Chapter 1

Nonalcoholic Fatty Liver Disease in Children: Role of the Gut Microbiota

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http://dx.doi.org/10.5772/64799

Abstract

Nonalcoholic fatty liver disease (NAFLD) has emerged as the most common cause of liver disease among children and adolescents in industrialized countries due to increasing prevalence of obesity. It is generally recognized that both genetic and environmental risk factors contribute to the pathogenesis of NAFLD. Convincing evidences have shown that gut microbiota alteration is associated with NAFLD pathogenesis both in patients and animal models. Bacterial overgrowth and increased intestinal permeability are evident in NAFLD patients and lead to increased delivery of gut-derived bacterial products, such as lipopolysaccharide and bacterial DNA, to the liver through portal vein and then activation of toll-like receptors (TLRs), mainly TLR4 and TLR9, and their downstream cytokines and chemokines, resulting in hepatic inflammation. Currently, the role of gut microbiota in the pathogenesis of NAFLD is still the focus of many active clinical/basic researches. Modulation of gut microbiota with probiotics or prebiotics has been targeted as a preventive or therapeutic strategy on this pathological condition. Their beneficial effects on the NAFLD have been demonstrated in animal models and limited human studies.

Keywords: nonalcoholic fatty liver disease (NAFLD), children, gut microbiota, probiotics, prebiotics

1. Introduction

A growing obesity epidemic over the past three decades has become a major public health concern in developed as well as developing countries. According to the 2012 National Health and Nutrition Examination Survey [1, 2], in the United States, 35.5% of men, 35.8% of women,

and 16.9% of children (2–19 years old) were considered obese. The worldwide prevalence of overweight and obesity increased from 28.8 to 36.9% in men, and from 29.8 to 38.0% in women between 1980 and 2013 [3]. Specifically, the prevalence for children increased from 16.9 to 23.8% for boys and from 16.2 to 22.6% for girls in developed countries, and from 8.1 to 12.9% for boys and from 8.4 to 13.4% for girls in developing countries as well [3].

Nonalcoholic fatty liver disease (NAFLD) has become the most common cause of liver disease in children in industrialized countries due to increasing prevalence of obesity [4]. NAFLD is defined as hepatic fat infiltration >5% of hepatocytes based on liver biopsy after excessive alcohol intake, viral, autoimmune, or drug-induced liver disease have been excluded. NAFLD is characterized by liver damage similar to that caused by alcohol but occurs in individuals that do not consume toxic quantities of alcohol. NAFLD includes a spectrum of liver diseases from simple fat infiltration (steatosis) through nonalcoholic steatohepatitis (NASH, steatosis with liver inflammation) to hepatic fibrosis and even hepatocellular carcinoma. The prevalence of NAFLD in the United States was 9.6% in normal weight children and 38% in obese ones based on liver biopsy at autopsy after accidents [5]. In the United States, the highest rates of pediatric NAFLD are in Hispanic and Asian children. In a study of 748 school children in Taiwan, the rates of NAFLD were 3% in the normal weight, 25% in the overweight, and 76% in the obese children determined by ultrasonography [6]. NAFLD in children is associated with severe obesity and metabolic syndrome, which includes abdominal obesity, type-2 diabetes, dyslipidemia, and hypertension. This chapter briefly summarizes the current understanding of the pathogenesis of NAFLD, role of gut microbiota, and potential new treatment strategies.

2. NAFLD pathogenesis: current understanding

Although the pathogenesis of NAFLD is not completely understood, considerable progresses have been made in recent years in explicating the mechanisms behind liver injury. As in other complex diseases, both genetic and environmental factors contribute to NAFLD development and progression. It is generally accepted that there is a genetic predisposition. In patients with NAFLD, genomic studies have identified many single nucleotide polymorphisms (SNPs) variants in genes controlling lipid metabolism, proinflammatory cytokines, fibrotic mediators, and oxidative stress. The most important one is the patatin-like phospholipase domain-containing 3 gene (PNPLA3) [7]. PNPLA3 rs738409 variant has been shown to confer susceptibility to NAFLD in obese children in different ethnic groups [8]. Other reported susceptible genes include glucokinase regulatory protein (GCKR), transmembrane 6 superfamily member 2 (TM6SF2), G-protein-coupled-receptor 120 (GPR120), farnesyl-diphosphate farnesyltransferase 1 (FDFT1), parvin beta (PARVB), sorting and assembly machinery component (SAMM50), lipid phosphate phosphatase-related protein type 4 (LPPR4), solute carrier family 38 member 8 (SLC38A8), lymphocyte cytosolic protein-1 (LCP1), group-specific component (GC), protein phosphatase 1 regulatory subunit 3b (PPP1R3B), lysophospholipase-like 1 (LYPLAL1), neurocan (NCAN), and polipoprotein C3 (APOC3) [9, 10]. To date, the strongest

SNP variants associated with pediatric NAFLD are the rs738409 in the *PNPLA3* gene, the 1260326 in the *GCKR* gene, and the rs58542926 in the *TM6SF2* gene.

Day and James initially proposed a two-hit hypothesis to explain the pathogenesis of NAFLD [11]. In individuals with genetic predisposition, the "first hit" results in liver fat accumulation (steatosis) due to environmental factors (e.g., western diet and lack of physical activity), obesity, insulin resistance, or metabolic syndrome. A subsequent "second hit", such as free fatty acids, adipokines/cytokines, oxidative stress (reactive oxygen species, lipid peroxidation), gut microbiota-derived endotoxins, mitochondrial dysfunction, and stellate cell activation, further amplify liver injury and NASH progression. A recent proposed multiple parallel hits hypothesis suggested that gut-derived and adipose tissue-derived factors may play a central role [12]. Both two-hit and multiple parallel hit hypotheses recognized that insulin resistance plays a crucial role in NAFLD pathogenesis and other factors including genetic determinants, nutritional factors, adipose tissue, and the immune system may be necessary for NAFLD manifestation and progression [11–13]. A new lipotoxicity hypothesis proposes that insulin resistance facilitates an excessive flow of free fatty acids to the liver, resulting in increased production of lipotoxic intermediates and eventually NASH, through oxidative stress, mitochondrial dysfunction, adiponectin, and other complex pathways [14, 15].

It has been well established that gut microbiota has been implicated in the development of NAFLD through the gut-liver axis [16–18]. An alteration of gut microbiota composition leads to bacterial overgrowth and increased intestinal permeability [19–21], resulting in translocation of gut micriobiota-derived products, such as lipopolysaccharide (LPS), bacterial DNA, and peptidoglycan, which would activate liver cell surface receptors (TLR4 and 9); a cascade of signal transductions is triggered and various cytokines and chemokines, such as TNF-α, TGF-β, IL-6, IL-10, CCL2, CCL5, and CxCL8, are released, leading to hepatic inflammation and fibrosis [22].

Evidences from both human and animal studies have supported important roles of gut microbiota-derived endotoxins, especially LPS, and their downstream signal pathways in the progression of NAFLD. Patients with NAFLD had increased serum endotoxin levels, with marked increases noted in NASH and early stage fibrosis. The increase in endotoxin level is related to IL-1α and TNF-α production [23–26]. In genetically obese fatty/fatty rats and obese/obese mice, Yang et al. showed that LPS contributes to the development of steatohepatitis by sensitizing TNF-α [27].

Toll-like receptors (TLRs) have been shown to play a crucial role in pathogenesis of NAFLD. Activation of TLRs and the adaptor molecule, MyD88, results in a cascade of signal transduction leading to release of various cytokines (TNF-α, TGF-β, interleukin-6 (IL-6), and IL-10) and chemokines (CCL2, CCL5, and CXCL8), which have been associated with NAFLD progression and hepatic fibrosis, as demonstrated in both human and animal studies [28]. TLRs are a class of pattern recognizing proteins that perceive bacterial and viral components. Gut microbiota is a source of TLR ligands, which can stimulate production of proinflammatory cytokines in the liver. TLRs are expressed on Kupffer cells, biliary epithelial cells, hepatocytes, hepatic stellate cells, epithelial cells, and dendritic cells in the liver. Among 13 known TLRs, TLR2, TLR4, and TLR9 have been implicated in NAFLD pathogenesis [17].

TLR4 is mainly activated by LPS, a cell component of Gram-negative bacteria. Elevated plasma and portal LPS levels are evident in human and animals with NAFLD [25, 29–32]. In methionine choline deficient diet(MCDD)-induced mouse model of NASH, liver injury and inflammatory cytokine production increased after challenge with LPS [33]. Rivera et al. further demonstrated histological change typical of steatohepatitis (extensive macrovesicular steatosis and necrosis), three-fold increase of portal blood endotoxin level, and enhanced TLR4 expression in wild-type mice fed with MCDD [31]. In a mouse model of high-fat diet-induced NAFLD, TLR4 signaling is involved in free fatty-acid-induced NF-kB activation in hepatocytes through release of free high-mobility group box1 (HMGB1), which is a key molecule for the activation of the TLR4/MyD88-dependent pathway [34]. TLR4 mutant mice fed with fructose-enriched diet had significantly less hepatic steatosis and lower TNFα levels in comparison to fructose-fed wild-type mice, indicating an important role of LPS/TLR4 signaling in fructose-induced NAFLD [35]. Plasma LPS levels are also markedly elevated in children and adults with NAFLD [25, 29, 30, 32]. Thus, gut microbiota-derived LPS/TLR4 signaling pathway is crucial for the progression of NAFLD in humans as well as animal models.

TLR9 is activated by bacterial DNA CpG motif and induces proinflammatory cytokine production. In a mouse model of CDAA diet-induced NASH, Miura et al. showed hepatic inflammation and fibrosis in wild-type mice, which was suppressed in mice deficient in TLR9 or MyD88, suggesting the critical role of the TLR9/MyD88 signaling pathway in the pathogenesis of NASH [36].

Inflammasomes have been shown to be major contributors to inflammation and are upregulated in mouse models of MCDD or high-fat-induced NASH and in livers of NASH patients. Stimulation of TLR4 by LPS can further activate inflammasomes [37]. In genetic inflammasome-deficiency mice, an altered gut microbiota configuration is associated with abnormal TLR4 and TLR9 agonist accumulation in the portal circulation, resulting in elevated hepatic TNF-α expression and exacerbation of hepatic steatosis and inflammation [38].

TLR2 recognizes components from Gram-positive and Gram-negative bacteria, as well as mycoplasma and yeast. In comparison to wild-type mice, TLR2-deficiency animals are substantially protected from high-fat diet-induced adiposity, insulin resistance, hypercholesterolemia, and hepatic steatosis [39]. In contrast, increased hepatic inflammation and TNF-α mRNA expression were observed in TLR2-deficiency mice fed with MCDD [33, 40]. The conflicting results of the role of TLR2 signaling in those studies could be due to different animal models used, different gut microbial ligands involved or compensation by other TLRs.

3. Modulation of gut microbiota: effects of prebiotics and probiotics on NAFLD

Given the accumulating evidence of the critical role of gut microbiota in the pathogenesis of NAFLD, microbiota manipulation has been targeted as a potentially therapeutic option for this pathological condition. Possible strategies for altering gut microbiota include probiotics,

prebiotics, synbiotics, antibiotics, dietary modification/supplementation, and microbiota transplantation. So far, only probiotics have been tested for the treatment of NAFLD in animal models and human subjects with promising effects.

Probiotics are live commensal microorganisms that have been shown to beneficially modulate the host's gut microbiota. In animal models of NAFLD, VSL#3 (a probiotic mixture containing *streptococcus*, *Bifidobacterium*, and *lactobacillus*) improved hepatic inflammation and decreased hepatic steatosis with reduction of serum alanine aminotransferase (ALT) levels. Those changes were associated with decreased hepatic expression of TNF-mRNA and reduced activity of Jun N-terminal kinase (JNK) [41–43]. In methionine choline deficient diet (MCDD)-induced NASH rats treated with probiotic mixture containing 6 or 13 bacterial strains, which were isolated from the healthy human stool samples, improved hepatic inflammation, likely in part through modulation of TNF-α activity [44]. Furthermore, the treatment of apolipoprotein E-deficiency mice with dextran sulfate sodium (DSS) induced histopathological features typical of steatohepatitis, which were prevented by 12-week VSL#3 administration, through modulation of the expression of nuclear receptors, peroxisome proliferator-activated receptor-γ, Farnesoid-X-receptors, and vitamin D receptor [45].

In human studies, Aller et al. reported that a 3-month treatment with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* improved liver aminotransferases in adult patients with NAFLD [46]. Alisi et al. performed a double-blind and placebo-controlled RCT to assess the effect of VSL#3 in 44 obese children with biopsy-proven NAFLD and demonstrated that VSL#3 supplement for 4 months significantly improved hepatic steatosis and BMI [47].

Prebiotics are nondigestible dietary fibers that stimulate the growth and activity of intestinal bacteria. In genetically obese mice, supplementation with prebiotics (oligofructose, a mix of fermentable dietary fibers) decreased plasma levels of LPS and cytokines (TNF-α, IL1b, IL1α, IL6, and INFγ) and reduced gut permeability through a mechanism involving glucagon-like peptide-2 [48]. Lactulose, as a prebiotic, can promote the growth of certain intestinal bacteria such as *Lactobacillus* and *Bifidobacterium*. In a rat model of high-fat diet-induced steatohepatitis, lactulose improved hepatic inflammatory activity and decreased serum endotoxin levels [49]. Human studies with prebiotics are very limited. In an earlier clinical pilot study in patients with biopsy-proven NASH, dietary supplementation of oligofructose 16 g/day for 8 weeks significantly decreased serum aminotransferases and insulin levels [50]. There have been no randomized, controlled, double-blind, prospective clinical trials of prebiotics on NAFLD, except a randomized controlled trial protocol, which will randomize adults with confirmed NAFLD to either a 16 g/day prebiotic supplemented group or isocaloric placebo group for 24 weeks (n = 30/group) [51].

4. NAFLD in children

4.1. Gut microbiota and NAFLD in children

Given the important role of gut microbiota in obesity and metabolic syndrome [52, 53], it is not surprising that ever-increasing literature in recent years suggested a potential role of gut

microbiota in NAFLD pathogenesis. An observation by Spencer et al. provided the initial evidence that gut microbiota and human fatty liver are closely linked [54]. In adult subjects with choline-deficient diet-induced fatty liver, gut microbiota compositions were associated with changes in liver fat in each subject during choline depletion. Subsequently, Mouzaki et al. showed that patients with NASH had a lower percentage of Bacteroidetes compared to both simple steatosis and healthy controls and higher fecal *Clostridium coccoides* compared to those with simple steatosis [55]. There was an inverse and diet/BMI-independent association between the presence of NASH and percentage of Bacteroidetes, suggesting a link between gut microbiota and NAFLD severity. Raman et al. reported an over-representation of *Lactobacillus* species and selected members of phylum Firmicutes (Lachnospiraceae; genera, Dorea, Robinsoniella, and Roseburia) in NAFLD patients [56]. A recent study identified Bacteroides as independently associated with NASH and Ruminococcus with significant fibrosis and further confirmed the association of NAFLD severity with gut dysbiosis [57].

In a pediatric cohort of 63 children, Zhu et al. determined the composition of gut bacterial communities of obese children with NASH [58]. They found that Bacteroidetes were significantly elevated (mainly *Prevotella*) in obese and NASH patients compared to lean healthy children and that an increased abundance of ethanol-producing *Escherichia* in NASH children was observed. Ethanol can promote gut permeability. A recent study by Michail et al. showed that children with NAFLD had more abundant Gammaproteobacteria and Prevotella and significantly higher levels of ethanol, with differential effects on short chain fatty acids [59]. Both studies demonstrated that the gut microbiota profile in pediatric NAFLD is different from lean healthy children, with more ethanol-producing bacteria, suggesting that endogenous alcohol production by intestinal microbiota may play a role in NAFLD pathogenesis. Engstler et al. also showed that fasting ethanol levels were positively associated with measures of insulin resistance and significantly higher in children with NAFLD than in controls [60]. Interestingly, with further animal experiments, they demonstrated that increased blood ethanol levels in children with NAFLD may result from insulin-dependent impairments of alcohol dehydrogenase activity in liver tissue rather than from an increased endogenous ethanol synthesis [60]. Taken together, human studies demonstrated significant differences in gut microbiota between normal subjects and patients with NAFLD. However, there were great variations in microbiota compositions among these human studies, likely due to patient's age, fatty liver disease stages, study design, methods used, and observation endpoints.

4.2. Current management guidelines

All children with BMI ≥ 95th percentile or 85–94th percentile with risk factors (e.g., central obesity, metabolic syndrome, and strong family history) are recommended to have liver function test and hepatic ultrasonography [4, 61]. Since infants and children < 3 years old with fatty liver are less likely to have NAFLD, tests should be performed to exclude genetic, metabolic, syndromic, and systemic causes, such as fatty acid oxidation defects, lysosomal storage diseases, and peroxisomal disorders. In older children and teenagers, metabolic, infectious, toxic, and systemic causes should also be considered for differential diagnosis.

Recommended common laboratory tests include viral hepatitis panel, α-1 antitrypsin phenotype, ceruloplasmin, antinuclear antibody, lipid profile, TSH, and celiac panel.

Ultrasonography is the only imaging technique used for NAFLD screening in children because it is safe, noninvasive, widely available, relatively inexpensive, and can detect evidence of portal hypertension. Liver biopsy is recommended to exclude other treatable disease, in cases of clinically suspected advanced liver disease, before pharmacological/surgical treatment, and as part of a structured intervention protocol or clinical research trial [4, 61].

Treatment options for children with NAFLD are limited by a small number of randomized clinical trials and insufficient information on the natural history of the condition to assess risk-benefit ratios [4, 62]. So far, weight loss, though hard to achieve, is still the cornerstone of treatment regimen. Koot et al. demonstrated that a lifestyle intervention (physical exercise, dietary change, and behavioral modification) of 6 months significantly improved hepatic steatosis and serum aminotransferases in 144 children with NAFLD [63]. A long-term follow-up study showed that the greatest decrease of NAFLD prevalence was observed in children with the greatest overweight reduction [64]. Grønbæk et al. assessed the effect of a 10-week "weight loss camp" (restricted caloric intake and moderate exercise for one hour daily) in 117 obese children and found that the children had an average weight loss of 7.1 ± 2.7 kg, with significant improvements in hepatic steatosis, transaminases, and insulin sensitivity [65].

In children with poor adherence to lifestyle changes, pharmacological interventions and dietary supplementations, including antioxidants (vitamin E), insulin sensitizers (metoformin), ursodeoxycholic acid (UDCA), omega-3 docosahexaenoic acid (DHA), and probiotics, may be tried, but no randomized clinical trials have proved their effectiveness in children with NAFLD.

5. Summary and future directions

The increase of pediatric NAFLD is attributed to the worldwide obesity epidemic. Current evidences suggest that both genetic and environmental risk factors play a crucial role in the pathogenesis of NAFLD in children and adolescents. Although human studies clearly showed significant differences in gut microbiota between normal subjects and patients with NAFLD, there were great variations in microbiota compositions among these studies [66]. Adult patients have altered gut microbiota with an increase in the relative proportion of Bacteroidales and Clostridiales, whereas in children with NAFLD, ethanol-producing bacteria are predominant. Bacterial overgrowth and increased intestinal permeability are evident in NAFLD patients and lead to increased delivery of gut-derived bacterial products (e.g., LPS and bacterial DNA) to the liver through portal vein and then activation of toll-like receptors (TLRs), mainly TLR4 and TLR9, and their downstream cytokines and chemokines, resulting in hepatic inflammation [17].

Given the accumulating evidence of the critical role of gut-derived microbial factors in the development and/or progression of NAFLD, modulation of gut microbiota with probiotics

and/or prebiotics has been targeted as a therapeutic option. Their beneficial effects on NALFD are promising based on studies in animal models and patients including children. However, before probiotics and prebiotics become prime-time therapeutic modalities for NAFLD in children, several issues need to be addressed. First, we still do not know whether all children with NAFLD are truly associated with altered intestinal microbiota, and if so, which microbiota is involved. Second, randomized clinical trials with appropriate powers are required to assess benefits of tailored interventions with probiotics and/or prebiotics in children with NAFLD. Finally, it is clinically important to know the best types of probiotics or prebiotics to be prescribed in children with NAFLD. Nevertheless, probiotics and other integrated strategies to modify intestinal microbiota are promising to become efficacious therapeutic modalities to treat NALFD, with emerging evidence to demonstrate that prebiotics and probiotics modulate the intestinal microbiota, improve epithelial barrier function, and reduce intestinal inflammation.

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