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The gut microbiome and liver cancer: mechanisms and clinical translation

Le-Xing Yu[1] and **Robert F. Schwabe**[1,2]

[1]Department of Medicine, Columbia University, 1130 St. Nicholas Ave, Room 926, New York, NY 10032, USA.

[2]Institute of Human Nutrition, 1130 St. Nicholas Ave, Room 926, New York, NY 10032, USA.

Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of worldwide cancer mortality. HCC almost exclusively develops in patients with chronic liver disease, driven by a vicious cycle of liver injury, inflammation and regeneration that typically spans decades. Increasing evidence points towards a key role of the bacterial microbiome in promoting the progression of liver disease and the development of HCC. Here, we will review mechanisms by which the gut microbiota promotes hepatocarcinogenesis, focusing on the leaky gut, bacterial dysbiosis, microbe-associated molecular patterns and bacterial metabolites as key pathways that drive cancer-promoting liver inflammation, fibrosis and genotoxicity. On the basis of accumulating evidence from preclinical studies, we propose the gut-microbiota–liver axis as a promising target for the simultaneous prevention of chronic liver disease progression and HCC development in patients with advanced liver disease. We will review in detail therapeutic modalities and discuss clinical settings in which targeting the gut-microbiota–liver axis for the prevention of disease progression and HCC development seems promising.

Studies from the past decade have shed light on the important contributions of the gut microbiota to key aspects of our health. Although the gut microbiota provides substantial benefit to the host, in particular with respect to metabolism and immunity[1,2], there is also increasing recognition of the involvement of the gut microbiota in disease processes [3]. In addition to bacteria, the gut microbiota contains Archaea, eukaryotes such as fungi, and viruses. As the role of the commensal nonbacterial gut microbiota is not as well known, we will exclusively focus on the bacterial gut microbiota in this Review. The bacterial gut microbiota promotes disease development not only via local effects, as in chronic IBD[4,5], but also at distant sites such as the liver, heart, brain and the haematopoietic system[6–10]. Likewise, there is accumulating evidence for an important contribution of the gut microbiota to carcinogenesis via local and long-distance effects[11]. Owing to its anatomic connection via the portal vein, the liver is closely linked to the gut. Not only does the liver receive nutrient-

Correspondence to R.F.S. rfs2102@cumc.columbia.edu.

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Both authors contributed equally to the Review

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rich blood from the intestine, but it is also the first target of the intestinal microbiota, microbe-associated molecular patterns (MAMPs) – which may elicit inflammatory responses via pattern recognition receptors (PRRs) - and microbial metabolites. The multi-layer intestinal barrier ensures that hepatic exposure to pro-inflammatory MAMPs is minimal. However, a failing gut barrier and alterations of the gut microbiota in chronic liver disease (CLD) contribute to chronic inflammation and the progression of liver diseases[12], and thereby increase risk for the development of hepatocellular carcinoma (HCC) as the final stage of the disease process [13–15]. Here, we will review how the gut microbiota promotes the development of HCC, focusing on alterations of the gut microbiota at different disease stages and mechanisms by which it contributes to disease progression and HCC development in different types of liver diseases. We will then review therapeutic opportunities to interrupt this disease-promoting signalling axis, with a focus on the most promising drugs and clinical settings to test these therapeutic strategies.

1. THE INTESTINAL EPITHELIAL BARRIER

Strict separation of microbial entities from the host compartment forms the basis for a symbiotic relationship between host and microbiota. In the intestine, this partitioning is achieved by a well-maintained, multi-layer barrier[16,17]. This barrier relies on an intact epithelial lining, a mucus layer, Paneth and goblet cells, mucosa-associated lymphoid tissue, as well as a number of secreted factors such as IgA and defensins[17]. With constant changes in intestinal luminal contents and high epithelial cell turnover, the gut barrier is a highly dynamic system and can rapidly adjust. Continuous sampling of gut microorganisms by specialized epithelial cells, termed M cells, regulates the microbiota through the secretion of antibacterial peptides by Paneth cells; vice versa, the intestinal barrier and epithelial cell growth are regulated by the microbiota[17]. Moreover, the intestinal microbiota also suppresses the growth of pathobionts, as demonstrated by the protective role of the commensal microbiota against *Clostridium difficile* infection[18] and the increased susceptibility of germ-free mice to infection with pathogens[19]. Bile acids represent another key factor in this complex system, regulating epithelial barrier function and the proliferation of intestinal epithelial cells via farnesoid X-activated receptor (FXR)-dependent and epidermal growth factor receptor (EGFR)-dependent pathways[20–22], and controlling the growth and adhesion of intestinal bacteria[16]. Of note, bile acids provide an important link between the liver, bacterial microbiota and the intestine. After being synthesized in the liver, bile acids are metabolized by bacteria and sensed by FXR expressed by intestinal epithelial cells (IECs), which in turn provide feedback to the liver via the FGF19 (known as FGF15 in mice) pathway[23].

Acute and chronic liver diseases exert major effects on the composition of the intestinal microbiota and intestinal barrier function, resulting in dysbiosis and a leaky gut, respectively. The majority of studies on the gut–liver axis in CLD have focused on lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and one of the most potent inducers of inflammation via binding to the transmembrane receptor Toll-like receptor (TLR) 4 (discussed in detail later). Mean portal vein LPS levels increase in chronic liver injury from <3 pg/ml in healthy volunteers to 4.9 pg/ml, 7.9 pg/ml and 10.2 pg/ml in patients with Child–Turcotte-Pugh cirrhosis stage A, B and C, respectively[24]. Likewise,

chronic alcohol intake increases endotoxin levels in peripheral blood from 2.5 pg/ml in healthy individuals to 14–19 pg/ml in patients with alcoholic liver disease (ALD)[25]. Increases in blood LPS levels reflect gut leakiness and are mirrored by a number of other alterations such as increased intestinal permeability to high molecular weight polyethylene glycol in patients with ALD[26] and to FITC-dextran in mouse models of alcoholic and biliary liver disease[27,28]. Moreover, there is an increase in bacterial DNA , a well-established TLR9 agonist, in the peripheral blood of patients with CLD[29]. Together, these findings demonstrate that the chronically injured liver is subject to increased exposure to a wide range of TLR ligands as well as other bacterial products and metabolites. These pro-inflammatory mediators not only promote the development of CLD but also set the stage for the development of HCC[13–15].

Mechanisms underlying the failure of the intestinal barrier and development of a leaky gut are not fully understood and are most likely multifactorial. Contributing factors include decreased bile acid secretion, bacterial dysbiosis and a subsequent increase in the expression of inflammatory cytokines in the intestine, a failing immune system and increased permeability of the gut–vascular barrier[16,30,31]. Although the development of a leaky gut has been demonstrated by a wide body of literature, the changes in the intestinal microbiota in patients with CLD are only beginning to be understood. Studies in the past few years have demonstrated profound alterations of the intestinal microbiota in patients with cirrhosis, showing increased *Enterobacteriacea* as well as strains that are typically found in the oral microbiota, such as *Veillonellaceae* and *Streptococcaceae*, consistent with an invasion of microorganisms from the mouth to the gut in liver cirrhosis[32,33]. At the same time, there is decreased abundance of beneficial bacteria in the gut, such as *Lachnospiraceae*[33]. These changes seem to develop progressively, as cirrhosis stage is positively correlated with *Enterobacteriaceae* and negatively correlated with *Lachnospiraceae*[33,34]. Likewise, a number of studies have demonstrated alterations of the gut microbiota in earlier stages of liver disease [35,36] as well as in animal models[16,31]. However, the current understanding of the alterations of the gut microbiota in patients with liver diseases remains incomplete and is complicated by several factors, including: changes in the gut microbiota might be disease-specific; patients with advanced liver disease often take drugs that alter the composition of the microbiota, such as antibiotics, lactulose or antacids; the faecal microbiota might not reflect some of the most characteristic alterations in CLD, such as bacterial overgrowth in the upper gastrointestinal tract; and there could be changes in the adherent microbiota that are not reflected by studying luminal microbiota. In addition, well-designed functional studies are needed to understand the contribution of dysbiotic microbiota to liver disease. Not only is it essential to confirm that dysbiosis is a driver of liver disease development and progression, but it is also important to determine whether dysbiosis contributes to gut leakiness in CLD.

2. GUT MICROBIOTA AND DISEASE PROGRESSION

HCC is typically the result of chronic disease processes in the liver and almost never occurs spontaneously in the absence of liver disease. Moreover, ~80–90% of HCCs occur in advanced fibrotic or cirrhotic livers, which translates to around one in three patients with compensated liver cirrhosis developing HCC in their lifetimes[37,38]. Hence, the presence of

liver cirrhosis represents the most important unifying risk factor for the development of HCC. However, additional factors are involved and each type of underlying liver disease entails a specific risk for the development of HCC in cirrhosis; diseases such as chronic hepatitis B and C or haemochromatosis entail a relatively high risk and diseases such as autoimmune hepatitis or ALD a relatively low risk[38–40]. To dissect the contribution of the failing gut barrier and alterations of the gut microbiota to HCC development, it is not only important to understand how they might affect the development of HCC within a cirrhotic liver but also how these factors drive the progression of liver disease to advanced disease stages (which entail a significant risk for HCC development). Below, we will summarize disease-specific mechanisms by which the gut microbiota promotes progression of liver disease.

2.1. ALD

ALD contributes to about half of all cirrhosis cases[41] and is a cofactor in liver disease induced by HBV, HCV and NASH. Although ALD might have a lower relative risk of causing HCC than other types of CLD[39], the sheer number of patients with alcoholic cirrhosis means that the absolute number of HCCs caused by ALD is high. Moreover, subgroups of patients, such as those with cirrhosis, men, patients >55 years of age, individuals positive for antibodies against hepatitis B core protein (anti-HBc) as well as patients with high cumulative consumption of alcohol, might have extremely high risk development for HCC development, risk that may be >40% in a 10-year period[42].

The key contribution of the gut microbiota to early stages of ALD has been firmly established in the past two decades. Even a single binge of alcohol is sufficient to increase bacterial translocation, as evidenced by an increase of LPS in portal blood rats from undetectable levels to 30–80 pg/ml after ethanol administration [16]. Likewise, serum LPS levels are increased in patients with chronic alcohol abuse[16]. The ability of ethanol and its metabolite acetaldehyde to disrupt tight junctions contributes to the high levels of bacterial translocation in ALD[43]. Moreover, mice receiving intragastric alcohol feeding show perturbations of the intestinal microbiota, with reduced synthesis of long-chain fatty acids[44]. A number of functional studies have shown a key contribution of the gut-microbiota–TLR4 axis to ALD[45]: Global TLR4 deficiency in mice as well as gut sterilization with nonabsorbable antibiotics in rats reduces hepatic steatosis, oxidative stress and inflammation[46–48].

Owing to difficulties in modelling advanced stages of ALD in rodents, the functional contribution of the intestinal-microbiota–TLR4 axis in advanced liver disease, such as in the development of cirrhosis and HCC, is not well known. In one study, ethanol-fed transgenic mice with global TLR4 deficiency, which additionally expressed the NS5A HCV protein, were protected from HCC development, suggesting that TLR4 signalling synergizes with HCV to promote HCC[49]. This finding fits well with the well-established clinical observation that alcohol abuse is an important cofactor in promoting liver disease development and HCC in patients with chronic HCV infection [50] and suggests a potential role for the LPS–TLR4 axis in the synergy between alcohol and HCV.

2.2. NAFLD

Although recognized as a disease only about two decades ago, NAFLD represents the most prevalent liver disease, and is projected to become the leading contributor to CLD and the development of HCC[51]. In comparison to other CLDs, NAFLD carries a low relative individual risk for HCC development, but makes a big population-wide contribution to HCC development owing to its high prevalence[51]. Studies in germ-free and gnotobiotic mice have revealed a key contribution of the gut microbiota to metabolism and energy harvest. As such, germ-free mice display decreased body weight despite increased food intake[52]. Metagenomic and microbiota transplantation studies have shown that the gut microbiota from obese individuals is more efficient at energy extraction and thereby contributes to obesity[53,54]. Hence, treatment with antibiotics ameliorates high-fat-diet-induced NAFLD in mice[55]. Moreover, patients with NAFLD display dysbiosis. However, bacterial abundance patterns were not consistent between studies, with levels of Bacteroidetes increased in some studies[36,56] and decreased in other studies[57,58], and a substantial overlap with healthy individuals[59]. Interestingly, dysbiotic microbiota from mice fed a high-fat diet metabolize and convert dietary choline into methylamines, resulting in low circulating levels of plasma phosphatidylcholine[60]. These low levels of phosphatidylcholine impaired secretion of VLDL, thereby reducing hepatic lipid export and contributing to fatty liver[61]. Thus, alterations in choline metabolism might link dysbiosis to the development of NAFLD.

The contribution of the gut microbiota to NASH is not as well documented as its role in earlier disease stages. High-fat diet increases intestinal permeability in mice with a two-to-three-fold increase in systemic LPS levels[62]. Likewise, intestinal permeability is increased in patients with NAFLD[63]. In a model of NASH triggered by high-fat and high-cholesterol diet given to ApoE-deficient mice, TLR4 deficiency reduced hepatic inflammation and injury[64]. To date, the functional role of pathways in NASH development has often been studied in mouse models such as the methionine-choline-deficient (MCD) diet — this diet results in a NASH phenotype strongly resembling human NASH but lacks other essential features of NASH, such as adiposity and insulin resistance, and therefore lacks clinical relevance. In the MCD diet model, the microbiota has a key role in NASH exacerbation as demonstrated by experiments, in which co-housing transmitted NASH risk and antibiotics reduced NASH risk[65]. Conversely, fecal microbiota transplantation from healthy mice attenuated steatohepatitis in high fat diet treated mice [66]. The gut microbiota also has an important part in promoting HCC development in a mouse model in which HCC is driven by the carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA) and subsequent high-fat diet[15]. However, this model does not incorporate key features of NASH such as liver fibrosis and insulin resistance. Hence, further studies are needed to determine the functional role of dysbiosis in the progression of NASH and HCC in mouse models that incorporate adiposity and insulin resistance.

2.3. Chronic viral hepatitis

In contrast to ALD and NAFLD, there is little information on the role of the gut microbiota in chronic viral hepatitis. Current data suggest that dysbiosis and alterations of the gut–liver axis in patients with end-stage viral hepatitis and cirrhosis are similar to alterations in patients with cirrhosis from other causes[67]. However, it is not known whether the gut

microbiota contributes to the pathophysiology of chronic viral hepatitis and its progression to more advanced stages. A recent study demonstrated that the gut microbiota controls immune responses and tolerance to HBV in adult mice, with 6 weeks of antibiotic treatment preventing the clearance of HBV[68]. Whether the impaired response to HBV is mediated by specific bacteria or the result of broad suppression of the bacterial microbiota remains an important unanswered question. Notably, HBV titres in patients positively correlate with risk for disease progression and HCC development[69]. Hence, the gut microbiota might control antiviral responses that affect disease progression and HCC development.

2.4. Liver fibrosis

Liver fibrosis is part of the hepatic wound healing response and common to all types of advanced CLD. Notably, there is a strong correlation between hepatic fibrosis and HCC development with 80–90% of HCCs developing in fibrotic or cirrhotic livers. Thus, fibrosis represents a risk factor for HCC development[70]. There is strong evidence for an important contribution of the microbiota–TLR4 axis to liver fibrosis. Studies from the past six decades have shown that antibiotics prevent hepatic injury and fibrosis induced by CCl_4 treatment, bile duct ligation or a choline-deficient diet, and that endotoxin enhances hepatic fibrosis induced by a choline-deficient diet[71–73]. Studies in knockout mice have highlighted a key role for TLR4 and other important mediators in the TLR4 signalling pathway, such as CD14 and lipopolysaccharide-binding protein (LBP), in experimental models of toxic and cholestatic liver fibrosis[73,74]. However, recent studies have demonstrated an increase in liver fibrosis in germ-free mice[75,76], which seemingly contradicts the decrease of liver fibrosis seen in gut-sterilized mice. It has become apparent that the endogenous commensal microbiota provides hepatoprotective signals and that complete absence of the gut microbiota results in increased liver injury — probably owing to an absence of TLR4-mediated activation of anti-apoptotic NF-κB signalling — and a subsequent increase in liver fibrosis, as demonstrated in several models[13,75,76]. Nonetheless, the bacterial microbiota has an important role in promoting HCC in the setting of liver fibrosis, as demonstrated by reduced HCC formation in TLR4-deficient, germ-free and antibiotic-treated mice in a diethylnitrosamine (DEN) plus CCl_4 model of HCC[13]. Consistent with previous studies[71–73], treatment with nonabsorbable antibiotics resulted in a strong reduction of fibrosis despite increased liver injury[13]. However, further studies are required to investigate how the intestinal microbiota affects HCC development promoted by chronic inflammation, injury and fibrosis, without preceding carcinogen exposure.

3. MECHANISMS BY WHICH THE MICROBIOTA PROMOTES HCC

As discussed above, alterations in gut permeability and the gut microbiota are highly characteristic not only of late stages of all types of CLD, but also occur early in several types of CLD. Thus, the gut microbiota contributes to disease progression at various stages and might promote the development of HCC throughout all these stages. Here, we will discuss mechanisms through which the gut–liver axis promotes HCC development and progression, focusing on the role of the leaky gut and dysbiosis (FIG. 1).

3.1. HCC promotion via a leaky gut and the MAMP–TLR axis

High circulating LPS levels in mice and patients with CLD as well as in HCC[14,24,77,78] demonstrate the presence of a leaky gut during multiple stages of CLD and hepatocarcinogenesis (FIG. 1). Functional experiments in germ-free, gut-sterilized, TLR-deficient and LPS-treated mice have provided evidence that the leaky gut, via LPS and its receptor TLR4, makes essential contributions to hepatocarcinogenesis. As such, HCC development induced by the combination of DEN and CCl_4 was attenuated in gut-sterilized and germ-free mice compared with their specific pathogen-free counterparts[13]. In addition to causing characteristic infectious complications in end-stage liver disease, increased bacterial translocation also generates a chronic inflammatory state in the liver. The inflammatory responses in the liver are mediated by interaction between MAMPs and host PRRs, specifically the TLRs[79]. Accordingly, chronic infusion of low-dose LPS via osmotic pumps promotes HCC development in mice[13]. Likewise, disruption of the gut barrier by administration of dextran sulfate sodium not only results in increased systemic LPS levels and increased liver fibrosis, but also promotes HCC formation in mice[80,81]. Conversely, inhibition of TLR4 signalling suppresses liver inflammation, fibrosis and HCC formation in mice and rats[13,14,73]. The majority of tumour-promoting signals from the leaky gut occur in late stages of DEN+CCl_4-induced hepatocarcinogenesis, as demonstrated by strong inhibitory effects of gut sterilization on HCC formation in late stages but only mild effects in early stages[13]. However, the relative contribution of the leaky gut at early versus late stages of hepatocarcinogenesis has not yet been tested in other models.

TLR4 is present in multiple hepatic cell types, including Kupffer cells, hepatic stellate cells (HSCs), endothelial cells and hepatocytes. Experiments in bone-marrow-chimeric mice demonstrated that TLR4 expressed on liver-resident cells (which include hepatocytes, HSCs and Kupffer cells) is responsible for promotion of fibrogenesis and hepatocarcinogenesis[13]. LPS from the leaky gut seems to promote hepatocarcinogenesis via multiple cellular targets, including HSCs, the hepatocyte–tumour compartment as well as liver-resident Kupffer cells. In HSCs, TLR4 activation leads to an NF-κB-mediated upregulation of the hepatomitogen epiregulin[13]. Epiregulin is an epidermal growth factor family member with a potent mitogenic effect on hepatocytes[82]. Accordingly, epiregulin-deficient mice displayed reduced hepatocarcinogenesis when treated with DEN+CCl_4[13]. Another key mechanism by which the LPS–TLR4 axis promotes HCC formation is via NF-κB-mediated prevention of hepatocyte apoptosis. Accordingly, expression of the apoptosis marker cleaved caspase 3 in TLR4-deficient and gut-sterilized mice is inversely correlated with the formation of tumours[13]. However, due to the lack of studies in mice with conditional TLR4 ablation, it remains unclear whether this survival pathway is directly activated in the hepatocyte–tumour cell compartment, or whether it might involve paracrine signals from neighboring TLR4-expressing cells such as HSC or Kupffer cells. Moreover, it has been demonstrated that activation of the LPS–TLR4 signalling pathway in Kupffer cells leads to TNF- and IL6-dependent compensatory hepatocyte proliferation as well as reduced oxidative stress and apoptosis[14]. In addition, TLR4 activation in HCC cell lines by LPS enhances their invasive potential and induces the epithelial–mesenchymal transition[83]. In order to delineate the contribution of TLR4 on specific cell types in the liver, further experiments in mice with conditional TLR4 ablation are required.

Together, these data clearly show that the leaky gut, via MAMP–TLR-mediated signals, contributes to hepatocarcinogenesis. Dysbiosis (discussed below) and the leaky gut are probably intimately linked; it is likely that intestinal dysbiosis contributes to a leaky gut by multiple mechanisms, such as dysbiosis-induced alterations of the intestinal barrier as well as a shift to bacterial species with increased propensity to translocate.

3.2. HCC promotion via dysbiosis, bacterial metabolites and immunosuppression

Increasing evidence supports a key role for dysbiosis in the development of CLD and HCC (FIG. 1). Metagenomic studies have revealed substantial alterations in the composition of the gut microbiota in a range of CLD as well as in patients with cirrhosis[12,32]. The gut microbiomes of patients with advanced liver disease and cirrhosis are characterized by an increase in potentially pathogenic bacteria, along with reduced numbers of bacteria with beneficial properties[32,84,85]. Studies conducted so far on the gut microbiota in liver cirrhosis have pooled patients with different underlying liver diseases[32], indicating that at least some of the microbial alterations in cirrhosis are common to different aetiologies, and suggesting that alterations are driven by characteristic features of end-stage liver disease, such as reduced bile output and changes to the intestinal secretion of antimicrobial peptides and IgA . Key changes in the composition of the intestinal microbiota in cirrhosis include enrichment of *Veillonella* or *Streptococcus* as well as decreased bacteria from the order *Clostridiales*[32]. Of note, the majority of the patient-enriched species were of buccal origin, suggesting an invasion of the gut from the mouth in liver cirrhosis[32]. The finding that the intestinal microbiota of patients with compensated cirrhosis differs from that of patients with decompensated cirrhosis[34] suggests that cirrhosis stage, rather than the underlying liver disease, drives gut microbiota changes. However, a recent small-scale study described differences in the gut microbiota between different types of underlying liver disease[86]. Therefore, sufficiently powered studies in large cohorts are needed determine disease-specific alterations of the gut microbiota in liver cirrhosis. In addition to alterations in bacterial composition, there is evidence for bacterial overgrowth in the upper gastrointestinal tract, which in turn is associated with increased circulating LPS levels [87]. Bacterial translocation in the upper gastrointestinal tract is relevant for the development of liver disease owing to the anatomic connection of the small intestine to the liver. Recent studies have demonstrated differences in the duodenal and salivary microbiota between healthy controls and patients with cirrhosis[86,88], suggesting that there are also qualitative and quantitative changes in the upper gastrointestinal tract that might be linked to changes in the more distal microbiota and contribute to the pathophysiology of CLD as well as the development of HCC.

Functional studies utilizing co-housing and faecal transplantation have provided evidence that dysbiosis is a transmissible driver of liver disease development and progression [65,89]. In one study, high-fat diet feeding in mice resulted in dysbiosis, with increased abundance of Gram-negative bacteria and a reduced ratio of Bacteroidetes to Firmicutes . Transplantation of these dysbiotic microbiota into control-diet-fed mice that had undergone bile duct ligation increased liver damage and fibrosis in the recipient[89]. Similarly, dysbiosis represented a transmissible risk factor in a genetic NASH model in which NASH was triggered by inflammasome deficiency; co-housing of dysbiotic inflammasome-deficient mice with

control mice resulted in the development of NASH in control mice[65]. Although studies demonstrating a transmissible HCC risk by dysbiotic microbiota are still missing, several functional studies point towards a contribution of dysbiosis. As such, perturbation of the gut eubiosis by penicillin increased HCC formation in rats[77], which could be suppressed by probiotics[77].

Recent evidence suggests that the effects of dysbiosis on the development of liver disease and HCC are mediated by bacterial metabolites, possibly in a disease-specific manner. In a mouse model of NASH-induced HCC, triggered by the combination of DMBA and high-fat diet, there was a strong increase in Gram-positive bacterial strains, in particular of specific *Clostridium* clusters[15]. At the same time, this treatment led to increased serum levels of deoxycholic acid (DCA), a secondary bile acid whose production depends on 7α-dehydroxylation of primary bile acids by the bacterial microbiota, notably *Clostridium* clusters. The key role of DCA in hepatocarcinogenesis was further demonstrated in experiments that showed increased HCC development in mice after supplementing diets with DCA, and decreased HCC formation after inhibition of 7α-dehydroxylation[15]. In concert with TLR2 agonist lipoteichoic acid, DCA promoted a senescence-associated secretory phenotype in hepatic stellate cells, which in turn suppressed anti-tumor immunity through a prostaglandin E2-dependent mechanisms[90]. Together, these studies link bacterial dysbiosis to altered immune responses via bacterial metabolites and MAMPs. Further studies are required to determine whether the procarcinogenic effects of dysbiotic microbiota may be mediated by additional pathways. The gut microbiota exerts a key role in a number of other metabolic pathways, including overall energy extraction from the diet as well as the generation of a wide range of important metabolites with beneficial effects for the host[91]. One example is the production of short-chain fatty acids (SCFAs), which are a primary energy source for intestinal epithelial cells[91], and might provide a link between dysbiosis and alterations of the intestinal barrier that lead to a leaky gut and increased risk for HCC development, as discussed above.

4. TARGETING MICROBIOTA TO PREVENT HCC

Currently, there are no therapeutic options for HCC prevention besides treating the underlying disease. On the basis of its important contribution to CLD progression and hepatocarcinogenesis in particular, the gut-microbiota–liver axis represents a promising target for preventative approaches (FIG. 1). With a complete lack of clinical studies testing this strategy, targeting the gut-microbiota–liver axis represents an exciting and understudied clinical opportunity, supported by a large number of studies preclinical studies showing a drastic (≈80%) reduction of HCC development in murine models[13–15]. Moreover, several small-scale clinical studies have suggested that antibiotics such as norfloxacin and rifaximin increase survival in patients with liver cirrhosis[92–95]. Targeting the gut microbiota axis for HCC prevention is particularly attractive as it may utilize currently FDA approved drugs with high safety profile in CLD patients, such as the nonabsorbable antibiotic Rifaximin, or other approaches with low risk for severe adverse effects, such as probiotics or faecal microbiota transplantation (FMT). Moreover, the gut-microbiota–liver axis has a key involvement in many complications of CLD and could be targeted to ‘kill several birds with one stone’: In addition to potentially reducing the risk for HCC development, targeting the

gut-microbiota–liver axis has been shown to reduce liver fibrosis [73,96] and portal hypertension[97] in rodents, and spontaneous bacterial peritonitis[98] and hepatic encephalopathy[99] in patients. As the strongest effects of antibiotics on HCC and complications of cirrhosis in mice and patients, respectively, have been observed in advanced disease stages, preventative strategies that target the gut–liver axis seem most promising in patients with cirrhosis and at high risk for HCC development, which would also reduce the number of patients that would be unnecessarily subjected to such treatments. Moreover, targeting the gut microbiota-liver axis is unlikely to have a major effect on patients in which the gut–liver axis is not a dominant driver of disease progression, HCC development and mortality, for example those with perinatal HBV infection, high HBV titres and minimal liver fibrosis. Although there is accumulating evidence that the gut microbiota modulates responses to chemotherapy[100,101] and immunomodulatory therapies[102,103], there is currently no data supporting the concept of targeting the gut-microbiota–liver axis for the treatment of HCC. With increased understanding of the underlying pathophysiology, the number of clinically feasible approaches to target the gut-microbiota–liver axis is continuously growing (Table 1).

4.1. Antibiotics

Because antibiotics target several pathways through which the gut microbiota promote HCC development (Table 1), they could represent one of the most efficient strategies to interrupt the tumor-promoting gut liver axis in CLD: Decreasing the overall number of bacteria in the gut and eliminating bacteria that have a high ability to translocate will reduce bacterial translocation and thereby inhibit proinflammatory signals coming from leaky gut. At the same time, selective antibiotics might also block the production of HCC-promoting bacterial metabolites, such as DCA[15], by reducing the number of bacteria that produce specific metabolites. Continuous gut sterilization by a cocktail of oral antibiotics, consisting of ampicillin, neomycin, metronidazole and vancomycin, effectively reduced the number and size of HCC induced by DEN+CCl_4 or DMBA+HFD in mice[13,15]. Moreover, this antibiotic cocktail also reduced liver fibrosis [73], which often precedes HCC and represents a risk factor for HCC development in CLD[70]. Of note, administration of antibiotics at late stages of carcinogenesis, when microscopic tumours already existed, was more efficient at reducing HCC in mice than administration at earlier stages[13]. These data support the concept that HCC prevention by antibiotic treatment could be applied even at late stages, i.e. in patients with advanced cirrhosis and high risk for HCC development. However, findings from mice cannot be translated directly to patients as long-term administration of the employed antibiotic cocktail would be deleterious due to the depletion of almost all detectable commensal microbiota (>99.5%)[73,104] and the inclusion of nephrotoxic drugs, such as neomycin. Moreover, HCC prevention with antibiotics would require long-term, possibly life-long administration. Therefore, the use of single antibiotics with a high safety profile in patients with CLD represents the only clinically feasible approach.

Currently, two antibiotics, norfloxacin and rifaximin, have shown beneficial effects in patients with CLD or murine HCC models and fulfill these criteria. Vancomycin, another antibiotic that has shown effectiveness as monotherapy in the prevention of HCC in the combined DMBA and high-fat diet mouse model[15], is rarely used for long-term therapy in

patients and may cause a number of potentially severe adverse effects. Gram-negative bacteria have been found to be the most adept at translocating to the mesenteric lymph nodes and are the most frequent cause of spontaneous bacterial infections in patients with cirrhosis[105–107]. Norfloxacin, a poorly absorbed quinolone, is currently one of the drugs of choice for the primary or secondary prophylaxis of spontaneous bacterial peritonitis and infections in high-risk patients with cirrhosis [108]. Clinical trials in patients with advanced cirrhosis have shown that long-term use of orally administered norfloxacin is safe, produces a marked reduction of gram-negative bacteria in the faecal microbiota[109], reduces the 1-year probability of developing spontaneous bacterial peritonitis and hepatorenal syndrome and improves 3-month survival[92]. Although these data show that norfloxacin can effectively reduce small intestinal bacteria overgrowth and bacterial translocation in patients with advanced cirrhosis, the effects of norfloxacin on HCC development in patients with liver cirrhosis are not known. A major problem with the use of norfloxacin is the development of antibiotic resistance[110–112], suggesting that it might be suitable for treatment lasting weeks to months but not for long-term or life-long application in patients with cirrhosis. Rifaximin is a nonabsorbable antibiotic with broad-spectrum antimicrobial activity and an excellent safety profile[113] that was initially approved for the treatment of traveller's diarrhoea but is increasingly used for the prevention of hepatic encephalopathy[99]. Moreover, rifaximin appears to reduce the development of spontaneous bacterial peritonitis and may improve portal hypertension, suggesting that it effectively targets the gut–liver axis in advanced liver disease[93,114,115]. Similar to norfloxacin, rifaximin has been noted to increased survival in patients with advanced liver cirrhosis in several small-scale trials[92–95]. Of note, rifaximin reduces HCC development in the DEN–CCl_4 model of HCC, albeit less efficiently than the quadruple antibiotics cocktail described above[13]. Despite the large number of patients receiving rifaximin for the prevention of hepatic encephalopathy, the effects of rifaximin on HCC development remain unknown. Therefore, studies that determine the effects of long-term rifaximin treatment on HCC development are urgently needed. In contrast to norfloxacin, clinically relevant development of resistance to rifaximin has not been reported, suggesting that it is well-suited for long-term or even life-long treatment. As data from murine studies show that non-absorbable antibiotics and norfloxacin improve ALD[48,116] and insulin resistance in NAFLD[117], they might be particularly attractive for HCC prevention in these patient groups.

4.2. Probiotics

Probiotics have been proposed as a means of re-equilibrating the gut microbiota in CLD by restoring beneficial bacteria. Although a large number of studies have demonstrated the effectiveness of probiotics in treating liver diseases both in animal models and in patients (reviewed elsewhere[118,119]), substantial controversy remains on the basis of: the inability of most probiotics to permanently colonize the gut; largely unknown mechanisms of action, in particular given the lack of permanent colonization; the large number of different combinations of bacteria within different probiotics that have not been systemically evaluated and compared for their efficacy in CLD; and in view of a lack of large-scale studies, potential publication bias towards studies reporting positive results . So far, probiotics have only been investigated in murine HCC models and data in patients are lacking (Table 1). In a rat model of DEN-induced hepatocarcinogenesis, administration of

VSL#3 (containing *Streptococcus thermophiles*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracase*i, *Lactobacillus delbrueckii* subsp. *Bulgaricus*) mitigated enteric dysbiosis, ameliorated intestinal inflammation and decreased liver tumour growth and multiplicity[77]. In a subcutaneous transplant mouse model, the probiotic mixture Prohep (comprising *Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917 and heat inactivated VSL#3) reduced tumour size and weight[120]. The authors suggested that a shift of the gut microbiota composition towards beneficial bacteria such as Prevotella and Oscillibacter, and production of anti-inflammatory mediators by these bacteria, decreased Th17 cell levels within tumors and thereby limited tumour growth. However, the effects of probiotics on endogenously arising tumours are not known. To date, there are several clinical trials on probiotics in patients with CLD but none in patients with HCC. A double-blind trial showed that daily intake of VSL#3 reduced the risk of hospitalization for hepatic encephalopathy, as well as Child–Turcotte–Pugh and model for end-stage liver disease (MELD) scores, in patients with cirrhosis[121]. Another randomized trial showed that 4-month supplementation with VSL#3 improves NAFLD in children[122]. The possible mechanisms of action include modulation of the host microbiota[123], improvement of gut barrier function and modulation of the immune system. However, further studies are required to confirm these data, extend human studies and investigate mechanisms of action.

4.3. FMT

FMT has successfully been used in patients with *C. difficile* infection, resulting in restoration of eubiosis and clinical improvements that were superior to standard antibiotic therapy[18]. Currently, FMT is being evaluated in clinical trials for a number of additional diseases including NASH[124] and cirrhosis[125]. A randomized controlled trial demonstrated amelioration of hepatic and peripheral insulin resistance in patients with metabolic syndrome who had received microbiota from lean donors[126]. However, one needs to keep in mind that patients receiving FMT for recurrent *C. difficile* infection have usually undergone multiple courses of antibiotic treatment, and present with a marked reduction of microbial diversity [18]. Thus, these patients not only represent an ideal ‘breeding ground’ for transplanted microbiota but also suffer from a disease that is clearly linked to reduced bacterial diversity and overgrowth of single and measurable pathogenic strain. Nonetheless, it is conceivable that FMT might also restore eubiosis in patients with CLD, similar to effect seen in the trial of Vrieze *et al.*[126], and that FMT might reduce or delay the development of HCC (Table 1). However, there are currently no data supporting this premise and a number of hurdles have to be overcome. Most importantly, it is not clear whether the severe alterations of the gastrointestinal ecology in cirrhosis would allow permanent restoration of the microbiota by FMT. It is possible that effects will be transient and the microbiota will ultimately revert to the pre-FMT state. Moreover, there is substantial concern that viral infections and other pathogens might be transmitted via FMT, which would be particularly harmful to patients with advanced liver disease owing to their immunosuppression. In the future, faeces might be substituted in favour of defined mixtures of cultured bacteria that resemble the human microbiota transplanted via FMT and confer the same beneficial effects. This approach will not only alleviate concerns regarding the inadvertent transmission of disease-causing pathogens through FMT, but also make intestinal microbiota therapy more

acceptable to patients and physicians[127]. Once this goal has been achieved, patients with advanced liver disease should be considered as potential candidates to study effects on disease progression and HCC development.

4.4. TLR antagonists

Several studies have shown a key role for the TLR4 pathway as a mediator of the disease-promoting effects of the gut–liver axis in CLD and hepatocarcinogenesis[13,14,73]. On the basis of these findings, blocking the TLR4 pathway might represent another avenue for HCC prevention (Table 1). With detailed knowledge about mechanisms by which LPS activates TLR4, a variety of TLR4 antagonist have been developed, which can be clustered into several groups: compounds binding and sequestering LPS, such as polymyxin B; compounds antagonizing LBP and CD14–LPS interactions[128]; compounds targeting LPS–MD-2 or LPS–MD-2–TLR4 interactions, such as E5531 and eritoran (E5564); compounds directly targeting TLR4, such as resatorvid (TAK-242); and molecules inhibiting TLR4 activity such as thalidomide (reviewed elsewhere[129]). Eritoran[130] and resatorvid[131] improved survival in animal models of sepsis, but did not reduce mortality in patients with severe sepsis[132,133]. So far, none of these agents have been tested in clinical trials in patients with CLD or HCC. Although TLR antagonists represent an exciting opportunity, long-term inhibition of TLR4 could result in immunosuppression, which might be deleterious due to the severely immunocompromised state of CLD patients. Therefore, the safety profile of TLR4 antagonists needs to be carefully evaluated before long-term studies for prevention of HCC and other complications of CLD can be considered.

4.5. Targeting the gut barrier

On the basis that the leaky gut is a major driver of liver disease progression and HCC development (FIG. 1), targeting the gut barrier seems an attractive therapeutic approach (Table 1) that might avoid some of the complications of targeting the microbiota (such as development of resistance and/or decreased microbial diversity) or receptors that mediate the disease-promoting effects of a leaky gut (such as immunosuppression resulting from TLR4 antagonism. Moreover, therapies that target the gut barrier could potentially be combined with other approaches that directly target the gut microbiota or liver. With improved understanding of the gut barrier and mechanisms that disrupt the gut barrier in cirrhosis, targeting the gut barrier via specific pharmacologic approaches seems to be realistic.

Bile acids are an important regulator of the gut barrier. Decreased bile secretion in rodents by either ligation of the common bile duct or induction of cirrhosis contributes to bacterial translocation, which is not only caused by intestinal bacterial overgrowth but also by increased gut permeability[20,28,134]. Notably, these effects are attenuated after oral administration of bile acids in different experimental cirrhotic models[20,134,135]. FXR is a receptor for bile acids that mediates their effects on the intestinal epithelial barrier as well as multiple effects on the liver, such as suppression of bile acid synthesis, inhibition of liver inflammation, promotion of liver regeneration and tumour suppression (reviewed elsewhere[136]). Many of the hepatic effects of FXR activation are mediated by intestinal FXR receptors, resulting in the release of FGF19, which then acts on targets in the liver[136–139]. *Fxr*-deficient mice exhibit compromised intestinal integrity, with further deterioration after

bile duct ligation[20], and a high incidence of HCC[140]. Accordingly, FXR activation by agonists GW4064 or obeticholic acid (OCA) attenuates mucosal injury, ileal barrier permeability, bacterial overgrowth and bacterial translocation in mice and rats [20,21,141,142]. Moreover, OCA improves portal hypertension (which might contribute to bacterial translocation in cirrhosis) in thioacetamide- or bile duct ligation-treated rats[143]. OCA has a high safety profile in patients with NASH as demonstrated in the FLINT trial, with the major adverse effects being pruritus and alterations of serum lipid profiles[144]. Thus, OCA seems to be a promising candidate for HCC prevention therapies, and could be particularly effective by correcting multiple abnormalities in the gut–liver axis that promote the development of chronic inflammation and HCC in patients with cirrhosis.

Increased production of TNF by monocytes in mesenteric lymph nodes constitutes one of the main factors increasing tight junction permeability[145,146]. TNF increases tight junction permeability by decreasing expression of tight junction proteins as well as by activating myosin light chain kinase (MLCK)[147]. Treatment with an anti-TNF monoclonal antibody decreases the incidence of bacterial translocation in experimental cirrhosis in rats[148]. However, translating these findings to patients might be difficult because of strong immunosuppressive effects of TNF inhibitors and increased rates of severe infection. Owing to these adverse effects, long-term anti-TNF therapy might confer more harm than benefit, and further efforts need to be made to develop therapies that act locally to improve gut barrier function without negatively affecting systemic immune responses.

4.6. Prokinetics

Another factor that contributes to intestinal bacteria overgrowth in liver cirrhosis is gut dysmotility[149]. The prokinetic drug cisapride not only decreases intestinal transit time but also inhibits intestinal bacterial overgrowth and bacterial translocation, both in animal models[150,151] and in patients with cirrhosis[150,152]. However, the long-term benefits of prokinetics such as cisapride have yet to be determined in patients with CLD (Table 1). One of the purported mechanisms for altered motility in cirrhosis is increased adrenergic activity. Accordingly, nonselective β-adrenergic blockers decrease intestinal transit time and reduce intestinal bacterial overgrowth, intestinal permeability and bacterial translocation in experimental models of cirrhosis as well as in patients[153–157]. Interestingly, a retrospective long-term observational study suggests that propranolol treatment might decrease HCC occurrence in patients with HCV cirrhosis[158], suggesting a potential role for HCC prevention.

Malnutrition is common in patients with CLD and is associated with increased morbidity and mortality (reviewed elsewhere[149]). Of note, malnutrition increases intestinal permeability and facilitates bacterial translocation[159]. Therefore, nutritional support represents an important aspect to correct dysbiosis in patients with CLD.

5. CLINICAL TRANSLATION

Current data from mouse models suggest that targeting the gut–liver axis has a potential role for the primary or secondary prevention of HCC, but not for the treatment of HCC (Table 2). Primary prevention seems the most appealing approach, and could be tested prospectively in

large cohorts of patients with liver cirrhosis and at high risk for HCC development. Alternatively, the efficacy of primary prevention could be evaluated retrospectively in cohorts of patients that already received treatment, for example rifaximin for the prevention of hepatic encephalopathy. Primary prevention most likely requires long-term, if not life-long treatment. As discussed in the previous sections, good safety and beneficial effects on non-HCC complications of CLD are the most important selection criteria for the best-suited drug candidates. In this regard, rifaximin is probably the candidate with the best safety profile, and there is strong evidence that it positively affects additional complications of CLD such as hepatic encephalopathy, portal hypertension and liver fibrosis, and possibly even improves overall survival. The efficacy of targeting the gut–liver axis for secondary prevention could be tested in patients who have undergone curative HCC resection. Although this strategy would require a well-defined cohort of patients with high risk for HCC relapse, one would still have to carefully distinguish between tumour recurrence and *de novo* tumour formation, as therapies such as rifaximin might positively affect one but not the other.

6. CONCLUSIONS

Overwhelming evidence from the past three decades support a key contribution of the gut microbiota to multiple aspects of liver disease progression, thereby contributing to a hepatic environment that promotes that development and progression of HCC. The mechanisms by which the gut microbiota promotes the development of liver disease and HCC include dysbiosis — which results in altered bacterial metabolites such as the cancer-promoting secondary bile acid DCA — as well as a leaky gut, which promotes chronic hepatic inflammation via TLR-mediated signals. Currently, it is not clear whether chronic inflammation driven by the translocation of MAMPs from the leaky gut is the dominant contributor to hepatocarcinogenesis, whether alterations of bacterial metabolites are restricted to specific diseases such as NAFLD, or whether both mechanisms work hand-in-hand to synergistically promote the development of HCC in most settings. Some alterations of the gut microbiota are probably disease-specific, and therefore some mechanisms by which the gut microbiota promotes the progression of liver disease and HCC could be — at least in part — disease-specific. Hence, better understanding of disease-specific alterations and thorough determination of their functional contributions to liver disease development are needed. Detailed knowledge about key pathways through which the gut microbiota affect CLD and HCC development could allow the development of broad or tailored therapeutic approaches that block the disease-promoting gut–liver signalling axis. Moreover, our current understanding of the contribution of the gut microbiota is largely based on animal models and faecal microbiota samples from patients. As many of the key changes in the gut–liver axis occur in the small intestine and possibly also within mucosa-adherent microbiota, better analysis of the human microbiome at different anatomic sites is needed. Finally, many types of CLD that confer a high risk for HCC development cannot be perfectly modeled in mice. Therefore, more effort should be put into translating our current knowledge on the HCC-promoting role of the gut–liver axis into well-designed trials in patients.

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- Intestinal dysbiosis and increased bacterial translocation contribute to the pathophysiology of chronic liver disease (CLD) and hepatocarcinogenesis
- A large body of literature has demonstrated that targeting the gut microbiota-liver axis can inhibit the development of hepatocellular carcinoma in mice and rats
- Translation of preclinical studies in mice and rats to clinical settings is missing and present a therapeutic opportunity
- Targeting the gut-liver axis by non-absorbable antibiotics such as Rifaximin may not only prevent the development of HCC in CLD patients, but additionally reduce other complications, and improve survival.

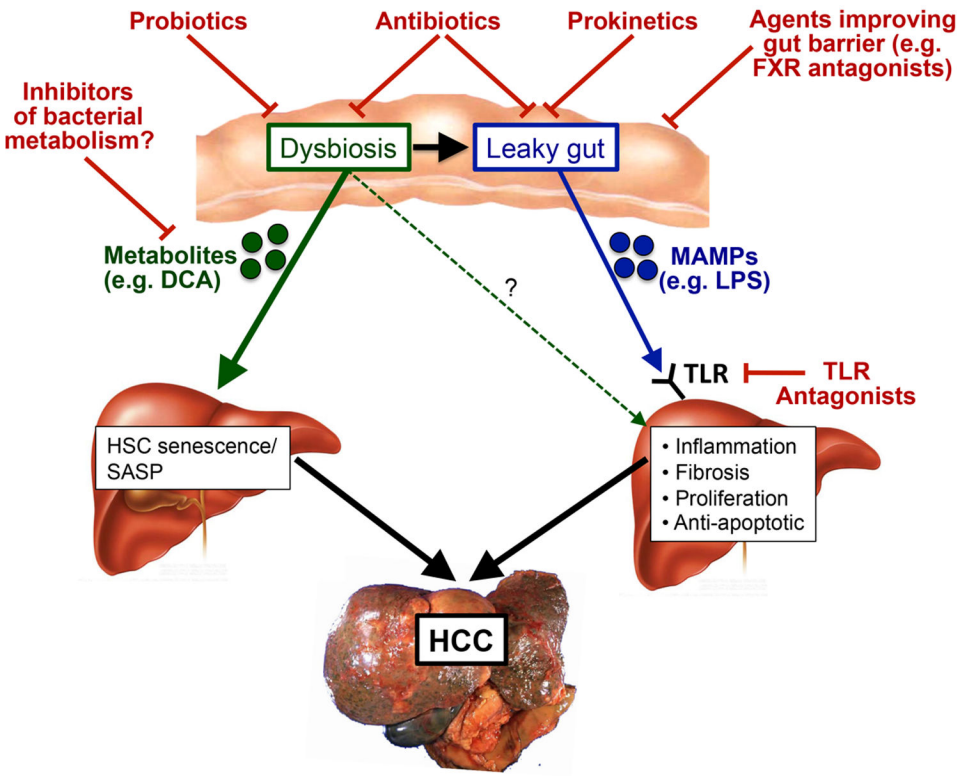

Figure 1. Contribution of the gut microbiota to hepatocarcinogenesis: mechanisms and therapeutic targets.

Dysbiosis and the leaky gut promote the progression of liver disease and the development of hepatocellular carcinoma (HCC) via multiple mechanisms, including the release of cancer-promoting and senescence-promoting metabolites such as deoxycholic acid (DCA) from the dysbiotic microbiota, and increased hepatic exposure to gut-derived microbe-associated molecular patterns (MAMPs) such as lipopolysaccharide (LPS), which in turn promote hepatic inflammation, fibrosis, proliferation and the activation of anti-apoptotic signals. These cancer-promoting signalling pathways can be interrupted at several levels: using probiotics to restore eubiosis; using antibiotics to eliminate disease-promoting bacteria and decrease the release of MAMPs and metabolites from the leaky gut; using agents to improve the gut barrier; and potentially using inhibitors of bacterial metabolism to reduce the production of cancer-promoting metabolites by the gut microbiota. HSC, hepatic stellate cell; TLR, Toll-like receptor; SASP, senescence-associated secretory phenotype; FXR, farnesoid X receptor.

Table 1.

Drugs targeting the gut-liver axis for prevention of CLD progression and HCC development.

Class of drug/treatment	Drug	Mechanism of action	Effects in mouse models	Effects in patients	Ref
Antibiotics	Rifaximin	A minimally absorbed oral antimicrobial agent when administrated orally; broad-spectrum activity against enteric bacteria by inhibiting the bacterial protein synthesis; low risk of inducing bacterial resistance	Reduced HCC development in the DEN+CCl_4 model of HCC Rifaximin reduces fibrosis, angiogenesis and portal hypertension in mice following bile duct ligation	Reduced the development of spontaneous bacterial peritonitis and portal hypertension, suggesting that it effectively targets the gut-liver axis in advanced liver disease Improved survival in Rifaximin-treated patients with chronic liver disease in several small-scale studies	13,93-96,113-115
	Norfloxacin	A poorly absorbable quinolone when administrated orally; selectively eliminates the intestinal gram-negative microbiota; low activity against anaerobic bacteria	Suppressed numbers of cecal aerobic and anaerobic bacteria in ob/ob mice and ameliorated glucose tolerance when combined with ampicillin	Long-term use reduced the 1-year probability of developing SBP and hepatorenal syndrome Improves 3-month survival in advanced cirrhotic patients Reduces the levels of bacterial translocation and proinflammatory cytokines in serum and ascites	92,109,117,160,161
Probiotics	VSL#3	Mechanisms not conclusively determined; possibly acting via modulation of the host's microbiota, improvement of gut barrier function and modulation of the immune system	Mitigated enteric dysbacteriosis, ameliorated intestinal inflammation, and decreased liver tumor growth and multiplicity in a DEN-induced rat HCC model	Reduced the risk of hospitalization for HE Reduced Child-Turcotte -Pugh and model for end-stage liver disease (MELD) scores, in patients with cirrhosis in one study Improved NAFLD in children	77,121,122
	Prohep		In a subcutaneous transplant model, it reduced tumor size and weight by 40%	Not studied.	120
TLR4 antagonists	E5564 (eritorin)	Binds to the hydrophobic pocket of MD-2, competitively inhibits the lipid A component of endotoxin from binding to the same site, and thereby prevents dimerization of TLR4 and intracellular signaling	Protective in animal models of sepsis No data on liver disease or HCC development.	In healthy volunteers, blocked symptoms of endotoxmia in a dose-dependent manner No data on liver disease or HCC development.	129
	TAK-242	Binds to the intracellular domain of TLR4 and inhibits interaction with TLR adapter molecules TIRAP and TRAM, thereby blocking TLR4 signaling	Protected mice against LPS-induced lethality No data on liver disease or HCC development.	Failed to suppress cytokine levels in patients with severe sepsis and septic shock or respiratory failure	129

				No data on liver disease or HCC development.	
FXR agonists	GW4064	Activating the FXR signaling in the ileum	Attenuated mucosal injury, ileal barrier permeability, bacterial overgrowth and bacterial translocation	Not studied.	20,21
	Obeticholic acid	Activating the FXR signaling in the ileum	Attenuated mucosal injury, ileal barrier permeability, bacterial overgrowth and bacterial translocation; improved portal hypertension (which might contribute to BT in cirrhosis) in TAA and bile duct ligation-induced cirrhosis models	Improved histological features of NASH	21,141-144
Prokinetics	Cisapride	Ameliorates gut dysmotility	Inhibited intestinal bacteria overgrowth and bacterial translocation in animal models	Inhibition of intestinal bacterial overgrowth and translocation in cirrhotic patients	150-152
Betablockers	Propranolol	Non-selective beta-adrenergic receptor antagonist that ameliorates gut dysmotility, reduces portal pressure and thereby might reduce bacterial translocation	Increased intestinal transit and reduced intestinal bacterial overgrowth, intestinal permeability and bacterial translocation in experimental models	Decreased HCC occurrence in patients with HGV-related cirrhosis	153-158
FMT		Restores in patients with antibiotics-induced and *C.difficile* infection	FMT improves NASH Cohousing and transplantation studies in mice have also shown worsening of NASH and liver fibrosis by dysbiotic microbiomes	Restoration of eubiosis and significant clinical improvements in patients with *C.difficile* infection Improved hepatic and peripheral insulin resistance	18,65,66,89,126

Table 2.

Clinical setting to studying therapeutic interventions in the gut-liver axis in HCC patients

Study design and participants	Treatment	Primary outcomes	Primary outcomes	Advantages	Disadvantages
Retrospective studies					
Patients with liver cirrhosis treated with rifaximin for the primary or secondary prevention of HE	Rifaximin	Reduction of HCC development	Difficult to study as available data might not be sufficient	Large number of patients that have received treatment with rifaximin	• Untreated control group needs to be well-matched in regards to disease stage • HCC surveillance might not have been ideal in the majority of patients • Additional treatments and interventions might not be recorded and could represent confounders
Prospective, primary HCC prevention trials					
Patients with liver cirrhosis and high risk of HCC development	Long-term or life-long treatment with drugs such as antibiotics, probiotics or FXR agonists	HCC development and HCC-related mortality	• Reduction of overall and liver-related mortality • Reduction of liver disease progression, for example, MELD score, synthetic liver function, portal hypertension	Allows for best study design and patient selection criteria	• Would probably require a very long treatment and observation period • Expensive owing to long treatment and observation period and large number of patients required to detect small differences in risk reduction
Prospective, secondary HCC prevention trials					
Patients that have undergone curative HCC resection	Long-term or life-long treatment with drugs such as antibiotics, probiotics or FXR agonists	HCC development, in particular late recurrence, and HCC-related mortality	Difficult to study as many patients will not have advanced liver disease	Patients are at high risk and many recurrences are early	• Not clear if targeting the microbiota or gut–liver axis can prevent recurrences or only *de novo* tumour formation • For the latter, long-term treatment and large patient numbers might be required