69462_Auto_EditedC.docx

WORD COUNT	6210	TIME SUBMITTED	02-FEB-2022 01:53PM
		PAPER ID	82094463

Name of Journal: *World Journal of Clinical Pediatrics*

Manuscript NO: 69462

Manuscript Type: REVIEW

Advances in pediatric non-alcoholic fatty liver disease: From genetics to lipidomics

Riccio S *et al*. Pediatric NAFLD pathophysiology

Simona Riccio, Rosa Melone, Caterina Vitulano, Pierfrancesco Guida, Ivan Maddaluno, Stefano Guarino, Pierluigi Marzuillo, Emanuele Miraglia del Giudice, Anna Di Sessa

Abstract

As a result of the obesity epidemic, non-alcoholic fatty liver disease (NAFLD) represents a global medical concern already in childhood with an increased cardiometabolic risk closely related. Knowledge in NAFLD pathophysiology has been largely expanded over the last decades. Beside to well-known key NAFLD genes (including the I148M variant of the *PNPLA3* gene, the E167K allele of the *TM6SF2*, the *GCKR* gene, the *MBOAT7-TMC4* rs641738 variant, and the rs72613567:TA variant in the *HSD17B13* gene), an intriguing pathogenic role has been also demonstrated for the gut microbiota. More interestingly, evidence has added new factors involved in the "multiple hits" theory. In particular, omics determinants have been highlighted as potential innovative marker for NAFLD diagnosis and treatment. In fact, different branches of omics including metabolomics, lipidomics (in particular sphingolipids and ceramides), transcriptomics (including micro RNAs), epigenomics (such as DNA methylation), proteomics, and glycomics represent the most attractive pathogenic elements in NAFLD development, by providing insightful perspectives in this field. In this perspective, we aimed to provide a comprehensive overview of the NAFLD pathophysiology in children, from the oldest pathogenic elements (including genetics) to the newest intriguing perspectives (such as omics branches).

Key Words: Fatty; Liver; Genetics; Lipidomics; Pediatric

Riccio S, Melone R, vitulano C, guida P, maddaluno I, Guarino S, marzuillo P, Miraglia del Giudice E, Di Sessa A. Advances in pediatric non-alcoholic fatty liver disease: From genetics to lipidomics. *World J Clin Pediatr* 2022; In press

Core Tip: A large body of evidence supported a complex non-alcoholic fatty liver disease (NAFLD) physiopathology with several factors involved in this tangled puzzle. Considering the cardiometabolic burden of NAFLD even in childhood, a better knowledge of NAFLD physiopathology is fundamental for novel therapeutic strategies.

INTRODUCTION

As the increasing rate in pediatric obesity worldwide, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease also in childhood[1,2]. Current pediatric estimates reported a prevalence of 3%-10% in the general population, while a dramatic increase (up to 50%) has been observed in children and adolescents with obesity[2]. Owing to its strong relationship with metabolic syndrome (MetS) and insulin resistance (IR), both metabolic and cardiovascular risks are increased in subjects with NAFLD already in childhood[2-4].

Hepatic fat accumulation represents the hallmark of the disease, that includes a wide spectrum of progressive forms ranging from simple steatosis through non-alcoholic steatohepatitis (NASH) to fibrosis and cirrhosis[5]. Lipolysis of the adipose tissue and *de novo* hepatic lipogenesis are the main biological pathogenic processes contributing to fatty liver and IR[3,6]. Taken together, they result in an increased flux of free fatty acids to the liver and skeletal muscle that might activate lipotoxic pathways responsible for more progressive forms of hepatocellular injury. Interestingly, recent studies have highlighted not only the role of lipotoxicity but also of the fatty acid composition as central players in NAFLD[7-9].

Pathophysiological hypotheses of NAFLD have been resumed in the "multiple hits" theory, by assuming the role of genetics, microbial, metabolic, and environmental factors through a complex interplay[1 2,10-12].

Key genetic factors for NAFLD are represented by the I148M variant of the *PNPLA3* gene[13], the E167K allele of the *TM6SF2*[14,15], the *MBOAT7-TMC4* rs641738 variant[16], and the rs72613567:TA variant in the *HSD17B13* gene[17] (Figure 1).

Recently, advances in the understanding of NAFLD pathogenesis have reported the role of specific lipid class (in particular sphingolipids and ceramides) and their correlation also with IR, by underscoring the strength of the tangled link between NAFLD and IR[9,18-21].

For this reason, we aimed to provide a comprehensive overview from the oldest to the newest pathophysiological evidence on pediatric NAFLD.

NAFLD PATHOGENESIS: THE "MULTIPLE HITS" THEORY

One of the most recurrent questions about NAFLD is about the potential progression to more severe forms in certain subjects. This seems to be relevant since hepatic inflammation or fibrosis determine the long-term prognosis of the disease, while simple steatosis does not seem to worsening the outcome worse[22,23], although some studies would seem to weaken this assumption[24,25].

In an attempt to explain NAFLD pathogenesis, Day et al. first proposed the ''two hit'' model theory, suggesting that after a first hit (*i.e.*, hepatic steatosis), another hit (*e.g.*, gut-derived endotoxin) contributed to NASH development[26]. Later, a more complex model called "multiple parallel hits model"[23] in which multiple factors (including genetics, obesity, insulin resistance, metabolic and environmental determinants) act together to induce NAFLD development and progression in genetically predisposed or high-risk individuals was proposed. In particular, increased lipid storage, lipogenesis, and adipokine synthesis in the adipose and liver tissue, may act as stress signals for the endoplasmic reticulum (ER) with subsequent hepatocellular damage[27]. More, certain genes (such as *PNPLA3, TM6SF2, GCKR, MBOAT7*, and *HSD17B13*) have been strongly related to NAFLD susceptibility.

Genetics

PNPLA3: The *PNPLA3* gene, discovered by Hobbs and colleagues in 2008, has been largely accepted as the most important genetic determinant in NAFLD development. *PNPLA3* is located on chromosome 22 and belongs to the patatin-like phospholipase family. Its expression seems to be influenced by several factors, including diet, obesity, insulin and glucose levels, and gene mutation[28]. *PNPLA3* encodes for a protein called adiponutrin, an enzyme found in liver and adipose tissue that appears to confer susceptibility to increased liver fat levels and liver inflammation[29]. The discovery of *PNPLA3* has brought new insights into the understanding of fatty liver, specifically lipid remodeling in intracellular droplets has been identified as a common mechanism

underlying disease progression independent of environmental trigger. In particular, *PNPLA3* is involved in the remodeling of triglycerides, phospholipids, and retinyl ester release, acting as a lipase on lipid droplets[30]. Adiponutrin is an enzyme with retinyl-palmitate lipase function that, in response to insulin, has been shown to be responsible for the release of retinol from lipid droplets in hepatic stellate cells in vitro and *ex vivo*[31]. It is induced by diet and IR[32] and exhibits lipolytic activity on triglycerides[33].

Several studies have investigated the major pathogenic role of the *PNPLA3* rs738409 (*PNPLA3* I148M) single nucleotide polymorphism (SNP) in NAFLD development. It is a non-synonymous variant in which there is a cytosine to guanosine change leading to an amino acid substitution of isoleucine to methionine at amino acid position 148 of the coding sequence, in the active site of the enzyme (I148M). This amino acid substitution affects the function of the enzyme (loss of-function), leading to intrahepatic triglyceride accumulation and consequent development of micro vesicular steatosis. On the other hand, adiponutrin might exhibit a gain of lipogenic function, which could further lead to hepatic fatty acid accumulation[34]. The I148M variant, due to the altered enzymatic activity, determines an altered lipid remodeling, with accumulation of polyunsaturated fatty acids in diacylglycerol and triglycerides, and a parallel depletion in phospholipids[30]. Several studies have reported that the *PNPLA3* SNP resulted in decreased retinol metabolism and decreased hepatic protein levels of retinol dehydrogenase 16, which correlate with fibrosis severity[31].

There is strong evidence in the literature for an association between the *PNPLA3* 148M allele and NAFLD in both adults and children. In 2008, Romeo *et al*[29] first reported the association between the *PNPLA3* gene polymorphism [rs738409C/G] and NAFLD in a multiethnic cohort of Hispanic, African American, and European American adults.

Similarly, a large body of evidence supported the role of this gene in NAFLD development in children. Santoro *et al*[35], in a multiethnic group of 85 obese youths with steatosis magnetic resonance imaging (MRI)-detected, demonstrated that the prevalence of the G allele was higher in subjects with hepatic steatosis. Another study investigating

1048 obese Italian children, reported that children carrying the 148M allele showed higher aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, in particular homozygous 148M carriers with high extent of abdominal fat (expressed as Waist/Height ratio greater than 0.62) had a higher OR for developing pathological ALT. Thus, it was observed for the first time that the extent of *PNPLA3* association with liver enzymes was determined by the size of abdominal fat[36].

Romeo *et al*[37], in a 2010 study of 475 obese/overweight children and adolescents with steatosis evaluated by liver ultrasound, reported that the I148M variant of the *PNPLA3* gene was associated with increased ALT/AST levels in obese children and adolescents, suggesting that it conferred a genetic susceptibility to liver damage at an early age.

In addition, it has been demonstrated that the frequency of the *PNPLA3* risk allele rs738409 was lower in African Americans, by suggesting some protection from hepatic steatosis in obese African American youths[38]. In a 2018 study, Hudert *et al*[39] in a cohort of Berlin adolescents aged 10-17 years with NAFLD observed that the *PNPLA3* rs 73844078G variant was significantly associated with the severity of steatosis, with an increased risk of progression to fibrosis.

The association between *PNPLA3* gene and the other major genetic variants of NAFLD was also evaluated. Viitasalo *et al*[40] demonstrated higher serum ALT levels in children carrying the risk alleles for the polymorphisms *PNPLA3, MBOAT7* and *TM6SF2*. Grandone *et al*[15] reported that homozygous subjects for the PNPLA3 148M allele carrying the rare variant of TM6SF2 showed an odds ratio of 12.2 (confidence interval 3.8-39.6, P = 0.000001) to have hypertransaminasaemia compared with the remaining patients. Of interest, an Italian pediatric study also confirmed the combined effect of the 3 major risk variants (*PNPLA3, TM6F2* and *MBOAT7*) on NAFLD risk[16].

Besides, the interaction of the *PNPLA3* 148M allele with environmental risk factors for NAFLD such as obesity, nutrients (including carbohydrate and polyunsatured fatty acids), physical activity, and sedentary behaviors has been demonstrated in children with NAFLD[41-45]. Dai *et al*[28], in a meta-analysis, reported a strong influence of the

PNPLA3 rs738409 polymorphism not only on fatty liver but also on histological damage.

More recently, compelling evidence has also supported an intriguing role of this gene in reducing the estimated glomerular filtration rate independently of common renal and metabolic factors both in adults and children[46-49]. This gene seems to promote both fibrogenesis and glomerulosclerosis through the activation of renal pericytes in which the 148M allele is highly expressed[47,48].

Considering its detrimental effect on renal function already in childhood[46-48], these findings demonstrated that the *PNPLA3* gene acts not only as one of the major genetic player in NAFLD development but also as a harmful factor beyond the liver[46-48].

GCKR

Several studies reported that variations at the *GCKR* gene locus are associated with NAFLD and appear to influence hepatic fat accumulation. GCKR protein has an inhibitory action on the activity of the enzyme glucokinase that regulates the hepatic storage and disposal of glucose. In particular, *GCKR* forms an inactive complex with the enzyme glucokinase and transports it from the cytoplasm to the nucleus, thus controlling both activity and intracellular localization of this key enzyme of glucose metabolism[49].

Fructose-6-phosphate (F6P) enhances GCKR-mediated inhibition. By controlling glucose influx into hepatocytes, *GCKR* regulates *de novo* lipogenesis. The mechanism responsible for liver injury is probably due to the lack of inhibition of glucokinase enzymatic activity by F6P and consequently uncontrolled lipogenesis[50].

GCKR gene polymorphisms (rs780094 and rs1260326) have been identified that appear to be important in the pathogenesis of NAFLD. In particular, Beer *et al*[51] and Valenti *et al*[52] reported that in the association with NAFLD and consequently in the accumulation of hepatic fat, the common missense loss-of-function *GCKR* mutation (rs1260326 C>T) encoding for the P446L protein variant plays an important pathogenic role. The P446L variant blocks the inhibitory activity of GCKR on the enzyme

glucokinase, resulting in a steady increase in hepatic glucokinase and glucose uptake by the liver. Hepatic glycolysis associated with the minor allele P446L results in lower levels of both glucose and insulin, but leads to increased levels of malonyl-CoA which in turn blocks fatty acid oxidation through inhibition of carnitine-palmytoyltransferase-1 and acts as a substrate for lipogenesis, thus promoting hepatic fat accumulation[53]. GCKR rs780094 C>T variant has been found to be associated with increased intrahepatic fat accumulation and progressive forms of NAFLD[54,55].

A pediatric study involving 70 obese adolescents demonstrated that the *GCKR* rs780094 C>T variant was associated with NAFLD and decreased levels of *GCKR* protein, while the GCKR rs780094C>T and rs1260326C>T variants were associated with fibrosis and decreased levels of GCKR protein[39]. Lin *et al*[56], in a study examining 797 obese Taiwanese children, reported that the *GCKR* rs780094T variant was associated with an increased risk of NAFLD, by further demonstrating that the *GCKR* and *PNPLA3* variants were common NAFLD risk genetic factors in obese individuals. In fact, several studies have also reported a combined effect of the *PNPLA3* and *GCKR* SNPs as NAFLD risk polymorphisms. In particular, Santoro et al. in a study of 455 obese children and adolescents reported that the *GCKR* rs1260326 variant was associated with hepatic fat accumulation along with large levels of very-low-density lipoprotein (VLDL) and triglycerides, further demonstrating that *GCKR* and *PNPLA3* synergistically act to convey susceptibility to fatty liver in obese youths[57].

More recent studies confirmed the strong association of the three major genetic variants such as *TM6SF2* rs58542926, *PNPLA3*rs738409, and *GCKR* rs1260326 with NAFLD in obese children and adolescents[58].

TM6SF2

TM6SF2 is responsible for the regulation of lipid metabolism in the liver[59]. In particular, *TM6SF2* gene contributes to the secretion of VLDL from the liver[60]. As suggested by recent evidence[61], *TM6SF2* is a polytopic membrane protein acting as a lipid transporter. It is predominantly expressed in the liver, small intestine, and kidney.

TM6SF2 encodes a 351 amino acid protein with 7-10 predicted transmembrane domains[60]. Sliz *et al*[62] reported an association of the *TM6SF2* rs58542926-T allele with lower-risk lipoprotein lipid profile and lower levels glycerol and glycoprotein acetylation. Specifically, authors reported that the *TM6SF2* variant was associated with lower concentrations of all lipoprotein particle subclasses [including VLDL and low-density lipoprotein (LDL)]. In addition, there was an inverse association between this variant and total serum triglycerides and triglycerides in all lipoprotein subclasses, including high-density lipoprotein (HDL) subclasses. Finally, the *TM6SF2* rs58542926-T allele did not appear to affect apolipoprotein A-I concentration, whereas it was associated with lower apolipoprotein B concentration. More, it was also found to impair the secretory pathway leading to hepatic lipid accumulation and reduced levels of circulating lipids and lipoproteins.

In the last years, a single nucleotide rs58542926 C>T polymorphism giving rise to the E167K *TM6SF2* variant was noted in the complex puzzle of NAFLD pathophysiology[34]. It was associated with increased liver fat content, NASH, advanced liver fibrosis, and cirrhosis[63]. This variant is characterized by an adenine-guanine substitution in nucleotide 499 that replaces glutamate at residue 167 with lysine (c.499A > G; p.Glu167Lys) leading to a loss of function in hepatic secretion of VLDL[61].

Another study on two large histologically characterized adult cohort (including steatosis, steatohepatitis, fibrosis and cirrhosis) reported an association of the *TM6SF2* gene with advanced liver fibrosis, regardless of the *PNPLA3* genotype presence[64]. This association was also independently validated in another large European cohort[65].

Thus, *TM6SF2* might be considered as a regulator of liver fat metabolism with the opposite effects on triglyceride-rich lipoprotein secretion and hepatic lipid droplet content[34].

Chen *et al*[59] in a recent meta-analysis, on associations of TM6SF2 polymorphisms with chronic liver disease, suggested that rs58542926 polymorphism may be significantly associated with chronic liver disease in both Asians and Caucasians. In addition, Holmen *et al*[66] showed in a longitudinal adult Norwegian study an

association of the E167K TM6SF2 variant with lower total cholesterol levels resulting in a reduced risk of myocardial infarction. Accordingly, Dongiovanni *et al*[65] showed an effect of this polymorphism on reducing the risk of carotid atherosclerosis in adults.

The effect of this polymorphism on ALT and cholesterol levels has also been confirmed in children and adolescents. Grandone *et al*[15] demonstrated in a cohort of 1010 obese Caucasian children and adolescents that the TM6SF2 167K allele in carriers was associated with hepatic steatosis, higher ALT levels and lower total cholesterol, LDL-cholesterol, triglycerides and non-high density lipoproteins. In addition, subjects homozygous for the *PNPLA3* 148M allele carrying the rare variant of *TM6SF2* showed an OR of 12.2 for presenting hypertransaminasemia compared with the remaining patients. Thus, the effect of *PNPLA3* and *TM6SF2* alleles appeared to be additive in determining pediatric NAFLD. As previously demonstrated in adults, authors found that the TMS6SF2 E167K variant predisposed to NAFLD in obese children, with a relevant beneficial effect on cardiovascular risk[15].

Noteworthy, recent data also showed a protective effect of the *TM6SF2* gene on renal function both in adults and children through the reduction of lipotoxicity[47,67].

In conclusion, the discovery of the E167K variant adds another piece not only in the complex pathophysiology of NAFLD but also in the larger context of cardiometabolic risk NAFLD-related.

MBOAT7

The pathogenic role of this gene in NAFLD susceptibility has been largely studied both in adults and children. Findings demonstrated its effect in increasing not only the risk (and the severity) of NAFLD but also of other chronic liver diseases (*e.g.* hepatitis B and C virus-related). *MBOAT7* encodes lysophosphatidylinositol acyltransferase, involved in the inflammation cascade through the regulation of arachidonic acid levels and leukotriene synthesis in neutrophils. A combined effect of this gene with the major NAFLD risk polymorphisms (such as *PNPLA3* and *TM6SF2*) has been also highlighted

in adult and pediatric studies[16]. Similarly to renal effects observed for *PNPLA3* and *TM6SF2*, a role for this gene in kidney dysfunction has been also demonstrated[47].

HSD17B13

The 17β-hydroxysteroid dehydrogenases (HSD17Bs) encompasses a large family of 15 members involved in various metabolic processes such as the metabolism of steroid hormones, cholesterol, fatty acids, and bile acids[68]. In 2008, Horiguchi *et al* identified *HSD17B13* as a novel lipid droplet (LD) associated protein. The human *HSD17B13* gene is located on chromosome 4 (4q22.1) and its expression is highly restricted to the liver, particularly in hepatocytes[69]. The human *HSD17B13* gene encodes a 300 aminoacid protein, hydroxyl-steroid 17-beta dehydrogenase 13, a liver-specific LD-associated protein which is localized to lipid droplets[70].

To date, the physiological function of *HSD17B13* remains largely unclear. *HSD17B13* appears to have a role in estradiol metabolism and enzymatic activity against bioactive lipid mediators, such as leukotriene B4, that are involved in lipid-mediated inflammation[71].

In a 2019 study, Ma *et al*[72] reported that HSD17B13 exerts retinol dehydrogenase activity *in vitro*, which is closely linked to the lipid droplet. Indeed, it was observed that *HSD17B13* catalyzes the oxidation of retinol to retinaldehyde, the rate -limiting step in all-transretinoic biosynthesis.

The fact that *HSD17B13* is highly abundant in the liver and selectively expressed on the lipid droplet surface suggests a potential critical effect in lipid droplet function, as supported by growing data demonstrating the key role of the *HSD17B13* gene in hepatic lipid homeostasis and NAFLD pathogenesis[73].

In contrast, inactivating variants in the *HSD17B13* gene have recently been linked with a reduced risk of chronic liver disease in several studies[63]. In 2018, Abul-Husn et al reported that a loss-of-function variation in the *HSD17B13* (rs72613567:TA) gene resulting in a truncated protein confers protection against chronic liver damage and attenuates the progression of NAFLD and alcoholic liver disease (ALD) in European

Americans through reduced enzymatic activity against several proinflammatory lipid species[71]. Sookoian *et al*[74] in an exome-wide association study, confirmed that the *HSD17B13* rs72613567 variant had an influence on the susceptibility and histological NAFLD severity. More, Pirola *et al*[75] observed a lower risk of progressive NASH in subjects carrying the rs72613567:TA variant compared to non-carriers. However, the exact role of *HSD17B13* in the NAFLD pathophysiology remains largely uncharacterized. Recently, interesting studies on the inactivation of *HSD17B13* in mice and the identification of an enzymatic active site that metabolizes retinol have been reported[76,77] but pathophysiological evidence on human models is still limited[74,78]. The rs72613567: TA *HSD17B13* variant seems to affect liver by modulating hepatic retinol metabolism and by reducing stellate cell activity[78]. Another study, examining a large adult population, reported a protective role of this variant against various liver diseases such as cirrhosis, and hepatocellular carcinoma (HCC). In particular, *HSD17B13* rs72613567 was associated with reduced inflammation and fibrosis, and milder disease severity of NAFLD. Thus, *HSD17B13* rs72613567 represents an important protective factor in distinct liver diseases (including ALD, cirrhosis, and HCC) and seems to be associated with milder histological progression of NAFLD[79,80]. In 2019, Yang *et al*[81] in a multicenter European study of a total of 3315 patients with HCC or without HCC but with chronic liver disease, reported that the *HSD17B13* loss-of-function variant rs72613567 is protective of HCC development in patients with ALD. Taken together, these findings suggested the potential therapeutic role of the *HSD17B13* inhibition[79] in patients at high risk for liver diseases. The rs72613567 variant also appears to interact with PNPLA3 I148M through the additional *HSD17B13* TA alleles that reduce the effect of the additional *PNPLA3* I148M alleles on serum ALT levels. It also mitigated liver damage in individuals genetically predisposed to hepatic steatosis by *PNPLA3* I148M[71]. The protective effect of the rs72613567:TA *HSD17B13* variant in reducing liver damage has been observed also in children[17]. By analyzing a large cohort of Italian obese children, carriers of the *HSD17B13* variant showed lower NAFLD risk than noncarriers. Noteworthy, this variant was found to protect against liver

damage even among patients stratified on the basis of the number of the steatogenic alleles of the three major NAFLD risk polymorphisms (such as *PNPLA3*, *TM6SF2*, and *MBOAT7*genes). More interestingly, recent pediatric evidences[47,48,82] showed a similar protective effect of this gene also on renal function, by supposing its role in retinol metabolism through modulation of both inflammation and fibrogenesis. Another variant (rs143404524) in the *HSD17B13* gene, resulting in a truncated protein has also been associated with a reduced risk of chronic liver disease in adult population[83]. Finally, it has been also demonstrated that the rs62305723 variant of the *HSD17B13* gene, a missense variant that confers loss of enzyme activity was associated with decreased steatohepatitis[72]. In conclusion, the *HSD17B13* gene represents a well-known genetic factor with a protective role against liver damage both in adults and children[68] that might be considered an important pharmacological target for NAFLD treatment[17,84].

NAFLD AND THE "GUT-LIVER AXIS"

Recently, compelling evidence has supported the close and interdependent relationship between liver and gut axis in the pathogenesis of numerous chronic liver diseases such as chronic hepatitis B and C, ALD, NAFLD, NASH, development of liver cirrhosis, and HCC (Figure 2).

Bäckhed *et al*[85] for the first time described the role of gut microbiota in the context of NAFLD and obesity, taking part in the processes of absorption and storage of energy but also in the triglycerides production, responsible for the infiltration of the hepatocytes.

The crosstalk among liver and gut occurs by means of the biliary tract, portal vein and systemic mediators[86]. The liver contributes to the maintenance of gut eubiosis through the transport of bile salts and antimicrobial molecules to the intestinal lumen. Conversely, the gut regulates bile acids (BAs) composition. In addition, BAs using farnesoid X receptor (FXR) in the enterocytes and G protein-coupled bile acid receptor 1 (also known as TGR5) are involved in the regulation of glucose and lipid metabolism,

anti-inflammatory immune responses and host energy expenditure[87-91]. Furthermore, the gut through the secretion of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide influences pancreas in regulating both insulin and glucagon secretion[92]. More, GLP-1 interaction with its receptor (located also on the hepatocytes) results in reduced hepatic fat deposition and IR. Finally, it promotes energy expenditure and peripheral utilization of triglycerides for energy production[93].

BAs synthesis is regulated by two hepatic ways such as the enterohepatic circulation (with a subsequent negative feedback loop on the expression of CYP7A1) and FGF19, (derived from the activation of FXR by BAs in ileum and provided with inihibitory effect on *CYP7A1* gene[94]).

An impaired FXR-FGF19 signaling and elevated circulating BA levels were described both in children and adults with NAFLD. However, experimental therapeutic interventions targeting BA signaling with FXR agonists (obeticholic acid) have produced contradictory results[95].

Some differences were reported in the composition of gut microbiota (*i.e.* dysbiosis) in healthy controls than subjects with simple fatty liver disease (FLD) and NASH[96]. In fact, many pediatric studies have reported a decreased gut microbiota alpha diversity, measured with Shannon index[45,97-99].

In the far 2006, Turnbaugh *et al*[100] found that the ratio of *Firmicutes* to *Bacteroidetes* increased in obese mice, by suggesting a putative role for *Firmicutes* as group of microbiomes obesity-related.

Loomba *et al*[101] in an elegant study showed that NAFLD patients exhibited more *Gram-negative* and fewer *Gram-positive bacteria* compared to healthy subjects, with an increase in *Proteobacteria* and a decrease in *Firmicutes* in more progressive NAFLD forms.

Michail *et al*[102] noted that children with NAFLD had more abundant *Gammaproteobacteria* and *Prevotella* compared to obese children without NAFLD and

healthy controls. In addition, no difference in *Firmicutes* and *Bacteroidetes* or their ratio was observed between the groups.

Del Chierico *et al*[97] in a complex study with an integrated meta-omics-based approach found a significant increment of *Actinobacteria* and a decrease of *Bacteroidetes* in NAFLD patients compared to healthy controls.

Stanislawski *et al*[102] examined 107 adolescents with hepatic steatosis MRI-detected and found that *Bilophila* was positively correlated with hepatic fat fraction (HFF), while *Oscillospira* and *Bacteroides* showed different patterns in relation with HFF.

Schwimmer *et al*[99] in a prospective, observational, cross-sectional study of 87 children with biopsy-proven NAFLD and 37 obese children without NAFLD noted that a high abundance of *Prevotella copri* was associated with more severe fibrosis.

In a metagenomic study of gut microbiota of Zhao *et al*[103] conducted on 58 children and adolescents with NAFLD diagnosis by magnetic resonance spectroscopy, authors found no significant differences in terms of alpha diversity among the study groups (NAFLD children, obese children without NAFLD and healthy controls). However, *Proteobacteria* were found to be more represented in NAFLD children than in the control group, while *Bacteroidates* (*Alistipes*) were significantly reduced.

Finally, Kravetz *et al*[45] in a cross sectional study including 73 obese children and adolescent with and without NAFLD, in which HFF was determined through MRI, NAFLD group showed a higher *Firmicutes* to *Bacteroidetes* ratio and lower levels of *Bacteroidetes*, *Prevotella*, *Gemmiger* and *Oscillospira*.

Altered gut microbial composition and increased intestinal permeability are linked to several factors (*e.g.* high-fat Western diet, chronic alcohol consumption, and genetic factors) and promote the influx of microbial-associated molecular patterns or pathogen-associated molecular patterns into the portal system reaching the liver. These molecular patterns are responsible for inflammatory responses mediated by the activation of pattern recognition receptors, like Toll-like receptor, in Kupffer cells and hepatic stellate cells, leading to liver injury and fibrosis[86,104-106].

Potential gut-microbiome-targeted therapies in hepatic diseases are represented by probiotics, prebiotics, antibiotics, fecal microbial transplantation and bacteriophage, but larger validation studies are needed[107].

THE ROLE OF "OMICS" IN NAFLD

Epigenomics

Several authors studied the role of epigenetic modifications in the natural history of NAFLD. The main epigenomic modification studied in NAFLD is the DNA methylation.

A recent systematic review[108] included twelve studies on DNA methylation and FLD of which two assessing the global DNA methylation, five the DNA methylation for specific candidate genes and other four that used the EWAS approach. The review suggested no consistent associations with FLD in the studies of the global DNA methylation evaluated in hepatic tissue sample by quantifying the methylcytosine (5-mC) present in the genome. One of the two studies assessing the global DNA methylation found mitochondrial encoded NADH dehydrogenase 6 hypermethylation in the liver of NASH patients compared to those with simple steatosis and that this methylation was significantly associated with NAFLD activity score[109]. On the other hand, another study reported that global liver methylation based on genome-wide methylation arrays was not associated with NAFLD or NASH but NASH was associated with long-interspersed nuclear element hypomethylation compared to simple steatosis or normal liver[110]. More, studies using a candidate gene approach found that NAFLD was associated with hypomethylation at *FGFR2*, *MAT1A*, *CASP1* and *PARVB* genes and hypermethylation at *PNPLA3*[111], *PPARa*, *TGFβ1*, *Collagen 1A1* and *PDGFa* genes[112]. Furthermore, PPARGC1A methylation status was significantly associated with NAFLD[113]. The epigenome-wide DNA methylation studies reported different association of distinct methylation compounds with NAFLD[114,115]. Finally, a single study reported the role in NAFLD of methylation in the expression of three genes (*NPC1L1*, *STARD* and *GRHL*) involved in lipoprotein particle composition[116].

A recent and interesting prospective cohort study analyzed epigenome-wide DNA methylation data of 785 newborns and 344 10-year-old children in relation to liver fat fraction (measured by MRI) at 10 years. No differential DNA methylation at age 10 years in newborns or 10-year-old children were found[117].

Despite some causative evidence, little is still known about the relationship between these changes in hepatic epigenome and their repercussion in the blood stream. As a result, the contribution of epigenomics in the non-invasive diagnosis of NAFLD is still very limited but promising.

Transcriptomics

A growing body of data derives from micro RNAs (miRNAs), highly conserved noncoding small RNAs, involved in gene expression modulation at the post-transcriptional level (Table 1). MiRNAs are resistant to degradation as well as to several freeze–thaw cycles, suggesting their potential role as ideal biomarkers for use in clinical practice.

Several studies highlighted the correspondence between miR-122 and the severity of steatosis[118]. A reduced hepatic expression of miR-122 was described[119,120], whereas miR-122 levels were upregulated in serum[120].

A systematic review reported 34 miRNAs associated with FLD. Among these, miR-122, miR-34a, miR-192, miR-21 and miR-99a were associated with FLD in two or more independent studies[108].

Specifically, circulating miR122 and miR192 not only reflected both histological and molecular processes occurring in the liver, but have been also considered to be able to differentiate simple steatosis from NASH[121].

A cross-sectional validation study disclosed that 15 specific circulating miRNAs were significantly deregulated in prepubertal obesity, including the decreased miR-221 and miR-28 -3p, and increased concentrations in plasma of miR-486 -5p, miR-486 -3p, miR-142-3p, miR-130b, and miR-423-5p[122].

Can *et al*[123] showed a significant association between circulating miR-370, miR-33, miR-378, miR-27, miR-335, miR-143 and miR-758 values, and childhood obesity. Low levels of miR-335, miR-143 and miR-758, and high levels of miR-27, miR-378, miR-33 and miR-370 may have been responsible for elevated triglycerides and LDL-C levels, and low level of HDL-C in obese subjects.

An interesting work of Cui *et al*[124] highlighted the specific role of three miRNAs, miR-486, miR-146b and miR-15b, by demonstrating their increased circulating expression in obese children and adult patients with type 2 diabetes mellitus (T2DM). In particular, miR-486 was implicated in accelerating preadipocyte proliferation and myotube glucose intolerance, miR-146b and miR-15b were engaged in the suppression of high concentration glucose-induced pancreatic insulin secretion, and they all contributed to the pathological processes of obesity and T2DM.

Iacomino *et al*[125] in a pilot study (FAMILY Study) conducted in 149 overweight/obese and 159 normal weight children and adolescent demonstrated a panel of miRNAs differentially expressed in these two groups (miR551a and miR-501-5p resulted upregulated; miR-10b-5p, miR-191-3p, miR-215-5p, and miR-874-3p resulted downregulated).

In a transcriptomic study of Sheldon *et al*[126] was proposed a new candidate marker for distinguishing steatosis from NASH, the soluble factor FCER2, produced from NOCTH2 activation in B cells, whose expression was increased in NASH patients.

Finally, in a recent study interleukin-32 was found as the most significantly upregulated transcript in advanced NAFLD and NASH, being linked to lipid accumulation and disease severity[127].

Although many studies have been investigating the role of miRNAs in the pathogenesis of NAFLD in view of their potential use as non-invasive biomarkers, results are still controversial and scarce. However, the innovative role of Transcriptomics in non-invasive diagnosis of NAFLD contributes to the new "omics" path of NAFLD.

Proteomics

So far, few studies on proteomic analysis in NAFLD have been performed, probably due to technical limitation in the correct detection and identification of proteins and to the changeling quantification of blood proteins[128].

Among these proteins, the caspase-generated cytokeratin-18 fragments (CK-18) have been proposed as a noninvasive alternative biomarker of NASH. CK-18 showed a relatively good specificity for NAFLD, NASH and fibrosis but limited overall sensitivity[129].

Another protein being studied is represented by the soluble intercellular adhesion molecule-1, with promising results also in NASH detection[130].

The mitochondrial enzyme carbamoyl-phosphate synthase 1 and the heat shock protein family A member 5 have been indicated as potential tool to stratify the different phenotypes associated with the liver disease severity[131-133].

In a recent study of Malecki *et al*[134], a proteome analysis in a group of 30 children (of which 16 with previous NAFLD diagnosis by ultrasound) identified a total of 297 proteins. Thirty-seven distinct proteins (responsible for inflammation, stress response, and regulation of these processes) were identified. Up-regulated proteins included afamin, retinol-binding protein-4, complement components, and hemopexin, while serum protease inhibitors, clusterin, immunoglobulin chains, and vitamin D binding protein were found in the down-regulated group[134].

Bălănescu *et al*[135] confirmed the role of the heat shock protein-90 (Hsp90) isoforms as biomarkers for NAFLD in obese and overweight children. While the Hsp90β isoform was higher, the Hsp90α isoform was lower in overweight and obese NAFLD patients.

Hence, proteomics represents one of the most challenging field that might contribute to the development of new noninvasive targeted tools for NAFLD diagnosis and treatment.

Glycomics

Most of the glycomics studies in NAFLD have tried to identify glycans or glycoproteins that can serve as blood biomarkers for differentiating between NAFLD and NASH or for detection of the presence of liver fibrosis and its stage.

Changes in glycosylation represent a potential good marker of liver damage because of the hepatic production of several serum glycoproteins[136].

Findings of these studies demonstrated that higher concentrations of fucosylated, sialylated and agalactosylated glycans were observed in NAFLD and its progressive forms. Circulating sialic acid levels were also positively associated with metabolic syndrome and with NAFLD[128].

More, changes in fucosylation were observed in others inflammatory conditions, such as in chronic pancreatitis, Crohn's disease, rheumatoid arthritis and sickle cell disease[137].

Finally, hypogalactosylation (especially of IgG) was also associated with some autoimmune diseases and inflammatory pathways[138].

The first glycomic analysis in a paediatric NAFLD population was conducted by Blomme et al. In agreement with adult findings, B cells were found to play a dominant role in the N-glycan alterations of paediatric NASH patients. Serum protein N-glycosylation patterns of 51 paediatric NAFLD patients were assessed with deoxyribonucleic acid sequencer-assisted fluorophore-assisted capillary electrophoresis and compared with histology. Analysis of the N-glycans on immunoglobulin G confirmed the undergalactosylation status typical for chronic inflammatory conditions[136].

Metabolomics and lipidomics

To date, both metabolomics and lipidomics represent the most investigated omics branches in NAFLD with promising results for new targeted strategies development (Figure 3). Of interest, robust and extensive changes were observed both in the hepatic as well as in the circulating lipidome, which have led to the development of numerous diagnostic models for NAFLD as well as to the identification of novel therapeutic

targets. Many studies have reported several diagnostic models based on metabolomics, lipidomics alone or combined with other biochemical and clinical parameters for the diagnosis and staging of NAFLD.

Lipidomic studies have described specific changes in hepatic lipidome in patients with NAFLD. The hepatic concentrations of triacylglycerols, saturated fatty acids (SFAs and specifically of palmitate acid, C16:0 and stearate acid, C18:0), free cholesterol, sphingolipids, glycerophospholipids and eicosanoids increase, whereas ω-3 polyunsaturated fatty acids (PUFAs) and specialized proresolving mediators of PUFAs decrease. Monounsaturated fatty acids, lysophosphatidylcholine (LPC) and ceramide are also increased[21].

SFAs accumulation is associated with liver disease severity. They work in two different ways: on the hepatocytes stimulating proinflammatory cytokine secretion, enhancing ossidative stress, inducing apoptosis and on nonparenchymal liver cells stimulating secretion of proinflammatory and profibrotic cytokines (Kupffer cells) and induce proinflammatory M1 polarization of macrophages. Finally, SFAs stimulate the secretion of chemokines from hepatic stellate cells that recruit more macrophages in the liver[128].

LPC also stimulate ER stress, cause mitochondrial dysfunction and increase apoptosis[139]. The increased activity of the enzyme phospholipase A2 that catalyzes the formation of LPC from PC, leads to the rapid depletion of PC which affects hepatocyte membrane integrity and results in hepatocyte apoptosis, high release of lipotoxic lipids and increased inflammation. Additionally, PC deficiency reduces VLDL secretion resulting in higher intrahepatic lipid degradation and formation of toxic intermediates[140].

Ceramides correlate positively with hepatic disease severity[141]. These lipids have been found to decrease insulin sensitivity in skeletal muscle and hepatocytes[142] and to be involved in increased oxidative stress, mitochondrial dysfunction, and cell apoptosis[142,143]. Finally, ceramide stimulates fibrogenesis and angiogenesis by

increasing extracellular matrix deposition and secretion of pro-angiogenic factors by hepatic stellate cells[144].

Given that, this attractive omics field might greatly contribute to improve not only the knowledge of NAFLD pathophysiology but also its management.

CONCLUSION

Given the global relentless spread of childhood obesity, NAFLD and its cardiometabolic burden (including MetS, IR, cardiovascular disease, prediabetes, and type 2 diabetes) represent a major health challenge for clinicians already in childhood[145]. More, the close relationship of NAFLD with metabolic milieu has been recently highlighted in the new definition of NAFLD as metabolic associated fatty liver disease[146,147].

To date, diet and lifestyle interventions remain the cornerstone of NAFLD treatment. Over the last years, promising approaches have been proposed, but larger validation studies are required. In particular, omics represents the most intriguing strategy in this field, because of its potential effectiveness in preventing NAFLD as noninvasive diagnostic and therapeutic tool.

Further novel therapeutic insights for this insidious disease might be provided only by advances in the knowledge of NAFLD pathophysiology.

69462_Auto_EditedC.docx

ORIGINALITY REPORT

26%
SIMILARITY INDEX

PRIMARY SOURCES

1	Nikolaos Perakakis, Konstantinos Stefanakis, Christos S. Mantzoros. "The role of omics in the pathophysiology, diagnosis and treatment of non-alcoholic fatty liver disease", Metabolism, 2020 Crossref	330 words — 5%
2	www.wjgnet.com Internet	275 words — 4%
3	www.ncbi.nlm.nih.gov Internet	258 words — 4%
4	www.science.gov Internet	190 words — 3%
5	Anna Di Sessa, Giuseppina Rosaria Umano, Grazia Cirillo, Pierluigi Marzuillo et al. "The rs72613567:TA Variant in the Hydroxysteroid 17-beta Dehydrogenase 13 Gene Reduces Liver Damage in Obese Children", Journal of Pediatric Gastroenterology & Nutrition, 2020 Crossref	122 words — 2%
6	Wen Su, Zhuo Mao, Yiao Liu, Xiaoyan Zhang, Weizhen Zhang, Jan-Ake Gustafsson, Youfei Guan. "Role of HSD17B13 in the liver physiology and pathophysiology", Molecular and Cellular Endocrinology, 2018 Crossref	79 words — 1%

7	Björn Carlsson, Daniel Lindén, Gabriella Brolén, Mathias Liljeblad, Mikael Bjursell, Stefano Romeo, Rohit Loomba. "Review article: the emerging role of genetics in precision medicine for patients with non-alcoholic steatohepatitis", Alimentary Pharmacology & Therapeutics, 2020 Crossref	76 words — 1%
8	onlinelibrary.wiley.com Internet	69 words — 1%
9	Carlos J Pirola, Silvia Sookoian. "Multiomics biomarkers for the prediction of nonalcoholic fatty liver disease severity", World Journal of Gastroenterology, 2018 Crossref	66 words — 1%
10	www.mdpi.com Internet	66 words — 1%
11	biblio.ugent.be Internet	64 words — 1%
12	repub.eur.nl Internet	53 words — 1%
13	www.tandfonline.com Internet	50 words — 1%

EXCLUDE QUOTES OFF
EXCLUDE BIBLIOGRAPHY OFF
EXCLUDE MATCHES < 48 WORDS