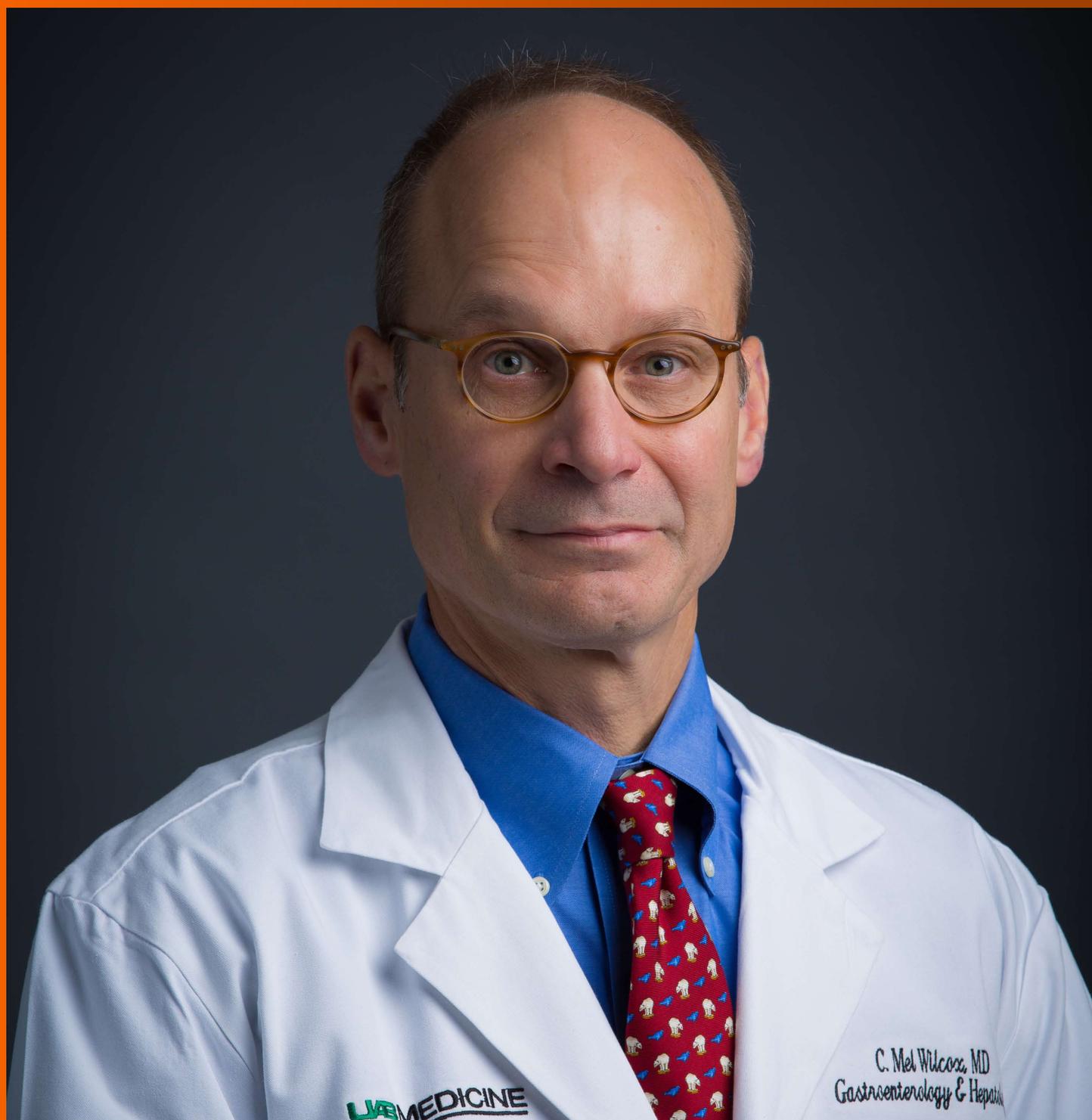


# World Journal of *Gastroenterology*

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## Pathogenesis of non-alcoholic fatty liver disease in children and adolescence: From “two hit theory” to “multiple hit model”

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) has become the dominant form of chronic liver disease in children and adolescents with the increasing prevalence of obesity worldwide. NAFLD represents a wide spectrum of conditions, ranging from fatty liver - which generally follows a benign, non-progressive clinical course - to non-alcoholic steatohepatitis, a subset of NAFLD that may progress to cirrhosis and end-stage liver disease or liver carcinoma. The underlying pathophysiological mechanism of “pediatric” NAFLD remains unclear, although it is strongly associated with obesity and insulin resistance. In this review we provide a general overview on the current understanding of NAFLD in children and adolescents, which underpins practice, enabling early diagnosis and appropriate therapeutic intervention for this life-threatening liver disease.

**Key words:** Non-alcoholic steatohepatitis; Children; Adolescents; Pathogenesis; Nonalcoholic fatty liver disease

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**Core tip:** Much work on nonalcoholic fatty liver disease (NAFLD) has been done, but an accurate understanding of its mechanism remains unclear. Our objective was to examine the current literature to better understand the

pathogenesis of NAFLD, thus showing how it evolved from the “two-hit theory” to a “multiple hit model”.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, is the most frequent form of chronic liver disease worldwide. Corresponding to abnormal fat accumulation in hepatocytes, it encompasses a spectrum of chronic liver diseases in the absence of excessive alcohol consumption, which may occur with or without hepatocyte inflammation or fibrosis<sup>[1]</sup>. Isolated steatosis, defined by abnormal accumulation of fat in more than 5% of hepatocytes, is a relatively benign condition.

In contrast, besides steatosis, non-alcoholic steatohepatitis (NASH) coexists with inflammation, hepatic cell injury, and deposition of collagen fibers<sup>[2]</sup>. NASH is a dynamic condition that can regress to isolated steatosis or cause progressive fibrosis leading to cirrhosis. The prevalence of NAFLD ranges from 10% to 30% depending on the study population and diagnostic methods used and is thought to be increasing worldwide<sup>[3,4]</sup>. Recently, a meta-analysis showed that the global prevalence of NAFLD is 25.24% (95%CI: 22.10%-28.65%) with the highest prevalence in the Middle East and South America and the lowest in Africa for the year 2016<sup>[5]</sup>. The “gold standard” test is liver biopsy, but this is neither feasible nor ethical for epidemiological studies aiming to screen NAFLD in the healthy population, and is also problematic in the clinical diagnosis. As so far, there was only one pediatric study which reported prevalence based on liver histology, and it reported that the prevalence of NAFLD in obese children (aged 2-19 years) was 38% and increased with age<sup>[6]</sup>.

Serum biomarkers such as alanine aminotransferase (ALT) and aspartate aminotransferase, as well as liver imaging (liver ultrasound and magnetic resonance imaging), are currently the most widely used tools for screening. Published guidelines vary about the frequency of ALT screening and use of imaging<sup>[7-9]</sup>. Ongoing developments of new technologies are improving the diagnosis; however, the specificity and sensitivity for the diagnosis of NAFLD have not yet reached an acceptable level.

As mentioned above, NASH is progressive, so early diagnosis and treatments are critical. However, many aspects of the pathogenesis of NAFLD remain unclear;

for example, the mechanism of the progression from steatosis to steatohepatitis. It is also unknown why NAFLD occurs only in a subgroup of obese subjects.

In the past decade, our team has researched the causes, pathogenesis, clinical diagnosis, and treatment of NAFLD<sup>[10-14]</sup>. In this review, we focus on the pathogenesis of NAFLD and explore new ways for improving both the diagnosis and treatment of NASH. The articles cited were identified based on a search of PubMed done in February 2018 using the criteria “NAFLD and pathogenesis and children and adolescent” with studies in humans and animals.

## PATHOGENESIS OF NAFLD

### *Evolution from the “two hit theory” to the “multiple hit model”*

During recent decades, the worldwide prevalence of obesity has increased in the pediatric population and the prevalence of NAFLD has more than doubled during the last 20 years in the United States<sup>[14]</sup>. The development of NAFLD is strongly influenced by age, sex, and ethnicity, and appears twice as often in boys than in girls<sup>[15-18]</sup>. NASH can progress to end-stage liver diseases such as hepatic cirrhosis or hepatocellular carcinoma. Conjeevaram PF *et al.*<sup>[19]</sup> analyzed the database and discovered that as the prevalence of NAFLD increased, the prevalence of NASH also increased, however, compared to adult the prevalence of liver fibrosis in children remained low, which indicated a possibly less aggressive NAFLD phenotype in children. Although the prevalence of NAFLD is increasing, most affected patients present with isolated steatosis with only a minority of cases progressing to liver cirrhosis in children, and it is not clear whether pediatric and adult NAFLD are two different pathologic entities or just age-dependent manifestations of the same disease, which implies that the pathogenesis of NAFLD may be related to the interplay among genetic, environmental, and individual factors. Early theories of the pathogenesis of NAFLD and NASH were described in terms of the “two hit hypothesis”<sup>[20]</sup>. At the onset of disease, the “first hit” is represented by an increase in liver fat, characterized by hepatic triglyceride accumulation and insulin resistance, and corresponding to hepatic steatosis once the accumulation of hepatic fat is more than 5%. Children, especially pre-pubertal boys, have a pattern of type 2 NAFLD characterized by a zone 1 distribution of steatosis, inflammation and fibrosis<sup>[21]</sup>. Liver fat accumulation is associated with a hypercaloric diet, sedentary lifestyle, and is perhaps genetically predisposed. Our team successfully established an *in vivo* NAFLD animal model induced by a high-fat diet, and reported that lifestyle interventions have an effect on NAFLD in obese children<sup>[22,23]</sup>. Subsequently, the “second hit” emerges, which includes inflammatory cytokines, adipokines, mitochondrial dysfunction, and oxidative stress. As the fatty liver is more susceptible

to this “second hit”, necroinflammation and fibrosis can develop and ultimately lead to cirrhosis<sup>[24,25]</sup>. However, with the development of new technology and further research, this view appears too simplistic for recapitulating the complexity of human NAFLD.

Now, the widely accepted theory is the “multiple-hit model”, involving more widespread metabolic dysfunction because of the interaction of genetic and environmental factors as well as changes in crosstalk between different organs and tissues, including adipose tissue, the pancreas, gut, and liver<sup>[24-27]</sup>. However, liver fat accumulation, caused by obesity and insulin resistance, still seem to represent the “first hits”.

## MULTIPLE FACTORS

### ***Fat accumulation and insulin resistance***

Fat accumulates in the liver of patients with NAFLD mainly in the form of triglycerides, which derive from the esterification of glycerol and free fatty acids (FFAs)<sup>[28]</sup>. Triglyceride accumulation is not hepatotoxic, in contrast with the excess of FFAs that undergo acetyl coenzyme A (acyl-CoA) synthase activity and form fatty acyl-CoAs which may trigger esterification or  $\beta$ -oxidation pathways<sup>[29]</sup>. Mitochondrial dysfunction, which consists of oxidative stress and production of reactive oxygen species and endoplasmic reticulum stress-associated mechanisms, also results from NAFLD<sup>[30,31]</sup>.

Physiologically, insulin controls hepatic glucose production by regulating lipolysis of adipocytes, leading to decreased fatty acid flux in the liver<sup>[32]</sup>. Consequently, the availability of hepatic acetyl coenzyme A (acyl-CoA) concentrations and the activity of pyruvate carboxylase are reduced, resulting in the decreased conversion of pyruvate to glucose (Figure 1).

Insulin resistance (IR) refers to a defective metabolic response to the effect of the hormone in the target cell (*e.g.*, muscle cell, hepatocyte, and adipocyte) or at the whole organism level. Systemic IR means that the ability of insulin to lower the serum glucose concentration to the appropriate level is hampered due to disrupted translocation of the GLUT4 receptor at the surface membrane of the muscle cell. As a result, glucose uptake (which depends on insulin) decreases. Hepatic IR consists of disturbed insulin mediated suppression of hepatic glucose production, but in the presence of preserved stimulation of lipogenesis<sup>[33]</sup>. In the adipose system, insulin resistance means that insulin is unable to suppress lipolysis. In humans, when the availability of lipids exceeds the lipid accumulation capacity, systemic IR and hepatic IR are likely to progress<sup>[34]</sup>.

### ***Inflammatory pathways***

Increased FFA levels can cause lipotoxicity and insulin resistance, and together with other factors (such as gut-derived endotoxins), activate the release of proinflammatory cytokines systemically and also locally in the liver. There are two main classical pathways

involved in the process of NAFLD inflammation: JNK-AP-1 and IKK-NF- $\kappa$ B<sup>[35]</sup>. JNK-AP-1 is a mitogen-activated protein kinase associated with apoptosis and NASH; IKK-NF- $\kappa$ B is a transcription factor regulating inflammatory activation. Previous studies have shown that persistent activation of NF- $\kappa$ B was found in NAFLD animal models as well as in humans with NASH<sup>[36,37]</sup>. Animal models demonstrated that hepatic exposure to high levels of proinflammatory cytokines could lead to histological changes mimicking NASH<sup>[38]</sup>.

The liver consists of parenchymal cells and nonparenchymal cells (NPCs); NPCs include sinusoidal endothelial cells. Kupffer cells (KCs) and hepatic stellate cells are less numerous than hepatocytes, but play a key role in the immune regulation of the liver, especially through substances released from KCs, which act as antigen presenting cells.

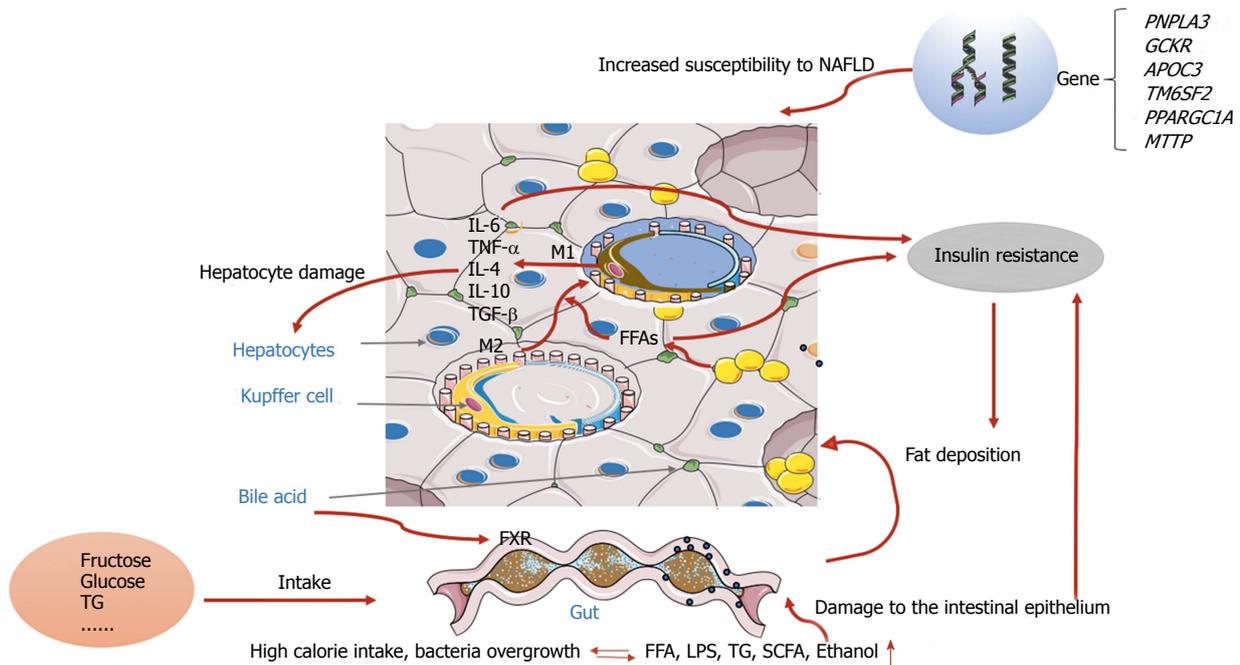
The hypothesis is that when the flow of FFAs or other pathogenic factors (such as endotoxins) from the gut into liver are excessive, KCs phagocytose the factors and present them through pattern recognition receptors (PRRs)<sup>[39]</sup>. PRRs include toll-like receptors (TLRS) such TLR4, TLR9, and nucleotide oligomerization domain-like receptors (NLRs)<sup>[40]</sup>. Inflammasomes, through NLR, activate the cascade events which finally generate mature IL-1, IL-8<sup>[41]</sup>, and IL-1, contributing to regulate the activation of the transcription factor NF- $\kappa$ B<sup>[42]</sup>. KCs *per se* will differentiate into either the M1 or M2 phenotype, depending on the environmental inducer; the former releasing cytokines like TNF- $\alpha$ , IL-1, and IL-12 and the latter, more heterogeneous, being able to stimulate the secretion of IL-4, IL-10, and TGF- $\beta$  according to different triggers<sup>[43]</sup>. IL-6 and TNF- $\alpha$  are the cytokines responsible for NASH progression. Patients with NASH have higher serum TNF- $\alpha$  levels, which play an important role in hepatic fibrosis through KC activation<sup>[38]</sup>.

Therefore, TLR suppression is thought to block the immune response, thereby alleviating liver inflammation. However, to date, despite some animal experiments aiming to reveal the links between TLRs and NAFLD pathogenesis, no investigations on TLR agonists have yet been conducted in humans<sup>[44,45]</sup>.

In summary, hepatocyte damage is an indicator of NASH progression. Different pathogens stimulate cell receptors thus activating the signaling pathway which contributes to cytokine production. Therefore, NASH might be detected at an earlier stage in the future by identifying an appropriate cytokine panel. Future studies should also focus on TLR modulation, which may provide a new target for NAFLD therapy.

### ***Gut-liver axis***

In recent years, many studies have been carried out on gut-liver axis (GLA) dysfunction (including intestinal dysbiosis, bacterial overgrowth, and alteration of mucosa permeability) intending to find the possible therapeutic target of NAFLD<sup>[46,47]</sup>. GLA is characterized by bidirectional traffic. Nutrients and factors derived



**Figure 1** Schematic mechanistic diagram of the “multiple hit model”. NAFLD: Nonalcoholic fatty liver disease.

from the gut lumen reach the liver through the portal circulation; bile acids, produced by hepatocytes, are released in the small intestine through the biliary tract<sup>[48]</sup>. Two of these components (intestinal barrier and gut microbiota) seem to play a key role in liver damage and its progression<sup>[49]</sup>. It is well known that trillions of microbes make up the gut microbiota. In normal conditions, only a small amount of bacteria products enter the liver through the portal circulation. However, bacteria dysbiosis or gut barrier alterations will increase the bacteria flow into the liver, thus stimulating inflammation *via* TLR and other pattern recognition receptor activation in KCs<sup>[50]</sup>. According to the bidirectional traffic of GLA, bile acid also impacts the gut environment, both directly by causing membrane damage and indirectly *via* the activation by bile acid metabolites of special receptors such as the farnesoid X receptor. Gut microbiota (GM) is specific to each individual, but humans share a core functional microbiome<sup>[51]</sup>.

Altered GM associated with NAFLD may occur through several mechanisms as follows: (1) GM digests and ferments the excessive energy into short-chain fatty acids (SCFAs); (2) GM bacteria can produce ethanol that may affect the liver in a similar way to chronic alcoholism; (3) bacteria/endotoxins translocate into the portal circulation and damage the liver *via* TLR signaling; and (4) disturbed lipid metabolism is mediated by increased bile acid synthesis and decreased choline metabolism<sup>[52]</sup>.

In addition, GM also plays a vital role in maintaining gut barrier integrity and intestinal permeability. GM dysbiosis can damage the intestinal epithelium and destroy tight junction proteins, which is important in

preventing harmful substances from the gut such as bacteria, ethanol, and endotoxins from entering portal blood<sup>[46,53]</sup>. Experiments on mice and humans have confirmed these data<sup>[54,55]</sup>. A recent study found that *E. coli* emerges as the predominant bacteria involved in small intestine bacterial overgrowth and that NAFLD may be related to the efficient translocation abilities of these patients<sup>[56]</sup>.

Hepatocyte triacylglycerol (TG) deposition is mainly due to three factors: lipolysis of adipose tissue, *de novo* lipogenesis, and TG dietary input, with contributions of 59%, 26%, and 15%, respectively. The excessive load of free fatty acid in the liver is the crucial cause of liver steatosis<sup>[57]</sup>.

#### **Dietary factors: Fructose and sugar**

Carbohydrates can be converted to TG, and fructose is more closely associated with NAFLD compared to glucose. Fructose consumption, largely in the form of high fructose corn syrup (HFCS), a mixture of fructose and glucose monosaccharides, has increased over the past several decades<sup>[58]</sup>. Recent data suggest that diets high in sugar (sucrose and/or HFCS) not only increase the risk of NAFLD, but also of NASH. Indeed, fructose intake from added sugars in processed foods correlates with the epidemic rise in obesity, metabolic syndrome, and NAFLD. Fructose induced-hepatic fat accumulation involves the stress pathway that results in gluconeogenesis, an increase in fat synthesis, and a decrease in fat oxidation<sup>[59-61]</sup>. Fructose may modulate the lipogenic enzymes by increasing the expression of sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP)<sup>[62]</sup>. Animal experiments<sup>[63]</sup> showed

that mice exposed to fructose with significant intestinal bacteria growth and increased intestinal permeability, as mentioned above, may trigger inflammation by increasing serum TNF- $\alpha$ .

Chronic fructose consumption induces leptin resistance prior to body weight, which accelerates high-fat induced obesity. Moreover, removal of fructose from this diet reverses leptin resistance and leptin augmentation, favoring a causal relationship<sup>[64,65]</sup>.

Therefore, GLA is involved in the pathogenesis of NAFLD, and GM dysbiosis promotes steatosis evolution to NASH. Special bacterial strains translocate more efficiently into the liver portal system. Further multicenter studies are required to test the bacterial genes of the normal population versus obese populations with IR (with and without NAFLD) with the aim of screening high-risk populations. In addition, improving intestinal dysbiosis and determining whether this improvement reduces the risk of NAFLD needs further investigation. These studies may pave the way for improving NAFLD diagnosis and treatment.

### Genetic factors

Genetic factors are also important in the development of NAFLD. A certain genetic background has been shown to predispose an individual to fatty liver<sup>[66,67]</sup>. Those genes are involved in inflammation, lipid metabolism, and oxidation, and are associated with progressive liver disease, IR, type 2 diabetes mellitus, and a higher risk for hepatocellular carcinoma.

**PNPLA3:** *PNPLA3* is the most documented NAFLD-related gene. A genome-wide association study (GWAS) showed that the hepatic fat content of *PNPLA3* I148M allele carriers was more than 2-fold higher than in non-carriers, and a new variant *PNPLA3* S453I allele was identified which was associated with a significantly lower liver fat content particularly in African Americans<sup>[68]</sup>. Several studies have shown that *PNPLA3* I148M increases the risk of NAFLD without a strong effect on metabolic syndrome (MS) components, but abdominal fat (which is closely correlated to MS components) can drive the effect of this polymorphism on liver damage<sup>[69,70]</sup>. In obese children, weight loss can weaken the effect of this polymorphism<sup>[71]</sup>.

McGeoch *et al.*<sup>[72]</sup> suggested that patients with *PNPLA3* p.I148M showed the greatest response to the fructose-restricted diet, whereas those lacking this variant exhibit minimal or no change from baseline. Wang *et al.*<sup>[73]</sup> revealed that physical activity and sedentary behavior can modulate the effect of the *PNPLA3* variant on childhood NAFLD. These evidences provide new clues to the function of the *PNPLA3* gene and are also useful for risk assessment and personalized treatment of NAFLD in the future.

**Glucokinase regulator protein:** Glucokinase regulator protein (GCKR) is an inhibitor of glucokinase

(GCK). GCK regulates glucose storage and disposal in the liver where its activity is regulated by GCKR. The GCKR genotype has been shown to modulate lipogenesis and fibrosis progression in NAFLD<sup>[74]</sup>. The combined effects of *PNPLA3* rs738409 and GCKR rs1260326 polymorphisms account for up to one-third of variability in liver fat content in obese children<sup>[75,76]</sup>.

**Apolipoprotein C-III:** Apolipoprotein C-III (*APOC3*) can inhibit the lipoprotein lipase and reduce the clearance of TG. In NAFLD, *APOC3* variants may lead to higher plasma concentrations of apolipoprotein C3 ending up in lower clearance. The consequence of the reduced TG clearance is an increase in residual particles of chylomicrons, that will lead to higher levels of circulating chylomicron remnants, which are especially cleared by the liver through a receptor-mediated process<sup>[77,78]</sup>. However, a recent study of *APOC3* transgenic mice suggested that *APOC3* dysregulation is not a predisposing factor for linking over-nutrition to NAFLD in obesity<sup>[79]</sup>.

**TM6SF2:** Transmembrane 6 superfamily 2 (*TM6SF2*) has been recognized to regulate plasma lipids. On the basis of sequence similarity to Emopamil-binding protein (an enzyme with sterol isomerase activity), *TM6SF2* has been hypothesized to play a role in sterol biosynthesis<sup>[80,81]</sup>. Smagris *et al.*<sup>[82]</sup> reported that *TM6SF2* is involved in the transfer of neutral lipids from cytoplasmic to luminal lipid droplets or very low density lipoprotein (VLDL) particles. Recently, variants of *TM6SF2* have been found to influence metabolic traits through alteration of protein stability<sup>[83-86]</sup>.

**PPARGC1A:** Peroxisome proliferator activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), expressing the *PPARGC1A* gene, is involved in the key steps of NAFLD development, such as insulin resistance, mitochondrial biogenesis, and oxidative phosphorylation<sup>[87,88]</sup>. In hepatocytes, PGC-1 $\alpha$  orchestrates broad energy programs, including gluconeogenesis and mitochondrial fatty acid  $\beta$ -oxidation<sup>[89]</sup>. Moreover, *PPARGC1A* has been shown to regulate several key genes in hepatic gluconeogenesis (*CREB*, *PPAR $\alpha$* , *FOXO1*, *TRB-3*)<sup>[90-93]</sup>. *PPARGC1A* knockout mice reportedly developed hepatic steatosis due to a combination of reduced mitochondrial respiratory capacity and increased the expression of lipogenic genes<sup>[94]</sup>.

**Human microsomal triglyceride transfer protein:** The human microsomal triglyceride transfer protein (MTTP) is involved in lipid transfer function and is critical for the assembly and secretion of VLDL to remove lipids from the liver. Thus, genetic polymorphisms in the *MTTP* gene may contribute to altered lipid metabolism by disrupting the assembly and secretion of lipoproteins, leading to reduced fat export from the involved hepatocytes and to NAFLD. Several genetic

polymorphisms in the *MTTP* gene have been identified; some are related to the pathogenesis of NAFLD while others interact with age, insulin resistance, and BMI and increase the risk for NAFLD<sup>[95-99]</sup>.

**Other genes:** Recently, Buch *et al.*<sup>[100]</sup> and Umamo *et al.*<sup>[101]</sup> identified the rs626283 variants in the *MBOAT7* gene as risk loci for alcohol-related cirrhosis in adults and obese youth<sup>[100,101]</sup>. In the Japanese population, the *SAMM50* gene (rs738491, rs3761472, and rs2143571), *PARVB* gene (rs6006473, rs5764455 and rs6006611), and *GATAD2A* gene (rs4808199) were found to be significantly associated with NAFLD<sup>[102,103]</sup>.

Meanwhile, Chinese children with NAFLD presented a higher prevalence of *UCP3* gene rs11235972 GG<sup>[104]</sup>. Adams *et al.*<sup>[105]</sup> reported that SNPs in two hepatic genes were associated with NAFLD in adolescents: The group-specific component and the lymphocyte cytosolic protein-1.

## CONCLUSION

The pathogenesis of NAFLD and its progression is a complex process, in which some questions remain unanswered. The initial "two-hit" theory can no longer completely explain the pathogenesis of NAFLD, which involves multiple factors. In recent decades, many experiments have suggested that the gut microbiome plays a key role in NAFLD pathogenesis *via* the GLA. More recently, with the development of technology (especially GWAS), increasing studies have focused on genetic predispositions and found various gene variants that may alter lipid and sugar metabolism in the liver as well as in other tissues such as adipose tissue. Given the multifactorial nature of the related diseases, it may not be possible to obtain a single indicator that could precisely differentiate NAFLD and NASH. However, data in the future could be more promising in terms of population screening, with the goal to identify individuals at risk for NAFLD.

Hopefully, the "multiple hit model" (once further refined) will pave the way for tailoring therapeutics to genetic predispositions to NAFLD and NASH.

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