# Gut–Liver Axis in Nonalcoholic Fatty Liver Disease: the Impact of the Metagenome, End Products, and the Epithelial and Vascular Barriers

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**Abstract**

Nonalcoholic fatty liver disease (NAFLD) is a systemic, dynamic, heterogeneous, and multiaxis entity, the pathogenesis of which is still uncertain. The gut–liver axis is regulated and stabilized by a complex network encompassing a metabolic, immune, and neuroendocrine cross-talk between the gut, the microbiota, and the liver. Changes in the gut–liver axis affect the metabolism of lipids and carbohydrates in the hepatocytes, and they impact the balance of inflammatory mediators and cause metabolic deregulation, promoting NAFLD and its progression to nonalcoholic steatohepatitis. Moreover, the microbiota and its metabolites can play direct and indirect roles in gut barrier function and fibrosis development. In this review, we will highlight findings from the recent literature focusing on the gut–liver axis and its relation to NAFLD. Finally, we will discuss the impact of technical issues, design bias, and other limitations on current knowledge of the gut microbiota in the context of NAFLD.

**Keywords**
- Microbiota
- Microbiome
- NAFLD
- NASH
- Gut–Liver axis

Nonalcoholic fatty liver disease (NAFLD) is a systemic, dynamic, heterogeneous, and multiaxis entity. NAFLD has been linked to extrahepatic malignancies such as chronic kidney disease, cardiac disease, sleep apnea syndrome, polycystic ovary syndrome, inflammatory disorders, brain aging, and cognitive impairment.[1–3] It is a dynamic entity and progression from steatosis to steatohepatitis and fibrosis seems to occur more often than regression, but both changes have been reported.[4,5] Moreover, it is a heterogeneous disease with several phenotypes depending on if people are obese or lean, have metabolic syndrome or are metabolically healthy, and have type 2 diabetes (T2D) or do not.

The pathogenesis of NAFLD remains poorly understood, but it is known to be related to multiple insults that occur synergistically, including triglyceride accumulation, insulin resistance, de novo lipogenesis, oxidative stress and mitochondrial dysfunction, altered mechanisms of apoptosis, and autophagy promoting inflammation and fibrosis.[6,7] Accumulating evidence has also revealed the prominent role of genetic variants, environmental factors, and changes in the gut microbiota (GM) under complex interactions that result in altered lipid metabolism and accumulation within the hepatocytes. Moreover, the microbiota plays a role in regulating the balance between pro- and anti-inflammatory signals, which may contribute to the progression to nonalcoholic steatohepatitis (NASH).[8–10]

Nevertheless, there is still an urgent need to understand the pathophysiologic implications of the altered GM in NAFLD, which could help improve diagnostics and identify patient subgroups and new targets in the era of personalized medicine. Throughout this review, we describe recently uncovered evidence for the role of the gut microbiome and its metabolites in the pathophysiology of NAFLD.

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## Gut–Liver Axis

### Anatomical Characteristics of the Gut–Liver Axis

The term gut–liver axis refers to the relationship between the gut, its microbiota, and the liver, which is a consequence of the close anatomical and functional bidirectional interaction through the biliary tract and the portal to the systemic circulation. The liver receives through the portal blood, in addition to blood and energy supply, gut-derived toxic elements, including bacteria with their metabolites and subproducts (pathogen-associated molecular pattern [PAMP] and damage-associated molecular pattern), which either actively or passively manage to cross from the gut barrier, reach the systemic circulation, and play a role in liver injury.[11] Conversely, the liver communicates with the intestine by releasing bile acids (BAs) and other metabolites into the biliary tract and systemic circulation, which in turn will control metabolic functions and the composition of the microbiota.[12] This relationship is regulated and stabilized by a complex network encompassing a metabolic, immune, and neuroendocrine cross-talk between the GM and the liver.[13]

### Gut Microbiota

The GM is a complex ecosystem consisting of bacteria, archaea, protists, fungi, and viruses, which plays important roles in physiological and pathological conditions in the human body. The GM exists in a precise and complex symbiosis among single organisms and with the human body, with its composition being shaped by environmental and host-related factors such as the diet, drugs, physical activity, circadian rhythms, and geography. Although the microbial profile varies among individuals, the composition and relative abundance of species are comparable between healthy people. Despite a wide diversity, four main phyla dominate: *Firmicutes, Bacteroidetes, Actinobacteria,* and *Proteobacteria,* the first two being 90% of the total. A rich and diverse microbiota has been characterized as a healthy microbiota, which is able to better withstand external threats. Several factors that challenge the composition of this ecosystem, such as the diet or antibiotic use, may promote dysbiosis, gut barrier dysfunction, and disturbances of the host–microbe homeostasis, which have been related to metabolic inflammation and fueling of metabolic perturbations, and are relevant events in T2D and NAFLD.[14] It is now acknowledged that this control has a role in NAFLD pathogenesis, as animal studies have suggested that the GM is involved in the development of adipose tissue and hepatic steatosis.[15] Indeed, high-fat diet (HFD)-fed germ-free mice accumulated less hepatic lipids than conventionally housed mice,[16] and fecal microbiota transplantation from donor mice exhibiting metabolic disorders led to NAFLD development in germ-free recipient mice.[9] Furthermore, another study found a positive correlation between the abundance of *Lactobacillus gasseri* and *Lactobacillus taiwanensis* and the accumulation of lipid droplets in the liver.[17] In humans, an intervention study consisting in 6 weeks of a low-choline diet found a correlation between microbiota changes, such as an increase in *Gammaproteobacteria* and a decrease in *Erysipelotrichia,* and the hepatic lipid content.[18] Of note, it has been suggested that the changes in microbiota composition are not stable during the progression of NAFLD, making it difficult to validate disease-specific microbiota signatures.[19]

The GM also contributes to the gastrointestinal health by controlling the integrity of the intestinal epithelial and vascular barriers as well as the mucus layer.[20,21] Moreover, the GM can contribute to liver fat accumulation through direct and indirect effects on the host, including appetite regulation, energy extraction from the diet, energy expenditure, and lipid handling through effects on insulin sensitivity.

### Metabolites Influencing Gut–Liver Axis

The GM contributes significantly to the pool of metabolites present in the human systemic circulation (up to 10%), featuring a systemic bioactive effect with both inflammatory and metabolic functions.[22] Thus, the liver is continuously challenged by the metabolic stress induced by bacteria and their metabolites, even when in a healthy state. However, it is not yet clear whether liver diseases such as NAFLD and T2D might affect the capacity of the liver to respond to bacteria and their byproducts. Other relevant metabolites are listed in ►**Table 1**.

#### Short-Chain Fatty Acids

Complex carbohydrates, such as fiber and resistant starch, and less commonly peptides are digested by different GM species leading to the release of short-chain fatty acids (SCFAs), an essential energy source for both the microbiota and the host (►**Fig. 1**). Acetate, propionate, and butyrate (also valeric and caproic acids) are the main SCFAs produced in the colon.[23] Nevertheless, a certain amount is transported to the bloodstream through the transporters MCT-1 and SMCT-1, reaching the liver via the portal vein. Once in the liver, they can enter the tricarboxylic acid cycle and be used as an energy source, but they can also function as signaling molecules by binding G-protein-coupled receptors such as GPR41, GPR43, and GPR109A.[24] Indeed, they have many bioactive roles, regulating lipid and carbohydrate metabolism and controlling gut immunity and microbiota homeostasis.[25]

SCFAs represent an additional link between GM, obesity, insulin resistance, and NAFLD, since intestinal recognition of SCFAs promotes the release of peptide YY (PYY) and glucagon-like peptide-1 (GLP1) through the activation of Ffar2 and Ffar3 receptors and AMP-activated protein kinase (AMPK) signaling.[26,27] They also increase the energy expenditure through the sympathetic nervous system; recent data from animal studies reflected an upregulation of thermogenesis genes in the liver and brown adipose tissue after stimulation with acetate and butyrate.[28] Moreover, in the skeletal muscle SCFAs lead to increased oxidation of fat and decreased lipogenesis through AMPK signaling and the enzyme fatty acid synthase.[29] They can also modify gene expression, since butyrate (and to a lesser extent propionate and acetate) can act as histone deacetylase inhibitors regulating, among others, genes involved in the synthesis of cholesterol.[30]

 

**Table 1** Effect of other microbial metabolites on NAFLD via the gut–liver axis

| Metabolites | Bacterial groups | Biological functions |
|---|---|---|
| Branched-chain amino acids | *Clostridium, Fusobacterium, Bacteroides, Actinomyces, Propionibacterium, Peptostreptococci* | Promote lipid metabolism and enhance intestinal barrier function.[159,160] |
| Indoles (tryptophan derivatives) | *Prevotella, Bacteroides, Fusobacterium, Escherichia, Clostridium* | Enhance tight junctions, modulate GLP-1 secretion, reduce liver inflammation and metabolic alterations induced by LPS, regulate lipogenesis mediated by cytokines and FFAs on hepatocytes[131,161,162] |
| Vitamins | *Bacteroidetes, Fusobacteria, Proteobacteria* | Dietary and microbiota-modified vitamins can strengthen innate immunity, regulate cell proliferation, and influence GM composition. Vitamin E and D have been proposed as treatments for NAFLD.[66,163] |
| Carotenoids and phenolic compounds | *Akkermansia muciniphila, Barnesiella* | GMs produce and improve the bioaccessibility of dietary phytonutrients with antioxidant and anti-inflammatory properties. Maintenance of gut homeostasis through agonism of AHR and PXR.[164,165] |
| Gases | Wide | Intestinal gases such as methane, hydrogen sulfide, and nitric oxide influence gut homeostasis, motility, and may act as inflammatory mediators.[166] |
| Neurotransmitters | *Enterococcus, Bifidobacterium, Bacillus, Escherichia, Streptococcus, Lactobacillus* | Neuroactive compounds (serotonin, dopamine, GABA, noradrenaline) can regulate the reward system, cognition, behavior, and motility. Microbes can also stimulate vagal signaling through GABAergic innervation and the HPA-axis via stress hormone regulation.[167] |

Abbreviations: AHR, aryl hydrocarbon receptor; FFA, free fatty acid; GABA, gamma aminobutyric acid; GM, gut microbiota; HPA, hypothalamic pituitary adrenal; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; PXR, pregnane X receptor.

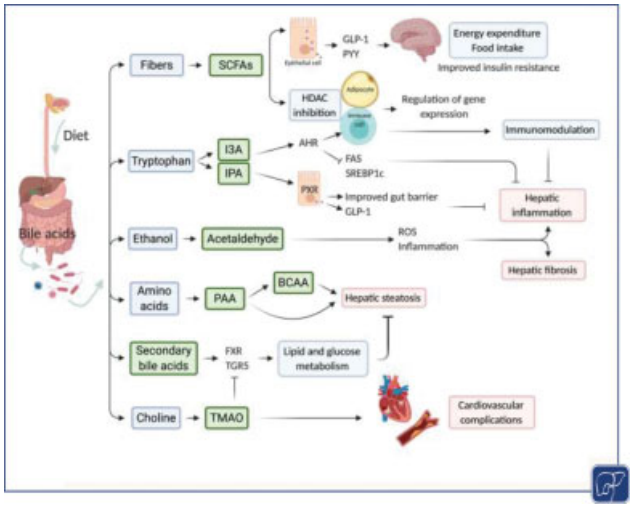


**Fig. 1 The role of relevant microbiota-derived metabolites.** Food components along with bile acids transformed by gut microbiota into active molecules that can elicit beneficial or detrimental responses in different organs of the body, contributing to the clinical phenotype.

Patients with T2D have a reduced abundance of butyrate-producing bacteria.[31] Recent evidence has shown an association between NASH and low-fiber diet, whereas NAFLD patients fed a high-fiber diet showed improved alanine aminotransferase, aspartate aminotransferase, and cholesterol levels, possibly through promotion of the abundance of butyrate-producing bacteria.[32,33] Moreover, direct supplementation with SCFAs improved diet-induced hepatic steatosis in a mouse model.[34] In a human study, propionate supplementation reduced weight gain and fat accumulation in the liver while improving insulin sensitivity.[35] However, obese patients had higher levels of propionate in the stool, which suggested that either its production was increased or its absorption is disturbed.[36] Further studies are needed to validate these observations and better elucidate the role of SCFAs in the pathogenesis of NAFLD.

## Bile Acids

BAs are direct intermediaries in the gut–liver communication. Primary BAs (mainly chenodeoxycholic acid and cholic acid) are synthesized as glycine or taurine conjugates from cholesterol in the liver. They are secreted and stored in the gallbladder and then released in the duodenum after food intake where they play an essential role in cholesterol metabolism, lipid digestion, and the absorption of fat-soluble vitamins.[37] The amount of the mainstream pool of BAs depends on the enterohepatic circulation in a two-way interaction, as the GM affects BA metabolism and the BA affects the GM composition. In this respect, the size and composition of the BA pool are controlled by the GM via the biotransformation of primary into secondary BAs (mainly deoxycholic acid and lithocholic acid), through several modifications: oxidation/epimerization, deconjugation, esterification, 7-dehydroxylation, and desulfation.[38] These processes can be disrupted in NAFLD patients due to dysbiosis. Specifically, a decrease in bacterial groups capable of triggering these transformations such as *Ruminococcaceae*, *Lachnospiraceae*, and *Blautia* (all having 7α-dehydroxylating activity) have been found in NAFLD cirrhosis fecal samples.[39] Moreover, NAFLD patients show a higher ratio of hydrophobic and

 

cytotoxic BA species with increased levels in the serum, urine, and liver tissue.[40]

Recent work has also shown that BAs also represent signaling molecules that influence metabolism in the host by binding nuclear and membrane receptors.[41] At least four nuclear receptors, ligand-activating transcription factors, recognize BAs and regulate the intestinal physiology by controlling gene expression: nuclear receptor subfamily 1 group I member 2, vitamin D3 receptor, nuclear receptor subfamily 1 group I member 3, and the most studied BA receptor (also known as farnesoid X receptor or FXR). The FXR participates in the crosstalk between the host and GM through the modulation of enterohepatic BA circulation, as it modulates the synthesis of BAs both in the intestine and the liver (in a tissue-dependent manner via FGF19 or SHP, respectively).[42] Additionally, a protective role for FXR in NAFLD has been studied, since its activation has been known to decrease triglyceride levels and thus suppress the synthesis and uptake of fatty acids in the liver. Moreover, it has demonstrated a major role in suppressing the mucosal immune response and in modulating glucose metabolism (i.e., reducing insulin resistance and gluconeogenesis, and increasing glycogenesis).[43] Activation of FXR would also protect against bacterial overgrowth, gut permeability, and bacterial translocation.[44] Thus, bacterial translocation from the gut might further decrease FXR activation in the liver, leading to decreased activity of the bile salt export pump. However, contradictory results have been obtained when using FXR-deficient mice, which are resistant to diet-induced obesity. One possible explanation for these findings could be found in the role of intestinal FXR and GM in regulating this process.[45–47]

Conversely, three major membrane receptors are known to interact with BAs: muscarinic acetylcholine receptor M3, sphingosine-1-phosphate receptor 2, and the G-protein-coupled BA receptor 1 (also known as TGR5). TGR5 activation promotes differentiation of intestinal L cells, a type of enteroendocrine cell responsible for the secretion of GLPs and PYY.[48] TGR5 signaling also increases colonic motility and it has been related to the anti-inflammatory response. Indeed, *Tgr5* knockout mice show a constipated phenotype with a decrease in the water content of the stool, an effect mediated by the effectors 5-HT and CGRP.[49] Furthermore, TGR5 activation by secondary BAs enhances energy expenditure in skeletal muscle and brown adipose tissue through increased thermogenesis.[50,51]

Thus, the reduction of secondary BAs ascribed to dysbiosis lowers the activation of FXR and TGR5 in the ileum, leading to bile salt accumulation, altered glucose and lipid homeostasis, gut permeability, bacterial overgrowth, and translocation, all of which contribute to liver disease progression.

### Endogenous Ethanol

Ethanol is a microbial metabolite that is constantly produced by saccharolytic fermentation and microbial cross-feeding even in the absence of alcohol consumption. The amount of ethanol produced depends on the carbohydrates consumed with the diet and it has been shown that obese NASH patients have a greater abundance of ethanol-producing bacteria in the feces as well as increased levels of ethanol in the circulation and breath compared with obese or healthy controls.[52–54] Previously, Cope et al demonstrated that *ob/ob* mice have a higher concentration of alcohol in breath that could be reduced after antibiotic treatment.[55] Additionally, an upregulation of the three major hepatic alcohol metabolizing pathways has been reported both in pediatric and adult NAFLD patients.[56,57] Although some researchers suggested that ethanol levels are increased due to insulin-dependent impairments of alcohol dehydrogenase (ADH) activity in the liver, recent evidence points to a role for microbiota-derived ethanol in the development of NASH.[58,59]

In addition to causing triglyceride accumulation in the liver, alcohol aggravates the inflammation and oxidative stress and, when it is metabolized by the ADH, produces acetaldehyde. CYP2E1 then converts it into acetate but this pathway can be saturated followed by acetaldehyde accumulation, which is toxic even in small quantities. The damage produced could be involved in NAFLD progression through the following: (1) direct toxicity on hepatic cells, (2) impairment of gut barrier function by downregulation of tight junction expression and dissolution of the lipids in the mucin layer, therefore resulting in the translocation of bacterial products into the systemic circulation, and (3) via nuclear factor kappa B (NF-kB) signaling pathways in peripheral cells.[60–62] In addition, it has been associated with changes in lipid metabolism in the liver: increased de novo lipogenesis, decreased fatty acid oxidation, and defective export of very low-density lipoprotein (VLDL) particles.[63,64] Moreover, this compound can induce an inflammatory and adaptive immune response by downregulating the expression of antimicrobial peptides in the gut. Recent studies have explored the role of α-defensin 5 and cathelicidin, antimicrobial peptides, in the suppression of lipid accumulation and the resolution of hepatic steatosis.[65,66] Future studies might confirm whether patients with NAFLD should modify their dietary patterns by replacing certain indigestible carbohydrates with others that do not increase ethanol levels and avoid ethanol-producing bacterial overgrowth.

### Choline/Trimethylamine

The nutrient choline is mainly obtained from the diet being stored and used in the liver for the biogenesis of phosphatidylcholine and for maintaining the S-adenosyl methionine cycle. Choline is essential for VLDL production and its deficiency can lead to NAFLD, causing the deposition of fatty acids and cholesterol, oxidative stress, and alterations in cytokine production[67]; thus, dietary choline deficiency has been linked to liver disease for a long time and is commonly used to induce NAFLD in animals. Moreover, the deletion of genes involved in choline metabolism also leads to NAFLD.[68]

The GM converts choline to methylamines (e.g., *Escherichia coli*, *Desulfovibrio desulfuricans*) and phosphatidylcholine, the demand for which can be increased in the context of NAFLD-associated bacterial overgrowth, lowering the availability of choline. Additionally, trimethylamine reaches the liver via portal vein, where it is oxidized to trimethylamine N-oxide

 

(TMAO) by hepatic flavin-containing monooxygenases.[69] TMAO has been found to contribute to the risk for atherosclerosis by reducing reverse cholesterol transport, promoting changes in BAs, and activating the inflammatory response to mediate foam cell formation; it also contributes to other metabolic disorders such as T2D and NAFLD.[70,71] A clinical study found that the severity of NAFLD was independently correlated with high serum levels of TMAO in Chinese adults.[72] Another study reported a correlation between serum levels of TMAO and the body mass index, also suggesting that a specific cut-off of TMAO levels could help to assess the risk for NAFLD.[73] Moreover, animal studies have shown that TMAO supplementation in HFD mice induces impaired glucose tolerance, obstructs the hepatic insulin signaling, and triggers adipose tissue inflammation. Furthermore, the inhibition of trimethylamine production revealed favorable effects in the study of cardiometabolic diseases.[74] Therefore, future studies are expected to explore and confirm the effects of trimethylamine on NAFLD.

## Microbiome-Induced Liver Inflammation

Current understanding of the pathophysiology of liver diseases relies on proinflammatory changes that take place in the host. Low-grade chronic inflammation is a hallmark of metabolic disorders such as obesity, T2D, and NAFLD, which contribute to patient outcomes within (liver cirrhosis) and outside the liver (atherosclerosis and cardiovascular complications). Several studies have revealed increased inflammatory molecules such as cytokines, acute-phase proteins, and adhesion molecules in the circulation of NASH patients.[75–77] This metabolic inflammation can be seen as a sterile process, as noninfectious factors, such as lipids, drive a low-grade inflammatory state via toll-like receptor (TLR) 4 in peripheral blood monocytes.[78] Moreover, lipotoxicity in the liver, muscle, or adipose tissue can trigger off a metabolic dysregulation leading to endoplasmic reticulum and oxidative stress, key drivers of the inflammatory response.[79,80] In a similar way, hypoxia in adipose tissue triggers inflammatory pathways while suppressing anti-inflammatory adipokine production.[81]

Conversely, dysbiosis and increased intestinal permeability facilitate the translocation of microorganisms and their subproducts including cell-wall components (endotoxins or B-glucan) or DNA (►**Fig. 2**). Any antigen that crosses the gut barrier, generated either from pathogenic microorganisms or directly from the diet, even small quantities of PAMPs (lipopolysaccharides [LPS], peptidoglycans or flagellin, ADP-heptose, and lipoteichoic acid), would lead to a proinflammatory response by innate and adaptive immune cells.[82–84] These molecules can be detected by tissue-resident dendritic cells and macrophages via TLRs and nod-like receptors (NLRs), which become activated and trigger the production of inflammatory cytokines and chemokines after activation of NF-kB.[13,85] This cascade of events influences hepatic stellate cell activation and causes bone-marrow-derived cells to infiltrate the injured liver.[86] Moreover, immune cells may be primed in the gut and migrate to other organs, such as the liver and the adipose tissue, to modulate metabolic inflammation.[87]

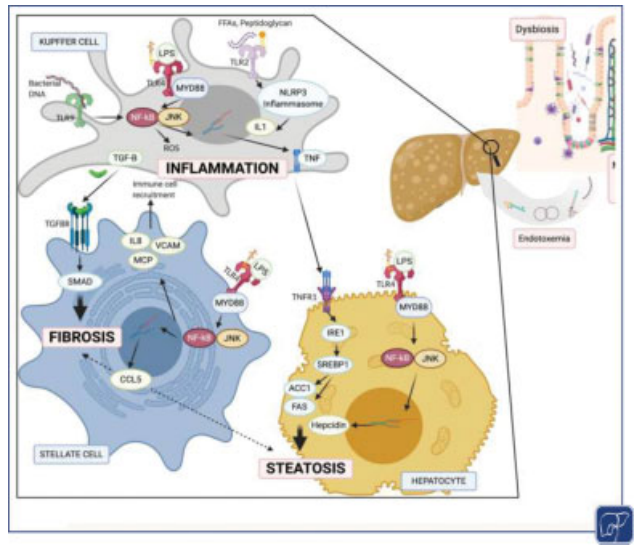


**Fig. 2 The mechanism of liver cell damage in NAFLD progression induced by endotoxemia.** Following dysbiosis, microbiota-derived products reach the liver through the portal vein. TLR signaling and communication between different cell types (Kupffer cells, hepatic stellate cells, and hepatocytes) sense these products and trigger not only cytokine production and inflammatory responses, but also lipid accumulation and fibrosis, key events in NAFLD progression. NAFLD, nonalcoholic fatty liver disease; TLR, toll-like receptor.

Recently, several studies have suggested a major role for endotoxemia as a driver of metabolic diseases; it has been associated with an increased risk of obesity and has been suggested to promote T2D.[88,89] Of note, injection of endotoxin into mice resulted in systemic and adipose tissue inflammation, along with insulin resistance, a dysregulation that could be prevented by antibiotic treatment in diet- or genetic-induced obese mice.[90,91] In humans, plasmatic endotoxin levels correlate with the degree of liver inflammation and fibrosis but also with high energy intake, revealing the impact of the diet in endotoxemia.[92,93] Similarly, HFD administration resulted in the expansion of LPS-containing bacteria and up to a 70% increase in endotoxin plasma levels.[94] Conversely, the administration of a prebiotic (oligofructose) or functional bacterial changes after calorie restriction reduces endotoxemia, further confirming the role of the GM in this process.[95,96] Although some bacterial LPS subtypes have higher immunogenicity than others, the exact contribution of every bacterial strain to the metabolic phenotype remains elusive.[97] In this regard, an opportunistic pathogen isolated from an obese human recapitulated the metabolic disorder in germ-free mice.[98] Similarly, peptidoglycan-based cell-wall compounds can affect inflammatory responses through the sensor NOD1, which promotes the release of interleukin (IL)-17A from the mouse intestine but also contributes to insulin resistance. Of note, HFD-fed mice showed levels of NOD1 activators in the circulation.[99] Moreover, NOD1 ligands induced inflammation in the liver and the adipose tissue while NOD1-deficient mice are protected from obesity-induced inflammation and showed reduced infiltration of proinflammatory macrophages.[100]

Various bacterial metabolites have been reported to play a role in the exacerbation of the inflammatory phenotype.

 

TMAO levels correlate with atherosclerosis and cardiovascular complications, glycemic control and NAFLD, being a driver of inflammation and platelet activation.[72,101,102] On the other hand, enhanced SCFA production has been reported to improve glucose homeostasis and to exert anti-inflammatory effects inducing regulatory T cells.[103,104]

## How the Microbiome Impacts on Gut-Barrier Function

The molecular mechanisms driving intestinal permeability alterations and their regulation by the host, microbiota, and lifestyle-related factors represent still an open question and require further elucidation. Most studies have only assessed epithelial tight junction expression, underestimating the influence of the supplementary barriers that prevent bacterial translocation. Also, neither the mucus abundance nor the production of immunoglobulins or antimicrobial peptides is normally tested, leading to the need for a better understanding of these additional processes and their regulation.

### Mucus Layer

The intestinal barrier is strengthened by the presence of mucins, which are highly glycosylated proteins that form a layer that plays three major roles: it prevents a direct connection between the microbiota and the epithelial cells, it provides support for bacteria to avoid its elimination during the intestinal peristaltic movements, and it is also used as a nutrient for some bacteria.[105] In the colon, the external layer offers nutrients for bacteria, and the internal layer confers protection to the host, being sterilized by the production of antimicrobial peptides and other proteins such as Lypd8 or ZG16.[106,107] An in vivo experiment showed that the composition of the mucus layer is commanded by the microbiota since after colonization, germ-free mice develop a mucus layer similar to the donor.[108] In fact, further experiments have shown that the GM can alter the intestinal barrier by degrading mucus or by inhibiting the production of mucus, both of which result in increased permeability of the epithelium.[109] Mice with a chronic or intermittent-fiber-deprived diet showed changes in the microbiota that favor the overgrowth of mucin-degrading bacteria. Consequently, the mucus layer permeability and susceptibility to infections were increased, a state reverted after microbiota transplantation from control mice.[110,111] Moreover, diet-induced changes in the balance between *Bacteroides* and *Firmicutes* can alter the glycosylation of mucins.[112]

However, the mucosal immune milieu also shapes the microbiota. During homeostasis, the commensal microbiota is sensed by the dendritic cells in Peyer's patches that, via the Mincle–SYK signaling pathway, produce IL-6 and IL-23 and stimulate intestinal T cells, which in turn produce IL-17 and IL-22.[113] Mucosal immune cells (such as RORγt-dependent TH17 cells) prevent bacterial translocation and systemic inflammation through the production of REG3γ and other antimicrobial peptides.[114,115] Martínez-López et al showed that mice with genetic disruption of the Mincle–SYK pathway presented liver inflammation and impaired lipid metabolism with accumulation of diacylglycerides and fatty acids in the liver.[113] Controversially, recent data showed that loss of mucin-2 protected mice from NAFLD and the features of metabolic syndrome possibly by activating the mucosal immune system.[116]

### Epithelial Barrier

The intestinal epithelium forms a tightly sealed physical barrier that separates the host from the contents of the gut, restricting access to toxins, antigens, and enteric flora to the circulation, while nutrients are selectively absorbed.[109] This barrier comprises enterocytes, goblet cells, and enterochromaffin cells that are bound to each other by transmembrane proteins including desmosomes, adherens junctions, and tight junctions granting them a physical sealing.[117] It also has immunological properties that help to maintain homeostasis between the microbiota and the host through a tolerogenic immune response. This equilibrium is achieved by pattern recognition receptors (PRRs), secretion of immunoglobulin A and antimicrobial peptides, and an immune environment formed by $CD103^+$ dendritic cells, regulatory T cells, and cytokines (IL-33, IL-10, and transforming growth factor beta).[118]

Recently, the connection between dysbiosis, barrier permeability, liver damage, and metabolic abnormalities has been established. Morphologically, obese patients display jejunal villus hyperplasia that leads to a greater surface area of exchange with alterations in the immune compartment.[119] Indeed, in a cohort of 39 pediatric NAFLD patients, Giorgio and colleagues showed, using a lactulose/mannitol test, the existence of intestinal permeability, which correlated with the severity of liver disease.[120] In another study, Miele et al reported evidence of intestinal permeability in patients with NAFLD, which correlated with small intestinal bacterial overgrowth (SIBO), decreased expression of ZO-1, and the severity of steatosis.[121] Accordingly, studies in mice fed high-fat or choline-deficient diets showed higher intestinal permeability, while they were protected in the absence of microbiota after antibiotic treatment.[122] It has also been demonstrated that a nutrition rich in fat can influence intestinal permeability and thus inflammation. Additionally, HFD resulted in a depletion of eosinophils in the small intestine, driving a greater paracellular permeability.[123] Consistently, obese patients showed an increased permeability in the jejunum after a lipid challenge and a higher density of epithelial CD8+ T cells, which migrate from the lamina propria to the epithelium.[124,125] In this setting, a decrease in regulatory T cells with an increase in interferon-γ-producing TH1 cells has also been found.[126] In vitro and in vivo experiments have shown that LPS can cross the epithelial barrier via the transcellular path by chylomicrons.[127,128]

It is not clear, however, if the disruption of the barrier is a cause or consequence of endotoxin exposure. Endotoxins from the outer membrane of gram-negative bacteria have been found to increase tight junction permeability by upregulating TLR4 expression.[129] A recent study using mice deficient in junctional adhesion molecule (JAM)-A on a high-fat high-fructose diet demonstrated increased bacterial translocation leading to liver inflammation and NASH. Also, NASH patients showed a decrease in JAM-A in colonic biopsies together with

 

increased mucosal inflammation.[130] By contrast, the tryptophan bacterial-produced metabolite indole propionic acid has shown to play a role in maintaining intestinal epithelial homeostasis, improving gut dysbiosis, reducing endotoxin leakage, and the production of proinflammatory cytokines in HFD-fed mice.[131]

Nevertheless, it remains to be established whether the capacity to cross the barrier after HFD exposure is only due to an increased leakiness of the epithelium caused by downregulation of tight junction proteins or if it is an acquired function of the microbiota due to an enrichment in pathobionts.

### Vascular Barrier

The gut vascular barrier (GVB) has been recently described as an intestinal barrier in addition to the epithelium, which actively prevents systemic bacterial dissemination from the gut, even in the case of mucoepithelial dysfunction. This barrier is anatomically located below the epithelial cell layer and is composed mainly of intestinal endothelial cells, sharing many characteristics with other vascular barriers, in particular the blood–brain barrier.[132] Indeed, endothelial cells forming the GVB harbor intercellular junctional complexes that reduce the paracellular trafficking and are in close contact with pericytes and enteric glial cells. Gut vascular endothelial cells present fenestrae covered by a diaphragm composed by the plasmalemmal-vesicle-associated protein 1 (PV-1) that determines pore size. Increased detection of PV-1 by the MECA antibody clone has been positively associated with and increased endothelial permeability reflecting a dysfunctional GVB.[132] Some enteric pathogens, such as *Salmonella typhimurium*, have developed tactics to penetrate the GVB and reach peripheral tissues by interfering with B-catenin activation in endothelial cells via Spi2 (Salmonella pathogenicity island 2).[133] In a healthy state, the GVB controls the selective translocation of immune cells and antigens across the blood endothelial cells, allowing only small molecules to extravasate from intestinal capillaries. By contrast, GVB disruption is responsible for the accessibility of bacteria and their subproducts to the portal-venous circulation, and their dissemination to the liver, contributing to the development of hepatic diseases. Therefore, the GVB plays a key role along the gut–liver axis[134] in both healthy and pathological states.

Recent data showed that changes in GM composition occurring during NASH pathogenesis are directly responsible for the disruption of both the gut epithelial and vascular barriers, being indeed a fundamental prerequisite for the development of the disease.[20,135] Genetic and pharmacological treatments sealing the GVB have been shown to block the accumulation of lipids in the liver and to exert therapeutic effects in NAFLD/NASH diet-induced mouse models. Moreover, pathological bacterial translocation is associated with GVB disruption in experimental models of cirrhosis, independently of portal hypertension and the lymphatic route.[136] In this way, it has been shown that part of the beneficial effects of FXR agonism for NASH and cirrhosis models might be driven by the sealing of the GVB through the activation the WNT/B-catenin pathway.[20,136]

## Role in Liver Fibrosis Development

The progression of NAFLD in terms of NASH, liver cirrhosis, and hepatocellular carcinoma is mostly driven by inflammatory events, which can impact the amount of fibrosis, defining the long-term prognosis of the liver disease.[137] Different alterations of the GM such as SIBO and dysbiosis have been associated with liver cirrhosis and treatment with nonabsorbable antibiotics (e.g., rifaximin) is recommended in certain cases.[138] Also, specific changes in the microbiota and its metabolic function have been associated with progression of the disease. Boursier et al identified, in a cohort of 57 biopsy-proven NAFLD patients, that the abundance of *Bacteroides* was associated with NASH and *Ruminococcus* with significant fibrosis.[139] Recently, it was reported that the existence of gut microbiome signatures was able to detect either advanced fibrosis, characterized by an increased abundance of *E. coli* and *Bacteroides vulgatus*, or cirrhosis.[8,19] Moreover, all experimental models of fibrosis result in dysbiosis and increased permeability, whereas the use of antibiotics in a choline-deficient, L-amino acid-defined (CDAA)-fed rat model decreased hepatic stellate cell activation and the severity of fibrosis.[140,141] The consumption of fructose can also exert hepatotoxic effects through its conversion to toxic metabolites by the microbiota. Indeed, it has been associated with hepatic fibrosis in NAFLD patients, but also with dysbiosis and endotoxemia.[142,143]

These events lead to the translocation of microbe-associated molecular patterns, which are recognized by immune receptors in intestinal cells, but also in liver cells. Fibrogenesis can then be triggered by direct activation of these receptors on hepatic stellate cells or indirectly by targeting hepatocytes and Kupffer cells where an inflammatory cascade starts ultimately leading to liver fibrosis promotion and progression.[144,145] In fact, Kupffer cells are more sensitive to LPS than hepatocytes, and activation of TLRs has been shown to contribute to the fibrotic process through NLR family pyrin domain-containing 3 (NLRP3) inflammasomes.[146] Accordingly, mice with genetic ablations of *Tlr2*, *Tlr4*, *Tlr9*, and *Nlrp3* are protected from experimental liver fibrosis.[147] However, a greater effect is suggested to be directly exerted on hepatic stellate cells, which express TLR4 even in a quiescent state. Available data suggest that TLR4-MyD88-NF-kB mediates fibrosis by upregulating cytokine production and a-SMA, TIMP1, and TGF-B expression. Moreover, continued activation of TLR4 has been shown to sensitize quiescent hepatic stellate cells for activation via downregulation of Bambi.[148] Other receptors, such as NOD-like and antifungal pattern-recognition receptors have been related to fibrosis. NOD1 stimulation can activate the NF-kB and MAPK pathway, inducing the production of CXCL1 and CCL5, further contributing to processes such as wound healing and fibrogenesis.[149] Lastly, it was proposed a role for STING in liver fibrogenesis which, beyond regulating insulin sensitivity, enhances macrophage proinflammatory activation leading to fibrosis via paracrine mechanisms in hepatic stellate cells.[150]

Finally, and given the endotoxemia observed in NAFLD patients, it is plausible that fibrosis develops as a direct consequence of dysbiosis.[151] The role of endogenous ethanol

 

in fibrosis is also being studied. Also, the expression of CYP2E1 correlates with the level of ethanol and is linked to the levels of oxidative stress, a potent profibrotic mechanism. Accordingly, a study by Zong et al reported increased expression of CYP2E1 in NASH patients.[152]

## Final Remarks and Future Prospects

This review has emphasized the connection between the liver and the gut, which consist of a complex balance of microbiota, its metabolites, and immunity, in the context of NAFLD. Addressing metagenomics is surrounded by several limitations in computational analysis, statistical assessments, standardization, and validation due to the vast variability of the cohorts themselves, experimental designs, and bioinformatics workflows, which we attempted to solve to reach conclusions. Here, we showed strong associations between microbiota changes and altered host metabolism. ►**Table 2** summarizes human studies that show a link between NAFLD and dysbiosis. However, it is still uncertain if liver disease state stimulates changes in the microbiota or if the microbiota exacerbates fibrotic and inflammatory progression. Indeed, much of this knowledge comes from cross-sectional or case–control association studies from which potential relationships can be demonstrated; however, it is almost impossible to isolate this association from secondary effects inherent to the condition and evidence toward causality is often missing. Additionally, an optimal healthy microbiota is unique for each individual, making the selection of control cohorts also a great source of bias since the microbiota composition is dynamic and influenced by lifestyle, medical history, and host genetics. Thus, matching cohorts based on age and sex is sometimes insufficient, and the differences detected in these studies could be owed to confounding factors. Regardless, it seems unlikely that single microbiota signatures can define the whole spectrum of NAFLD phenotypes. In general, there is a lack of reproducibility between cohorts, with an absence of a mechanistic explanation for dysbiosis on NAFLD. In this concern, discrepancies in studies assessing GM may also arise from technical issues: sample collection, storage, primer selection, and analysis techniques. Therefore, unified research standards should be established, and efforts to address sources of variation, such as the Microbiome Quality Control Project, are needed.[153]

Researchers have attempted to avoid some of these issues by performing twin studies, analyzing the heritability of hepatic steatosis and fibrosis. However, the sample size required for these studies to reach proper statistical power is usually challenging.[154,155] Nevertheless, ongoing longitudinal studies will help to examine the role of the gut bacterial communities in the development of complex metabolic disorders. As with humans, data extracted from animal models must be carefully interpreted. First, the rodent and human microbiota present differences. Second, some models, such as germ-free, gnotobiotic, or specific pathogen-free mice can be useful in the understanding of the impact of some bacteria on the experimental hypothesis. However, germ-free mice have an immature immune system with GVB damage, the exact composition of the gnotobiotic mice microbiota is still unknown, and specific pathogen-free mice lack strains that are potentially pathogenic. Indeed, it has been reported that the complete absence of microbiota may confer protection or exacerbation of liver diseases, possible due to an accumulation of constitutive androstane receptor ligands and a more efficient xenobiotic metabolism.[156] Antibiotic treatment to create microbiota-free mice has also been used. However, they can create resistance and overgrowth of certain strains, which is why the use of several models to address with precision the role of microbiota has been recommended.

The consequences of a disrupted gut–liver axis include altered microbiota, gut barrier damage, and reduced intestinal FXR signaling due to impaired BA metabolism. These events drive functional changes that promote the exposure of liver innate immune cells to bacterial subproducts and metabolites, resulting in liver inflammation and metabolic disturbances. Promising therapeutic approaches are underway, which can be classified in the way they target the gut–liver axis (►**Table 3**). One approach is based on the use of bacteria to alter the composition of the GM, such as FMT (fecal microbiota transplantation), although there are no published results in patients with NASH to date, and there are some concerns about its safety and efficacy alone without lifestyle interventions. Also, the effect of probiotics, prebiotics, or synbiotics has been under investigation, with trials showing reductions in liver enzymes, steatosis, and liver stiffness; however, no recommendation can be made yet due to the variability of the studies. Besides, some studies have revealed the potential use of engineered bacteria (e.g., *Lactobacillus reuteri* producing IL-22) to restore homeostasis to the gut–liver axis.[157,158] Other approach under study is to directly target the microbiota either with antibiotics, currently nonspecific and with potential side effects, or with bacteriophages, viruses that can defeat specific species of bacteria. Another approach that has gained attention lately is the supplementation with microbe-derived products (so-called postbiotics) to replace metabolic activities lost due to dysbiosis. Other interventions relay on pharmacological modulation of gut peptides, such as GLP-1, or BA pathway.

Finally, an active area of research is the profiling of the microbiome by multiomics analysis, which is expected to provide markers of liver damage and disease progression, predicting hospitalizations and complications. However, we must be aware of the complex relationship between the host, the microbiota, and the external environment and how the limitations of each study impact the observations. In this regard, there are still some pending issues to address in the future: (1) to establish specific alterations in the composition of the microbiota and microbial functions that take place in NAFLD patients. To date, an accurate definition of a healthy microbiota and the precise associations between GM and NAFLD is still lacking. (2) Despite a plethora of scientific evidence demonstrating that the GM is a contributing pathogenic factor in NAFLD, there is still the need to demonstrate the molecular mechanism involving the gut–liver axis underlying the pathogenesis of NAFLD, thus confirming a direct causal role of the GM alterations in the development of metabolic and liver dysfunction. (3) In the future, noninvasive serum biomarkers and individualized treatments targeting the

 

**Table 2** List of human studies that correlated changes in intestinal microbiome to NAFLD/NASH

| Population | Method | BMI | NAFLD diagnosis | Main outcome | Ref. |
|---|---|---|---|---|---|
| 26 Controls, 11 obese, 13 NAFLD | 16S rRNA, shotgun | Obese | Ultrasonography or biopsy | Obese children with NAFLD showed increased *Gammaproteobacteria*, *Epsilonproteobacteria*, and *Prevotella*. | Michail et al 2015[168] |
| 30 NAFLD vs. 30 controls | Pyrosequencing | Obese | Ultrasonography and blood test | Increased *Ruminococcaceae*, reduced *Lactobacillaceae* and *Lachnospiraceae*. | Raman et al 2013[169] |
| 16 Controls, 25 obese, 22 NASH | Pyrosequencing | Obese | Biopsy | Obese and NASH children versus healthy controls had increased *Bacteroidetes* and *Prevotella*. | Zhu et al 2013[53] |
| 16 NASH vs. 22 controls | Pyrosequencing | Overweight | Biopsy | Decreased *Firmicutes*. No changes in *Bacteroidetes*. | Wong et al 2013[170] |
| 37 NASH vs. 20 NAFLD | 16S rRNA | Obese | Biopsy | Increased *Bacteroides* in NASH patients. *Ruminococcus* abundance correlated with fibrosis stage. | Boursier et al 2016[139] |
| 98 NAFLD, 105 relatives | 16S rRNA | Obese | Biopsy | Increased gram-negative in advanced fibrosis. *Streptococcus* was enriched in NAFLD patients. A signature with 27 bacterial groups allowed the identification of NAFLD cirrhosis. | Caussy et al 2019[8] |
| 105 Obese women | Shotgun | Obese | Biopsy | Morbid obese women with steatosis had fewer *Lachnospiraceae* and *Ruminococcaceae*. *Escherichia* and *Bacteroides* were associated with insulin resistance. | Hoyles et al 2018[171] |
| 25 NAFLD vs. 22 controls | 16S rRNA | Overweight | Biopsy | Lower diversity with increased *Blautia* and *Lachnospiraceae*, and decreased *Prevotella*. | Shen et al 2017[172] |
| 123 NAFLD-mild fibrosis, 28 NAFLD advanced fibrosis | Shotgun | Obese | Biopsy | A decrease in *Firmicutes* and an increase of *Proteobacteria* in NASH patients with advanced fibrosis. Increased prevalence of gram-negative taxa. | Loomba et al 2017[19] |
| 54 Controls, 61 NAFLD or obese | 16S rRNA | Obese | Ultrasonography and biopsy | No differences observed among NAFL, NASH, and obese children. NASH showed increased *Ruminococcus*, *Blautia,* and *Dorea* compared with controls. | Del Chierico et al 2017[173] |
| 17 Controls vs. 11 steatosis vs. 22 NASH | qPCR | Obese | Biopsy | Lower *Bacteroidetes* in NASH patients (adjusted by BMI and fat intake). | Mouzaki et al 2013[174] |
| 32 NASH vs. 181 non-NASH cirrhotic | Pyrosequencing | Obese | Blood test | Cirrhotic NASH had increased *Porphyromonadaceae* and *Bacteroidaceae*; reduced *Veillonellaceae*. | Bajaj et al 2014[175] |
| 43 NAFLD vs. 83 controls | Pyrosequencing | Lean | Ultrasonography | Increased *Bacteroidetes* and decreased *Firmicutes* in nonobese NAFLD patients. | Wang et al 2016[176] |
| 30 NAFLD vs. 37 controls | Pyrosequencing | Overweight | Ultrasonography | Differences only at family or genus levels. Increased *Lactobacillaceae* and *Veillonellaceae.* Central obesity and insulin metabolism related to changes in microbiota. | Li et al 2018[177] |
| 53 NAFLD vs. 32 controls | 16S rRNA | Overweight | Ultrasonography or biopsy | Within *Firmicutes*, increased *Peptostreptococcaceae, Lactobacillaceae*, and *Streptococcus* with decreased *Ruminococcaceae.* In *Bacteroidetes*, decreased *Porphyromonadaceae* and *Prevotella.* | Jiang et al 2015[178] |
| 28 Controls vs. 15 steatosis vs. 24 NASH | 16S rRNA | Obese | Biopsy | No differences between simple steatosis and NASH. Increased *Lactobacillaceae* and decreased *Ruminococcus, Faecalibacterium*, and *Coprococcus* in NAFLD compared with controls. | Da Silva et al 2018[179] |
| 90 NAFLD vs. 21 controls | 16S rRNA | Obese | Ultrasonography or biopsy | Decreased *Bacteroidetes* and *Ruminococcaceae*, increased abundance of *Lactobacillaceae, Veillonellaceae,* and *Dorea* in NAFLD patients. | Demir et al 2020[180] |

Abbreviations: BMI, body mass index; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal ribonucleic acid.

**Table 3** Selected studies targeting the gut–liver axis in NAFLD

| Place of action | Type | Subjects | Intervention | Mechanism | Outcomes | References |
|---|---|---|---|---|---|---|
| Intestinal microbiome | Nonabsorbable antibiotics | $n = 50$ patients with NASH | Randomized, double-blind study. Rifaximin 1,100 mg/d for 6 months | Bactericidal effect, reduces endotoxin levels and has anti-inflammatory action. | Reduction of serum endotoxin, proinflammatory cytokines, and NAFLD-liver fat score. | NCT02884037 |
| | | $n = 15$ patients with NASH | Single-arm study. Rifaximin 800 mg/d for 6 weeks plus 6 weeks follow-up. | | No beneficial effect. | NCT01355575 |
| | FMT | $n = 20$ patients with NAFLD | Randomized, double-blind study. Allogenic or autologous infusion of feces at 0, 3, and 6 weeks by gastroscopy. | Restore healthy microbiome composition. | Ongoing | NCT04465032 |
| | | $n = 15$ patients with NASH | Single-arm study. One allogenic infusion by gastroscopy plus 72 weeks follow-up. | | Ongoing | NCT03803540 |
| | Probiotics | $n = 44$ obese pediatric patients with NAFLD | Randomized, double-blind study. Two sachets/d VSL#3 for 4 months. | Restoration of normal gut flora. | Decrease in BMI and increase in GLP-1. | NCT01650025 |
| | Prebiotics | $n = 14$ patients with NASH | Randomized, single-blind study. Oligofructose for 9 months vs. placebo. | Increase in *Bifidobacterium* and decrease in *Clostridium* cluster XI and I. | Improve in liver steatosis and NAS score independently of weight loss. | NCT03184376 |
| | Synbiotics | $n = 104$ patients with NAFLD | Randomized, double-blind study. Fructo-oligosaccharides 8 g plus *Bifidobacterium animalis* subsp. lactis BB-12 1 capsule/d for 1 year. | Reduce dysbiosis by promoting survival and colonization of healthy microbiota. | No reduction in fat or markers of liver fibrosis was observed. | NCT01680640 |
| Intestinal content | Carbon nanoparticles | $n = 70$ patients with NASH | Randomized, double-blind study. Yaq-001 8 g/d vs. placebo. | Carbon particles adsorb bacterial toxins from the intestine. | Ongoing | NCT03962608 |
| | Hydrogel technology | $n = 300$ obese patients with/without T2D | Randomized, double-blind study. Gelesis200 vs. placebo. | Modified cellulose mimicking natural fibers absorbs water in the intestine and increases satiety. | Ongoing | NCT03058029 |
| Intestinal mucosa | Postbiotics | $n = 60$ overweight adults | Randomized, double-blind study. Inulin-propionate ester vs. inulin alone for 24 weeks. | SCFAs stimulate the release of anorectic gut hormones. | Reduced weight gain, liver fat, and deterioration of insulin sensitivity. | NCT00750438 |
| | Duodenal mucosal resurfacing | $n = 60$ patients with T2D | Single-group study. Endoscopic DMR procedure with 24 week follow-up. | Hydrothermal ablation of the damaged mucosa induces its regeneration. | Improved glycemic control independent of weight loss, decrease in liver enzymes. | NCT02413567 |
| Bile acid pathway | FXR agonist | $n = 931$ patients with NASH | Interim analysis of a randomized, double-blind study. OCA 25 mg/d for 18 months vs. placebo. | Restore microbiota composition, barriers function while reducing inflammation and translocation. | Improved fibrosis and components of NASH disease activity. | NCT02548351 |
| | FGF19 analog | $n = 43$ patients with NASH | Open-label study. NGM282 1 mg and 3 mg/d for 12 weeks. | Inhibits de novo bile acid synthesis, improving insulin sensitivity and reducing inflammation. | Reduced NAS and fibrosis scores, improvements in serum and imaging markers. | NCT02443116 |

Abbreviations: BMI, body mass index; DMR, duodenal mucosal resurfacing; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SCFA, short-chain fatty acid; T2D, type 2 diabetes.

alterations of the GM or microbial metabolites should be further investigated to establish the basis for precision medicine for NAFLD.

## Main Concepts and Learning Points

- A disrupted gut–liver axis contributes to NAFLD development through alteration in microbiota, changes in microbial-derived metabolites, appearance of translocation and endotoxemia due to gut barrier damage, and changes in hormones and bile acid signaling. These changes lead to immune and metabolic disturbances inducing steatosis, inflammation, and fibrosis, key events in the progression of NAFLD.
- The complex relationship between the host, the microbiota, and the external environment limits the impact of every study and must be taken into account along with other limitations in the experimental design; unified research standards are needed.
- Promising therapeutics targeting the gut–liver axis for NAFLD are underway, and they, together with noninvasive predictive biomarkers obtained by current multiomics approaches, will establish the basis of future precision medicine for NAFLD.

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Conflict of Interest

Dr. Rescigno reports grants from Intercept Pharmaceuticals, during the conduct of the study; grants from Gelesis, grants from Kraft-Heinz, outside the submitted work. Dr. Romero-Gómez reports grants from Intercept, grants from Gilead Sciences, personal fees from Shionogi, personal fees from Alfa Wassermann, personal fees from Prosciento, personal fees from Kaleido, personal fees from Novo Nordisk, personal fees from MSD, personal fees from BMS, personal fees from Allergan, personal fees from Boehringer-Ingelheim, personal fees from Zydus, personal fees from Intercept Pharma, personal fees from Gilead Science, outside the submitted work.

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