



Review Article



Nonalcoholic Fatty Liver Disease and the Intestinal Microbiome: An Inseparable Link

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Abstract

Nonalcoholic fatty liver disease (NAFLD) particularly affects patients with type 2 diabetes and obesity. The incidence of NAFLD has increased significantly over the last decades and is now pandemically across the globe. It is a complex systemic disease comprising hepatic lipid accumulation, inflammation, lipotoxicity, gut dysbiosis, and insulin resistance as main features and with the potential to progress to cirrhosis and hepatocellular carcinoma (HCC). In numerous animal and human studies the gut microbiota plays a key role in the pathogenesis of NAFLD, NAFLD-cirrhosis and NAFLD-associated HCC. Lipotoxicity is the driver of inflammation, insulin resistance, and liver injury. Likewise, western diet, obesity, and metabolic disorders may alter the gut microbiota, which activates innate and adaptive immune responses and fuels hereby hepatic and systemic inflammation. Indigestible carbohydrates are fermented by the gut microbiota to produce important metabolites, such as short-chain fatty acids and succinate. Numerous animal and human studies suggested a pivotal role of these metabolites in the progression of NAFLD and its comorbidities. Though, modification of the gut microbiota and/or the metabolites could even be beneficial in patients with NAFLD, NAFLD-cirrhosis, and NAFLD-associated HCC. In this review we collect the evidence that exogenous and endogenous hits drive liver injury in NAFLD and propel liver fibrosis and the progressing to advanced disease stages. NAFLD can be seen as the product of a complex interplay between gut microbiota, the immune response and metabolism. Thus, the challenge will be to understand its pathogenesis and to develop new therapeutic strategies.

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Abbreviations: ALD, alcoholic liver disease; CCL, chemokine ligand; CLD, chronic liver disease; DNL, de novo lipogenesis; FMT, fecal microbiota transplantation; GF, germ-free; HSC, hepatic stellate cell; HCC, hepatocellular carcinoma; HFD, high fat diet; LPS, lipopolysaccharide; MAMP, microbe associated molecular pattern; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PRR, pattern recognition receptor; SCFA, short chain fatty acids; TLR, toll like receptor; TMAO, trimethylamineoxide; T2DM, type 2 diabetes mellitus.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide and affects almost 25% of the population worldwide.¹ NAFLD presents as phenotypes of varying severity ranging from steatosis to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma. One-third of patients present with inflammation and/or fibrosis,¹ but liver histology can distinguish NASH from simple steatosis in most patients. NAFLD, especially NASH, has a crucial role in many systemic diseases, especially cardiovascular disease and malignancy.²⁻⁴ It increases the long-term complications of these diseases and results in increased mortality.^{5,6} Thus far, no medical therapies have been approved for the treatment of NAFLD.⁷ This is reflected by an increasing need for liver transplantation because of NAFLD-associated cirrhosis and/or hepatocellular carcinoma (HCC),⁸ in high-income and medically advanced countries.

The underlying mechanisms for development and progression of NAFLD are complex and multifactorial. Initially a two hits hypothesis was proposed, in which the first hit was the hepatic accumulation of lipids as result of lack of physical activity along with a high-fat diet (HFD) and insulin resistance, making the liver more sensitive to further insult. The second hit was activation of the inflammatory cascade and stimulation of fibrogenesis.⁹ That hypothesis was supported by a model of obesity in ob/ob mice in which a second hit after increased hepatic lipid accumulation,¹⁰ was necessary to initiate inflammation and fibrosis. Many human studies have shown that the complexity of the NAFLD was not explained by this hypothesis. Multiple co-influencing factors are involved in the development and progression of this disease. As a result, a multiple hit hypothesis has replaced the two-hit hypothesis for the progression of NAFLD.¹¹ The view that steatosis always precedes inflammation has also changed. It is not the sum of hepatic hits, but more important, genetic, external, internal, and intracellular factors trigger different pathways that lead to steatosis and NASH.¹² In this review, we discuss two novel players in the pathophysiology of NAFLD, the altered gut microbiome and the related modification of its metabolites.

Pathogenesis of NAFLD

Insulin resistance has a crucial role in NAFLD and is more pronounced in NASH than in simple steatosis.¹³ Patients without type 2 diabetes mellitus (T2DM) but with hepatic steatosis and NASH have decreased insulin sensitivity.^{14,15} Resist-

ance to insulin appears to predispose to the development of NAFLD and further propels the progression to NASH,⁹ by activating the inflammatory cascade, inducing oxidative stress, and improving lipotoxicity.⁹ In addition, environmental and genetic factors interact with the insulin receptor signaling cascade. Interaction of these factors contributes to the worsening of insulin resistance in patients with NAFLD.¹⁶ Inflammatory signal transducers such as c Jun N-terminal protein kinase 1 (JNK1), nuclear factor B kinase inhibitor (IKKb),¹⁶ nuclear factor-kappa B activation (NF-kB) or suppressors of cytokine signaling all affect insulin signaling in patients with NAFLD.¹⁷ Activation of transcription factors such as carbohydrate response element-binding protein (ChREBP), sterol regulatory element-binding protein-1 (SREBP-1), and peroxisome proliferator-activated receptor (PPAR)- γ increase *de novo* hepatic lipogenesis (DNL).¹⁸ In patients with NAFLD, both DNL and nocturnal plasma free fatty-acid levels are increased compared with controls, and importantly, are not suppressed by fasting.¹⁹

Activated insulin receptors substrate 2 (IRS-2) influences DNL by regulating of SREBP-1c.²⁰ In insulin resistance, SREBP-1c is overexpressed and DNL is up-regulated, but IRS-2 is down-regulated.²¹ Enhanced insulin levels in insulin resistance also inhibit β -oxidation of free fatty-acids and promote hepatic lipid accumulation.²² In this vicious cycle, free fatty-acids in hepatocytes alter insulin signaling by activating serine kinases that increase insulin resistance.²³ As insulin suppresses lipolysis in adipose tissue, insulin resistance results in an increased efflux of free fatty-acids to the liver.²⁴

In addition, endoplasmic reticulum (ER) stress promotes DNL and steatohepatitis via different pathways.^{25,26} Reduced synthesis or secretion of very low density lipoprotein (VLDL) and other fat disposal pathways, such as impaired hepatic fatty acid oxidation, are thought to be of less importance for fat accumulation and lipotoxicity in NAFLD,²⁷ but they are not irrelevant nor neglectable. It is also important to mention that lipid regulation by autophagy is decreased in liver steatosis, which contributes to a vicious cycle of the suppression of autophagy and lipid accumulation.^{28,29} Other factors, including mitochondrial dysfunction, genetic determinants, adipose tissue dysfunction, and dietary factors in this context have been extensively reviewed elsewhere.^{30,31}

NAFLD and microbiota: preclinical and human evidence

The gut microbiome is increasingly seen as participating in NAFLD pathogenesis through the gut-liver axis. The evidence points toward involvement of the microbiome-gut-liver axis in NAFLD pathogenesis,³² and the microbiome-gut-liver axis seems to have a pivotal role in the progression of NAFLD to more advanced disease.³² Data describing the relationship of the gut microbiota and NAFLD development derive from fecal transplantation and murine studies. Housing of mice with genetically modified inflammasome pathways together with wild-type mice demonstrated that NASH developed following coprophagia.³³ In another study, fecal microbiota transplantation (FMT) from weight-matched obese mice with or without steatosis to germ-free (GF) controls led to increased expression of genes involved in lipid uptake, altered lipogenesis, fatty acid catabolism, and VLDL export in liver tissue and increased hepatic triglycerides.³⁴ These phenotypes were traced to an increase in *Lachnospiraceae* and the relative abundance of *Barnesiella intestinae hominis*.³⁴ Transfer of these findings from bench to bedside is challenging because the microbiota of mice and humans differ substantially.³⁵ First, some genera and species in humans are not present

in mice. And some that are present in mice are absent in humans.³⁵ Second, the digestive tracts of mice and humans have differences that influence the composition of the gut microbiota.³⁵ To avoid those problems, FMT from NAFLD patients to GF mice was performed to produce the patient hepatic phenotype.³⁶ FMT led to hepatic steatosis and inflammation in the mice, and the artificial phenotype was promoted by the feeding of an HFD.³⁶ Inflammation and immunologic balance influence development of metabolic disease, but GF mice,³⁷ does not have such a balance. To study the role of microbiota in murine models, conventional mouse models for FMT studies might be an alternative. Interestingly, hepatic triglycerides were increased within 14 days in conventional mice fed a chow diet after FMT from obese women.³⁸ Despite some limitations, the existing evidence from mouse studies supports the idea that the gut microbiota contributes to NAFLD development. The results of these studies indicate that increased intestinal permeability leads to lipopolysaccharide (LPS) release, which triggers tissue and systemic inflammation. In the long run, that enhances production of microbial metabolites such as trimethylamine N-oxide (TMAO), choline, or ethanol and bile acid signaling, which also interact with the host immunity.^{39,40}

Bacterial dysbiosis in NAFLD

Based on the above murine studies, the composition of the gut microbiota and microbiota-related metabolite signatures were studied in patients with NAFLD, NASH, and NAFLD cirrhosis and compared with each other and healthy controls.⁴¹ The microbiota of NAFLD patients compared with healthy controls had consistently altered microbiome signatures at the phylum,^{38,42–45} family,^{42,45} and genus levels.^{38,46} When comparing them with patients with NASH,^{38,43,47} some concordant microbial signatures were observed at the,^{42–44} family,^{42,43,45} and genus levels.^{42,44,45} The signatures overlapped in NASH and NAFLD patients. The microbiome signatures in NAFLD fibrosis have not been extensively studied. The role of the gut microbiome in NAFLD fibrosis progression was investigated in a randomized trial,⁴² and distinct microbial patterns have been found in cases with advanced fibrosis.^{42,43,48} *Bacteroides vulgatus* and *Escherichia coli* were the most abundant species,⁴² and increases of *B. vulgatus* correlate with mild-to-moderate to advanced fibrosis in NAFLD.⁴² Interestingly, in the presence of metabolic alterations, the same signature of *B. vulgatus* has been reported, with increased abundance correlated with body mass index, hemoglobin A1c level, and insulin resistance.⁴⁹ Similarly, an abundance of *E. coli* has been seen in patients with T2DM and there was a strong connection between NAFLD and metabolic disorders.⁵⁰ Dysbiosis and NAFLD seem to create a complex network and are linked to each other. This is consistently shown and in several studies,^{39,44,46,51,52} that highlighted external factors such as socioeconomic status.⁵³ *Ruminococcaceae* and *Veillonellaceae* were identified in a recent study as the main microbiota species associated with fibrosis severity in 171 Asian nonobese subjects.⁵⁴ In addition, a Finnish study including more than 6,000 patients found a strong association between the fatty liver index and a specific microbiome signature mostly belonging to order *Lachnospirales* and *Oscillospirales*.⁵⁵ Frost et al.⁵⁶ showed that fatty liver disease and diabetes mellitus, which are cofactors of the metabolic syndrome, were associated with the greatest microbiome signature variability. *Enterobacteriaceae* or *Escherichia/Shigella* were more abundant in metabolic syndrome-associated diseases. High initial alpha diversity identified the greatest microbial stability. The gut microbiome has shown prom-

ise as a predictive biomarker for various diseases, and the potential clinical validity of gut metagenomic sequencing to complement conventional risk factors for prediction of liver diseases was convincingly demonstrated in a recent study.⁵⁷ Therefore, clinical trials to modulate the gut microbiome, for instance with FMT, and improve NAFLD have been performed. Unfortunately, FMT did not improve insulin resistance or increase the hepatic proton density fat fraction, but it did improve intestinal permeability.⁵⁸ Similar effects were shown with symbiotics in NAFLD,⁵⁹ and physical exercise.^{60,61} A Mediterranean diet, restricted in processed and/or red meat and enriched with green plant-based proteins/polyphenols like green tea, and walnuts, seems to be the best strategy for intrahepatic fat loss compared with other diets. It has been shown to reduce NAFLD by half.⁶² Furthermore, disulfiram, a drug commonly used to treat chronic alcoholism, had promising results in the treatment of NAFLD.⁶³

Bacterial dysbiosis in liver cirrhosis

In patients with liver cirrhosis, there is convincing evidence that the progression of NAFLD, alcoholic liver disease, or viral hepatitis is strongly associated with gut microbiome dysbiosis. Cirrhotic microbial signatures are characterized by an increase in pathogenic taxa and a decrease in metabolically beneficial taxa.^{64–66} In multiple preclinical NAFLD and alcoholic liver disease studies, a clear association between the degree of liver disease and dysbiosis was described.^{67–71} Some human and animal studies demonstrated that the microbiome also influenced the progression from early chronic liver disease (CLD) to cirrhosis, pointing out a key role of dysbiosis in the development of end-stage liver disease.^{68,69,72,73} As mentioned above, the microbial composition in patients with advanced NAFLD and cirrhosis is characterized by a decrease of beneficial bacteria and an increase in potentially pathogenic bacteria.^{66,74} The gut microbial composition was studied in patients with cirrhosis caused by different underlying liver diseases. Some of the microbial alterations overlapped in cirrhosis of different etiologies. This suggests that features of end-stage liver disease drive the microbial alterations. An abundance of *Veillonella* or *Streptococcus* and a decrease of order *Clostridiales* are commonly found in patients with end-stage liver disease and cirrhosis.⁶⁶ The gut microbiome of patients with cirrhosis presents with a relative reduction in *Bacteroidetes* and an increase in *Proteobacteria* and *Fusobacteria*, but changes in *Firmicutes* mimicked the microbiome from healthy individuals.⁷⁵ Furthermore, there are differences at the family level, with *Streptococcaceae* and *Veillonellaceae*. *Streptococcaceae* positively and *Lachnospiraceae* negatively correlated to cirrhosis severity. Another research group has confirmed these differences in a large population of cirrhosis patients.^{64,75}

Microbial composition differs between patients with compensated or decompensated cirrhosis, which suggests that microbial alterations are more influenced by cirrhosis stage rather than by the underlying liver disease.⁷² Bacterial's overgrowth in the upper gastrointestinal tract has a pivotal role along with shifts in microbial signatures when it comes to the increase of circulating LPS levels.⁷⁶ Various studies investigated the qualitative and quantitative bacterial changes in the duodenal and salivary microbiota, comparing healthy individuals and patients with cirrhosis. Bacterial shifts in the upper gastrointestinal tract may influence the microbial signature in the lower gastrointestinal tract and might therefore have a key role in the pathophysiology of CLD as well as in the development of HCC.⁷⁷

These cirrhosis-related alterations in the microbiome are

not only evident in feces, but also in serum, saliva, small intestine mucosa, ascites, colon mucosa, and liver tissue.^{64,75,78,79} The intestinal metabolic shift in cirrhosis seems to influence a cascade of mucosal immune changes and vice versa. It is also associated with the main cirrhosis comorbidities like spontaneous bacterial peritonitis, hepatic encephalopathy, organ failure and finally death.^{64,72,80,81} The most common components of the microbiota are bacteria, but there is evidence of the importance of fungi, archaea, and viruses, especially bacteriophages.⁸² In a recent study, fungal diversity in patients with cirrhosis was linked to bacterial diversity, and suggests that fungi can affect hospitalizations in conjunction with bacterial indices.^{83,84}

Recent studies indicate that specific changes of the gut microbiota are promising markers in different stages of liver disease and liver disease progression. The findings underline the hypothesis that the microbiota is a key factor in the complex pathophysiology of NAFLD disease and disease progression from mild fibrosis to severe fibrosis, cirrhosis and finally HCC.^{42,43,48} It was already proposed to use microbiome signatures for the diagnosis of NAFLD fibrosis, but confirmation and validation in independent cohorts and across geographical regions is necessary.⁶⁵ Further studies are needed to accurately and precisely describe the constituents of the entire microbiome in liver disease. Nevertheless, studies including patients with non-NAFLD, NAFLD without advanced fibrosis, or NAFLD cirrhosis are needed to define potentially diagnostic microbial signatures (Fig. 1).^{42,71}

HCC and microbiota: preclinical evidence

Evidence that changes in the gut microbial composition participate in the development of liver disease and HCC is increasing. High levels of circulating LPS in mouse models and humans with CLD or HCC indicate the presence of an altered intestinal barrier during multiple stages of CLD and hepatocarcinogenesis.^{85–87} Functional experiments in GF mice lacking the toll-like receptor (TLR) 4 and treated with LPS had evidence of a leaky gut essentially contributes to hepatocarcinogenesis.⁸⁸ Furthermore, chronic liver inflammation and increased rates of infectious complications in end-stage liver disease, are associated with a leaky gut and increased bacterial translocation. Microbe-associated molecular pattern (MAMPs) and host pattern recognition receptors (PRRs), specifically TLRs, interact in the hepatic inflammatory cascade.⁸⁹ The development of HCC in a murine model can be triggered by chronic infusion of low-dose LPS via osmotic pumps.⁸⁸ Furthermore, the administration of dextran sulfate sodium leads to a disruption of the intestinal barrier and higher levels of systemic LPS, which worsened liver fibrosis and promoted HCC development.^{90,91} In accordance with these findings, liver inflammation, fibrosis and HCC formation in mice and rats can be suppressed via inhibition of TLR4 signaling.^{92,93} TLR4 is expressed in multiple hepatic cell lines, including hepatic stellate cells (HSCs), endothelial cells, Kupffer cells, and hepatocytes. TLR4 in hepatocytes, HSCs and Kupffer cells drive hepatic fibrogenesis and hepatocarcinogenesis.⁸⁸ Activation of the TLR4 cascade leads to NF-κB-mediated upregulation of the potent hepatomitogen epiregulin (EPR), an epidermal growth factor family member, in HSCs.^{88,94} Increased LPS levels resulting from a disrupted gut barrier have multiple cellular targets, such as Kupffer cells and HSCs, participate in hepatocarcinogenesis.

NF-κB mediated prevention of hepatocyte apoptosis,⁸⁸ has a key role in hepatocarcinogenesis and is promoted via the LPS-TLR4 pathway. Hepatocyte proliferation, reduced oxidative stress, and apoptosis in Kupffer cells can also be medi-

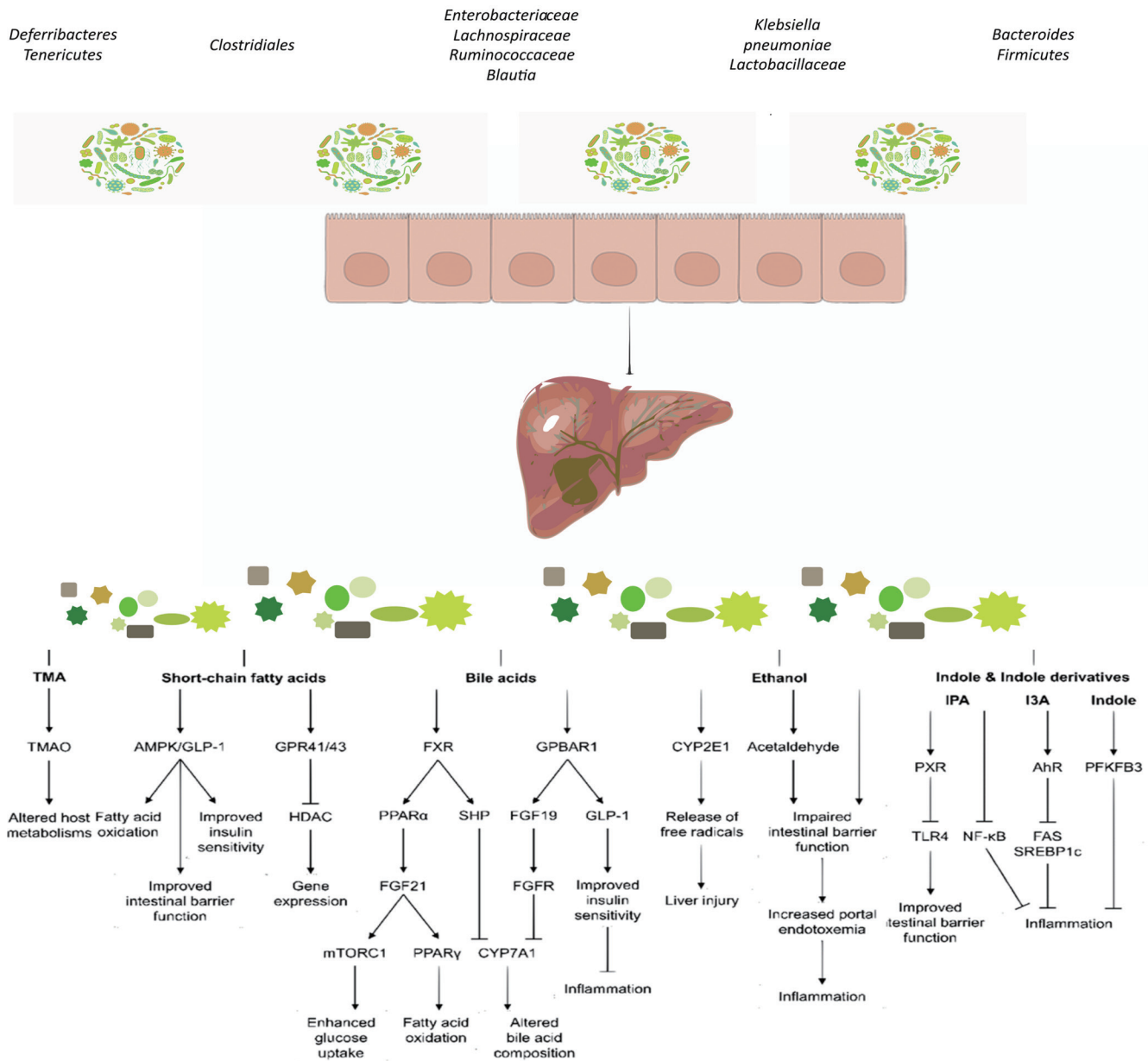


Fig. 1. Gut microbiota-derived metabolites are involved in the progression of nonalcoholic fatty liver disease. AhR, aryl hydrocarbon receptor; AMPK, AMP-activated protein kinase; CYP2E1, cytochrome P450 2E1, CYP7A1, cytochrome P450 7A1; FAS, fatty acid synthase; FGF21, fibroblast growth factor 21; FXR, farnesoid X receptor; FGF19, fibroblast growth factor 19; GPBAR1, G-protein-coupled bile acid receptor 1; GLP-1, glucagon-like peptide-1; GPR41/43, G-protein-coupled receptor 41 and 43; HDAC, histone deacetylase; I3A-indole-3-acetic acid; IPA, indole-3-propionic acid; mTORC1, mammalian target of rapamycin complex 1; NF- κ B, nuclear factor-kappa B; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PPAR α , peroxisome proliferator-activated receptor α ; PXR, pregnane X receptor; SHP, short heterodimer partner; SREBP1c, sterol regulatory element-binding protein 1c; TLR4, Toll-like receptor 4; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

ated by tumor necrosis factor (TNF) and interleukin (IL)6, where transcription and release of TNF and IL6 are activated by the LPS-TLR4 pathway.⁹³ In HCC cell lines TLR4 is activated by LPS. This trigger promotes invasiveness and epithelial mesenchymal transition in these cell lines.⁹⁵ Together, these data show that an impaired intestinal barrier via MAMP-TLR-mediated signals contributes to hepatocarcinogenesis.⁹⁶ Dysbiosis and the impaired intestinal barrier are closely related. Furthermore, in HCC there seems to be a shift toward bacterial species with an increased likelihood of translocation across the gut barrier.⁹⁷ In a murine HCC model, antibiotic

depletion of the host microflora suppressed tumor formation with a significant reduction in the number and size of HCC nodules in treated mice compared to control animals.⁹³

Furthermore, dysbiosis promotes development of HCC by altering the bile acid metabolism. In a model of NASH-associated HCC, an HFD rich in saturated fatty acids and cholesterol (STHD-01), was fed to specific pathogen-free (SPF) C57BL/6J mice. The accumulation of cholesterol and secondary bile acids caused hepatic inflammation and injury, which in turn contributed to carcinogenesis.⁹⁸ In another mouse model of HCC, tumor growth was significantly reduced by

administering probiotics, thus decreasing the number of activated Th17 cells and their production of the proinflammatory IL17. Probiotic treatment also slowed the growth of established tumors and reduced tumor size.⁹⁹

Inflammation has a key role in carcinogenesis and dysbiosis can create a proinflammatory environment that favors HCC development. Regulatory T cells (Tregs) are a subpopulation of T cells that act to suppress immune responses not only by producing anti-inflammatory IL10. The number of Tregs in peripheral blood and liver tissue has been associated with increases of *Alistipes*, *Butyricimonas*, *Mucispirillum*, *Oscillibacter*, *Parabacteroides*, *Paraprevotella*, and *Prevotella*. *Parabacteroides* are known to inhibit inflammation by inhibiting the secretion of inflammatory cytokines and promoting the release of anti-inflammatory cytokines like IL10.^{99,100} Such microbial changes are found in the feces of SPF mice having a normal spectrum of commensal microorganisms but not in microbiota-deficient mice treated with broad-spectrum antibiotics or in GF mice.⁹⁹ In a murine model of streptozotocin-high fat diet (STZ-HFD) induced NASH-HCC, *Akkermansia muciniphila*, *Bacteroides fragilis*, *Parabacteroides distasonis*, and *Alistipes shahii* were significantly enriched.^{101,102} *Alistipes shahii* tends to modulate the gut by ablating tumor growth and *Bacteroides fragilis* acts by stimulating Treg cells via induction of IL10.¹⁰³

HCC and microbiota: human evidence

There are only a few clinical trials correlating microbiota with HCC, and they found different alterations of the gut microbiota in patients with HCC. In one study the presence of HCC in cirrhotic patients was associated with increased fecal *E. coli*, and intestinal overgrowth of these bacteria was thought to have contributed to hepatocarcinogenesis.¹⁰⁴ In a more recent study, HCC patients with hepatitis B virus/hepatitis C virus infection harbored increased populations of potential pro-inflammatory bacteria (*Escherichia*, *Shigella*, *Enterococcus*) and had reduced populations of *Faecalibacterium*, *Ruminococcus*, and *Ruminoclostridium*, which resulted in a decrease of potentially anti-inflammatory short-chain fatty acids (SCFAs).¹⁰⁵ Patients with NAFLD-related cirrhosis and HCC, NAFLD-related cirrhosis without HCC, and healthy controls were also compared. Plasma IL8, IL13, chemokine ligand (CCL) 3, CCL4, and CCL5 were increased in the HCC group and were associated with activation of circulating monocytes. The fecal microbiota of patients with cirrhosis had a higher abundance of *Enterobacteriaceae* and *Streptococcus* and a reduction in *Akkermansia*. *Bacteroides* and *Ruminococcaceae* were increased in the HCC group and *Bifidobacterium* was reduced.¹⁰⁶ In another study 75 patients with early HCC were compared to patients with cirrhosis and healthy controls. In this investigation, fecal microbial diversity increased from cirrhosis to cirrhosis with early HCC. Specifically, phylum *Actinobacteria* and 13 genera including *Gemmiger* and *Parabacteroides* were increased in early HCC. The microbiota pattern showed an increase in LPS-producing species, such as *Parabacteroides*, and a decrease in butyrate-producing species, such as *Actinobacteriae*, compared with healthy individuals. Therefore, current evidence suggests that a specific microbiota pattern for patients with HCC might exist.¹⁰⁷

Gut-derived metabolites and pathways in NAFLD

Numerous studies have defined metabolomic signatures associated with NAFLD.¹⁰⁸ The signatures include molecules produced by bacteria such as LPS100 or SCFAs. The SCFAs, butyrate, propionate and acetate,³⁹ and products de-

rived from bile acid metabolism act on farnesoid X receptors (FXRs) within the liver or the intestine.¹⁰⁹ Changes in the composition of these metabolites are thought to be involved in the pathophysiology of liver injury (Fig. 1). Multiple studies found that these metabolites have an effect in obesity and metabolic alterations in T2DM. The activation of insulin resistance pathways can be promoted by LPS in obesity.¹¹⁰ Furthermore, SCFAs may increase weight gain by energy harvesting despite their benefits in metabolic health and disease.¹¹¹ SCFAs were found to be increased in fecal samples of obese individuals compared with a healthy cohort.¹¹²

TMAO

Higher liver fat accumulation and modified gut bacteria composition can be evoked by dietary choline reduction in mice.¹¹³ In a murine model of HFD-induced NAFLD, standard choline levels led to a decrease of systemic phosphatidylcholine along with NAFLD progression.¹¹⁴ The gut microbiota metabolizes dietary choline into trimethylamine (TMA), and the hepatic enzyme FMO3 converts TMA to TMAO.^{115,116} Increased TMAO levels are a biomarker of cardiovascular events and are positively correlated with increased abundance of fecal *Deferribacteres* and *Tenericutes* in murine models.¹¹ Increased urinary levels of TMA and TMAO were associated with NAFLD severity in a murine model.¹¹⁴ However, other studies reported that dietary intake of choline and phosphatidylcholine were associated with increased TMA and TMAO production.^{115,116} These divergent data suggest that the role of TMA and TMAO in NAFLD is not fully understood and needs further investigation. One of the most likely hypotheses is that modification of the microbiota metabolism leads to reduced host choline bioavailability with methylamine production and as its urinary excretion.¹¹⁴

Bile acids

The gut microbiota deconjugates primary bile acids into secondary bile acids. Both, primary and secondary bile acids have endocrine functions in multiple metabolic pathways through different receptors.¹¹⁷ For example, primary bile acids facilitate the absorption of dietary fat and fat-soluble molecules and are involved in cholesterol metabolism.^{117,118} The G-protein-coupled bile acid receptor 1 (TGR1) participates in energy, glucose, and lipid metabolism. Secondary bile acids are the preferential ligands of TGR1. The gut microbiota interacts with bile acid pathways on multiple levels. In regulating the secondary bile acid metabolism, the microbiota reduces FXR inhibition, which in turn reduces hepatic synthesis of lipids.¹¹⁹ Hence, its composition influences the homeostasis and bile acid composition because it deconjugates, dehydrogenates, and dehydrates bile acids, which promotes NAFLD and NASH progression, as suggested by various studies.¹⁰⁹ NAFLD is associated with decreased bile acid levels via TMAO production. CYP7A1 and CPY27A1, two enzymes involved in bile acid metabolism, are inhibited by TMAO. TMAO thus induces a decrease of total bile acids.¹²⁰ Accordingly, patients with advanced cirrhosis have a gut microbiota composition that decreases the conversion of bile acids including abundant *Enterobacteriaceae* and relatively less abundant *Lachnospiraceae*, *Ruminococcaceae*, and *Blautia*.^{121,122}

Ethanol

Gut microbiota-derived ethanol production may also participate in NAFLD pathophysiology. In children with NAFLD, the gut microbiota contains more ethanol-producing bacteria than obese or healthy children.⁴⁴ A recent study assayed ethanol concentration in the peripheral blood and in the por-

tal vein while fasting and 120 min after a mixed meal test. In the presence of NAFLD, the ethanol concentration in the median portal vein was increased by 187 times, and continued to increase with disease progression.¹²³ The abundance of *Lactobacillaceae* was positively correlated with postprandial peripheral blood ethanol concentrations in a prospective study.¹²³ The results of these studies suggest that microbial ethanol production is associated with gut microbial disturbances acts as a hepatic toxin in the progression of NASH and NAFLD.³¹ A study in mice and humans identified *Klebsiella pneumoniae* as an ethanol-producing bacteria in the absence of any alcohol consumption.¹²⁴

SCFAs

SCFAs have a key role in increasing liver triglyceride levels, which serve as energy storage and promote weight gain.¹²⁵ In patients with NAFLD or NASH fecal levels of SCFAs were increased compared to healthy controls, going along with an increase in SCFAs producing bacteria. Accordingly, patients with NASH had reduced levels of resting regulatory T cells (CD4+, CD45RA+, CD25+) and an increased Th17/Treg ratio in peripheral blood, previously recognized as systemic immunological features of NASH. Decreased T cells were found to be associated with increased fecal SCFAs and changes in the microbial signature.¹²⁶ However, SCFAs pathways are not fully understood. For example, the benefits of SCFAs include activation of G-protein-coupled receptor 43 (GPR43), which decreases hepatic T-cell infiltration and production of pro-inflammatory cytokines. Accordingly, GF and GPR43^{-/-} mice express lower levels of SCFAs together with increased circulating T cells and colonic inflammation, a feature usually seen in NASH.¹²⁷

Products of microbial protein fermentation

Some strains of the gut microbiota that ferment proteins may also have a role in NAFLD progression and proinflammatory pathways.^{32,128} Ammonia, hydrogen sulfide and phenolic compounds be involved in NAFLD progression by translocating proinflammatory compounds toward the enterohepatic pathway. A murine model of GF mice suggested there was a connection between products of gut microbial protein fermentation and NAFLD.³⁴ GF mice fed an HFD and colonized with microbial strains from diabetes model mice developed hepatic macrovesicular steatosis. The control animals received microbiota from normoglycemic mice and developed only low-level steatosis. In the mice with macrovesicular steatosis, cecal concentrations of the branched-chain fatty acids isovalerate and isobutyrate were present and mainly derived from the microbial fermentation of branched-chain amino acids (BCAAs). Furthermore, insulin resistance and leptinemia were detected in those mice.³⁴ On the other hand, the microbial metabolite indole, which is derived from the aromatic amino acid L-tryptophan by the microbiota-associated enzyme tryptophanase, preserved gut barrier dysfunction and decreased inflammation in the gut.^{129,130} Furthermore, orally administered indole reduced LPS-linked upregulation of pro-inflammatory cytokines in this murine model and proteins active in the hepatic NF- κ B pathway were down-regulated in these experiments.¹³¹ However, there is a need to replicate the results of this study into a setting with patients with NAFLD, to determine potential beneficial effects. In women with morbid obesity, but without evidence of T2DM, fecal metagenome and the hepatic transcriptome were analyzed and the plasma and urine metabolomes were studied.³⁸ In this setting, the grade of steatosis was significantly associated with dysregulation of microbial aromatic amino acid

and BCAA metabolism. Another study identified a product of phenylalanine catabolism, phenylacetic acid, as a driver of steatosis progression in a murine model and in human hepatocytes. BCAA utilization in the tricarboxylic acid cycle increases lipid accumulation in the liver, potentially via phenylacetic acid.³⁸ The development of hepatic steatosis seems fueled by proteolytic fermentation products, as shown in these studies. Accordingly, LPS and TMAO were identified as key players in the development in steatosis in this study. The findings support the microbial multihit hypothesis in metabolic diseases.

Preclinical and clinical studies in the last decade support the key role of the gut microbiome/metabolome in NAFLD. These studies suggest that a specific microbial signature is associated with NAFLD and provide an opportunity to uncover important mechanistic insights (Table 1).^{26,70,126,132–40} Ongoing research guided by previous results might identify distinct microbiome signatures and new bacterial metabolites as key players in liver disease. Because the gut microbiota plays this role, research was focused on the intestinal microbiota. It offered opportunities as well as challenges in understanding the pathogenesis and developing treatment options of NAFLD. Most published studies focused on 16sRNA sequencing with low resolution and limited to the genus level. With metagenomics sequencing techniques NAFLD-related microbes at a strain level can be identified, offering functional information of the gut microbiota in this expanding disease. Future research will aim to find direct causal relationships of NAFLD with intestinal microbial changes in murine and human studies. These future data should aim at microbiota-targeted therapeutics. The microbiome–host interaction in the development and progression of NAFLD, despite enormous advances in correlating microbial intestinal changes with NAFLD, remains largely elusive. Therefore, further studies to understand host-microbial interactions in patients with NAFLD are needed. These novel bacterial-derived pathways in disease progression will uncover novel treatment strategies.

NAFLD, as a multifactorial disease, needs novel clinical approaches. Zeevi *et al.*¹⁴¹ reported that individual glycemic responses can be predicted by combining personal, dietary, and microbiome characteristics, successfully targeting personalized nutrition. A individualized precision medicine based on diet and intestinal microbiota profiles could facilitate risk stratification and diagnostic accuracy, predict variable clinical phenotypes, and hopefully the therapeutic response of NAFLD. Gut-derived metabolites and metabolomic signatures have a pivotal role in NAFLD, and the gut microbiota may thus be a promising marker in diagnosis and progress prediction in NAFLD. Influencing gut microbial alterations will be a novel therapeutic strategy in the NAFLD pandemic in the future. To summarize, the underlying mechanisms for development and progression of NAFLD are multifactorial and very complex. Gut dysbiosis has a key role that is supported not only by preclinical studies, but also by large clinical datasets. However it is still not clear which bacterial strains are the big players in this context.

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Table 1. Changes in the gut microbiome and metabolites in different stages of NAFLD, including potential therapeutic options

Patient cohort	Control cohort	Microbiome increase	Microbiome decrease	Metabolites	Potential therapy
NASH ¹²⁶	NAFLD	<i>Eubacterium biforme</i> , <i>Fusobacteria</i> , <i>Fusobacteriaceae</i> , <i>Fusobacterium</i> , <i>Prevotella</i>	α -diversity↓,	Stool: propionate↑, butyrate↑, acetate↑	Tributylin. (Butyrate prodrug) ¹³²
NASH ¹²⁶		<i>Fusobacteriaceae</i> ; <i>Prevotellaceae</i>		Stool: propionate↑, butyrate↑, acetate↑	Tributylin ¹³²
NAFLD ¹²⁶		<i>Prevotellaceae</i>		Stool: propionate↑, butyrate↑, acetate↑	Tributylin ¹³²
NAFLD G3 ²⁶	NAFLD G0		β -diversity, <i>Christensenellaceae</i> , <i>Coprococcus</i> , <i>Odoribacter</i> , <i>Odoribacteraceae</i> , <i>Oscillospira</i> , <i>Ruminococcaceae</i> ,	Not available	
NAFLD cirrhosis ⁷⁰		<i>Catenibacterium</i> , <i>Faecalibacterium prausnitzii</i> , <i>Gallibacterium</i> , <i>Megasphaera</i> , <i>Mogibacterium</i> , <i>Rikenellaceae</i> , <i>Streptococcus</i> , <i>Peptostreptococcaceae</i>	<i>Bacillus</i> ↓, <i>Lactococcus</i> ↓,	Not available	
Lean NAFLD ¹³³		<i>Dorea</i>	<i>Christensenellaceae</i> , <i>Marvinbryantia</i>	Total BA↑, total primary BA↑, total secondary BA↑, CDCA↑, DCA↑	FXR; ^{134–136} NGM282 ¹³⁷
Increased NAFLD severity ¹³⁸		<i>Actinobacteria</i> , <i>Actinomycetaceae</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Lachnospiraceae</i> <i>Proteobacteria</i> ,	α -diversity <i>Bacteroidaceae</i> , <i>Bacteroidetes</i> ↓	Total BA↑, primary conjugated BA↑, GCA↑, secondary conjugated BA↑; stool: total BA↑, DCA↑	FXR; ^{134–136} NGM282 ¹³⁷
NAFLD-HCC ¹³⁹		<i>Enterobacteriaceae</i> , <i>Proteobacteria</i>	<i>Erysipelotrichaceae</i> , <i>Oscillospiraceae</i>	Stool: oxaloacetate↑, acetylphosphate↑, isocitrate↑, acetate↑, butyrate↑, formate↑ Serum: butyrate↑, propionate↑	Tributylin ¹³²
NAFLD-cirrhosis ¹³⁹		<i>Eubacteriaceae</i>	<i>Coriobacteriaceae</i> , <i>Muribaculaceae</i> , <i>Odoribacteraceae</i> , <i>Prevotellaceae</i>	Not available	
NAFLD-HCC ¹³⁹	NAFLD cirrhosis	<i>Bacteroides caecimuris</i> , <i>Veillonella parvula</i>		Not available	
Nonobese F2-4 fibrosis ¹⁴⁰		<i>Babjeviella inositovora</i> , <i>C. albicans</i> , <i>Cyberlindnera jadinii</i> , <i>Mucor sp</i> , <i>Salinispora sp.</i>		Not available	

BA, bile acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic, HC, healthy control; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SS, simple steatosis.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Design and writing of the paper (ME, HT), provision of critical feedback (HT), and manuscript preparation (CG, ME, FG, HT). All authors have made a significant contribution to this study and have approved the final manuscript.

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