Journal of Clinical and Translational Hepatology **2022**
DOI: 10.14218/JCTH.2022.00136

Review Article

The Gut Microbiome and Ferroptosis in MAFLD

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Received: 21 March 2022 | **Revised:** 22 May 2022 | **Accepted:** 12 June 2022 | **Published:** 14 July 2022

Abstract

Metabolic-associated fatty liver disease (MAFLD) is a new disease definition, and is proposed to replace the previous name, nonalcoholic fatty liver disease (NAFLD). Globally, MAFLD/NAFLD is the most common liver disease, with an incidence rate ranging from 6% to 35% in adult populations. The pathogenesis of MAFLD/NAFLD is closely related to insulin resistance (IR), and the genetic susceptibility to acquired metabolic stress-associated liver injury. Similarly, the gut microbiota in MAFLD/NAFLD is being revaluated by scientists, as the gut and liver influence each other via the gut-liver axis. Ferroptosis is a novel form of programmed cell death caused by iron-dependent lipid peroxidation. Emerging evidence suggests that ferroptosis has a key role in the pathological progression of MAFLD/NAFLD, and inhibition of ferroptosis may become a novel therapeutic strategy for the treatment of NAFLD. This review focuses on the main mechanisms behind the promotion of MAFLD/NAFLD occurrence and development by the intestinal microbiota and ferroptosis. It outlines new strategies to target the intestinal microbiota and ferroptosis to facilitate future MAFLD/NAFLD therapies.

Citation of this article: Ji J, Wu L, Wei J, Wu J, Guo C. The Gut Microbiome and Ferroptosis in MAFLD. J Clin Transl Hepatol 2022. doi: 10.14218/JCTH.2022.00136.

Introduction

Because of its close association with metabolic diseases and the many challenges faced by previous diagnostic strategies of exclusion, a new disease nomenclature, metabolic-associated fatty liver disease (MAFLD), has been proposed to replace the previous name, nonalcoholic fatty liver disease (NAFLD).[1] Globally, MAFLD/NAFLD is the most common liver disease, with an incidence rate between 6% and 35% in adult populations.[2] Studies have shown that the long-term existence of NAFL and NASH are important causes of liver cirrhosis and hepatocellular carcinoma (HCC).[3] Indeed, the predominant HCC etiology in the USA is MAFLD/NAFLD. NASH is the second most frequent reason for liver transplantation in the USA, and is likely to supersede hepatitis C as the most common cause of transplantation in the future.[4] Although MAFLD/NAFLD is not inherently serious, its complications are, and include liver cirrhosis and HCC which seriously affect quality of life or even endanger patient lives. MAFLD/NAFLD occurrence is an extremely complex pathological process that involves a variety of hepatic cells and multiple extrahepatic signals.[5] In recent years, immunoinflammatory responses, genetic metabolism, insulin resistance (IR), ferroptosis, and the gut microbiome have been closely associated with MAFLD/NAFLD.[2,5]

Bacteria, viruses, phages, and archaea collectively colonize the human intestines, and are known as the gut microbiome. More than 1×10^{14} microorganisms are found in healthy individuals and comprise more than nine million genes, which is approximately 150 times larger than the human genome.[6] Although the human gut microbiome is closely related to host physiological activities, its importance to human health and disease has long been neglected because of inadequate research methods. However, in recent years, technical advances in DNA/RNA sequencing, bioinformatics data analysis, and culture-based microbiology have increased our understanding of microbes in health and disease.[7,8] Simultaneously, the increased gut microbiome literature has been instrumental in delineating metabolic diseases, including NAFLD, obesity, cardiovascular disease, carcinoma, and type 2 diabetes mellitus.[9,10] Thus, rather than existing as individual pathogens, microbes exist as complex consortia with myriad interactions with their hosts.

The liver and intestinal tract are anatomically and functionally related, having both developed from the same germ layer in the embryo.[11] Since the gut-liver axis was first proposed by Marshall in 1998, it has attracted much interest in the relationships between liver disease and the intestinal tract.[12] The portal vein connects the gut to the liver and provides 70% of its blood supply. The unique anatomical structure of the liver increases its susceptibility to gut bacteria, bacterial products, endotoxins, and microbiome inflammatory molecules.[13] Under normal physiological conditions, the intestinal mucosal barrier is the first bodily defense against external pathogen invasion.[14] The liver also produces specific antibodies and inflammatory factors that monitor the intestinal mucosa.[15] However, under some pathological conditions,

Keywords: Gut microbiota; Ferroptosis; Nonalcoholic fatty liver disease; Gut-liver axis.

Abbreviations: BAs, bile acids; FXR, farnesoid X receptor; FOS, fructo-oligosaccharide; GLP-1 RA, glucagon-like peptide-1 receptor agonists; HFD, high fat diet; IR, insulin resistance; LPS, lipopolysaccharide; MAFLD, metabolic-associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; SIBO, small intestinal bacterial overgrowth; SGLT2i, sodium/glucose cotransporter-2 inhibitors; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

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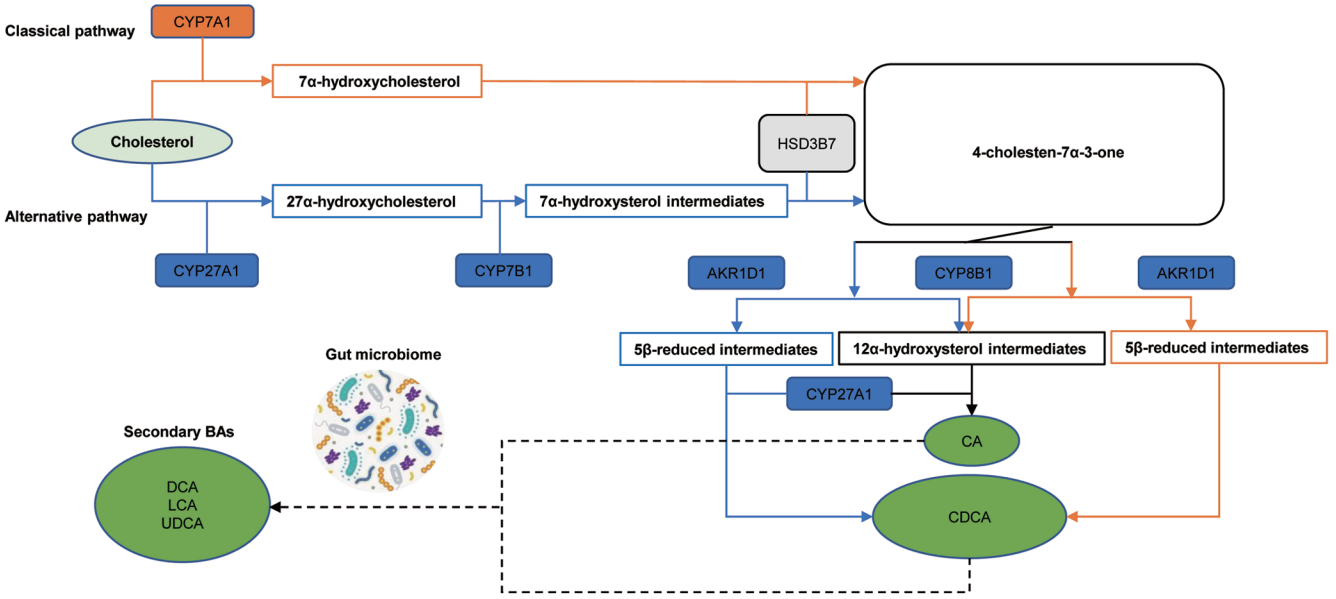

Fig. 1. Bile acid metabolism in the liver and intestine. CYP7A1, cholesterol 7a-hydroxylase; CYP27A1, sterol-27-hydroxylase; CYP7B1, hydroxysterol 7a-hydroxylase; AKR1D1, aldo-keto reductase family 1, member D1; HSD3B7, hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 7; CA, cholic acid; CDCA, chenodeoxycholic acid, DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.

these defense mechanisms become disrupted, thereby facilitating bacterial migration outside the gut. In patients with NAFLD, intestinal bacteria migrate through the portal vein into the liver and cause abnormal activation of the immune system, leading to inflammation responses and injury.[16] In addition, interactions between the intestine and liver are bidirectional, and hepatogenic inflammatory cytokines thus impair intestinal mucosal barrier function, disrupting tight junctions of the intestinal epithelium, and forming a malignant liver-gut cycle during NAFLD.[17,18]

Ferroptosis is a novel form of cell death characterized by iron overload and reactive oxygen species (ROS)-dependent accumulation of lipid peroxides. Ferroptosis, morphologically manifests as mitochondrial shrinkage, reduction or disappearance of mitochondrial cristae, and increased mitochondrial membrane density. Like other cell death modes, ferroptosis is tightly regulated by a variety of intracellular metabolic processes, including glutathione (GSH) synthesis, lipid peroxidation, cysteine transport, iron homeostasis, and NADPH.[19] In recent years, many studies have found that ferroptosis is involved in the progression of NAFLD, and preliminarily confirmed that ferroptosis of hepatocytes and intrahepatic macrophages can trigger NASH.[20] Inhibition of ferroptosis may become a new therapeutic strategy for NAFLD in the future. In this review, we focus on how the gut microbiota and ferroptosis promote NAFLD development via the gut-liver axis and explore gut microbiome potential as a novel diagnostic biomarker and therapeutic strategy for NAFLD.

Interaction between the Gut-Liver axis and the gut microbiome

The liver and intestinal tract are physiologically bidirectional organs. In one direction, the liver excretes bile and other bioactive mediators into the intestinal cavity via the bile duct, while in the other direction, metabolic nutrients are transported into the liver via the portal vein after reabsorption from the small intestine.[11] Simultaneously, intestinal bacteria and their products, e.g., vitamins, short-chain fatty acids (SCFAs), lipopolysaccharide (LPS), endogenous ethanol, and other metabolites are transported through the portal vein, exposing the liver to intestinal microenvironments and pathological changes.[17,21]

Bile acids (BAs) and enterohepatic circulation

BAs are small molecules synthesized from cholesterol via cholesterol 7a-hydroxylase (CYP7A1) catalysis by liver cells.[22] They not only participate in lipid digestion and absorption, but are also important signal regulators that affect energy metabolism, inflammation, and development of liver disease.[23] Recent studies have reported that interactions between BAs and intestinal microbiota are closely related to NAFLD.[24] BA synthesis is highly complex and includes multistep reactions involving at least 17 different catalytic enzymes (Fig. 1).[25] Synthesis occurs in the liver and is accomplished via two different steps. Under normal physiological conditions, at least 75% of BA is synthesized by the classical pathway, which is initiated by cholesterol 7a-hydroxylation catalyzed by CYP7A1.[26] CYP7A1 is the rate-limiting enzyme in the process and determines total BAs production.[27] The selective pathway is initiated by sterol-27-hydroxylase (CYP27A1) and is further hydroxylated by hydroxysterol 7a-hydroxylase (CYP7B1).[28] Studies have shown that the gut microbiota regulates the expression of key enzymes in BA synthesis, including CYP7A1, CYP7B1, and CYP27A1.[29] Sayin *et al.*[30] confirmed that liver-based CYP7A1 was regulated by gut microbiota via farnesoid X receptor (FXR)-dependent mechanisms throughout the enterohepatic system in germ-free and conventionally raised mice.[30] Moreover, recent research confirmed that inhibiting the intestinal microbiota of hamsters up-regulated CYP7B1 in the alternative BAs synthesis pathway, increased BAs hydrophilicity, and increased tauro-β-muricholic acid (TβMCA).[31]

Intestinal barriers and permeability

The intestinal barrier is an important bodily defense mecha-

Table 1. Changes in the gut microbiota in fatty liver disease

Disease	Species	Increased gut microbiota	Decreased gut microbiota	Reference
ALD	Mouse	*Candida spp*	Intestinal fungi	51
	Human	*Bifidobacteria, Lactobacilli, Proteobacteria, Fusobacteria*	*Faecalibacterium, prausnitzii, Coprococcus, Roseburia spp*	52,53
NASH/NAFLD	Mouse	Bacteroides and Firmicutes	A. muciniphila	45,48
	Human	*Proteobacteria, Enterobacteriaceae, Escherichia, Bacteroides, Ruminococcus*	*Ruminococcaceae, Anaerospacter, Coprococcus, Eubacterium, Faecalibacterium, Prevotella*	49,50,51

ALD, alcohol-related liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

nism and is composed of mechanical, chemical, biological, and immune barriers.[14] Under normal physiological conditions, large numbers of anaerobic bacteria grow in the intestinal lumen or intestinal mucosa surfaces and include Bifidobacterium that adhere closely to the intestinal epithelium to form a membrane barrier that resists and repels invasion by foreign pathogens.[32] Studies have shown that the intestinal microbiota maintain intestinal barrier stability by producing a series of metabolites and instigating signal pathways. Ijssennagger *et al.*[33] reported that sulfide produced by sulfate-reducing bacteria dissolved the mucin polymer network, thinned the mucus layer, and changed the mechanical barrier of the intestinal mucosa. In addition, the *Bacteriodes fragilis* toxin had proteolytic enzyme-like activity that degraded mucin and destroyed mucus layer structures.[34] Furthermore, SCFAs like acetic, propionic, and butyric acids, which are the main metabolites of colonic bacteria required for carbohydrate fermentation, protect the chemical barrier of the intestinal mucosa. Researchers transplanted the butyric acid-producing bacteria, *Butyrivibrio fibrisolvens* into sterile mice and observed that bacteria restored energy metabolism to colonic epithelial cells and restored cell oxidative phosphorylation and ATP levels, maintained energy homeostasis, inhibited autophagy, and protected colonic epithelial cell integrity.[35] More important, the intestinal microbiota are important elements of the intestinal biological barrier; their mechanism of action toward intestinal barrier function is to primarily secrete bacteriocins to kill pathogenic bacteria,[36] antagonize pathogen colonization,[37] and compete for oxygen and nutrients.[38]

Destruction of one or more of the barriers affects intestinal barrier integrity. The main driving factors for increased permeability are intestinal inflammation and dysbiosis,[39] which are related to long-term antibiotic use,[40] chronic alcohol intake,[41] continuous high-fat diets,[42] and immune-mediated inflammatory disease.[43] *Akkermansia muciniphila* is a Gram-negative anaerobic bacterium that colonizes intestinal mucus layers and is an important link between the intestinal microbiota, inflammation, and intestinal barrier integrity.[44] Decreased abundance of *A. muciniphila* is related to thinning of the mucus layer and increased inflammation, which promotes alcoholic and nonalcoholic liver damage.[45] When intestinal permeability increases, microorganisms and microorganism-derived molecules are transferred to the liver through the gut-liver axis causing inflammation and liver damage.[46] Some translocated intestinal metabolites may directly interact with host factors, leading to liver disease.[18,47] The next section discusses the influence of the gut microbiota on NAFLD and underlying mechanisms.

Gut microbiota in NAFLD

During embryological development, the gut and liver are intrinsically connected, with the liver budding directly from the foregut during this period. Increasing evidence shows that the intestine and liver have multiple interdependence levels and that dysbiosis and metabolic changes in intestinal microbiota are closely associated with NAFLD (Table 1).[45,48–53] This includes observations that patients with NAFLD experience increased intestinal permeability when compared with non-NAFLD patients,[54] exhibit correlations between liver disease and microbiota changes,[55] and the impact of flora manipulation on liver injury.[16]

Dysbiosis

Dysbiosis refers to the destruction of the normal intestinal microbiota, including the loss of beneficial bacteria, changes in bacterial abundance, and increased pathogen levels.[6] The condition is induced by factors that include drastic environmental changes, immune or host factors, changes in bile composition, gastric pH, and intestinal motility disorders.[6,56] In recent years, studies linking dysbiosis with NAFLD pathogenesis have rapidly increased, focusing on the metabolism of intestinal microbes and their metabolites. However, the exact mechanism by which the gut microbiota promotes the progression of NAFLD needs additional study, and it is also necessary to discover more effective new treatments for gut microbes in NAFLD. A 2001 study by Wigg *et al.*[57] was the first to describe the link between gut dysbiosis and liver disease. Using a ^{14}C-D-xylose-lactulose breath test, the study showed that small intestinal bacterial overgrowth (SIBO) was present in 50% of patients with nonalcoholic steatosis, but in only 22% of control subjects (p=0.048). However, low participant numbers and excluded diseases potentially affected SIBO, such as diabetes and anemia, were major study limitations. In addition, subsequent studies showed that SIBO was related to low intestinal motility and other factors such as the inhibition of gastric acid secretion, decreased secretion of intestinal enzymes, and decreased bile flow, which is a causative factor in NAFLD.[58,59] Furthermore, patients with SIBO experienced increased intestinal permeability with more severe portal endotoxemia that may have exacerbated NAFLD progression.[48]

Animal studies where the microbiome is manipulated provide powerful evidence of dysbiosis in NAFLD. Turnbaugh *et al.*[60] reported that obesity was related to changes in the relative abundance of two main bacteria, *Bacteroides* and *Firmicutes* by comparing the gut microbiota of genetically ob/ob mice with lean littermates. They also showed that the ability that the obese microbiota to obtain energy from the diet was partially transmissible, for a significant increase in total body fat after colonizing obese flora in sterile mice compared with the lean flora group. Furthermore, transgenic mouse models have been used to study NAFLD-related intestinal dysbiosis to unravel mechanisms underpinning liver disease progression. Rahman *et al.*[61] used F11r ($^{-/-}$) mice encoding junctional adhesion molecule A (JAM-A) found that

JAM-A deficiency led to more severe NASH. Associated inflammation was reduced by antibiotics, which emphasized the contribution of microbial dysbiosis to NASH development.

Although some animal studies have emphasized the role of gut microbiota in NAFLD, the literature on intestinal dysbiosis in human NAFLD is scarce, especially on the full spectrum of NAFLD lesions. An obesity study reported that an increased abundance of *Bacteroides* was related to percentage weight loss but not to changes in dietary calorie levels. The study was performed by sequencing 16S ribosomal RNA genes from stool samples, suggesting obesity displayed correlations with gut microbiota changes.[49] Another study using 16S ribosomal RNA gene sequencing in 57 patients with biopsy-proven NAFLD, revealed that *Bacteroides* and *Ruminococcus* were significantly increased, whereas *Prevotella* abundance was decreased in those with NASH compared with those without the condition.[50] Indeed, studies of fecal microbiota transplantation have provided direct evidence. In one study, obese patients with metabolic syndrome received small intestinal infusions of allogenic microbiota from a thin male donor with a body mass index (BMI) <23 kg/m^2 or autologous microbiota. Six weeks after infusion, recipient insulin sensitivity and intestinal butyrate-producing microbiota levels were both significantly increased.[62] The findings suggest that gut microbiota changes may be used to improve human insulin sensitivity, indicating the potential benefit for NAFLD treatment.

Leaky gut

As the hepatic portal vein collects blood supplies from the intestine, the liver is often exposed to potentially harmful intestinal metabolites, including translocated bacteria, LPS, endotoxins, and secreted cytokines.[63] Therefore, leaky gut, previously associated with liver disease, has attracted considerable attention in recent decades, and has been widely associated with complementary/alternative medicine approaches.[64] Leaky gut is typically caused by several pathogenic factors, including high-fat diet, gut microbiota dysbiosis, and reduced BAs secretion. The conditions change the intestinal mucosal barrier, which increases intestinal mucosa permeability, causing leakage of bacteria, toxic digestive metabolites, and bacterial toxins into the blood, inducing liver immune responses.[6,63,65] Dysbiosis changes tight junction proteins in the intestinal mucosa, increases mucosa permeability, and exposes intestinal mucosal cells and the liver to potentially pro-inflammatory bacterial products. Cani *et al.*[66] reported that gut dysbiosis induced by obesity increased lower plasma LPS and cytokine levels and increased the expression of inflammatory and oxidative stress markers associated with higher intestinal permeability and tight junction integrity changes. Meanwhile, gut microbiota are reported to have positive effects on intestinal barriers and permeability. For example, *Bifidobacteria* was shown to enhance barrier function in experimental necrotizing enterocolitis in mice and the yeast *Saccharomyces boulardii* had beneficial effects on altered intestinal microbiota and epithelial barrier defects in different pathologies.[67]

Products from translocated microorganisms may participate in NAFLD pathogenesis through a variety of mechanisms. LPS is the central component of the outer membrane of Gram-negative bacteria and is an endotoxin related to NAFLD pathogenesis. Studies have shown that plasma LPS-binding proteins in patients with NAFLD are significantly increased.[68] LPS binds to LPS-binding proteins than then bind to toll-like receptor 4 (TLR4), triggering IR and inflammation.[69] During NAFLD occurrence and development, gut dysbiosis leads to increased LPS secretion. SIBO, changed intestinal barrier, and increased permeability promotes circulating LPS level, which then elevated portal levels of gut-derived TLR ligands. Activated TLR4 on hepatic Kupffer cells and stellate cells further stimulated pro-inflammatory and profibrotic pathways via a range of cytokines, including interleukin-1 (IL1), IL6, and tumor necrosis factor (TNF).[56,70,71] TLR signal proteins have complex and cooperative interactions with inflammasomes in metabolic diseases.[72] Henao-Mejia *et al.*[72] reported that inflammasome-deficient mice had an increased expression of TLR4 and TLR9 agonists and more severe liver steatosis, which were closely related to an imbalance of intestinal microbiota. In fact, TLR signaling enhanced NASH progression by increasing the expression of pro-inflammatory cytokines, such as TNF-α. Specifically, TNF-α regulates liver cell death and prevents insulin signal transduction by inhibiting the insulin receptor and insulin receptor substrate-1, leading to IR.[73] Inflammasomes have also been shown to activate several liver processes, including cleavage of pro-caspase 1 to active caspase 1 leading to cell apoptosis.[74] Another downstream effect mediated by inflammasomes is the release of IL1β, which promotes NAFLD progression. IL1β regulates lipid metabolism by inhibiting peroxisome proliferator-activated receptor alpha (PPARα) and downstream molecules, leading to accumulation of triglycerides in the liver and promoting steatosis.[73]

Microbiota metabolism

Studies that evaluated the metabolic characteristics associated with NAFLD or NAFLD-fibrosis and are summarized elsewhere.[75] Changes in metabolites, including molecules produced by intestinal microorganisms, e.g., ethanol,[76] SCFAs such as butyric, propionic, and acetic acid,[56] and BA metabolites that target FXR in the liver or intestine,[17,77,78] all have important roles in liver injury pathophysiology. Here, we discuss the role of intestinal microbial metabolic substrates and circulating intestinal microbial-derived metabolites in promoting NAFLD progression.

BAs

BAs are synthesized by hepatocytes and are discharged into the intestinal tract via the large papilla of the duodenum. Their physiological functions include promoting fat digestion, increasing pancreatic lipase and lipoprotein esterase activity, and regulating the intestinal microbiota.[79] BA metabolism (enterohepatic circulation) and associated interactions with gut microbes are extremely complex and have been discussed earlier. In recent decades, BA functions in the pathogenesis and treatment of the fatty liver have received considerable attention and are discussed in several reviews.[29,80,81] As a signal regulator molecules, BAs regulate bodily immune homeostasis and inflammatory responses via the FXR (also known as NR1H4) and the G protein-coupled BA receptor, Gpbar1 (TGR-5; also known as GPR131, GPBAR1, M-BAR, and BG37), and further affect the physiological processes of liver cell fatty degeneration, cell damage, and apoptosis.[82] FXR is a nuclear receptor believed to be the master regulator of BA metabolism. It is involved in all phases of the biosynthetic pathway and is expressed in a variety of tissues and organs, with the highest expression in liver and ileum cells.[83] In addition, the FXR is activated by BAs to inhibit NLRP3 inflammasome activation by interacting with caspase-1, and to reduce release of IL-1B and other inflammatory factors to relieve NAFLD.[84] A recent study reported that FXR knockout mice had an increased proportion of secondary BAs and infiltration of lymphocytes and neutrophils, whereas FXR overexpression alleviated liv-

er damage caused by inflammation and infection.[85] Indeed, FXR signaling is modulated by the gut microbiota. Li *et al*.[86] used the antioxidant, Tempol, to alter the microbiota and BA distribution, resulting in increased TβMCA levels and suppressed FXR signaling.

TGR-5 is another BA response receptor involved in host metabolism. Functioning as a plasma membrane-bound G protein-coupled receptor (GPCR), the protein is generally highly expressed in the gallbladder, placenta, lung, spleen, intestine, liver, brown and white adipose tissue, skeletal muscle, and bone marrow.[87] Recently, TGR-5 was shown to have a key role in anti-inflammatory effects,[88] reinforcing barrier functions,[89] and regulating BA metabolism in participation with intestinal microbiota.[23,90] TGR-5 knockout mice had conventional phenotypes and reproductive abilities, but their BA pool was significantly reduced, suggesting that the TGR-5 receptor had important roles in maintaining BA homeostasis.[91] In addition, it was demonstrated that treating obese *db/db* mice with INT-767, a TGR-5 agonist, reduced liver steatosis and inhibited the expression of pro-inflammatory cytokines, indicating the TGR-5 signaling pathway had the potential to treat NAFLD.[77]

SCFAs

SCFAs are organic fatty acids with 1–6 carbon atoms that are produced by microbial carbohydrate fermentation in the intestinal tract. The most common SCFAs are acetic acid, produced by both the host and bacteria; propionic acid, butyric acid, produced by bacterial fermentation; isovaleric acid, and valeric acid. Acetic, propionic, and butyric acid account for more than 95% of the entire SCFA complement.[92] Butyrate is an energy source for intestinal cells and helps maintain the intestinal barrier.[6] Recently, it was shown that SCFAs inhibited cell proliferation,[93] induced cell differentiation and apoptosis,[94] and is closely associated with inflammatory bowel disease (IBD),[95] irritable bowel syndrome (IBS),[96] colon cancer,[97] NAFLD,[56,98] and other digestive diseases. SCFA types and levels in the intestine vary with carbohydrate consumption and gut dysbiosis, but they promote NAFLD progression via several mechanisms such as binding to GPCRs. Using isotope-labeled SCFA enemas in rats, Besten *et al.*[99] found that acetic acid, propionic acid, and butyric acid were involved in the expression of fat metabolism-related genes. SCFAs protected the liver by reducing intestinal mucosa permeability through the gut-liver axis and inhibiting endotoxin translocation.[99] A recent study by Mollica *et al.*[100] reported that butyric acid and its synthetic derivative, N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), regulated mitochondrial function, efficiency, and kinetics, and proposed it as a new therapeutic strategy to combat obesity and IR. Specifically, butyric acid and FBA improved respiratory capacity and fatty acid oxidation, activated the AMPK acetyl-CoA carboxylase pathway, and promoted inefficient metabolism, thereby reducing intracellular lipid accumulation and oxidative stress.[100] Moreover, in another study, acetic acid inhibited liver fat accumulation without changing food consumption or skeletal muscle weight, and was also associated with the PPARα and AMPK pathways.[101] Notably, an NAFLD study demonstrated statistically significant differences in *Clostridium* and *Bacteroidetes* percentages compared with normal groups. Indeed, the changes between *Clostridium* and *Bacteroidetes* may adjust the proportion of SCFAs that affect the energy supply and demand in the liver, altering the progress of NAFLD.[102]

The GPCRs, GPR41 and GPR43 are the main targets of SCFAs acting on intestinal endocrine cells, and produce a variety of effects that may lead to NAFLD. The exact mechanisms are related to the slowing of gastric emptying and intestinal transit and improved nutrient absorption,[103] inhibiting lipolysis and promoting fat cell differentiation,[16,104] and increasing intestinal inflammation and permeability to participate in NASH pathogenesis.[105]

Bacterially-derived ethanol

Endogenous alcohol refers to ethanol produced by dietary sugar fermentation, with intestinal microbiota being the main source of this alcohol. Under normal physiological conditions, the body's metabolism will continuously produce ethanol.[106] After eating nonalcoholic food, the blood ethanol concentration also increases. Bacterially-derived ethanol is quickly and completely eliminated in the portal vein by liver alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol oxidizing system. When ADH is inhibited, blood ethanol concentrations increase. The fact that the human liver and digestive tract both have the highest ADH activities proves that the intestinal tract produces alcohol.[107]

NAFLD and alcoholic fatty liver disease are pathologically similar and may have common pathogenic mechanisms. Studies have confirmed that blood ethanol concentrations are higher in obese patients or obese mice than in lean individuals, suggesting intestinal alcohol may be related to the occurrence of NASH.[108] In addition, excess growth of small intestinal bacteria and gut dysbiosis (e.g., increased *Escherichia coli*) may lead to increased levels of endogenous alcohol. Zhu *et al*.[109] reported significantly increased *E. coli* levels in patients with NASH compared with obese patients. As *E. coli* is the main alcohol-producing bacteria, differences in blood ethanol concentrations were observed, suggesting a role for alcohol-producing microbiota in this condition. Moreover, recent studies reported the increased expression of alcohol-metabolizing enzymes (i.e. ADH) in patients with NASH. Specifically, increased ADH activity increased acetaldehyde levels, which further increased small intestine mucosa permeability. The absorption of intestinal microbiota metabolites increased, which augmented acetaldehyde levels and promoted NASH.[110]

Ferroptosis in NAFLD

Iron overload is prevalent in NAFLD patients, and it is widely accepted that iron-induced lipid peroxidation is one of the major triggers of NAFLD.[111] In addition, iron imbalance is associated with obesity and IR, both of which are typical features of patients with NAFLD.[112] In general, people tend to speculate that ferroptosis may be involved in the pathogenesis of NAFLD, which has been confirmed by numerous studies.[113] Fortunately, some drugs that act on ferroptosis targets (e.g., sorafenib, sulfasalazine, and artesunate) have been widely reported, making it possible that ferroptosis could be a key target for the treatment of NAFLD (Table 2).[114–126]

Dietary Fe^{3+} is absorbed by duodenal intestinal epithelial cells and reduced to Fe^{2+} by divalent metal-ion transporter-1 (DMT1). Fe^{2+} absorbed into the blood is oxidized to Fe^{3+} by ceruloplasmin, bound by transferrin, and then transported to tissues. However, because of the first-pass effect of the hepatic portal circulation, iron exposure of the liver is much greater than that of other tissues, resulting in liver damage and various complications.[127] Serum ferritin is a clinical biomarker for detecting iron homeostasis in the body. When the serum ferritin content is abnormal, the overload operation of the liver as an organ responsible for removing serum ferritin further aggravates liver damage. The expression of ferritin is influenced by iron stores and inflammation, and elevated ferritin levels are common in NAFLD.[128] In a study

Table 2. Drugs targeting ferroptosis in liver disease

	Drug	Target	Mechanism	Reference
Ferroptosis promoters	Erastin, glutamate, ulfasalazine, sorafenib	System Xc^-	Inhibits system Xc^-, resulting in GSH depletion	177,116
	FIN56	GPX4	Depletes CoQ_{10}, resulting in lipid peroxidation	117
	$FINO_2$	GPX4	inhibits GPX4 enzymatic function and directly oxidizes iron, ultimately causing widespread lipid peroxidation	118
	Statin	HMG-CoA reductase	Inhibits CoQ_{10}, resulting in lipid peroxidation	119
	Artesunate	Ferritinophagy	Activates ferritinophagy	120
Ferroptosis inhibitors	Ferrostatins, liproxstatins-1, vitamin E	PUFAs, GPX4	Inhibits lipid peroxidation	121–123
	Ginkgolide B	Nrf2	Activates Nrf2, leading to reducing lipid peroxidation	114
	Deferoxamine (DFO), deferiprone	Iron	Chelate iron ions	124,125
	Dihydrobiopterin (BH2), tetrahydrobiopterin (BHA)	Lipid Remodeling	Selectively preventing depletion of phospholipids with two polyunsaturated fatty acyl tails	126

CoQ10, coenzyme Q10; GPX4, glutathione peroxidase 4; GSH, glutathione; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; Nrf2, nuclear factor erythroid 2-related factor 2; PUFAs, polyunsaturated fatty acids.

of 628 adult patients with biopsy-proven NAFLD, a serum ferritin (SF) 1.5 times the upper limit of normal has been associated with a diagnosis of NASH, higher steatosis grade, and lobular inflammation. Elevated SF was also found to be an independent predictor of advanced hepatic fibrosis in patients with NAFLD.[129] Also, a study of 25,597 participants in Korean National Health and Nutritional Examination Surveys between 2007 and 2012 and confirmed that people with higher SF levels were more likely to have NAFLD. An increase in SF of 10 ng/mL increased the likelihood of NAFLD by 3–10%.[130] Therefore, many researchers have proposed that in equivocal circumstances, SF measurement can be used to assess the risk of NAFLD.[131] However more long-term studies are needed to assess the relationship between SF levels and complications of liver disease (e.g., HCC) and liver-related mortality.

Iron overload is prevalent in NAFLD patients.[132] In a retrospective study, patients with biopsy-proven NAFLD and iron overload had poor long-term outcomes[133] that may have been the result of increased IR, excess hepatic lipid peroxidation, and accelerated liver fibrosis progression caused by iron overload.[134] Loguercio *et al*.[135] found that more than 90% of NAFLD patients had increased levels of lipid peroxidation markers, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which were significantly higher in NASH patients than in steatosis patients. Qi *et al.*[113] studies the impact of ferroptosis on the progression of NASH induced by a methionine/choline-deficient diet (MCD) for 10 days. RSL3, a ferroptosis activator, aggravated symptoms, including serum biochemical index levels, liver steatosis, and inflammation) in mice with NASH induced by the MCD diet. Sodium selenite, a GPx4 activator, rescued RSL3-induced lipid peroxidation and cell death. Similarly, Li *et al*.[20] used RNA-seq analysis to show that arachidonic acid metabolism promote ferroptosis in the MCD diet-induced NASH mouse model, suggesting that ferroptosis may be a therapeutic target for NASH treatment. Consistently, other studies found that some drugs like Ginkgolide B and dehydroabietic acid alleviated NASH severity by inhibiting ferroptosis. In that context, Nrf2 and GPx4 stand out as major protective mechanisms.[114,136] Overall, the results imply that the regulation of ferroptosis in the context of NAFLD is an intriguing notion that deserves further investigation.

Targeting the gut microbiota to prevent NAFLD

As discussed, gut dysbiosis and associated metabolites such as BAs, SCFAs, and endogenous ethanol, and inflammatory responses and damage of the intestinal barrier are important factors during NAFLD occurrence and development. If those conditions are corrected, NAFLD progression can be slowed and possibly reversed. This section focuses on gut microbiota regulation as a therapeutic target for NAFLD prevention, including lifestyle and diet therapies, antibiotics, probiotics, and prebiotics, glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1 RA), and sodium/glucose cotransporter-2 inhibitors (SGLT2i; Table 3).[137,–146]

Lifestyle and diet therapy

NAFLD is closely related to obesity.[147] Studies have shown that eating foods rich in fat and fructose alters the intestinal microbiota, changes intestinal barrier function, and causes endotoxemia and inflammatory reactions, all of which promote obesity and NAFLD.[148] Therefore, the most important treatment goal for patients with NAFLD is weight reduction and maintenance of a healthy lifestyle to reduce liver fat deposition and inflammatory responses. In addition, a balanced diet, adequate sleep, and appropriate exercise are essential to maintain intestinal microbiota stability and health, and to reduce the risk of other diseases. Dietary interventions are effective in the treatment of NAFLD patients. Even a modest 3–5 kg weight gain predicts the development of NAFLD independent of baseline BMI. In addition, patients with NAFLD were found to experience a 75% remission rate with a weight loss of 5% or more from baseline.[149] Much evidence suggests that the Mediterranean diet can reduce liver fat, even without weight loss. It is the most recommended diet for NAFLD.[150] The Mediterranean diet in-

Table 3. Gut microbiota-targeted therapies of NAFLD

Interventions		Main effects	Experimental model	Clinicaltrials.gov ID	Reference
Lifestyle and diet therapy	Keeping circadian rhythm	Circadian rhythm disorders increase intestinal permeability, promoting hepatic steatosis	High-fat diet (45% fat)	–	137
	Weight loss/ exercise	Increasing gut microbe diversity, improving metabolic capacity, and reducing *Bacteroides* to *Firmicutes* ratio	Mice with HFD (45% kcal from fat) for 12 weeks	–	138
Antibiotic	Rifaximin (1,200 mg/daily)	Reduction in serum AST, ALT, and endotoxin	Biopsy-proven NAFLD, *n*=42	NCT02009592	139
	Cidomycin	Lowering serum levels of ALT, AST and alleviating the severity of NASH	Rats with NASH	–	143
Probiotics	Lactobacillus (LcS)	Suppressing NASH development	MCD diet-induced NASH in mice	–	144
	Bifidobacterium (Bif)	Ameliorating visceral fat accumulation and insulin sensitivity	HFD-fed rats	–	145
	VSL#3	Improving the degree of liver disease in children	44 obese children with NAFLD	NCT01650025	146
Prebiotic	Fructo-oligosaccharides (FOS)	Restoring normal gastrointestinal microflora and decreasing steatohepatitis	MCD diet-induced NASH in mice	–	140
	Lactulose	Ameliorating the hepatic inflammation and decreasing serum levels of ALT and AST	HFD-induced NASH in rats	–	141
GLP-1 RA	liraglutide	decreasing *Proteobacteria* and increasing *Akkermansia muciniphila*	HFD mice	–	142

NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; HFD, high-fat diet; MCD, methionine-choline-deficient; GLP-1 RA, glucagon-like peptide-1 (GLP-1) receptor agonists.

cludes nuts, fruits, legumes, olive oil, vegetables, and fish, in which consumption of sugar and refined carbohydrates is decreased and consumption of monounsaturated fatty acids and omega-3 fatty acids is increased. Dietary micronutrients also greatly influence the progression of NAFLD. Studies have shown that the intake of micronutrients such as vitamin C, vitamin D, and choline is significantly negatively correlated with the prevalence of NAFLD, which may be related to their antioxidant and antifibrotic activity.[151] However, randomized controlled trials have not resulted in clear evidence that high-dose vitamin D supplementation is beneficial for hepatic steatosis or IR in NAFLD.[152] Although the Mediterranean diet advocates moderate alcohol consumption, whether or not alcohol should be allowed in NAFLD patients remains controversial. Regular alcohol consumption increases the risk of developing HCC in NASH patients with cirrhosis, so alcohol should be avoided in such patients.[153] However, in patients without cirrhosis, uncertainty about the impact of moderate drinking (e.g., 12 ounces of beer, 4 ounces of wine, or 1 ounce of liquor) is not clear, so prudent drinking is advised.

Sleep is an essential physiological process required for normal function, and adequate sleep patterns help maintain normal homeostasis. In recent decades, studies have shown that sleep-related factors, especially sleep time, influence the risk of obesity.[154] A prospective study by Nielsen *et al*.[155] reported that patients with obesity slept for significantly shorter periods than nonobese individuals. Also, Gildner *et al.*[156] observed that in middle-aged individuals, short sleep duration was significantly correlated with elevated BMI and waist circumference. However, obesity-associated mechanisms caused by shortened sleep times remain unclear. A recent study showed that a chronic lack of sleep decreased leptin and increased ghrelin levels, resulting in a "hyperappetite."[157] Increased eating rate caused by prolonged wakefulness was also a cause of obesity.[158] People with short sleep times are prone to fatigue that leads to reduced exercise, increased weight gain, or obesity. In addition to sleep time, changes in circadian rhythm influence development of obesity and NAFLD progression.[137] Voigt *et al*.[159] reported that reversing circadian rhythms in mice changed the Firmicutes and Bacteroidetes composition in those fed a high-sugar diet, but the microbiome in mice fed normal diets did not change. Summa *et al.*[160] also found that circadian rhythm disorders increased intestinal permeability in mice, promoting alcohol-induced steatohepatitis.[160]

Weight loss is recognized as a basic and key measure for NAFLD management, and exercise is an effective and safe way to lose weight. For patients with NAFLD, exercise not only directly reduced liver fat content, but also reduced fatty acid absorption, improved insulin sensitivity,[161] reduced liver transaminase indicators, and improved other metabolic indicators.[162] In 2009, George *et al*.[162] compared the low intensity exercise, medium intensity exercise, and control groups. After a 3-month intervention, patients who maintained more than 150 min/week had decreased serum transaminase levels, independent of changes in body mass, effectively illustrating physiological exercise advantages for these patients. Previous studies have also shown that exercising changes the composition of intestinal microbes and affects NAFLD progression.[163] Munukka *et al.*[164] recruited 17 overweight adult women for a 6-week bicycle endurance training study and found that exercises decreased Proteobacteria, but significantly increased *Akkermansia* levels.

Animal studies have also confirmed the effect of exercises on gut microbiota. Petriz *et al.*[165] reported that treadmill exercises changed the composition and abundance of microorganisms, and training increased lactobacilli, (beneficial bacteria) numbers in obese rats. Denou *et al.*[138] conducted a 6-week high-intensity exercise regime in rats fed a high-fat diet, and found that the exercise intervention increased gut microbe diversity, improved metabolic capacity, and reduced the *Bacteroides* to *Firmicutes* ratio.

Antibiotics

Therapeutic antibiotics inhibit excessive proliferation of intestinal microbes and bacterial translocation. They alter disease-related microbial communities to ensure healthy homeostasis. Antibiotics eliminate harmful microbiota, and are effective in several digestive-system models, including hepatic encephalopathy,[166] IBS,[1167] IBD,[168] and NAFLD.[47,169] The therapeutic effects of antibiotics in NAFLD are attributed to (1) improving leaky gut by reducing pathogens and potential pathogens and suppressing liver inflammation and (2) reducing harmful bacterial metabolites which promote NAFLD. In an observational study, Gangarapu *et al.*[139] reported that after 28 days of with rifaximin treatment of NASH patients, circulating endotoxin levels were significantly reduced. Short-term rifaximin treatment acted on intestinal bacteria to achieve the desired therapeutic effectiveness. However, more clinical trials are required to confirm effective rifaximin treatment cycles for patients with NAFLD.

Animal studies have reported that antibiotics quickly and significantly altered the intestinal microbiota. Broad-spectrum antibiotics, such as ampicillin, neomycin, metronidazole, and vancomycin reduced hepatitis by regulating free secondary BA levels and improving liver steatosis by inhibiting the FXR pathway to downregulate liver SREBP1C expression.[85,170] However, another study found that penicillin G and erythromycin aggravated liver lipid metabolism and inflammatory reactions.[171] Antibiotics also promoted liver lipid accumulation, inflammatory responses, and liver fibrosis by increasing liver immune damage.[172] At the same time, we cannot ignore the impact of antibiotics on the beneficial intestinal microbiota, therefore correct use of effective antibiotic treatment requires more comprehensive research.

Probiotics and prebiotics

Probiotics are living microorganisms that benefit host health.[6] Studies show that they regulate the intestinal microbiota,[173] enhance intestinal barrier function,[174] reduce intestinal permeability,[175] alleviate immune and metabolic damage,[176] up-regulate fatty acid oxidation,[177] and reduce liver steatosis and inflammatory-response damage.[178] A recent meta-analysis confirmed that *Lactobacillus*, *Bifidobacterium*, *Streptococcus* probiotics, when used for 8–24 weeks were beneficial for the recovery of liver enzymes and IR in patients with NAFLD.[179] A clinical study reported that after a 6 month intervention with the probiotic, *Lepicol* in 10 patients with NASH, intrahepatic triacylglycerol levels were reduced by more than 30% compared with baseline levels and serum AST levels were significantly reduced.[180] A randomized controlled trial of 42 patients with NAFLD found that fasting blood glucose, insulin, IR, TNF-a, and IL6 were significantly reduced after 8 weeks of probiotic treatment (two capsules/day).[181] Other studies have reported an association of probiotics on liver fibrosis or death in NAFLD patients, therefore results are inconsistent. A liver biopsy clinical study reported that *Bifidobacterium longum* supplementation significantly improved liver steatosis, but not liver fibrosis.[182] In a long-term survey of 39 biopsy-confirmed patients with NAFLD, the continuous use of the probiotic, VSL#3 for 1 year significantly improved NAFLD activity scores, hepatocyte swelling, and liver fibrosis. Prebiotics are dietary supplements that benefit the host by selectively stimulating the growth and/or activity of one or several bacterial colonies.[183] Matsumoto *et al*.[140] studied the effects of fructo-oligosaccharides (FOSs) on intestinal barrier function and steatohepatitis in mice with methionine-choline deficiency. Liver inflammation and hepatocyte steatosis in FOS-treated mice were significantly reduced ($p<0.01$), suggesting that 3 weeks of treatment improved NAFLD and restored barrier functions in the intestinal tract.[140] The probiotic lactulose promoted *Bifidobacteria* and lactic acid bacteria growth. Fan *et al.*[141] used it to treat mice with NAFLD induced by a high-fat diet, and showed that liver inflammation indicators such as AST and ALT in the lactulose treatment group (0.9 mL/kg/day for 8 weeks) were significantly reduced, but hepatocyte steatosis was not significantly improved, suggesting that lactulose reduced liver inflammation but did not improve fat degeneration in liver cells.[141]

Glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1 RAs) and sodium/glucose cotransporter-2 inhibitors (SGLT2is)

GLP-1 is an incretin secreted by L cells in the distal small intestine and colonic mucosa after meal stimulation in a glucose concentration-dependent manner. It promotes insulin secretion and participates in the regulation of blood glucose homeostasis. GLP-1 has a very short half-life *in vivo*, and is degraded by dipeptidyl peptidase-4 (DPP-4), so it cannot be used for disease treatment.[184] GLP-1 RA belongs is an incretin drug with pleiotropic effects such as lowering blood glucose and blood lipids and reducing body weight.[185] Recent studies have found that GLP-1 RA improved IR in NAFLD, reduced liver steatosis, and improved liver fibrosis. It is of great significance for the treatment of NAFLD, especially NAFLD complicated with T2DM.[186] A randomized, multicenter, double-blind, placebo-controlled phase 2 trial in the UK that evaluated the safety and efficacy of subcutaneous liraglutide, an acylated GLP-1 RA, 1.8 mg daily compared with placebo in patients with biopsy-confirmed NASH. Liraglutide significantly improved hepatic steatosis by 83% in the liraglutide group and 45% in the placebo group, and hepatocyte swelling by 61% in the liraglutide group and 32% in the placebo group. which indicated that the patient's NASH was in remission. The histological effects of liraglutide on NASH were not entirely mediated by its action on the improvement of glycemic control.[187] A study by Moreira *et al*.[142] showed that liraglutide not only reduced hepatic fat accumulation by 78% in ob/ob mice and reversed steatosis in HFD mice, but also altered the overall gut microbial composition. *Proteobacteria* decreased and *Akkermansia muciniphila* increased in the mice fed the HFD. The studies suggest that GLP-1 RA contributed to the improvement of NAFLD by the regulation of gut microbiota, which offers a new perspective for us to find gut microbiota-targeted therapies of NAFLD.

Sodium-glucose cotransporter 2 (SGLT-2) inhibitors are a class of hypoglycemic drugs that is commonly used in clinical practice to reduce the reabsorption of glucose by the kidneys, intestines, and heart. Several studies showed that SGLT-2i was associated with improvement of hepatic steatosis.[143] In an open-label, randomized, active-controlled trial, Ito *et al*.[189] of 66 patients with type 2 diabetes and NAFLD found that ipragliflozin 50 mg significantly reduced body weight and visceral fat area. A similar study by Nasiri-Ansari *et al*.[190] in mice fed an HFD found that empagliflozin reduced fasting glucose, total cholesterol, and serum tri-

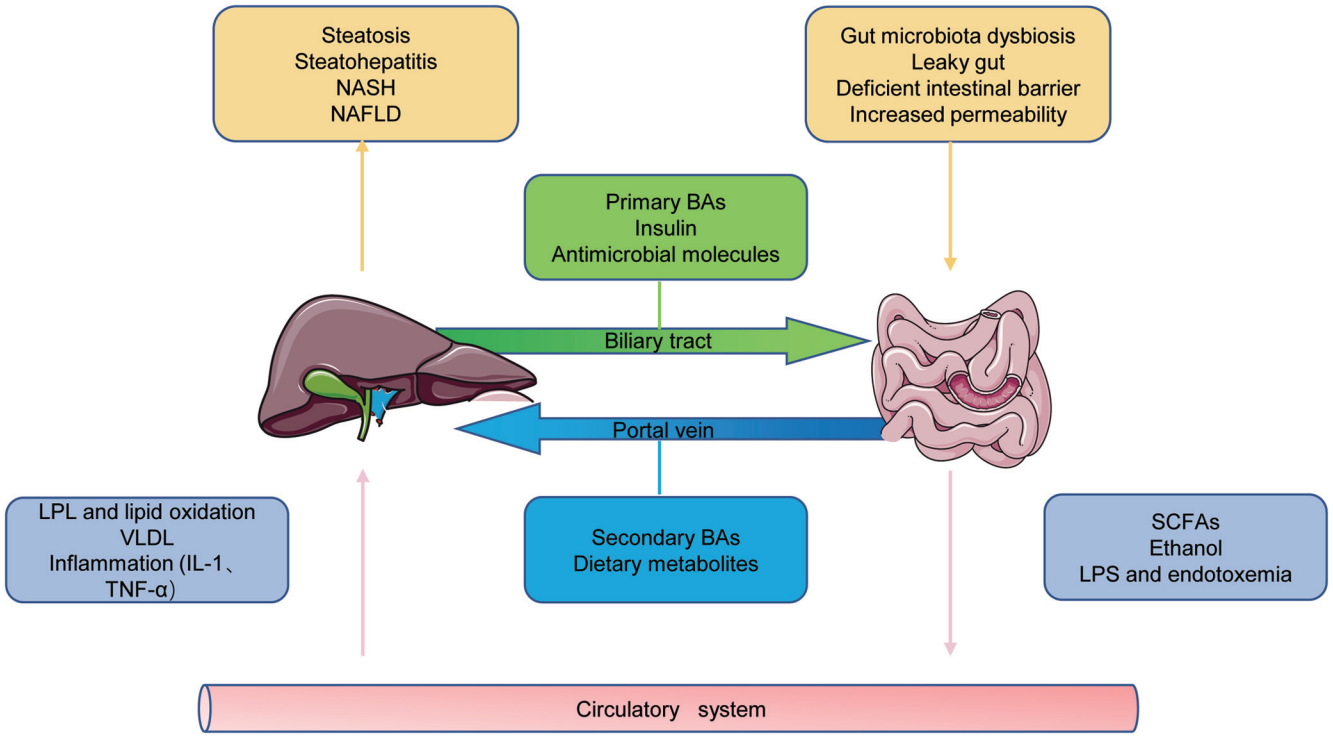

Fig. 2. Promotion of gut microbiota in NAFLD. Gut microbiota dysbiosis includes the reduction of beneficial bacteria and SIBO, change of the small intestine mucosal barrier, increase of intestinal permeability and microbial metabolites including LPS and SCFAs, leads to an increase in endotoxins and inflammatory factors that enter the liver through the gut-liver axis that induces immune and inflammatory reactions, leading to the progression of NAFLD NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; BAs, bile acids; SCFAs, short-chain fatty acids; LPS, lipopolysaccharide; VLDL, very low-density lipoprotein; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α.

glyceride levels; and decreased the NAFLD activity score, expression of lipogenic enzymes, and inflammatory molecules. However, side effects associated with SGLT-2 inhibitors, such as increased risk of urinary and genital infections cannot be ignored. The increased risk may be explained by the fact that persistent diabetes may promote the growth of pathogenic microorganisms. A meta-analysis showed that gliflozins were associated with an increase in genitourinary infections,[191] and they have also been reported to increase the risk of malignancy, particularly of the breast or bladder, but no studies have confirmed that possibility.[192]

Conclusions

Evidence that the gut microbiota has important mechanistic roles in NAFLD occurrence and progression is increasing. Gut microbiota dysbiosis usually reduces beneficial bacteria and SIBO, changes small intestine mucosal barriers, and increases intestinal permeability and microbial metabolites (e.g., LPS and SCFAs). That increases endotoxins and inflammatory factors that enter the liver via the gut-liver axis, inducing immune and inflammatory reactions, and culminating in NAFLD (Fig. 2).

No drugs are currently licensed for NAFLD therapy, but diet and exercise have proven to be effective treatments. Because of the relationship between NAFLD and T2DM, many diabetes drugs have achieved positive results in relieving NASH. In addition, experimental drugs targeting intermediate metabolism in NAFLD have also been shown to be beneficial, but adverse effects may limit their use. This review focuses of the gut microbiota and ferroptosis treatments for NAFLD, as well as proposing new treatment strategies. Lifestyle and diet, antibiotics, regulation of ferroptosis, probiotics, and prebiotics, GLP-1 RA, and SGLT2i may become effective and safe treatments to alleviate NAFLD. However, to effectively transform and apply animal model findings to humans, well-designed large clinical trials, spanning multiple disease etiologies and patient characteristics, are required.

Acknowledgments

We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

Funding

This study was supported by: (1) the National Natural Science Foundation of China (grant number: 82002539); (2) the Natural Science Foundation of Shanghai (grant number: 19ZR1447700); (3) the Health System Innovation Project of Shanghai Putuo Science and Technology Commission (grant numbers: PTKWWS201903); (4) The Yangfan Plan of Shanghai Science and Technology Commission (grant no. 21YF1435400); (5) Shanghai Municipal Health Commission, China (grant no. 201840233).

Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Study concept and design (JJ, LW), drafting of the manuscript (JJ, LW), critical revision of the manuscript for important intellectual content (JJ, JW), administrative support (GC, JW), and study supervision (CG). All authors have made a significant contribution to this study and have approved the final manuscript.

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