |
| PI | Reduced in liver[22], increased in plasma[29] | No available data |
| PL | No change in liver[56]; decreased in plasma of NASH patients[25,57] | No available data |
| SM | Conflicting results in NAFLD and NASH patients[22,28,29,31,37,51] | Increased SM (d39:0), SM (d41:0) in serum[19] |
| CE | Increased in liver and in plasma[51,64,65] | Increased in serum[20] |

20-COOH AA: 20-carboxy arachidonic acid; CE: Ceramides; DAG: Diacylglycerols; dhk PGD2: 13,14-dihydro-15-keto prostaglandin D2; diHETrE: Dihydroxy-eicosatrienoic acid; EDP: Epoxyeicosapentaenoic acid; EET: Epoxyeicosatrienoic acid; EEQ: Epoxyeicosatetraenoic acid; FC: Free cholesterol; HETE: Hydroxyeicosatetraenoic acid; LCPUFA: Long chain polyunsaturated fatty acid; LPC: Lysophosphatidylocholine; LPE: Lysophosphatidylethanolamine; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; MUFAs: Monounsaturated fatty acids; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PL: Plasmalogens; PS: Phosphatidylserine; PUFAs: Polyunsaturated fatty acids; SFAs: Saturated fatty acids; SM: Sphingomyelin; TAG: Triacylglycerols; TG(O): Alkyl-diacylglycerols.

**Table 2 Main findings of lipidomic studies in paediatric nonalcoholic fatty liver disease**

|  |  |  |  |
| --- | --- | --- | --- |
| **Ref.** | **Study design and methods** | **Population (*n*)** | **Main findings** |
| Wasilewska *et al*[20], 2018 | Prospective study | 80 children at median age 12 (7-17 yr) | Higher total serum CE concentration in NAFLD patients, compared to the controls and of certain CEs (C14:0, C16:0, C16:1, C18:0, C18:1, C22:0, C24:0). Total CE concentration was positively correlated with HOMA-IR and insulin levels |
| Draijer *et al*[8], 2020 | Case-control study | 21 children with obesity and steatosis and 21 with only obesity. Mean age of NAFLD patients: 14.8 yr; mean age of non-NAFLD patients 14.7 yr | Statistically significant alterations in 5 major lipid classes [TG(O), PE, PE(O), LPE(O), PC(O)] and 12 individual lipid species |
| Kalveram *et al*[32], 2021  | Prospective study | 40 children with biopsy-proven NAFLD. Mean age 14.2 ± 2.3 yr | Hepatic epoxyeicosanoids levels increased with higher degrees of steatosis. CYP epoxygenase activity increased, protein level, and activity of sEH decreased. In contrast, hepatic epoxyeicosanoids decreased with higher stages of fibrosis, with a decrease of CYP epoxygenase activity and protein expression |

CE: Ceramides; CYP: Cytochrome P450; HOMA-IR: Homeostasis model assessment; NAFLD: Nonalcoholic fatty liver disease; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; PC(O): Alkyl/alkenyl-phosphatidylcholine; PE: Phosphatidylethanolamine; PE(O): Alkyl/alkenyl-phosphatidylethanolamine; sEH: Soluble epoxide hydrolase; TG(O): Alkyl-diacylglycerols.