

A 360-degree overview of paediatric NAFLD: Recent insights

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Summary

Non-alcoholic fatty liver disease (NAFLD) is a multi-faceted disorder, which ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) with/without fibrosis. The effects of specific risk factors, such as obesity and sedentary lifestyle, on predisposing genetic settings eventually lead to the development of NAFLD in children. The complex interplay between genes and environment in NAFLD pathogenesis is sustained by multiple mechanisms that involve liver crosstalk with other organs and tissues, especially gut and adipose tissue. Unfortunately, natural history of paediatric NAFLD is lacking, and the etiopathogenesis is still in the process of being defined. Potential early predictors and suitable non-invasive diagnostic tools can be discovered based on the pathogenetic mechanisms and histological patterns. This will also help design novel treatments and a comprehensive and successful management strategy for patients.

In this review, we discuss the recent advances made in genetics, etiopathogenesis, diagnosis, and therapeutic management of NAFLD, focusing especially on the obesity-related steatotic liver condition.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major health problem that integrates several liver conditions ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH), which is sometimes associated with fibrosis that may evolve into cirrhosis and result into hepatocellular carcinoma [1,2]. Furthermore, as underlined in a recent meta-analysis, NAFLD is also associated with increased overall morbidity and mortality for cardiovascular disease [3–5].

Key Points

- Analysis of genetic background may help paediatrics identify children susceptible to NAFLD
- Novel findings about pathogenetic mechanisms leading to paediatric NAFLD may improve therapeutic performance
- Adequate diagnostic and therapeutic management is crucial to prevent and counteract progression of paediatric NAFLD

As the prevalence of obesity in children increases, so does the prevalence of paediatric NAFLD [4]. This rise is worrisome because of its close association with the development of metabolic syndrome (MetS) [6–8].

Although etiopathogenesis of paediatric NAFLD remains unknown, it is conceivable that, such as in adults, a network of interactions among multiple factors is involved in both the development and progression of the disease [9,10]. However, as obesity-related NAFLD in children is rarely influenced by secondary causes (e.g., severe weight loss, drug, and alcohol consumption), it may be an excellent model to elucidate the factual origins of primary NAFLD, including the contribution of susceptibility genes and environment [11,12].

NAFLD is suspected mainly by the analysis of anthropometrical and biochemical parameters and/or ultrasound liver brightness [13]. Histological analysis of liver biopsy remains the masterful method to differentiate simple steatosis from NASH and to perform staging and grading of the disease in children [14]. However, recently, advances have been made in the field of non-invasive evaluation of paediatric NAFLD [15].

There are no current specific therapeutic indications for the treatment of paediatric NAFLD. However, as overweight/obesity is often coupled with NAFLD, weight loss by diet and exercise programs is widely accepted as the first line of intervention in children [16]. Poor adherence to lifestyle modifications often fails to halt or revert the occurrence of liver damage during pathogenesis of paediatric NASH. Therefore, at this time, developing possible multi-targeted therapies to halt disease progression, restoring

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liver cell homeostasis and repairing the damage are all fundamental objectives in the treatment. In fact, during the last 5 years, a series of fairly novel pharmacological treatments have been tested, and a wide range of newer drugs are now being developed [17].

Here we provide an extensive overview of NAFLD in children while discussing the most up-to-date literature in the field. Particularly, we focus on the many interesting breakthroughs in genetics, etiopathogenesis, diagnosis, and therapeutic management of paediatric obesity-related liver disease.

Epidemiology

The epidemics of overweight and obesity in paediatric population has reached worldwide proportion over the last two decades [4,13]. As a consequence, NAFLD has now become the most common cause of chronic liver disease in children and adolescents in the USA [18], and most probably, in the rest of the industrialized countries. The true prevalence of NAFLD, however, remains largely elusive. In fact, extrapolation of correct epidemiological data is hampered by the large variability of available non-invasive and invasive diagnostic tests. None of these tests are exempt from criticisms in terms of misdiagnosis and/or invasiveness and costs.

Serum alanine aminotransferase (ALT) activity is a widely accessible and cheap test for the screening and initial evaluation of NAFLD. The sensitivity of this biochemical marker, however, remains low because some adult and paediatric patients with biopsy-proven NAFLD may present ALT values in the normal range [19]. In an Italian study, 72 obese children presented with ultrasonographic fatty liver (50% of cases) and/or hypertransaminasemia (25% of cases) [20].

To add to the difficulties, histology – the gold standard for the diagnosis of NASH – may be subject to the higher probability of staging sampling error [14]. Furthermore, epidemiological data of a paediatric population may be influenced by a series of crossed risk factors such as peri-pubertal age, male gender (for NAFLD, but not for NASH), hispanic ethnicity, non-black race and individual genetic predisposition. NAFLD and/or its progression to severe disease have, in fact, been linked to several inherited variants that will be discussed extensively in the next paragraph on genetic risk factors [21]. Familial clustering of obesity, insulin resistance (IR), NAFLD or type 2 diabetes are frequent and should raise suspicion of NAFLD in children from such families [22,23].

Obesity and MetS are the major risk factors for paediatric NAFLD. NAFLD prevalence is higher in overweight (gender and age specific BMI >85th percentile) or obese (>95th percentile) children as compared with normal weight age matched pairs. Today, it seems clear that waist circumference (i.e., abdominal fat or central obesity) plays a pivotal role and correlates with NAFLD diagnosis more than BMI alone [24].

Population-based studies conducted with ALT or ultrasonography in several countries have shown that paediatric NAFLD prevalence remains in a range of 2.6–7.1% of children [10]. An autopsic study in California, conducted in children deceased for accidents, showed a prevalence of histological NAFLD ranging from 0.7% in 2–4 year old to 17.3% in 15–19 year old subjects [25]. In cohorts of children of various nationalities selected for overweight or obesity, the prevalence of elevated ALT is higher and ranges from 8 to 42%, whereas the prevalence of bright liver

ranges from 1.7 to 77% [26]. In an autopsic study by Schwimmer *et al.*, prevalence of histological NAFLD in obese individuals was 38% [25].

Genetic risk factors

In recent years, an exciting step towards the understanding of the pathogenesis and risk factors in the development and progression of paediatric NAFLD has been taken as a result of genetic studies. It is indeed well known that NAFLD has a major genetic component [23]. Because of the lower numbers of confounding factors (e.g., the duration of disease, presence of obesity, lifestyle habits, co-morbidities, and drugs) and the more important role of inherited factors in early-onset disease, this is especially true for children [27].

The major advance has come from the finding, first obtained by genome-wide association studies (GWAS) conducted in the general adult population [28,29], that genetic variants of the patatin-like phospholipase domain-containing protein-3 (PNPLA3) and, in particular, the common rs738409 C>G single nucleotide polymorphism (SNP) encoding the I148M variant, are not only associated with hepatic fat content and increased serum liver enzymes but also increase the risk of NASH and fibrosis progression [30–32]. The I148M PNPLA3 variant influenced liver triglyceride content without apparently affecting body mass, serum lipid levels and systemic IR [31,33]. PNPLA3 is regulated by the lipogenic program [34] and is highly expressed in the liver and adipose tissue at the level of the endoplasmic reticulum and the surface of lipid droplets, where it seems to be involved in the metabolism of triglycerides [35]. Although the mechanism and physiological substrates remain unclear, the common I148M variant disrupts the activity of the enzyme, thereby altering lipid catabolism, and it might also acquire new and still unknown functions [35]. Indeed, the association of I148M variant with progressive liver disease and hepatocellular carcinoma is independent of the predisposition to increased steatosis, thus suggesting that it influences the regulation of pro-inflammatory lipid mediators [27,30,32].

The association between I148M variant and both liver enzymes and steatosis was soon confirmed in obese children of different ethnicities [36–39] and in one family study in Italian trios [30], indicating that it exerts its effect early in life and that the magnitude of the association between I148M PNPLA3 variant and serum levels of liver enzymes was related to the size of abdominal fat [40] and to the high dietary carbohydrate and sugar consumption [41]. In addition, an interaction was reported between dietary fatty acids composition, PNPLA3 genotype and hepatic fat accumulation. Indeed, the omega-6/omega-3 polyunsaturated fatty acids ratio was correlated with hepatic fat fraction and ALT levels only in children homozygous for I48M PNPLA3 variant and at risk of steatosis [42].

Furthermore, PNPLA3 genotype influenced the histological severity of NASH alterations and fibrosis in obese paediatric patients who underwent biopsy because of persistently altered liver enzymes. Interestingly, the association with fibrosis was stronger in children than in adults, in that each I48M allele increased the risk of fibrosis by almost twofold [27].

A more recent GWAS, conducted in a larger population, was able to identify a wider set of genetic variants influencing steatosis besides I148M of PNPLA3 [43]. Of these variants, rs2854116

Review

SNP of glucokinase regulator (GCKR), involved in the regulation of uptake of monosaccharides and lipogenesis, was confirmed to predispose to fatty liver and dyslipidemia in obese children and adolescents independent of PNPLA3 [44], although the effect on histological progression of liver disease remains unknown, especially in view of the ameliorating effect on IR.

Besides, additional SNPs of genes implicated in NASH pathogenesis have been shown to influence liver damage and fibrosis progression in candidate gene case-control studies including paediatric patients. These include genetic variants regulating insulin receptor activity, namely the ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (*ENPP1*) Lys121Gln and the insulin receptor substrate-1 (*IRS-1*) Gly972Arg functional SNPs [45], thus underscoring the causal role of IR in the progression of liver damage in NAFLD. The manganese superoxide dismutase (*SOD2*) C47T rs4880 SNP, regulating *SOD2* mitochondrial import and antioxidant activity [46], and the Kruppel-like factor 6 (*KLF6*) IVS1-27G>A SNP, regulating alternative splicing isoforms of the transcription factor *KLF6*, involved in the regulation of metabolism in hepatocytes and fibrogenesis in hepatic stellate cells, seem to be involved as well [47]. In contrast, variants in the hemochromatosis gene (*HFE*), regulating iron metabolism and in the apolipoprotein-C3 (*APOC3*), regulating very low density lipoprotein metabolism, were not confirmed to influence susceptibility steatosis and NASH [48,49].

Finally, there is a growing awareness that the expression of some genetic variants may be age-dependent, i.e., the phenotype may be more (or less) marked or involve different traits during the developmental age. For example, a common variant (rs13412852) influencing the expression of lipin-1 (*LPIN1*), another lipid phosphatase involved in adipogenesis and regulating the flux of free fatty acids between the adipose tissue and the liver, whose expression is deregulated during steatosis [50], was associated with lipid levels, NASH severity, and hepatic fibrosis in children with NAFLD, whereas it influenced body mass in adults of the same ethnicity [51]. In Table 1, we have summarized genetic polymorphisms influencing the susceptibility to paediatric NASH.

Metabolic risk factors and organ crosstalk for NAFLD development

It is widely accepted that genetic susceptibility, epigenetic mechanisms, physical inactivity, and excess caloric intake by diet influence visceral fat accumulation inducing an obese and metabolically dysfunctional phenotype [52]. This pattern, characterized by abdominal obesity, IR, impaired glucose tolerance, dyslipidemia, and hypertension, defines a subject with MetS. NAFLD is generally associated with at least one of these MetS features in adults, and emerging evidence confirms the existence of a similar relationship in children as well [52,53,12]. An Italian study demonstrated that 65.8% of 120 children (age range: 3–18 years) with biopsy-proven NAFLD presented with MetS, which was also associated with fibrosis grade [54]. Approximately 26% of 254 children adolescents (age range: 6–17 years) enrolled in the Non-alcoholic Steatohepatitis Clinical Research Network (NASH CRN) met specified criteria for MetS diagnosis [55]. These data, as well as other contributions [6–8], highlight the potential role of NAFLD as co-factor of other obesity-related co-morbidities in a paediatric population.

In the last decade, the concept that increased consumption of obesogenic food may have a role in the pathogenesis of NAFLD has spread. In fact, apart from the impact of the genetic background, hypercaloric diets (particularly those enriched in fat and fructose/sucrose) may act by favouring the occurrence of systemic IR and, in turn, a dangerous hepatic free fatty acid (FFA) accumulation or causing visceral fat deposition and consequent hepatic IR, accountable for development of fatty liver [56,57]. According to the 'multiple hits' hypothesis, IR and FFA accumulation may predispose the fatty liver to secondary hits, including the imbalance of production/release of hormones derived from adipose tissue (adipocytokines), oxidative stress, activation of specific nuclear receptors and fibrogenesis [58,59]. In order to counteract the IR, the number of pancreatic β cells increases, resulting in a compensatory hypersecretion of insulin. This overflow of circulating insulin accelerates liver fat storage leading to NAFLD.

Table 1. Genetic variants influencing the susceptibility to NAFLD and NASH during the developmental age.

Gene	Function	SNP	Protein variant	Activity	MAF	Effect on steatosis	Effect on fibrosing NASH
<i>PNPLA3</i> , patatin-like phospholipase domain containing 3	Lipid remodelling, lipogenesis	rs738409 C>G	Ile148Met	Increased lipogenesis ?	0.19	↑↑	↑↑
<i>GCKR</i> , glucokinase regulatory protein	Glucose uptake, lipogenesis	rs780094 C>T	Pro446Leu	Loss of regulatory function	0.45	↑	?
<i>SOD2</i> , superoxide dismutase 2, mitochondrial	Anti-oxidant response	rs4880 C>T	Ala16Val	Loss of mitochondrial import	0.49	none	↑
<i>ENPP1</i> , ectonucleotide pyrophosphatase/phosphodiesterase 1	Insulin signalling	rs1044498 A>C	Lys121Gln	Loss of inhibitory function	0.29	none	↑
<i>IRS1</i> , insulin receptor substrate 1	Insulin signalling	rs1801278 G>C	Gly972Arg	Loss of function	0.05	none	↑
<i>KLF6</i> , Kruppel-like factor 6	Fibrogenesis, glucose metabolism and lipogenesis	rs3750861 G>A	IVS1-27G>A	Altered splicing	0.06	?	↑
<i>LPIN1</i> , lipin-1	Lipogenesis, adipogenesis	rs13412852 C>T	Non coding SNP	Unknown	0.35	↑	↑

SNP, single nucleotide polymorphism; MAF, minor allele frequency, in European healthy subjects.

The number of arrows is related to the strength of both the genetic association and the available evidence.

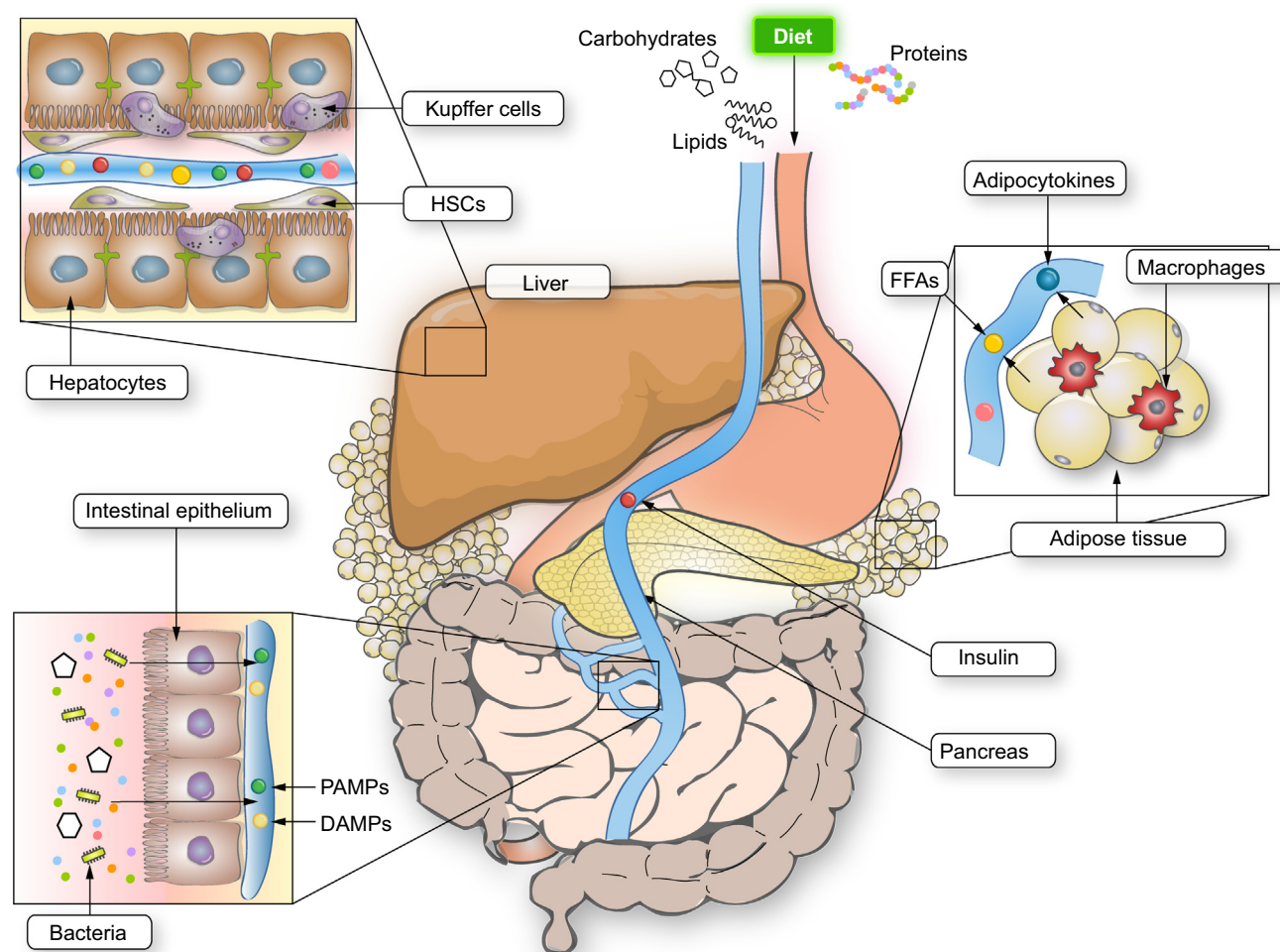


Fig. 1. Schematic representation of the crosstalk among different organs during NAFLD development.

In this already complex network of organ crosstalk, a crucial role of the gut has been recently considered. In fact, it has been suggested that the diet- and/or gut-microbiota-dependent increase of gut-derived products, and the consequent release of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), may cross a reduced gut permeability barrier, activate molecular mechanisms of innate immune response, and act as possible inducers of necroinflammatory lesions and severe fibrosis in the progression of simple fatty liver to NASH [60,61]. Interestingly, the lipopolysaccharide (LPS), one of most studied PAMPs, in association with plasminogen activator inhibitor-1 (PAI-1), has a mild to moderate correlation with steatosis and NAS [62].

All these findings suggest that, although the pathogenesis of NAFLD remains the subject of intense debate, fatty liver development and eventually its progression to NASH are the result of a crosstalk among different organs, including adipose tissue, pancreas, gut, and liver (Fig. 1).

Cellular and molecular signatures in the pathogenesis of NASH

NASH represents a histological progressive condition and a major cause of what was previously considered as cryptogenic cirrhosis [63–65]. Progression of simple fatty liver towards a condition of

inflammation and fibrosis is characterized by the contribution of multiple mechanisms that differently regulate the physiological liver response, although why this occurs in some patients only, remains unknown [66–69].

The cellular basis of liver fibrosis in NASH

Fibrogenesis mainly occurs in extracellular matrix (ECM) remodelling, which is regulated by three different cell types: (1) endogenous (resident) fibroblast or myofibroblast-like cells, mainly represented by hepatic stellate cells (HSCs) and also by portal fibroblasts; (2) fibrocytes recruited from the bone marrow to the liver during the development of chronic hepatic injury; (3) the potential engraftment and differentiation of bone marrow-derived mesenchymal stem cells into myofibroblast-like cells [70]. In addition to the previous points, another source of collagen-producing cells might be derived from the process of ductular reaction and the so-called epithelial-mesenchymal transition (EMT), via which epithelial cells can transdifferentiate into mesenchymal cells to acquire the ability of producing collagen [70,71]. Although HSCs are considered the main ECM-producing cells during the development of NASH, detailed studies to dissect the real contribution of each fibrogenic phenotype are lacking. Considering this, the possibility that the elements of the ductular

Review

reaction might undergo EMT has been recently questioned [71]. However, the elements of the ductular reaction represent the hepatic progenitor cell (HPC) compartment of the liver, and it has been recently shown that the HPC compartment, especially in children with NASH, was expanded and independently associated with the degree of fibrosis [72].

Role of adipocytokines

Key players in the progression from simple steatosis to NASH are the so-called adipocytokines including adiponectin, leptin, resistin, tumour necrosis factor (TNF) α and interleukins (ILs) secreted by adipocytes or the inflammatory cells that infiltrate the adipose tissue in insulin-resistant conditions.

Leptin downregulates insulin signalling in the liver and reduces fibrosis in experimental models of fibrosis and NASH. Leptin may activate HSC through the NADPH oxydase system [73–77]. In contrast, it has been clearly demonstrated in a previous study that adiponectin can counteract the IR effect of leptin, exert anti-inflammatory actions and inhibit HSC proliferation, thus interfering with fibrogenesis [78].

Cytokines, particularly TNF α , IL-6, and IL-1, are involved in the recruitment and activation of Kupffer cells and transformation of HSC into myofibroblasts. Levels of TNF α and IL-6 are often elevated in the liver and blood of patients with NASH, and inhibition of these cytokines has been shown to improve NAFLD in rodents [79,80].

Role of oxidative stress and lipotoxicity

In a normal liver, antioxidant systems such as superoxide dismutase and catalase efficiently remove reactive oxygen species (ROS) produced during cellular metabolism and maintain normal cell homeostasis [81]. Oxidative stress is increased in patients with NASH [82]. Excess of non-esterified fatty acids (NEFA) supply to the liver increases mitochondrial and peroxisomal oxidation, promote microsomal induction of CYP4A1 and CYP2E1, and cause elevated production of ROS [83]. Among ROS producing systems, the NADPH oxidase (NADPH) complex plays a major role in hepatic fibrogenesis and collagen production. This system also plays a crucial role in ROS production in both Kupffer cells and HSCs during hepatic fibrogenesis [84,85]. Kupffer cells in the liver mainly produce ROS through the phagocytic form of NADPH, which plays an important role in host defence and inflammation [86,87]. HSCs express the non-phagocytic form that plays an important role in regulating cell signalling and can be stimulated by fibrogenic molecules such as leptin [88,89].

The pathogenesis of NASH also takes into consideration the concept of lipotoxicity. The most recent hypothesis for this is that excessive intra-hepatic lipid accumulation triggers a local necro-inflammatory response, subsequently producing free radicals that can result in damage to the cell membrane and DNA [90–92]. Thus, the cytotoxic products of lipid peroxidation induce apoptosis signals in hepatocytes and play a role in liver fibrogenesis by modulating HSCs behaviour in a paracrine manner [93,94].

Role of nuclear receptors in the pathogenesis of NASH

Nuclear receptors (NRs) are ligand-activated transcription factors that regulate the expression of specific genes controlling a broad range of cellular and metabolic functions [95,96].

Among NRs, the peroxisome proliferator-activated receptors (PPARs) have been studied the most because of the presence of pharmacological ligands [96]. PPARs are involved in the activation of HSCs [97,98]. In addition to PPARs, other NRs, such as the farnesoid X receptor (FXR), xenobiotic sensors (CAR and PXR), liver X receptor (LXR), and hepatocyte nuclear factor 4 (HNF4), have also been implicated in the pathogenesis of NAFLD, but their role in the development of NASH remains to be established [99].

Diagnostic tools

Considering the lack of specific symptoms and/or signs of NAFLD, early detection of NAFLD in children may be effective to identify subjects with potential silent progressive fatty liver [5]. In addition, the screening for NAFLD should be recommended to overweight and obese children [100–102].

In the first assessment of children with suspicion of fatty liver, clinicians should take an accurate clinical history with particular attention to information regarding the nutritional habits and possible drug consumption.

Routine laboratory tests

ALT elevation is common in boys and girls with fatty liver [103,104]. In epidemiological studies, elevated ALT seems to be associated with MetS in children and adolescents [105,106]. In a tertiary centre setting, NAFLD associated hypertransaminasemia was responsible for about 20% of all cases [107].

Recently, the American Academy of Paediatrics recommended screening with liver function tests (ALT, AST) in children aged 10 years in case of BMI \geq 95th percentile or between 85th and 94th percentile with risk factors. The biochemistry test panel should also include lipid profiling and fasting glucose determination [14,108].

Some considerations concerning the significance of liver enzymes are mandatory. Serum aminotransferases that are influenced by dietary habits and hyperalimentation, may reflect, in some case the presence of steatosis [109]. However, as the serum levels of aminotransferases may be reduced even in the presence of NASH and fibrosis, liver function test cannot represent the severity of NAFLD [110]. Another major issue with aminotransferases lies in the variability among centres and the need of 'biology-based thresholds' to increase sensitivity, as recently underlined in the SAFETY study [111]. In this study, the thresholds of 25.8 U/L in boys and 22.1 U/L in girls have been proposed as a reliable index for suspicion of a chronic liver disease. Obese children who become hepatopathic should definitely be tested for causes of liver diseases other than NAFLD, particularly for those conditions that are rapidly progressive if not adequately treated (e.g., autoimmune hepatitis and Wilson disease). The so-called NASH trash bin should also be carefully taken into consideration, especially in early-onset NAFLD among young children [14].

US and radiology

Liver ultrasound (US) can detect fatty liver when steatosis involves $>30\%$ of hepatocytes [112]. US has several advantages for use as a screening tool: relative low cost, large diffusion in

medical community and feasibility. Recently, a large prospective paediatric cohort showed a good correlation between ultrasonographic steatosis score and the severity of steatosis on liver biopsy [113]. Moreover, US has been used to assess the outcome of efficacy in paediatric trials with good compliance between the outcomes of children and parents [114].

Computed tomography (CT) scan is not recommendable in paediatric setting because of the unjustified radiation exposure involved in the process. In contrast, magnetic resonance imaging (MRI) has been demonstrated to have good sensitivity in quantification of fat in the liver [115,116].

Progression indexes

The main challenge for paediatric hepatologists is the identification of children with higher probability of progression to a more severe form of liver disease.

Ultrasound-guided or assisted needle liver biopsy remains the gold standard method for defining diagnosis, severity, and rate of progression of NASH to its clinical sequelae. Because it is an invasive tool for monitoring therapeutic responses and disease evolution, research on clinical parameters or serum markers that can identify subjects with steatohepatitis and who are prone to further progression has been undertaken.

Several studies have aimed at identifying clinical parameters that can predict the progression of a liver disease. The presence of necro-inflammation at liver biopsy is associated with a possible rapid progression of fibrosis [54]. Results of clinical studies have indicated BMI, lipid profile, IR, and fasting glucose as predictors of NAFLD in children [14]. Moreover, waist circumference alone seems to be correlated with liver fibrosis in the paediatric setting [117]. In conclusion, simple clinical parameters such as age (expression of length of disease), IR surrogate clinical marker acanthosis nigricans, anthropometrical data (BMI, waist), lipid, and glucose profile can allow a clinician to recognise children with potential severe NAFLD. The Paediatric NAFLD Fibrosis Index (PNFI) algorithm was developed on the basis of waist circumference, triglycerides, and age [118].

The need of a simple and reliable tool for the assessment and monitoring of liver fibrosis has boosted the research of 'non-invasive' markers. Unfortunately, limited sample size, lack of adequate information regarding the control groups and quality of liver specimen, and lack of validation in large and independent cohorts have together reduced the application of this tool in clinical practice [119].

Several molecules are involved in ECM remodelling during fibrogenesis: the matrix metalloproteinases (MMPs) that participate in ECM degradation; their specific tissue inhibitors, the tissue inhibitor of metalloproteinases-1 (TIMPs), and various cytokines that stimulate HSC conversion into myofibroblast-like cells that synthesize hyaluronic acid (HA), which has been found as a reliable marker of fibrosis deposition in children [120].

However, a recent paper suggests that the more reliable serum marker of steatohepatitis is cytokeratin-18 (CK18) fragment levels, which are increased in the bloodstream according to the presence and severity of NASH [121].

Recently, the enhanced liver fibrosis (ELF) panel of serum markers (tissue inhibitor of matrix metalloproteinase 1, HA, and aminoterminal peptide of pro-collagen III) was tested in a paediatric cohort to show the capability of prediction of fibrosis [122].

In a recent study, the combination of ELF and PNFI showed good accuracy in predicting fibrosis in children with histologically confirmed NAFLD [123].

Transient elastography appears to be a more promising tool for non-invasive assessment of fibrosis, and its role in paediatric NAFLD has been recently reviewed elsewhere, unfortunately the lack of capability to discriminate between intermediate degrees of fibrosis represents the major limitation to avoid liver biopsy for the diagnosis of fibrosis in NASH [124].

Treatments

The aim of this section is to evaluate established and other possible novel NAFLD treatment options in the paediatric age group by providing recent evidence from the literature. Fig. 2 illustrates a synoptic summary of most pathogenetic mechanism-based treatments.

Diet and lifestyle changes

The goal of lifestyle interventions is a gradual and controlled weight loss achieved by diet and physical exercise. Unfortunately, this aim is difficult to achieve and the results are disappointing, with an extremely low percentage of individuals who are able to steadily lose weight and exercise regularly [16]. Weight loss in NAFLD patients on diet improves hepatic insulin sensitivity by reducing hepatic FFA supply, improves extra-hepatic insulin sensitivity through better glucose utilization and reduces ROS generation and adipose tissue inflammation. Exercise and outdoor activities are also promoted because they may improve substrate utilization in muscles and contribute to obtaining better insulin sensitivity, irrespective of weight loss [125].

Weight loss (approximately 5–10% of the basal weight) should be gradual, since extreme slimming diets may lead to the onset of severe metabolic disorders and promote liver damage. Indeed, some studies in children confirmed an improvement in serum aminotransferase levels and several metabolic parameters including lipids, fasting glucose, insulin, and insulin sensitivity indices [126–128]. Interestingly, Nobili *et al.* showed that a repeated biopsy at 24 months displayed significant improvement of liver histology with reduction of the grade of steatosis, hepatic lobular inflammation, hepatocyte ballooning, and NAFLD activity score [129].

Currently, there are no precise evidence-based guidelines establishing the optimal dietary interventions. Reduction in sugar/sucrose and in soft drinks rich in fructose, most probably not only acts through a reduction in IR and lipogenesis but also counteracts the recently evidenced hepatic pro-inflammatory/fibrogenetic role of fructose [130].

Diet in childhood must be balanced to allow a healthy and harmonic growth, including wellness of bone structures. Reduced dietary intake of saturated/*trans* fat, increased intake of polyunsaturated fat (omega-3) [131], and increased fibres intake [132] have been proposed as other valuable measures. Diets rich in fibres, however, should be approached with caution because poor information is available on the possible side effects of fibres when used in large amounts in children. Unfortunately, lifestyle intervention has only a very moderate effect on weight loss, with <10% success rate 2 years after the onset of intervention. A reduction of >0.5 SDS-BMI (indicating stable weight over 1 year in

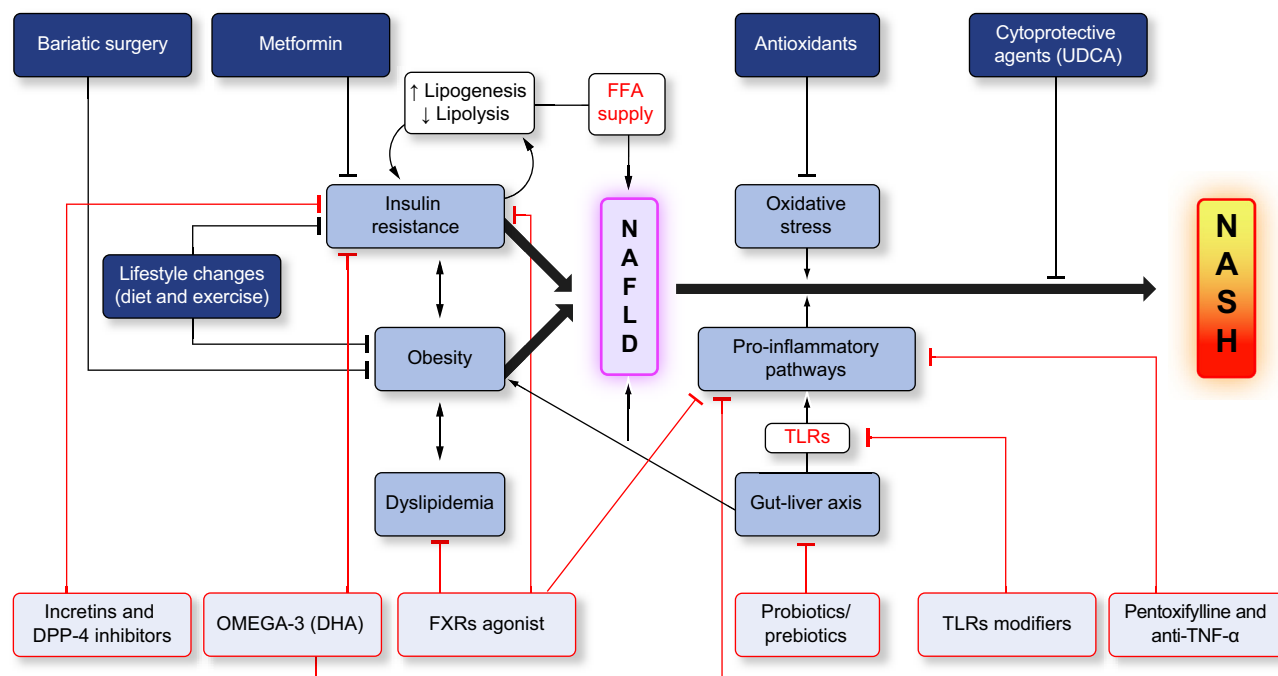


Fig. 2. Schematic representation of the pathogenetic mechanism-based NAFLD armamentary. Established and potential therapies targeting a given pathway are depicted in the upper and lower part of the cartoon, respectively.

growing children) is associated with an improvement of cardiovascular risk factors, while improvements in quality of life seem independent of the degree of weight loss. Younger children and less overweight children particularly profit from lifestyle interventions as compared with extremely obese adolescents [133].

Durable weight loss can be achieved with bariatric surgery, especially in adolescents; however, the guidelines for eligibility remain to be standardized. In adults, this therapeutic option improves liver damage [134]. However, there are very scarce data on the NAFLD adolescent population, and a comparison with untreated natural history data is required [135,136].

Pharmacological interventions for NAFLD

The pharmacological approach, in NAFLD children poorly adherent to or being unresponsive/partially responsive to lifestyle changes, is aimed at acting upon specific targets involved in etiopathogenesis (Fig. 2). The following drugs have been more extensively used and include antioxidants, insulin sensitizers, and cytoprotective agents.

Antioxidants, by reducing oxidative stress, protect susceptible components of biological membranes from lipid peroxidation, and may therefore be able to prevent the progression of simple steatosis to NASH. The most studied antioxidant in children with NAFLD is alpha-tocopherol (vitamin E). The first open-label paediatric trial with vitamin E (400–1,200 IU/day), involving 11 children with NAFLD, showed a decrease of serum aminotransferase levels not associated with a reduction in BMI values and bright liver at ultrasonography [137]. Other studies comparing the effect of vitamin E vs. that of single weight loss, did not find a higher efficacy of antioxidant treatment as compared with exclusive lifestyle changes [138,139]. Nobili *et al.* demonstrated that adding

ascorbic acid to vitamin E was still not better than lifestyle intervention alone [129].

Lavine *et al.* [140], in a more recent large, multicenter, randomised double-blind placebo-controlled trial (TONIC study), evaluated the effect of a 96-week antioxidant therapy with vitamin E (400 IU twice daily), insulin sensitizer metformin (500 mg twice daily) or placebo in children with NAFLD. A total of 173 patients (aged 8–17 years) with biopsy-confirmed NAFLD and persistently elevated levels of ALT, without diabetes or cirrhosis, were randomly assigned to 1 of 3 groups. At 96 weeks, vitamin E treatment was not better or worse than the placebo treatment in reducing ALT and was only able to improve histological hepatocellular ballooning in NAFLD and NASH. Other antioxidants, particularly betaine and silymarin, are possible promising therapeutic tools for NAFLD [141], but more data are required for the paediatric age.

For its pathogenetic role, IR is a rational therapeutic target. Metformin is the only insulin-sensitizing agent evaluated in children. A single-arm, open-label, pilot study on metformin (500 mg twice daily for 24 weeks), conducted in 10 non-diabetic children with biopsy-proven NASH and elevated ALT levels, showed reduction of hepatic steatosis, as evaluated with Magnetic Resonance Spectroscopy (MRS), and low serum ALT levels [142]. On the other hand, a subsequent study in the paediatric age by comparing metformin with lifestyle interventions did not confirm the advantages of using this drug [143], whereas another study [144] demonstrated a noteworthy improvement in fatty liver prevalence and severity in a selected cohort of 50 obese and insulin-resistant adolescents and in fasting insulin in metformin compared with placebo patients. Finally, the most recent TONIC study showed that metformin, like vitamin E, was no better or worse than placebo in reducing ALT and was able to improve histological hepatocellular ballooning only in NAFLD [140]. As compared

to placebo, both vitamin E and metformin significantly improved ballooning, but neither of them led to a significant improvement in other histological features.

There are still insufficient data about the possibility of use of the glitazones, drugs believed to have a role in the treatment of adult NAFLD [145], in NAFLD-affected children.

UDCA (ursodeoxycholic acid) is a hydrophilic bile acid that normally constitutes 3% of the human biliary pool [146]. UDCA might theoretically interfere with the progression of NAFLD/NASH through the following different pathways: (1) protecting hepatocytes from mitochondrial membrane injury mediated by bile salts, (2) playing an immunomodulatory function, and (3) activating anti-apoptotic signalling pathways. A pilot randomised controlled trial involving 31 NAFLD-affected children [147] showed that conventional dosage of UDCA is not effective on ALT levels and ultrasonographic bright liver improvement.

Data from NAFLD animal model studies suggests that gut microbiota manipulation with probiotics reduces liver inflammation and improves gut epithelial barrier function, thus representing a novel advantageous therapeutic approach in NAFLD patients [148–150].

In human NAFLD, Loguercio *et al.* evaluated the effects of chronic therapy with a probiotic (VSL#3) in patients affected by several types of chronic hepatopathies, including NAFLD. Results of the study suggested that probiotics warrant consideration as an additional beneficial therapy in some types of chronic liver disease, such as NAFLD [151]. In obese children with persisting hypertransaminasemia and ultrasonographic bright liver, a double-blind, placebo-controlled, short-term pilot study showed that after 8 weeks of treatment, patients receiving probiotics therapy vs. placebo attained a significant improvement of serum ALT and antipeptidoglycan-polysaccharide antibodies levels, a surrogate test for gut microbiota dysbiosis evaluation, independently of weight, waist, and abdominal fat changes [152]. Overall, these results, in association with their excellent tolerability, suggest the use of probiotics as a promising therapeutic tool in paediatric NAFLD.

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. This class includes many important compounds, such as essential fatty

acids, like omega-3 and omega-6 acids. The term 'essential fatty acid' refers to fatty acids that the body needs, but cannot produce. Essential fatty acids serve multiple functions; in each of them, the balance between dietary ω -3 and ω -6 strongly affects function. PUFAs can be found in several natural foods. PUFAs have a variety of health benefits when they are consumed in moderation and as a part of a diet high in fibre [153].

Recent pharmacological studies in NAFLD animal models and in human adults, focusing on the effect of oral treatment with omega-3 fatty acids, showed both anti-inflammatory and insulin-sensitizing properties, suggesting a role of this lipid in the treatment of NAFLD [153]. In NAFLD children, a recent double-blind RCT [131] investigated the effect of ω 3-docosahexaenoic acid (DHA) supplementation on the decrease of liver fat content, evaluated by ultrasonographic bright liver after 6 months of treatment. DHA taken orally for 6 months improves bright liver and insulin sensitivity, without significant differences between doses of 250 and 500 mg/day. Because ω 3 fatty acids are well tolerated by the paediatric population, therapy with DHA warrants consideration in the management of paediatric patient with NAFLD. Randomised placebo-controlled trials are therefore needed.

Promising novel therapeutic approaches

Here, we discuss other interesting approaches that have hitherto been explored only in NAFLD animal models or in adults (Table 2), and which will perhaps become the object of study in the paediatric population in future.

Incretin mimetics and DPP-4 inhibitors increase insulin secretion through different pharmacological mechanisms. Exenatide and liraglutide are glucagon-like peptide (GLP)-1 receptor agonists, resistant to DPP-4 degradation [141]. Sitagliptin is a selective DPP-4 inhibitor that enhances GLP-1 and glucose-dependent insulinotropic peptide (GIP) serum levels. An extra-pancreatic protective role of sitagliptin in diet-induced adipose tissue inflammation and hepatic steatosis has also been shown in diabetic mice [154].

FXRs are NRs seemingly involved in the NAFLD pathogenesis [155]. Bile acids act as endogenous ligands for these receptors, mediating a variety of functions as follows: (1) control of lipids homeostasis with a beneficial effect on dyslipidemia; (2) glucose metabolism regulation (mechanism is still unknown, but FXR ablation in mice led to glucose intolerance); (3) reduction of hepatic inflammation and fibrogenesis through different mechanisms. Therefore, FXR agonist might have a role in the pharmacological therapy of NAFLD/NASH. However, future studies are expected, especially in adults, due to their narrow therapeutic range or poor safety profile [156].

Cysteamine bitartrate is a potent antioxidant that readily traverses cellular membranes and can increase the levels of all adiponectin multimers [157]. In a recent pilot study, enteric-coated cysteamine was shown to be of potential benefit in NAFLD-affected children [158].

Some studies in NASH-affected adults showed a role of pentoxifylline, a phosphodiesterase inhibitor that exerts its immunomodulatory functions antagonizing the pathway of TNF- α , in reduction of serum ALT levels and in histological features such as steatosis, lobular inflammation, and fibrosis stage [159]. More research is needed, especially in paediatric patients, where the

Table 2. Novel potential NAFLD treatment targets still partially explored in adults and/or children.

Drug class	Target/mechanism of action	References
Probiotics	Gut-liver axis	Vajro <i>et al.</i> , 2011
Omega-3 DHA (docosahexaenoic acid)	Dyslipidemia/insulin resistance	Nobili <i>et al.</i> , 2011
Pentoxifylline and anti-TNF- α	TNF- α pathway	Li <i>et al.</i> , 2011
Incretins and dipeptidyl dipeptidase (DPP)-4 inhibitors	Insulin resistance	Nguyen <i>et al.</i> , 2012 Shirakawa <i>et al.</i> , 2011
Agonist of the farnesoid X receptor (FXR)	FXRs pathway	Fuchs <i>et al.</i> , 2012
Toll like receptors (TLRs) modifiers	Pro-inflammatory pathway in Kupffer cells and natural killer T cells	Miura <i>et al.</i> , 2010
Bariatric surgery	Obesity	Pardee <i>et al.</i> , 2009 Weiner <i>et al.</i> , 2010

Review

treatment seems interesting especially in view of the good tolerability reported in NASH-affected adults [160].

TLRs are a group of receptors that recognise PAMPs and DAMPs. TLR2, TLR4, and TLR9 seem to be involved in NAFLD pathogenesis [61,161]. TLRs stimulation results in activation of the transcriptional factor NF- κ B, crucial for the inflammatory response and implicated in the progression of NAFLD to NASH. Upon stimulation, hepatic immune cells produce various mediators (cytokines and chemokines) that alter lipid metabolism, insulin signalling, cell survival, and fibrogenesis. For their ability to antagonize TLRs pathway in Kupffer cells and natural killer T cells, antagonists of TLRs may represent a novel tool in NAFLD therapy [10,161].

Conclusions

NAFLD in children is a new global challenge for liver disease researchers and an important burden for healthy systems.

Paediatric NAFLD has a cause-and-effect complex relationship with several metabolic abnormalities that increases the risk of MetS and cardiovascular diseases. Therefore, fundamental research studies are urgently required to investigate the mechanisms by which these inherited or acquired traits influence NAFLD. These advances may have a high clinical translationality in diagnosis as well as in therapy. In fact, although diagnosis of NAFLD in children is currently based upon invasive and non-invasive approaches, concomitant efforts should be directed in the identification of novel tools for large-scale screening of paediatric populations at risk. Finally, as no exact guideline exists on NAFLD optimal therapy, in case of poor compliance to diet and lifestyle changes and/or in case of partial or no response, integrated multi-disciplinary programs for prevention and multi-targeted treatment in children are required to combat the escalation and progression of this disease.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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