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## REVIEW ARTICLE OPEN

## Gut microbiome, liver immunology, and liver diseases

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The gut microbiota is a complex and plastic consortium of microorganisms that are intricately connected with human physiology. The liver is a central immunological organ that is particularly enriched in innate immune cells and constantly exposed to circulating nutrients and endotoxins derived from the gut microbiota. The delicate interaction between the gut and liver prevents accidental immune activation against otherwise harmless antigens. Work on the interplay between the gut microbiota and liver has assisted in understanding the pathophysiology of various liver diseases. Of immense importance is the step from high-throughput sequencing (correlation) to mechanistic studies (causality) and therapeutic intervention. Here, we review the gut microbiota, liver immunology, and the interaction between the gut and liver. In addition, the impairment in the gut–liver axis found in various liver diseases is reviewed here, with an emphasis on alcohol-associated liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), and autoimmune liver disease (AILD). On the basis of growing evidence from these preclinical studies, we propose that the gut–liver axis paves the way for targeted therapeutic modalities for liver diseases.

**Keywords:** Gut–liver axis; Alcohol liver disease; Nonalcoholic fatty liver disease; Autoimmune liver disease; Microbiome

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## INTRODUCTION

Although the past decade has witnessed major efforts in fighting liver disease, it remains one of the top ten causes of death worldwide.<sup>1</sup> The prevalence and incidence of nonalcoholic fatty liver disease (NAFLD) and alcohol-associated liver disease (ALD) continue to rise, which contribute to the burden of cirrhosis and liver cancer.<sup>2–4</sup> Increasing epidemiological trends have also been observed in autoimmune liver disease (AILD), albeit less so in developing countries.<sup>5,6</sup> Fine dissections of the pathophysiology of these liver diseases should be elucidated, which boosts the development of new therapeutic modalities and tackles the socioeconomic burden of these liver diseases.

The gut and liver communicate extensively through the biliary tract, portal vein and systemic circulation. This bidirectional crosstalk is called the gut–liver axis.<sup>7,8</sup> The liver is a key and frontline immune organ. Positioned to receive various gut-derived signals (bacterial products, environmental toxins and food antigens), the liver's default state is a balance between immunity and tolerance, which is essential to its function. Disruption of gut homeostasis leads to an altered immune state and various liver diseases—sterile liver inflammation caused by an excessive immune response even in the absence of pathogens; chronic infection and cancer caused by an insufficient immune response. Liver-derived factors, such as bile acids (BAs) and antibodies, also regulate the gut microbiota. The missing links in the gut–liver axis are being discovered piece by piece via well-designed experiments (detailed later). The identification of these altered elements

in the gut–liver axis offers possibilities for intervention.<sup>9</sup> Here, we first provide a basic overview of the gut microbiome (bacteria, fungi, viruses) and the microbiota-immune interaction. We then summarize the gut–liver axis with an emphasis on changes in liver immunology. Finally, we examined distinct microbial profiles and mechanistic studies in ALD, NAFLD, AILD, and other types of liver diseases.

## THE GUT MICROBIOME

The gut microbiota is a dense and diverse consortium of microorganisms composed of bacteria, fungi, viruses, archaea, and protozoa. The gut microbiome includes the assemblage of these microorganisms, their genome, and the environmental factors of a certain habitat. Following the first wave of intestinal microbiome studies mainly focusing on bacteria,<sup>10,11</sup> knowledge of the gut microbiome has substantially increased. One could expect that  $\sim 10^{14}$  microbial cells reside in the human gut, comparable to human cells.<sup>12</sup> Spatially, the majority (>99%) of the intestinal microbiome resides in the distal segments of the gastrointestinal tract (GIT), occupying different ecological niches. Temporally, an expansion in bacterial diversity is observed in infancy. It slows in early childhood and becomes stable in adults.<sup>13</sup> Healthy adult intestinal bacteria are dominated by *Bacteroidetes* and *Firmicutes*, with smaller proportions of *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*.<sup>14</sup> To date, crosstalk between the gut and distal organs has been increasingly recognized.

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Physiologically, this diverse consortium has emerged as a potent modulator of the host's immune system and metabolism. Pathologically, intestinal dysbiosis (i.e., qualitative and quantitative alterations of the gut microbiota) are associated with diseases including inflammatory bowel disease (IBD)<sup>15</sup>, type 1 diabetes (T1DM)<sup>16</sup>, obesity<sup>17</sup>, cardiovascular diseases<sup>18</sup> and autism<sup>19</sup>. Methodologically, studies of the gut microbiota are divided into three stages: (1) descriptive/correlative studies to demonstrate the composition and diversity of the intestinal bacteria in the disease state, fueled by high-throughput sequencing methods, including 16S rRNA and metagenomics; (2) mechanistic/causal studies to elucidate the role of the intestinal bacteria in the pathogenesis of certain diseases, fueled by humanized animal models colonized by certain bacterial strains and/or bacterial communities isolated from patients or bacterial metabolites,<sup>20,21</sup> and (3) microbe-based interventions to alleviate diseases, including fecal microbial transplantation (FMT), synthetic cocktails, and microbe-derived compounds.<sup>22</sup>

While intestinal bacteria have received considerable attention, there is a growing interest in gut-associated fungi and viruses. Fungi-relevant studies flourished with the advent of internal transcribed spacer (ITS) ribosomal sequencing. It is estimated that there are  $10^5$ – $10^6$  fungal cells per gram of feces.<sup>12</sup> The major phyla of fungi in the gut are *Ascomycota* and *Basidiomycota*. The “core mycobiota” within the human gut includes *Candida*, *Saccharomyces*, *Penicillium*, *Aspergillus*, and others.<sup>23</sup> In the GIT, fungi interact with bacteria via mutualism, commensalism, or competition. Fungi elicit the host immune response by activating receptors, including Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors (NLRs), and galectin 3, expressed on various immune cells, followed by the release of antimicrobial molecules.<sup>24</sup> Recent studies have demonstrated the roles of the gut mycobiota in different diseases, including IBD<sup>25</sup>, colorectal cancer (CRC)<sup>26</sup>, irritable bowel syndrome<sup>27</sup>, ALD<sup>28</sup>, and AILD<sup>29</sup>. Bacteriophages (phages), which infect bacteria, constitute the majority of gut viruses (reviewed elsewhere<sup>30,31</sup>). The total counts of phages in human feces reach  $10^9$ – $10^{10}$  per gram of feces in the form of virus-like particles.<sup>32</sup> Metagenomics has further revealed the complexity and richness of human intestinal phages.<sup>33</sup> Morphologically, intestinal phages are mostly members of the *Caudovirales* order, comprising the *Siphoviridae*, *Podoviridae*, and *Myoviridae* families. Functionally, temperate phages, constituting at least 20–50% of free phages in the gut, play a major role in interacting with bacteria. CrAssphage is the most abundant phage in the human gut replicating on its host *Bacteroides intestinalis*.<sup>34</sup> Ample evidence reveals the role of intestinal phages in diseases: (1) specific and durable changes in intestinal phages are observed in IBD<sup>35,36</sup>, CRC<sup>37</sup>, T1DM<sup>38</sup>, and rheumatic arthritis<sup>39</sup>; (2) selective engraftment of intestinal phages in FMT promotes therapeutic effects and safety;<sup>40</sup> and (3) the delivery of phages targeting certain pathogens loaded with pharmaceutical nanoparticles could augment the effect of chemotherapy against cancer.<sup>41,42</sup>

The microbiota plays a significant role in the induction and education of the host immune system, shaping the functional diversity, and repertoires of various immune cells.<sup>43</sup> During hematopoiesis, intestinal bacterial metabolites (such as short-chain fatty acids (SCFAs)) can penetrate into the circulation and then tune immune cells (such as dendritic cell (DC) precursors) in the bone marrow.<sup>44</sup> Commensals regulate both innate and adaptive immune systems to establish sustained tolerance to innocuous antigens. Innate lymphocytes are often located in peripheral tissues and are regulated by microbiota.<sup>45</sup> Adaptive lymphocytes are also shaped by gut microbes, such as B cells generating IgA controlled by microbes,<sup>46</sup> Th17 cells regulated by segmented filamentous bacteria,<sup>47</sup> regulatory T (Treg) cells modulated by *Clostridia*,<sup>48</sup> and T follicular helper cells influenced by *Akkermansia muciniphila*.<sup>49</sup> Translational investigations have been conducted, which permits the design of certain microbes

that activate or suppress programs of the immune system to treat infections,<sup>50</sup> autoimmunity,<sup>51</sup> allergies<sup>52</sup> and cancer<sup>53</sup>.

## GUT MICROBIOME AND LIVER IMMUNOLOGY

Immune cells in the liver communicating with the gut

The liver has been proposed as an innate immune organ<sup>54</sup> because it is responsible for producing the majority of immune molecules in the circulation and contains a wealth of resident innate immune cells. Here, we highlight recent studies on those immune cells enriched in the liver that have a close relationship with gut microbiota.

**Macrophages.** Macrophages are key components of the innate immune system, and in the liver, they comprise subsets of different cell populations, including resident Kupffer cells (accounting for 80–90% of the total population of resident macrophages in the body) and recruited monocyte-derived macrophages.<sup>55</sup> Located in the liver reticulo-endothelial system as the primary line of defense against invading microorganisms, macrophages regulate liver immune homeostasis via phagocytosis or serve as antigen-presenting cells.<sup>56</sup> Traditionally, gut-derived endotoxins, such as LPS, sensed by TLRs expressed on liver macrophages, are one of the main factors contributing to macrophage activation. Physiologically, constitutive exposure to LPS educates liver macrophages, forming tolerance to LPS and downregulating TLRs.<sup>57</sup> Pathologically, growing evidence suggests a key role for the excessive activation of liver macrophages induced by the LPS-TLR axis (in particular, TLR4) in the NAFLD process.<sup>58,59</sup> Increased susceptibility of liver macrophages to LPS might be further mediated by lipids (called lipotoxicity) via enhanced release of proinflammatory cytokines<sup>60</sup>, recruitment of effective immune cells<sup>61</sup>, and ROS activation.<sup>62</sup> In addition, early colonization of the infant gut microbiota originating from obese mothers increases susceptibility to NAFLD via impaired phagocytosis of hepatic macrophages.<sup>63</sup> By targeting the LPS-TLR axis, one could expect the potential to prevent inflammatory macrophage activation and alleviate liver diseases.<sup>64,65</sup> In addition to LPS, some bacterial metabolites also regulate the immune state of hepatic macrophages. Tryptophan metabolites, including tryptamine and indole-3-acetate (IAA), reduce proinflammatory cytokines in macrophages by activating aryl hydrocarbon receptor<sup>66</sup> or upregulating PFKFB3 (a key regulatory gene of glycolysis).<sup>67</sup> Granisetron, a 5-hydroxytryptamine 3 (5-HT<sub>3</sub>) receptor antagonist derived from gut microbiota, suppresses the proinflammatory cytokine release by macrophages after LPS, alleviating sepsis-induced liver injury in mice.<sup>68</sup> Recently, one study revealed that commensal-derived D-lactate could protect against pathogen dissemination by upregulating the phagocytic capability of Kupffer cells, thereby generating an intravascular immune firewall.<sup>69</sup> BAs, including chenodeoxycholic acid and deoxycholic acid (DCA), could upregulate NLRP3 in hepatic macrophages, contributing to cholestatic liver diseases.<sup>70</sup> In turn, hepatic macrophages could increase intestinal permeability and alter the microbiome composition by activating NLRP3, which aggravates the flux of endotoxin into the cholestatic liver.<sup>71</sup>

**NKT cells.** Natural killer T (NKT) cells are a heterogeneous group of innate-like T cells that recognize lipid antigens presented by a class I MHC-like molecule, CD1d (reviewed elsewhere<sup>72</sup>). NKT cells are predominantly found in the liver compared with other immune organs.<sup>73</sup> On the basis of the diversity of the TCR repertoire, NKT cells are classified into two subtypes: type I or invariant NKT (iNKT) cells and type II NKT cells. Mice orally inoculated with *Novosphingobium aromaticivorans*, which expresses conserved PDC-E2 epitopes, could develop NKT cell-mediated destruction of small bile ducts resembling PBC, suggesting the role of NKT cells in the activation of organ-specific autoimmunity. One study also

suggested that NKT cells facilitate ConA-induced hepatitis mediated by intestinal bacterial antigens presented by DCs.<sup>74</sup> Another study demonstrated that ConA treatment failed to trigger the activation of hepatic NKT cells in GF mice, and liver injury could be restored by supplementation with killed intestinal bacteria, suggesting that gut-derived glycolipid antigens are necessary for NKT cell activation.<sup>75</sup> Mice harboring the gut microbiota from a patient with severe ALD developed more severe liver inflammation with increased NKT cells, greater intestinal permeability and higher translocation of bacteria.<sup>76</sup> In the scenario of liver cancer, one study suggested that the application of certain antibiotics targeting Gram-negative bacteria causes the primary/secondary BA ratio to reach a balance, which then increases the level of the chemokine CXCL16 of sinusoidal endothelial cells and thus promotes the accumulation of CXCR6-positive NKT cells in the liver, inhibiting tumor growth.<sup>77</sup>

**$\gamma\delta$  T cells.**  $\gamma\delta$  T cells are enriched in the liver at a frequency of 3–5% among total hepatic lymphocytes.<sup>54</sup> This group of cells is characterized by the expression of  $\gamma\beta$  TCRs that recognize lipid antigens resembling NKT cells and the rapid secretion of proinflammatory cytokines such as IL-17A upon stimulation.<sup>78</sup> Growing evidence suggests that  $\gamma\delta$  T cells expand in response to invading bacterial pathogens and modulate tissue injuries.<sup>79,80</sup> Recent studies further revealed  $\gamma\delta$  T cells at the nexus of the gut–liver interaction. Li et al. showed that hepatic  $\gamma\delta$  T cells are the major producers of IL-17A, which is quantitatively modulated by the commensal bacterial load.<sup>81</sup> Tedesco et al. showed that in *Mdr2*<sup>-/-</sup> mice (an animal model resembling PSC), hepatic  $\gamma\delta$  T cells are activated by exposure to *L. gasseri*, leading to fibrosis and inflammation of the liver, suggesting that  $\gamma\delta$  T cells are a promising candidate for immunotherapies for liver diseases.<sup>82</sup>

**MAIT cells.** In a healthy adult, the frequency of mucosa-associated invariant T (MAIT) cells is 6–15% of the total T cells in the liver,<sup>83</sup> serving as the most abundant innate-like T cells. Classically, MAITs can be activated by microbial riboflavin (vitamin B2) derivatives, which include 5-(2-oxopropylideneamino)-6-D-ribitylaminoouracil (5-OP-RU), presented in the context of an MHC class I-related molecule (MR1).<sup>84</sup> Two studies further suggested that the intrathymic development of MAITs in early life requires 5-OP-RU.<sup>85,86</sup> MAITs can also be activated in an MR1-independent but cytokine-dependent manner, such as IL-12 and IL-18. Growing evidence suggests a wide range of functional options for MAITs in the context of different liver diseases (reviewed elsewhere<sup>83,87</sup>). In patients with acute hepatitis A, liver MAIT cells exert cytotoxicity and cause liver injury and are activated by IL-15 in the absence of TCR/MR1 engagement.<sup>88</sup> In patients with decompensated liver cirrhosis, MAIT cells are enriched in ascites and display increased functional responses to stimulation with *Escherichia coli* and proinflammatory cytokines.<sup>89</sup> MAITs could alleviate NAFLD by inducing anti-inflammatory macrophage polarization.<sup>90</sup> In AILD, MAITs are regulated by cholic acid-induced IL-7<sup>91</sup> and promote HSC activation<sup>92</sup>, aggravating disease progression.

### BILE ACID-MICROBIOTA CROSSTALK AND RELATED SIGNALING PATHWAYS

BA is a classic component in the gut–liver axis. The process of BA metabolism is thoroughly reviewed elsewhere.<sup>93</sup> BA is involved in multiple processes connecting the gut–liver axis. First, the ratio of different BAs influences gut homeostasis and the immune system (reviewed in detail elsewhere<sup>94,95</sup>). Unconjugated BAs have stronger antibacterial activity than conjugated BAs.<sup>96</sup> Gram-positive bacteria are more sensitive to BAs than Gram-negative bacteria, while some probiotics (such as *Lactobacillus*, *Bifidobacterium* and 7 $\alpha$ -dehydroxy bacteria) show bile acid resistance related to glycolysis activation.<sup>96</sup> Compared with patients with familial

adenomatous polyposis, patients with ulcerative colitis (UC) have lower levels of lithocholic acid and DCA (the two most abundant secondary BAs in the intestinal tract), a reduction in the genes that convert primary BAs into secondary BAs, and a decrease in the abundance of the secondary BA-producing *Ruminococcus*.<sup>97</sup> Second, BAs have a bidirectional relationship with immune cells. BA can regulate regulatory T (Treg) cells in multiple ways, including agonizing VDR,<sup>98</sup> antagonizing receptors expressed on DCs,<sup>99</sup> and activating the reactive oxygen species (ROS) process.<sup>100</sup> BA can also be regulated by immune cells. One study suggested that liver-infiltrating T cells could modulate the synthesis and metabolism of BAs in a TNF- and IFN- $\gamma$ -dependent manner.<sup>101</sup> Third, certain BA receptors have received attention. FXR (also known as NR1H4), which is located in intestinal epithelial cells, initiates the transcription process of fibroblast growth factor 19 (FGF19) after binding to BA. FGF19 enters the liver through the portal vein and downregulates the level of BA and maintains homeostasis by inhibiting the hepatocyte cholesterol 7 $\alpha$ -monooxygenase (CYP7A1) enzyme, forming a feedback system for regulating BA production.<sup>102</sup> FXR engagement mediates the restoration of homeostasis in the intestinal barrier, gut vascular barrier<sup>103</sup> and energy metabolism<sup>104</sup>. Therapeutically, obeticholic acid, an FXR agonist, has proven useful in patients with PBC with poor response to UDCA.<sup>105</sup> FGF19-52, an analog of FGF19, could also modulate the pool size and composition of BA and protect mice from intestinal inflammation.<sup>106</sup> The probiotic *Lactobacillus rhamnosus* GG (LGG) could decrease hepatic BA by increasing intestinal FXR-FGF-15 signaling pathway-mediated suppression of BA synthesis and could prompt BA excretion, which alleviates excessive BA-induced liver injury and fibrosis in bile duct ligation and *Mdr2*<sup>-/-</sup> mice.<sup>107</sup> Fourth, bile salt hydrolase (BSH) also has the potential to influence gut–liver crosstalk. The gut microbiota initiates BA metabolism via a critical first step catalyzed by BSH, that is, deconjugating glycine or taurine from primary BAs in preparation for various transformations into secondary BAs.<sup>108</sup> Growing evidence shows the potency of BSH in modulating health and disease (beautifully reviewed elsewhere<sup>109</sup>), ranging from recurrent *Clostridioides difficile* infection (rCDI) to autism spectrum disorder. Therefore, BSH enzymes are promising for targeted modulation of the gut–liver axis.

### THE GUT MICROBIOME AND ALD

#### Introduction of ALD

Chronic alcohol consumption leads in most cases to hepatic steatosis, which is reversible following a short period of abstinence. Approximately 10–20% of patients with alcohol-use disorder develop progressive liver disease, which is characterized by steatohepatitis, fibrosis and cirrhosis. Alcoholic hepatitis is an acute chronic form of ALD that develops mostly in patients with underlying cirrhosis and is associated with high mortality. Known factors that predispose to a more progressive type of ALD include the amount of consumed alcohol, female sex, the pattern of alcohol drinking (in particular, binge drinking and drinking outside of meals), cigarette smoking, obesity, concurrent other chronic liver diseases (CLDs) and genetic polymorphisms.<sup>110</sup>

#### The gut microbiome signature in ALD

Preclinical and clinical evidence supports an important role of the gut microbiota in ALD. Germ-free mice transplanted with stool from patients with severe alcoholic hepatitis develop more ethanol-induced liver inflammation (increased number of liver T lymphocyte subsets and NKT lymphocytes) and liver disease than germ-free mice colonized with fecal material from patients without alcoholic hepatitis.<sup>76</sup> Steroid-ineligible patients with severe alcoholic hepatitis treated with daily fecal microbiota transplantation via a nasojejunal tube for 8 days had an increased survival rate compared with historical controls.<sup>111</sup> Thus, the

intestinal microbiota might be an additional factor that contributes to the progression of ALD, at least in a subset of patients.

Patients with alcohol-use disorder and liver disease show compositional changes in the bacterial fecal microbiota (Table 1). These changes are characterized by a decrease in bacterial diversity and lower proportions of several bacteria that are considered beneficial, including *Lactobacillus* spp., *Bifidobacterium* spp., *Faecalibacterium prausnitzii*<sup>112</sup> and *Akkermansia muciniphila*<sup>113,114</sup>. In addition, patients with alcoholic cirrhosis have lower proportions of *Bacteroidaceae* and *Prevotellaceae*.<sup>115</sup> Patients with alcoholic hepatitis show increased proportions of pathogens, including *Veilonella*<sup>114</sup> and *Enterococcus faecalis*.<sup>116</sup> Quantitative changes are largely ignored when analyzing microbiota sequencing data, which focus on the relative proportions of bacteria. Patients with alcohol-use disorder show bacterial overgrowth in the luminal<sup>117</sup> and mucosa-associated compartment of the small intestine<sup>118</sup>. Patients with alcohol-use disorder and ALD not only show bacterial dysbiosis but also changes in the gut mycobiome. They have a decrease in fungal diversity and a proportional and absolute increase in *Candida* spp. in the fecal mycobiota.<sup>28,119,120</sup> We also recently described increased viral diversity in fecal samples from patients with ALD, with the most significant changes in samples from patients with alcoholic hepatitis. *Escherichia*-, *Enterobacteria*-, and *Enterococcus* phages were overrepresented in fecal samples from patients with alcoholic hepatitis, along with significant increases in mammalian viruses such as *Parvoviridae* and *Herpesviridae*.<sup>121</sup> Taken together, changes in the fecal bacterial microbiota, mycobiota, and virome are observed in patients with alcohol-use disorder and ALD.

#### The role of the gut microbiome in ALD

How does the gut microbiota increase susceptibility to ALD in patients? Dysbiosis-induced intestinal inflammation, acetaldehyde as a product of ethanol metabolism, changes in circadian rhythm, and alterations in intestinal bile acids and possibly other metabolites contribute to a disrupted gut barrier (Fig. 1).<sup>122–124</sup> Since intestinal venous blood is drained into the portal vein, the first organ in our body that gut-derived microbial products and metabolites reach is the liver. Several pathogen-associated molecular pattern receptors, such as TLRs and NLRs, are expressed on parenchymal and nonparenchymal cells in the liver and recognize components of Gram-positive bacteria, the lipid A portion of LPS, flagellin and bacterial DNA. Once Kupffer cells and infiltrating macrophages are activated, they can produce a variety of inflammatory cytokines and chemokines, which contribute to disease progression.<sup>125</sup> Chemokines such as CC-chemokine ligand 2 (CCL2) and IL8 recruit other immune cells, such as macrophages and neutrophils, to the liver. Another important cytokine is IL1 $\beta$ , which is induced by NF- $\kappa$ B following activation of TLR4 by LPS.<sup>126</sup> Release and secretion of active IL1 $\beta$  require cleavage of pro-IL1 $\beta$  and production of mature IL1 $\beta$  by activating the canonical NOD-, LRR- and pyrin domain-containing 3 (NLRP3) and caspase-1 inflammasome pathways. In ALD, uric acid, and ATP levels are increased<sup>127,128</sup>, and fungal  $\beta$ -glucan activates the NLRP3 inflammasome in liver macrophages<sup>119</sup>. IL1 $\beta$  recruits other immune cells to the liver, induces lipid accumulation in hepatocytes and primes hepatocytes for cell death.<sup>3</sup> Blocking IL1 $\beta$  reduces ethanol-induced liver disease in mice<sup>129</sup> and is currently being evaluated in clinical trials for the treatment of alcoholic hepatitis (ClinicalTrials.gov Identifier: NCT04072822 and NCT03775109). Hepatic stellate cells undergo an activation process that can be triggered by microbial products and leads to liver fibrosis. Hepatocytes also recognize microbial components via TLRs, which could contribute to cell death.<sup>125</sup> However, only ~50% of patients with alcohol-use disorder and mild liver disease show increased intestinal permeability.<sup>112</sup> Future studies are required to determine whether the proportion of patients with

increased intestinal permeability also shows progressive liver disease.

Several other mechanisms have been identified regarding how the gut microbiota contributes to ALD beyond increased intestinal permeability. We recently showed that patients with alcoholic hepatitis have elevated proportions of fecal *E. faecalis* compared with nonalcoholic subjects or patients with alcohol-use disorder.<sup>116</sup> A higher severity of liver disease and mortality were seen in patients with alcoholic hepatitis who had cytolytic-positive (cytolytic) *E. faecalis*. Cytolysin is a two subunit toxin secreted by *E. faecalis* that can directly induce hepatocyte death presumably via pore formation.<sup>116</sup> The importance of cytolytic *E. faecalis* for clinical outcome might be specific for alcoholic hepatitis, since the presence of cytolytic *E. faecalis* in patients with NAFLD was low and did not correlate with liver disease severity.<sup>130</sup> In addition, we also discovered that the *Candida albicans* exotoxin candidalysin promotes ALD.<sup>120</sup> Similar to cytolysin, candidalysin can damage primary hepatocytes and is associated with liver disease severity and mortality in patients with alcoholic hepatitis.<sup>120</sup>

In addition to toxins secreted by microbes, bacteria can metabolize primary bile acids into secondary bile acids in the gut. Alcohol-associated changes in the bacterial metagenome result in a dysregulation of bile acid metabolism, which is characterized by increased systemic bile acid levels and ethanol-induced liver disease in mice.<sup>131</sup> The binding of bile acids to the nuclear receptor FXR impacts gut barrier function and host metabolic functions, including hepatic lipid metabolism. Modulation of intestinal bile acid signaling reduces ethanol-induced liver disease in mice.<sup>131</sup> Total and conjugated bile acids are increased in patients with alcoholic hepatitis compared with controls.<sup>124</sup>

Bacteria can ferment nondigestible carbohydrates into SCFAs, with butyrate, propionate, and acetate being the most abundant in the intestine. Fecal levels of SCFAs are reduced in patients with chronic alcohol consumption.<sup>132</sup> Similarly, the concentrations of SCFAs and numbers of SCFA-producing bacteria were lower in fecal samples from patients with alcoholic hepatitis than in samples from heavy drinkers.<sup>133</sup> Supplementation with butyrate (tributyryn) protects mice from acute ethanol-induced gut injury.<sup>134</sup>

Taken together, changes in the gut microbiota contribute to ALD via different mechanisms, which include disruption of the intestinal barrier, secretion of toxins, and metabolism of microbial molecules. Alcohol-associated dysbiosis represents an attractive target to reduce ethanol-induced liver inflammation and hepatocyte damage.

#### THE GUT MICROBIOME AND NAFLD

The gut microbiome signature in NAFLD

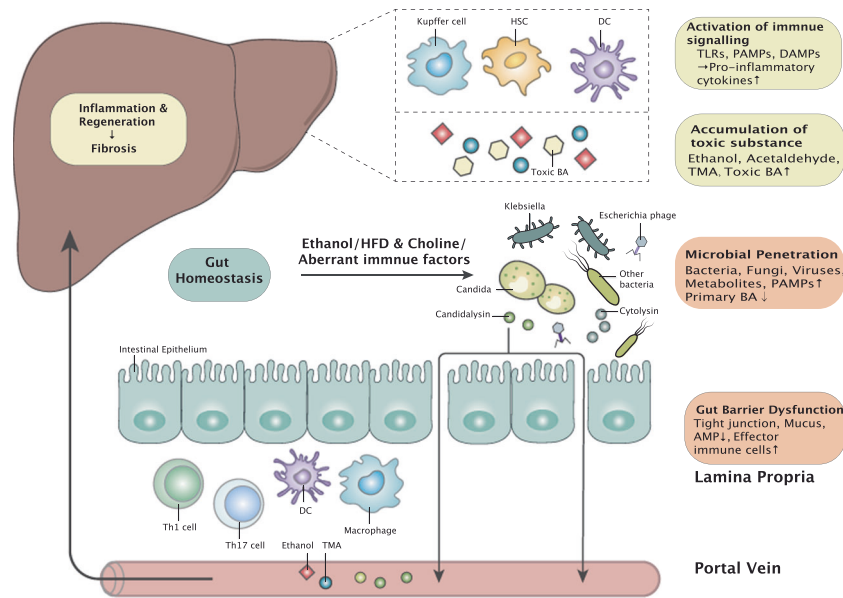
Early studies have provided evidence that the gut microbiome might contribute to the development of fatty liver disease.<sup>135</sup> In a landmark study from Anna Mae Diehl's group, the authors observed that VSL#3, a multistrain probiotic, improved hepatic steatosis in ob/ob mice, suggesting that bacterial components contribute to liver steatosis. Whereas the exact pathomechanisms by which bacterial components might contribute to such a clinical phenotype remain unclear, many clinical studies from the last decade have convincingly demonstrated that patients with NAFLD exhibit a gut microbiome signature characterized mainly by reduced bacterial diversity.

The largest study on microbiome assessment in NAFLD was recently reported.<sup>136</sup> Here, 472/1355 participants in the Rotterdam cohort exhibited evidence of hepatic steatosis, and steatosis was associated with lower microbial diversity and the presence of *Coprococcus* and *Ruminococcus gnavus*. Earlier smaller studies suggested that the gut microbiome in patients with NAFLD differed from that in healthy controls, with blooms of *Proteobacteria*, *Enterobacteriaceae*, and *E. coli*.<sup>137,138</sup> Loomba et al. went a

**Table 1.** Studies of the gut microbiome in ALD patients

Study	Country	Samples	Groups	Method	ALD-enriched Taxa	Controls-enriched Taxa
<b>Bacteria</b>						
Bode et al. <sup>117</sup>	Germany	Jejunal aspirate	Chronic alcoholics (27) vs. HC (13)	Culture	<i>Coliform</i> microorganisms, Gamma-negative anaerobic bacteria, endospore-forming rod	/
Mutlu et al. <sup>115</sup>	USA	Mucosa	ALD (19) vs. ALC (28) vs. HC (18)	16S rRNA	<i>Bacilli</i> , <i>Gammaproteobacteria</i>	<i>Clostridia</i> , <i>Bacteroidetes</i>
Wang et al. <sup>118</sup>	USA	Mucosa	Chronic alcoholics (8) vs. HC (5)	16S rRNA	Mucosa-associated bacteria	/
Leclercq et al. <sup>112</sup>	Belgium, Denmark and Sweden	Stool	AD high IP (26) vs. AD low IP (34) vs. HC (15)	16S rRNA	(At the family level) <i>Lachnospiraceae</i> , <i>Incertae sedis XIV</i> (At the genus level) <i>Dorea</i> , <i>Blautia</i> , <i>Megasphaera</i>	(At the family level) <i>Ruminococcaceae</i> , <i>Incertae sedis XIII</i> (At the genus level) <i>Faecalibacterium</i> , <i>Ruminococcus</i> , <i>Subdoligranulum</i> , <i>Oscillibacter</i> , <i>Anaerofilum</i> , <i>Clostridia</i> , <i>Bifidobacterium</i> , <i>Akkermansia muciniphila</i>
Grander et al. <sup>113</sup>	France	Stool	Severe ASH (15) vs. ASH (21) vs. HC (15)	16S rRNA	/	
Duan et al. <sup>116</sup>	USA, Mexico, UK, France and Spain	Stool	Alcoholic hepatitis (75) vs. AUD (43) vs. HC (14)	16S rRNA	<i>Veillonella</i> , <i>Escherichia/Shigella</i> , <i>Megasphaera</i>	Unclassified <i>Ruminococcaceae</i> , unclassified <i>Lachnospiraceae</i> , <i>Clostridiales</i> , <i>Ruminococcus</i>
Lang et al. <sup>114</sup>	Multiple centers (USA, Mexico and Europe)	Stool	Alcoholic hepatitis in high MELD (54) vs. alcoholic hepatitis in low MELD (18)	16S rRNA	(Compared to alcoholic hepatitis in low MELD) <i>Veillonella</i> , <i>Enterococcus</i>	(Compared to alcoholic hepatitis in high MELD) <i>Akkermansia</i>
<b>Fungi</b>						
Yang et al. <sup>119</sup>	Unclassified	Stool	AUD (10) vs. alcoholic hepatitis (6) vs. alcoholic cirrhosis (4) vs. HC (8)	ITS	<i>Candida</i>	<i>Epicoccum</i> , unclassified fungi, <i>Galactomyces</i> , <i>Debaryomyces</i>
Lang et al. <sup>28</sup>	Multiple centers (USA, Mexico, Canada, UK, France and Spain)	Stool	AUD (15) vs. alcoholic hepatitis (59) vs. HC (11)	ITS	<i>Candida</i>	<i>Penicillium</i>
Chu et al. <sup>120</sup>	Multiple centers (USA, Mexico, Canada, UK, France and Spain)	Stool	AUD (42) vs. alcoholic hepatitis (91) vs. HC (11)	Culture + qPCR	<i>Candida</i>	/
<b>Viruses</b>						
Jiang et al. <sup>121</sup>	Multiple centers (USA, Mexico, Canada, UK, France and Spain)	Stool	AUD (36) vs. alcoholic hepatitis (89) vs. HC (17)	Metagenomics	<i>Escherichia</i> phage, <i>Enterobacteria</i> phage, <i>Enterococcus</i> phage, <i>Parvoviridae</i> , <i>Herpesviridae</i>	/

AD alcohol-dependent, IP intestinal permeability, ASH alcoholic steatohepatitis, MELD model for end-stage liver disease, AUD, alcoholic use disorder



**Fig. 1** Interplay between the liver and gut microbiota in chronic liver diseases. Intestinal dysbiosis is the cornerstone of the impaired gut–liver axis, with different altered profiles in ALD, NAFLD, and AILD. Significant changes include (1) increased microbial invasion and impaired intestinal barrier and (2) activation of immune signalling and accumulation of toxins (especially in ALD and NAFLD) found in the liver. Changes in the gut and liver are connected by the portal vein, systemic circulation and bile acid metabolism. Finally, chronic inflammation and recurrent proliferation within the liver lead to cirrhosis. HFD high-fat diet, PAMP pathogen-associated molecular pattern, BA bile acid, AMP antimicrobial peptide, DC dendritic cell, TMA trimethylamine, TLR Toll-like receptor, DAMP damage-associated molecular pattern, HSC hepatic stellate cell

**Table 2.** Studies of the gut microbiome in NAFLD patients

Study	Country	Samples	Groups	Method	NAFLD-enriched Taxa	Controls-enriched Taxa
<b>Bacteria</b>						
Zhu et al. <sup>137</sup>	USA	Stool	NASH (22) vs. obese (25) vs. HC (16)	16S rRNA	<i>Proteobacteria</i> , <i>Enterobacteriaceae</i> , <i>Escherichia</i>	/
Mouzaki et al. <sup>138</sup>	Canada	Stool	Simple steatosis (11) vs. NASH (22) vs. HC (17)	PCR	<i>Clostridium coccooides</i>	<i>Bacteroidetes</i>
Alferink et al. <sup>136</sup>	Netherlands	Stool	No steatosis (883) vs. steatosis (472)	16S rRNA	<i>Ruminococcus gauvreauii</i> group, <i>Ruminococcus gnavus</i> group	<i>Coprococcus3</i>
Loomba et al. <sup>139</sup>	USA	Stool	NAFLD (72) vs. advanced fibrosis (14)	Metagenomics	<i>Proteobacteria</i> , <i>Escherichia coli</i>	<i>Fimicutes</i>
<b>Viruses</b>						
Lang et al. <sup>140</sup>	Germany	Stool	NAFLD (73) vs. PBC (13) vs. HC (9)	16S rRNA + Metagenomics	<i>Escherichia phage</i> , <i>Enterobacteria phage</i> , <i>Lactobacillus phage</i>	/
NAS NAFLD activity score						

step further and demonstrated a gut microbiome signature in fibrotic NASH.<sup>139</sup> In this study, patients with NASH exhibited a bacterial signature allowing us to differentiate between early and advanced liver fibrosis. Importantly, advanced liver fibrosis was paralleled by an increase in *Proteobacteria* and *E. coli*, whereas *Firmicutes* and *F. prausnitzii* were significantly decreased.<sup>139</sup> Whereas several studies so far have assessed the gut microbiome, much less is known about the virome. A first report provided evidence from 73 patients with biopsy-proven NAFLD that more advanced disease shows a decrease in viral diversity and the respective proportion of bacteriophages.<sup>140</sup> Overall, evidence is now substantial that the gut microbiome is affected even at the early stages of NAFLD and becomes more disturbed when this disease progresses towards a more advanced stage (Table 2).

What remains one of the most important questions is whether using certain probiotics could not only affect the gut microbiome

but also influence hepatic steatosis. A large placebo-controlled study using a symbiotic (fructo-oligosaccharide plus *Bifidobacterium animalis* subspecies lactis BB-12) was recently reported.<sup>141</sup> In this 10–14-month study, the symbiotic affected the fecal microbiome but failed to alter hepatic steatosis in subjects with NAFLD. A prebiotic-antibiotic strategy might also have the potential to affect NAFLD. When metronidazole was administered over 1 week followed by 11 weeks of inulin therapy, patients with NAFLD exhibited a reduction in ALT activity.<sup>142</sup> Many studies have now been performed both preclinically and clinically studying the impact of various pre- and probiotics in NAFLD.<sup>143</sup>

Does an altered microbiota contribute to liver inflammation in NAFLD?

Whereas it seems well established that advanced liver disease with its complications, such as hepatic encephalopathy, is driven

by microbial-derived products, it is still unclear whether dysbiosis observed at earlier stages of liver diseases might contribute to liver inflammation. Obviously, one of the most important and relevant parts protecting the host from bacterial invasion is an intact intestinal epithelial barrier (Fig. 1). It is now rather well established that the intestinal barrier is disrupted in many chronic inflammatory disorders, including NAFLD, and the gut microbiome might play a crucial role in the physiology of an intact epithelial barrier.<sup>8</sup> Numerous factors might contribute to an impaired intestinal barrier,<sup>144</sup> and various dietary factors, including a typical Western diet, might disturb this barrier. In mice with genetic impairment of the intestinal epithelial barrier, i.e., junctional adhesion molecule A-deleted mice, barrier disruption was accompanied by severe NASH.<sup>145</sup> Targeting this defective barrier seems possible, and in addition to various beneficial diets, manipulation of the gut microbiome, e.g., by FMT, might reflect another possibility, as shown in a small clinical trial.<sup>146</sup>

Various bacteria-derived metabolites might play different roles at certain stages of liver disease.<sup>147</sup> Several recent studies have identified gut bacteria-derived metabolites that might be involved in the development of hepatic steatosis. The serum levels of N,N,N-trimethyl-5-aminovaleric acid (TMAVA, a metabolite of gut bacteria) are increased in NAFLD, levels are reduced in mice treated with antibiotics or germ-free mice, and when administered to mice on a high-fat diet, this metabolite worsens hepatic steatosis.<sup>148</sup> Gut microbes metabolize trimethyllysine to TMAVA, which inhibits butyrobetaine hydroxylase, thereby promoting steatosis. Hoyles et al. identified phenylacetate, a mainly bacteria-derived metabolite, as a molecule causing hepatic steatosis<sup>149</sup>, and fecal transfer from obese women into mice resulted in hepatic steatosis, as did feeding phenylacetate to mice.<sup>149</sup> Goldstein et al. discovered another metabolite, imidazole propionate, a microbially produced histidine-derived metabolite,<sup>150</sup> and serum and portal vein levels were increased in patients with type 2 diabetes. Furthermore, imidazole propionate regulated insulin signaling by activating p38 MAPK and phosphorylating p62, resulting in activation of mechanistic target of rapamycin.<sup>150</sup>

One important microbiota-derived driver of liver disease, especially in more advanced stages of disease, might be endotoxin. Endotoxin was considered a key pathogenetic factor in NAFLD more than two decades ago.<sup>151</sup> Several bacteria, such as *Enterobacter cloacae* B29, *E. coli* PY102, and *Klebsiella pneumoniae* A7, all endotoxin producers, induced NAFLD in germ-free mice on a high-fat diet.<sup>152</sup> Patients with NASH show higher circulating endotoxin levels than patients with simple steatosis, and hepatocytes in NASH livers are positive for endotoxin accompanied by an increased number of TLR4+ hepatic macrophages.<sup>153</sup> High-ethanol-producing *K. pneumoniae* strains isolated from patients with NAFLD may cause fatty liver disease in mice.<sup>154</sup> All these studies are important, as they suggest that bacterial components such as endotoxin are of importance in various aspects of early and late liver disease, finally resulting in fibrosis and cirrhosis. As discussed in the chapter on ALD, similar mechanisms might take place in NAFLD, at least at a more advanced disease stage, when certain TLRs and NLRs expressed in the liver by various cell types are activated by gut-derived microbial components. Activation of TLRs and NLRs results in the production of numerous cytokines and chemokines driving liver inflammation. Microbially derived metabolites may especially affect patient outcomes in liver cirrhosis, as demonstrated by a large recent US study.<sup>155</sup> Various metabolic pathways, such as bile acids, choline metabolism, aromatic amino acid metabolism, and xenobiotic pathways, correlated with the development of acute-on-chronic liver failure and death.<sup>155</sup> Metabolomic approaches provide a large array of potentially involved molecules in the pathophysiology of liver diseases, many of which are released and regulated by gut microbes. Characterization of such key metabolites might have potential for future therapeutic targeting.

Extraintestinal microbiome signature in NAFLD: from adipose tissue to liver bacterial components

Whereas it had until recently been believed that bacterial signatures can only be observed in the intestine or other mucosal surfaces, there is increasing evidence that such signatures can be found in various tissues. The liver is continuously exposed to gut-derived bacteria and bacterial components, especially in advanced stages of disease. A circulating microbiome has been detected in central, hepatic, and portal venous blood and peripheral blood from patients with cirrhosis undergoing a transjugular portosystemic shunt.<sup>156</sup> However, even in the early stages of liver disease, bacterial components might be present in the liver, as shown recently by Sookoian et al. when investigating liver tissue in NAFLD for the presence of bacterial DNA.<sup>157</sup> In particular, liver *Proteobacteria* were increased in severe obesity, whereas in the case of moderate obesity, *Gammaproteobacteria* and *Alphaproteobacteria* as well as *Deinococcus-Thermus* dominated.<sup>157</sup> This seems to be in accordance with other NAFLD studies assessing the gut microbiome where *Proteobacteria* also dominated.<sup>139</sup> Another study investigated plasma and tissue (liver and 3 different adipose tissues) microbiomics in obese subjects + type 2 diabetes.<sup>158</sup> In omental adipose tissue, the authors detected a high bacterial load with a decrease in certain Gram-positive bacteria, such as *F. prausnitzii*, and an increase in *Enterobacteriaceae*.<sup>158</sup> Although all these "tissue microbiomic studies" are descriptive and probably detect only certain fragments of bacteria, such a phenomenon might contribute to local inflammatory processes in certain tissues, including the liver.

## THE GUT MICROBIOME AND AILD

### Introduction of AILD

AILD is a group of chronic and inflammatory liver diseases that is characterized by circulating autoantibodies and pathological inflammatory damage in the liver. On the basis of the involved cells (hepatocytes and/or biliary epithelial cells) in the liver, AILD can be categorized into four types: autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and IgG4-related cholangitis (IgG4-SC). Variant forms of AILD (i.e., overlap syndromes) are not discussed here. Regarding pathogenesis, AILD is caused by environmental triggers in a genetically prone individual, involving the autoantigen, the major histocompatibility complex (MHC) and receptors expressed on various effective immune cells.<sup>5</sup>

### The gut microbiome signature of AILD

Microbial composition and functional changes have been described in patients with AIH, PBC, and PSC (Table 3). For patients with AIH, our group revealed that Chinese patients with AIH are characterized by a decreased abundance of obligate anaerobes and increased potential pathobionts.<sup>159</sup> Among the 11 changed bacterial genera, *Veillonella dispar* displays the strongest correlation with disease severity.<sup>159</sup> Another study confirmed disease-specific fecal microbial alterations in patients with AIH in Germany.<sup>160</sup> Moreover, a decreased abundance of *Bifidobacterium* is observed in this group of patients with AIH and is associated with increased disease activity. For patients with PBC, one cross-sectional study demonstrated correlations of the gut microbiome, metabolism, and immune changes in patients with PBC in China.<sup>161</sup> Our group then revealed that the bacterial diversity decreased significantly in patients with PBC.<sup>162</sup> The increased abundance of eight bacterial genera and the decreased abundance of four genera were also observed. This longitudinal study also revealed that gut microbial alterations in patients with PBC are associated with treatment with ursodeoxycholic acid (UDCA).<sup>162</sup> Our group further analyzed serum and fecal BA profiles in patients with PBC and the association between BA and intestinal microbiota.<sup>163</sup> This study demonstrated abnormal BA



**Table 3.** Studies of the gut microbiome in AILD patients

Study	Country	Samples	Groups	Method	Certain AILD-enriched Taxa	Controls-enriched Taxa
AIH Wei et al. <sup>159</sup>	China	Stool	AIH (91) vs. HC (98)	16S rRNA	<i>Veillonella</i> , <i>Klebsiella</i> , <i>Streptococcus</i> , <i>Lactobacillus</i>	<i>Clostridiales</i> , <i>RF39</i> , <i>Ruminococcaceae</i> , <i>Rikenellaceae</i> , <i>Oscillospira</i> , <i>Parabacteroides</i> , <i>Coproccoccus</i> , <i>Faecalibacterium</i> and <i>Bifidobacterium</i>
Liowski et al. <sup>160</sup> PBC	Germany	Stool	AIH (72) vs. HC (95) vs. PBC (99) vs. UC (81)	16S rRNA	<i>Veillonella</i> , <i>Klebsiella</i> , <i>Streptococcus</i>	<i>Acidobacteria</i> , <i>Lachnobacterium</i> , <i>Bacteroides</i> and <i>Ruminococcus</i>
Lv et al. <sup>161</sup>	China	Stool	PBC (42) vs. HC (30)	16S rRNA	<i>Proteobacteria</i> , <i>Enterobacteriaceae</i> , <i>Neisseriaceae</i> , <i>Spirochaetaceae</i> , <i>Veillonella</i> , <i>Streptococcus</i> , <i>Klebsiella</i> , <i>Actinobacillus</i> , <i>Anaeroglobus</i> , <i>Enterobacter</i> , <i>Haemophilus</i> , <i>Megasphaera</i> , <i>Paraprevotella</i>	<i>Oscillospira</i> , <i>Faecalibacterium</i> , <i>Sutterella</i> and <i>Bacteroides</i>
Tang et al. <sup>162</sup>	China	Stool	Treatment-naïve PBC(60) vs. HC (80) Treatment-naïve PBC vs. UDCA-treated PBC (37, longitudinal)	16S rRNA	<i>Haemophilus</i> , <i>Veillonella</i> , <i>Clostridium</i> , <i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Pseudomonas</i> , <i>Klebsiella</i> , <i>Enterobacteriaceae</i>	
Furukawa et al. <sup>165</sup>	Japan	Stool	PBC (76) vs. HC (23) UDCA responder (43) vs. UDCA non-responder (30)	16S rRNA	<i>Lactobacillales</i>	<i>Clostridiales</i>
PSC Rossen et al. <sup>170</sup>	Netherlands	Mucosa	PSC-IBD (12) vs. UC (11) vs. HC (9)	16S rRNA		<i>Clostridiales II</i>
Kevans et al. <sup>171</sup>	Canada and Norway	Mucosa	PSC-UC (31) vs. UC (56)	16S rRNA		/
Torres et al. <sup>172</sup>	USA	Mucosa	PSC (20, 19 with IBD) vs. IBD (15, 13 with UC and 2 with CD) vs. HC (9)	16S rRNA	<i>Barnesiellaceae</i> , <i>Blautia</i> , <i>Ruminococcus</i>	/
Quraishi et al. <sup>173</sup>	UK	Mucosa	PSC-IBD (11) vs. IBD (10) vs. HC (9)	16S rRNA	<i>Lachnospiraceae</i> , <i>Escherichia</i> , <i>Megasphaera</i>	<i>Prevotella</i> , <i>Roseburia</i> , <i>Bacteroides</i>
Pereira et al. <sup>169</sup>	Finland	Bile	PSC (80) vs. controls (46)	16S rRNA	<i>Streptococcus</i>	<i>Bacteroides</i>
Kummen et al. <sup>166</sup>	Norway	Stool	PSC (85, 55 with IBD) vs. UC (36) vs. HC (263)	16S rRNA	<i>Veillonella</i>	<i>Coprococcus</i> , <i>Phascolarctobacterium</i> , <i>Lachnospiraceae</i> , <i>Christensenellaceae</i>
Sabino et al. <sup>168</sup>	Belgium	Stool	PSC (52, 39 with IBD) vs. UC (13) vs. CD (30) vs. HC (52)	16S rRNA	<i>Veillonella</i> , <i>Streptococcus</i> , <i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Fusobacterium</i>	/
Iwasawa et al. <sup>174</sup>	Japan	Stool	PSC (13, all pediatric-onset) vs. UC (15) vs. HC (23)	16S rRNA	<i>Veillonella</i> , <i>Streptococcus</i> , <i>Enterococcus</i>	/
Bajer et al. <sup>175</sup>	Czech	Stool	PSC (43, 32 with IBD) vs. IBD (32) vs. HC (31)	16S rRNA	<i>Veillonella</i> , <i>Rothia</i> , <i>Streptococcus</i> , <i>Enterococcus</i>	<i>Coprococcus</i>
Torres et al. <sup>176</sup>	USA and Portugal	Stool	PSC-IBD (15) vs. IBD (15)	16S rRNA	<i>Ruminococcus</i> , <i>Fusobacterium</i>	<i>Blautia</i> , <i>Roseburia</i> , <i>Veillonella</i> , <i>Doria</i>
Rühlmann et al. <sup>167</sup>	Germany and Norway	Stool	PSC (73, 38 with IBD) vs. UC (88) vs. HC (98)	16S rRNA	<i>Veillonella</i> , <i>Streptococcus</i> , <i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Parabacteroides</i> , <i>Gammaproteobacteria</i>	<i>Coprococcus</i>
Lemoine et al. <sup>29</sup>	France	Stool	PSC (49, 27 with IBD) vs. IBD (33) vs. HC (30)	16S rRNA and ITS	<i>Exophiala</i> (fungal), <i>Veillonella</i> , <i>Sphingomonadaceae</i> , <i>Alphaproteobacteria</i> , <i>Rhizobiales</i>	<i>S. Cerevisiae</i> (fungal), <i>Ruminococcus</i> , <i>Ruminiclostridium</i> , <i>Faecalibacterium</i> , <i>Lachnospiraceae</i> , <i>Blautia</i>

metabolism in patients with PBC, characterized by blockage of the conversion from conjugated to unconjugated and primary to secondary BAs. UDCA treatment leads to a decline in the level of certain conjugated BAs, therefore reversing the conjugated/unconjugated ratio in PBC. Moreover, the level of certain secondary BAs inversely correlates with upregulated bacteria in patients with PBC (e.g., *Veillonella* and *Klebsiella*) but positively correlates with upregulated bacteria in healthy controls (e.g., *Faecalibacterium* and *Oscillospira*).<sup>163</sup> Interestingly, one study suggested that *Veillonella* might be a bile acid-sensitive bacterium and enable aldafermin (an FGF19 analog)-mediated suppression of bile acid synthesis, indicating its role as a novel marker for treatment response in patients with NASH.<sup>164</sup> Recently, one study elucidated the relationships among clinical profiles, response to UDCA treatment, and gut microbiome composition in patients with PBC,<sup>165</sup> indicating a decrease in *Faecalibacterium* as a novel prognostic factor in PBC. For patients with PSC, various studies show coherent changes in fecal and biliary bacteria in patients with PSC independent of IBD, characterized by low diversity and higher abundances of *Enterococcus*, *Fusobacterium*, and *Lactobacillus*.<sup>29,166–176</sup> These studies suggested specific alterations in gut bacteria and bacterial metabolites in patients with AILD, suggesting the potential for using these gut microbiomes as novel biomarkers and targets in the diagnosis and treatment of AILD.

#### The role of the gut microbiome in AILD

The mechanisms of how the gut microbiota contributes to the predisposition, initiation, and progression of certain autoimmune diseases include the following (summarized elsewhere<sup>177,178</sup>): (1) molecular mimicry; (2) translocation of gut commensals to distal organs; and (3) migration of gut immune cells to distal organs. A comprehensive understanding of the role of gut microbiota in AILD paves the way for therapeutic avenues to restore immune tolerance and homeostasis (Fig. 1).

**Expression of autoantigen orthologs by the gut microbiome.** Molecular mimicry could induce an autoimmune response.<sup>179</sup> Multiple studies have suggested that autoantibodies isolated from patients with AILD can react with specific microbial proteins. The anti-mitochondrial antibody from patients with PBC can bind to certain *E. coli* proteins.<sup>180</sup> The IgG3 autoantibody from patients with PBC can interact with *Lactobacillus delbrueckii*  $\beta$ -galactosidase, and its similarity with the PBC autoantigen PDC-E2 subunit is up to 67%.<sup>181</sup> The peripheral antineutrophil cytoplasmic antibody (p-ANCA) derived from the PSC antibody can bind to the bacterial cell division protein FtsZ.<sup>182</sup>

**Translocation of gut commensals to the liver.** Physiologically, intestinal epithelial cells and their neighbors (mucus layer, antimicrobial peptides, immunoglobulins, symbiotic bacteria, etc.) jointly construct the gut barrier, while the mesenteric lymph nodes and the liver in turn act as immune firewalls to limit the translocation of gut microbiota to distal organs. Pathologically (named "gut barrier dysfunction"), bacteria and/or their metabolites can enter the liver through the portal vein, causing inflammation and damage in the liver. One study pointed out that *Enterococcus gallinarum* can pass through the intestinal epithelium and reach multiple distal organs, including the mesenteric lymph nodes and the liver, thereby causing autoimmune diseases (such as AIH and systemic lupus erythematosus).<sup>183</sup> The application of antibiotics or specific vaccines in vivo can inhibit the growth of *E. gallinarum* and alleviate the disease. Another study showed that *K. pneumoniae* in the intestines of patients with PSC can also destroy the intestinal epithelium, prompting other bacteria, such as *E. gallinarum* and *Proteus mirabilis*, to jointly cross the intestinal barrier, leading to Th17 cell-mediated liver inflammation.<sup>184</sup>

**Migration of immune cells from the gut to the liver.** Gut lymphocyte homing can cause inflammation and damage in the liver. One study revealed that in the state of inflammatory liver disease including PSC, hepatic sinusoidal endothelial cells overexpress MADCAM-1, and CCL25, which recruit intestinal-derived lymphocytes into the liver and cause damage.<sup>185</sup> Mechanistically, liver vascular adhesion protein (VAP)-1 is associated with the overexpression of MADCAM-1 in the liver. Cysteamine, the substrate of VAP-1, originating from intestinal bacterial metabolism and the diet, acts on VAP-1 in the liver after entering the liver through the portal vein.<sup>186</sup> Another study revealed that the TCR $\beta$  chain expressed on T cells in the intestine and liver is clonally related and recognizes the same antigen.<sup>187</sup> B cells also participate in the gut–liver axis. One study showed that the subgroup of B cells that produce IgA in the liver is derived from intestinal lymphoid tissue and can recognize intestinal commensal bacterial antigens.<sup>188</sup>

#### The gut microbiome and other types of liver diseases

In the scenario of etiologically different CLDs, the vicious cycle of liver injury (inflammation and regeneration that spans decades) drives cirrhosis and liver cancer as the final stage of the disease process. Given the growing body of literature, it is becoming increasingly clear that intestinal dysbiosis plays a key role in this progression. Here, we will briefly review the role of the gut microbiome in viral hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). More detailed reviews have recently been published elsewhere for further exploration.<sup>189–191</sup>

#### Viral hepatitis

Hepatitis B virus (HBV) is one of the most common causes of chronic viral hepatitis, especially in developing countries. Intestinal dysbiosis has been found in patients with HBV. Opportunistic pathogenic bacteria (e.g., *E. coli*, *Clostridium difficile*, and *Clostridium perfringens*)<sup>192</sup> and some fungi (e.g., *Candida*)<sup>193</sup> are enriched in patients with HBV and HBV-related cirrhosis. Other bacteria, such as *Bifidobacteria*, display a shift from beneficial species to pathogenic species.<sup>194</sup> Studies also demonstrate the interaction between gut microbiota and the elimination of HBV within the host. Using an HBV-infected mouse model, Chou et al. found that the establishment of intestinal bacteria via a TLR4-dependent pathway promotes liver immunity, resulting in rapid HBV clearance.<sup>195</sup> Given the potential role of gut microbiota in the pathogenesis of HBV, randomized clinical trials of FMT for the treatment of HBV have been conducted (NCT02689245 and NCT03429439). Recently, one nonrandomized pilot trial of FMP for HBV treatment displayed safety and efficacy in viral suppression and HBeAg clearance.<sup>196</sup>

Other types of viral hepatitis also exhibit changes in the gut microbiota. Studies focusing on patients with hepatitis C virus demonstrated a reduction in microbial diversity and composition in the patient group by 16S rRNA<sup>197,198</sup> and high levels of urease gene expression by metagenomics<sup>199</sup>. Hepatitis E virus infection, as one type of acute viral hepatitis, might lead to acute liver failure. One study illustrated a perturbation of intestinal microbiota effector T lymphocytes, the serum international normalized ratio and the severity of hepatic encephalopathy.<sup>200</sup>

#### Cirrhosis

Changes in the composition and function of the gut microbiota have been observed in cirrhosis. Regarding composition, the fecal microbiome in patients with cirrhosis is characterized by a decrease in diversity, an increase in immunostimulatory pathogens (e.g., *Enterococcaceae* (the major causative organism releasing endotoxin) and *Staphylococcaceae*) and a decline in potentially beneficial *Firmicutes* (e.g., *Lachnospiraceae* and *Ruminococcaceae*).<sup>201,202</sup> Similar changes can be detected in the colon mucosa<sup>203</sup>, serum<sup>204</sup> and saliva<sup>205</sup> of patients with cirrhosis.

Interestingly, the change in the gut microbiota composition is associated with cirrhosis patient outcomes (i.e., compensated vs uncompensated<sup>206</sup>, inpatient vs outpatient, and noninfected and infected patients<sup>203</sup>), suggesting its potential as a novel biomarker. Regarding function, leaky gut is a well-established feature of patients with cirrhosis and relevant animal models, rendering potentially pathogenic bacteria and their metabolites permeable to the circulation.<sup>207</sup> In addition to the physical destruction of the gut barrier, intestinal infiltration with effective immune cells is also observed in cirrhosis, exemplified by the expansion of TNF- $\alpha$ - and IFN- $\gamma$ -expressing lymphocytes and the depletion of Th17 cells.<sup>208</sup> Moreover, reduced bile flow<sup>209</sup> and impaired FXR signaling pathways<sup>210</sup> are also detected in cirrhosis and parallel the disease severity.

A common gut microbiome signature in cirrhosis independent of the etiology of liver disease might exist. Oh et al. characterized a gut microbiome signature when combined with age that precisely detected NAFLD-related cirrhosis.<sup>211</sup> Importantly, by using a machine-learning-based approach and when comparing their findings with liver cohorts from Italy and China comprising various etiologies of liver cirrhosis, a similar microbiome signature was detected independent of the etiology of cirrhosis. These findings suggest that at least in advanced stages of liver disease, the etiology of liver disease seems less relevant regarding the gut microbiome disturbances.<sup>212</sup> The authors proposed that such an approach might allow the noninvasive diagnosis of liver cirrhosis in the near future.<sup>211</sup> More studies are needed to clarify whether gut microbiome signatures differ between various etiologies at earlier stages of liver diseases and whether, in advanced stages, such differences might disappear.

## HCC

Sustained liver injury and regeneration promote HCC, which is the third leading cause of cancer mortality worldwide. Recent studies have revealed changes in the gut microbiota in patients with HCC, suggesting a correlation of specific microbial profiles in patients with HCC with different etiologies, geographical distributions, and nutritional states.<sup>213,214</sup> The contribution of gut microbiota to HCC is multifaceted: (1) A disrupted gut barrier brings a series of TLR ligands (LPS<sup>215</sup> and bacterial DNA<sup>216</sup>) to the liver and triggers inflammation. The TLR signaling pathways mediate hepatocarcinogenesis via downregulation of hepatocyte apoptosis and upregulation of hepatic stellate cell proliferation<sup>64</sup>. (2) Impaired immunosurveillance is associated with abnormal gut microbiota in HCC. As mentioned before, a negative correlation between BA-metabolizing *Clostridium* cluster XIV and CXCR6-positive antitumor NKT cells is observed in mice with liver cancer.<sup>77</sup> Moreover, the composition of the gut microbiota could potentially predict the response rates in patients with liver cancer treated with certain immune checkpoint inhibitors (such as PD-L1 inhibitors),<sup>217</sup> suggesting the potential to harness the microbiome for HCC immunotherapy.

## Concluding remarks and perspectives

An accumulating body of research suggests the close relationship between the gut and the liver, called the gut–liver axis. They communicate with each other anatomically and functionally via the portal vein, biliary tract, and systemic circulation. Extensive interplay between the gut and the liver is exemplified in specialized bacteria and their metabolites, immune cells in the liver, and BA signaling pathways. Pathologically, preliminary correlative studies have revealed different microbiota (bacteria, fungi, and viruses) phenotypes in different liver diseases, including ALD, NAFLD, AILD, and other types of liver diseases. More longitudinal studies are anticipated to provide persistent and accessory microbiomes within individuals. Mechanistic studies are the second step to explore potential microbiota-mediated causality by moving from human data and samples to animal

models. Culturomics fuels functional studies of the key microbial member(s) identified in the disease state<sup>218</sup>. To gain a complete picture of the gut–liver axis, multiomics provides high-dimensional measurements to capture a comprehensive profile of both the microbiota and the immune system.<sup>219,220</sup> There is also a trend to integrate environmental factors with genetic factors to better understand pathogenesis<sup>221,222</sup>.

Advances in knowledge of the gut–liver axis promote the development of novel diagnostic, prognostic and therapeutic modalities for liver diseases. FMT with precise modulation based on the prior selection of beneficial microbial members could bring benefits with reduced risks. Synthetic biology (i.e., engineered probiotics and human microbial members) also provide a sustainable therapeutic approach.<sup>223,224</sup> Bacteriophages, which target specific bacterial hosts, also show efficacy as novel therapeutic modalities to treat liver disease.<sup>116</sup> Thus, as the role of the gut microbiome in liver disease is increasingly recognized, more effort should be put into translating our current knowledge of the disease-modulating role of the gut–liver axis into well-designed trials in patients.

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## AUTHOR CONTRIBUTIONS

B.S. was responsible for writing the chapter “Contribution of the gut microbiota to alcohol-associated liver disease”. H.T. wrote the chapter “NAFLD and the Microbiome”. R.W., R.T. and X.M. wrote the chapters “The Gut Microbiome”, “The Gut Microbiome and Liver Immunology”, “The Gut Microbiome and AILD”, and “The Gut Microbiome and Other Types of Liver Diseases”. B.L. was responsible for draft calibration.

## ADDITIONAL INFORMATION

**Competing interests:** B.S. has been consulting for Ferring Research Institute, Intercept Pharmaceuticals, HOST Therabiomics, Mabwell Therapeutics and Patara Pharmaceuticals. B.S.’s institution (UC San Diego) has received grant support from BiomX, NGM Biopharmaceuticals, CymaBay Therapeutics, Synlogic Operating Company and Axial Biotherapeutics. H.T. and X.M. have no competing interests.

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