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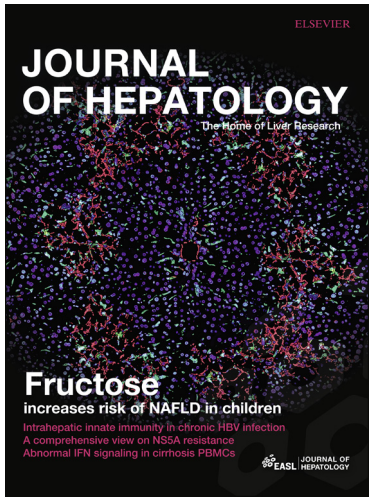

Seminar

The gut-liver axis in liver disease: pathophysiological basis for therapy

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SEMINAR ARTICLE

Title:

THE GUT-LIVER AXIS IN LIVER DISEASE: PATHOPHYSIOLOGICAL BASIS FOR THERAPY

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Abstract

The gut-liver axis refers to the bidirectional relationship between the gut and its microbiota, and the liver, resulting from the integration of signals generated by dietary, genetic and environmental factors. This reciprocal interaction is established through the vascular route of the portal vein that carries gut-derived products directly to the liver, and the liver feed-back route of bile and antibody secretion to the intestine. The intestinal mucosal and vascular barrier is the functional and anatomical structure that serves as a playground for the interactions between the gut and the liver, limiting the systemic dissemination of microbes and toxins while allowing nutrients to access the circulation and to reach the liver. The control of microbial communities is critical to maintain homeostasis of the gut-liver axis, and as part of the two-way communication the liver shapes intestinal microbial communities. Alcohol disrupts the gut-liver axis at multiple interconnected levels, including the gut microbiome, mucus, epithelial barrier and antimicrobial peptides production, which increases the microbial exposure and the pro-inflammatory environment of the liver. Growing evidences indicate the pathogenetic role of microbe-derived metabolites, such as trimethylamine, secondary bile acids, SCFA and ethanol, in the pathogenesis of non-alcoholic fatty liver disease. Cirrhosis by itself is associated to profound alterations in gut microbiota and damage at the different levels of defense of the intestinal barrier, including the epithelial, the vascular and the immune barriers. The relevance of the severe disturbance of the intestinal barrier in cirrhosis has been linked to translocation of live bacteria, bacterial infections and disease progression. The identification of the elements of the gut-liver axis primarily damaged in each chronic liver disease offers possibilities to intervention. Beyond antibiotics, upcoming therapies centered in the gut include new generations of probiotics, bacterial metabolites (postbiotics), fecal microbial transplantation, and carbon nanoparticles. FXR-agonists target both the gut and the liver and are currently being tested in different liver diseases. Finally, synthetic biotic medicines, phages that target specific bacteria or therapies that create physical barriers between the gut and the liver offer new approaches of treatment.

Keywords: microbiome, intestinal barrier, cirrhosis, bile, farnesoid X receptor

The gut-liver axis refers to the bidirectional relationship between the gut along with its microbiota and the liver, and arises from interactions among signals generated by dietary, genetic and environmental factors. The interdependence between the gut and the liver sets the stage for the fact that disturbances of the intestinal barrier result in increased portal influx of bacteria or their products to the liver, where they cause or worsen a range of hepatic diseases. The part played by intestinal microbes in liver diseases such as alcoholic liver disease (ALD) or bacterial infection in advanced liver disease has long been known [1, 2]. However, the critical role of a disrupted gut-liver axis in the pathogenesis of many liver diseases has only been recently accepted, as knowledge has gradually accumulated on intestinal microbiome composition and function, intestinal barrier homeostasis and the role of bile in gut-liver communications.

This Seminar article focuses on the most recent advances in knowledge of i) homeostasis of the gut-liver axis in health, including the role of epithelial, immunological and vascular barriers, ii) distinctive gut-liver axis disruption patterns in the prevalent chronic liver diseases ALD and non-alcoholic fatty liver disease (NAFLD), including their end-stage form cirrhosis, and iii) innovative therapies to restore gut-liver axis homeostasis. Most of the advances were brought up during the EASL Monothematic Conference held in Leuven in June 2018 on the state of the art of gut/liver communication and liver disease, and a complete view of the contents of the conference can be watched at https://livertree.easl.eu/

THE GUT-LIVER AXIS IN HEALTH

The reciprocal interaction between the microbiome and the liver is established through the vascular route of the portal vein that carries to the liver gut-derived products, and the liver feed-back route of bile and antibody secretion to the intestine. In fact, besides interacting with nuclear receptors to regulate metabolic functions, bile acids critically control the gut microbiota. The ground where the microbiome-liver axis takes place is the gut mucosal barrier, which is constructed by intestinal epithelial cells, that maintain gut homeostasis by segregating gut microbiota and host immune cells.

Stratification of the microbiota by mucus

The mucus physically separates the microbiota from the epithelial linen and allows to avoid an exaggerated inflammatory response to it (**Figure 1**). Only in a few cases, such as segmented filamentous bacteria, which in the human gut is present only in the early life [3], the microbiota is capable of crossing the mucus, and interact with epithelial cells in a host-specific manner [4]. For the rest of the microbes, the interaction with the host is indirect and is mediated by their metabolic products, also called postbiotics, that are released during food fermentation [5-7].

The mucus thickness varies in the different segments of the gut and is higher where the microbiota is residing, such as in the terminal ileum and colon [8]. In the colon, the mucus is formed by two layers, the inner one which is firmer and is in close proximity to the epithelium, and the outer one which is colonized by the bacteria [8]. The outer mucus layer offers a shelter for microbial strains to attach and to avoid being washed out by the peristaltic movements. Those bacteria that are unable to bind to the mucus layer can attach via the intervention of mucin-IgA interactions [9]. The inner mucus layer is almost sterile because of the restricted size of the meshes and because of the presence of antimicrobial peptides [10] and of microbiota-excluding proteins such as lypd8 and the lectin-like protein ZG16 (zymogen granulae protein 16) [11]. These proteins interact with several groups of bacteria and hinder their penetrance in the inner mucus layer.

The composition of the mucus is dictated by the microbiota as shown by experiments in germ-free mice that when colonized with the microbiota develop a mucus similar to that of the mice from which the microbiota was originally collected [12]. This is probably due to the capacity of goblet cells to sense the presence of bacterial products and to produce Muc2 after activating the NLRP6-inflammasome pathway [13]. On the other hand, the mucus is a source of nutrients for several bacteria, Akkermansia municiphila for instance, can degrade mucins and uses them for its growth [14]. In the absence of fibers in the diet, mucin-degrading bacteria overgrow at the expenses of mucus thickness [15]. In

addition, the balance between Bacteroides and Firmicutes can alter mucin glycosylation [16]. Hence, both mucus and the diet can affect microbiota composition. This can have huge consequences on health as it has been shown that an alteration in mucus composition as that observed in patients with inflammatory bowel disease, and in particular ulcerative colitis, can lead to a direct interaction between the microbiota and the epithelium thus contributing to the establishment and maintenance of the inflammatory response [8]. Thus, the mucus is not only a first line of defense towards the microbiota, but it also serves as a nutrient source and as a niche for microbiota colonization to avoid wash out by peristaltic movements.

Physical elements of the intestinal barrier: the epithelial and the gut-vascular barriers

Just below the mucus layer the gut barrier is formed by a monolayer of epithelial cells that are composed of enterocytes, Goblet cells, Tuft cells and enterochromaffin cells [17]. All of these cells cooperate to protect the gut from insult coming from the microbiota and eventual infectious agents. This barrier is simultaneously physical as the epithelial cells are sealed one to the other by tight junctions, electrical as the brush border is negatively charged and opposes a negative charge to the microbiota and chemical as a series of antimicrobial peptides are released by epithelial cells. In addition, a series of mucosal immune cells patrol the epithelium, see below. Further the lamina propria is enriched in plasma cells that release immunoglobulins of the A type (IgA) that further protect the barrier. In case the epithelium is breached by bacteria, either via active mechanisms employed by invasive pathogens or pathobionts, or after an injury, then bacteria are found within the lamina propria.

However, only a minority of them will be able to disseminate systemically. Some will reach the mesenteric lymph nodes that act like a firewall to avoid entrance of microbes into the systemic blood circulation. This is because of the existence of another barrier, the gut vascular barrier (GVB) that prevents bacteria from entering the portal circulation and reaching the liver [18, 19]. However, some pathogenic bacteria and probably some pathobionts have evolved strategies to elude this barrier. Indeed, during Salmonella infection, the barrier is disrupted and

the bacteria are found systemically [19]. Interestingly, this barrier is disrupted in some pathologic conditions such as celiac disease [19], anchylosing spondylitis [20], and non-alcoholic steatohepatitis NASH [21].

Immune system control of the microbiota

The intestinal mucosal barrier is further reinforced by the presence of a series of immune cells that contribute to the establishment of the barrier. These cells can be distinguished into intraepithelial cells and lamina propria cells. Intraepithelial cells consist of intraepithelial lymphocytes including conventional and unconventional $\alpha\beta$ and $\chi\delta$ T cells and intraepithelial mononuclear phagocytes [22-25]. Intraepithelial lymphocytes share common properties as they all express type I cytokines, are cytolytic and release antimicrobial peptides upon activation by intestinal epithelial cell-released cytokines or engagement with NK cell receptor activating ligands [22]. Intraepithelial mononuclear phagocytes extend protrusions that allow direct sensing of the intestinal lumen [23-25] and development of oral tolerance after delivering food antigenic peptides to lamina propria dendritic cells [26]. Together, these cells act like a first line soldiers in the case of infection and participate to induce tolerance to food and microbial antigens. In the lamina propria, immune cells play a second line of defense and foster tissue regeneration in case of damage. Besides lymphocytes, mostly CD4+ T cells, one can distinguish innate-like cells such as iNKT cells and mucosa-associated invariant T cells. These cells are highly specialized in the type of microbial antigens or metabolites that they recognize. NKT cells recognize lipids presented on CD1 molecules [27], while mucosa-associated invariant T cells recognize raboflavin metabolites presented by MR1 molecules [28-30]. CD4+ T cells comprise primarily Th17 cells and T regulatory cells. Th17 cells release IL17 A, IL17-F and IL-22 which is involved in reinforcing tight junction molecules between epithelial cells and foster epithelial cell regeneration [31]. Th17 cells are induced after adhesion of segmented filamentous bacteria to the intestinal epithelium [4, 32, 33]. They also drive IgA production and the expression of pIgR which allows their translocation into the intestinal lumen [4, 34]. T regulatory cells can be distinguished into thymic derived and peripheral derived T regs [35]. The first recognize self-antigens and control the function of autoreactive T cells while the second recognize food antigens (small intestine) or microbial antigens (large

intestine) where they control tolerance to innocuous non self-antigens [35]. In the intestine one can find also innate lymphoid cells which can release type 1, 2 and 3 cytokines in a rapid response to infection before the action of adaptive T cells [36, 37]. Hence the whole intestinal barrier is formed in response to the microbiota by the coordinated action of structural elements (mucus, epithelial cells), immune cells (intraepithelial and lamina propria immune cells) and soluble mediators (IgA, antimicrobial peptides). Any changes in this asset can alter the intestinal barrier. The microbiota on the other hand can also affect treatment efficacy through an action on the immune system. For instance during immune check point blockade (anti-PD1) in cancer immunotherapy, the composition of the microbiota of the treated patients has an impact on the outcome of the therapy [38, 39].

Postbiotic control of the intestinal barrier

As mentioned earlier the intestinal epithelium is covered by the mucus whose thickness and tightness depends on the segment of the gut that is analyzed [8] . In particular in the colon, where most of the bacteria reside, the mucus layer is composed by an external structure which is colonized by the microbiota and offers nutrients for their growth and metabolic activity, and a firmer inner structure which is almost devoid of the microbiota, conferring protection to the host [40-42]. This area is also called demilitarized.

Thus, most of the interactions occurring between the host and the microbiota are indirect and are mediated by soluble factors which include bacterial products and metabolites, also called postbiotics [7, 43]. Examples of postbiotics are short chain fatty acids (SCFAs) such as acetate, propionate and butyrate that are released during the degradation of dietary fibers, peptides with immunomodulatory activities, biosurfactants, vitamins, etc. These can have several activities on both the intestinal epithelial barrier, the immune system and the microbiota. SCFAs, for instance, have been shown to control the differentiation of several immune cells and in particular T regulatory cells [36], the microbicidal activity of macrophages [44] and several other immune cell functions [45]. The fibers and their postbiotics can also impact on the brown versus white adipose tissue ratio [46].

Bile acids control of microbiota: role of farnesoid X receptor signaling

Bile acids are molecules synthesized in the liver from cholesterol that are then released in the gut where they can be further metabolized by the microbiota (beautifully reviewed in [47]). The amount of bile acids produced depends on an active feedback loop from the gut to the liver which is called the enterohepatic circulation. Before being excreted, primary bile acids are conjugated with the aminoacid glycine and to a lesser extent with taurine in humans and then released in the bile [48]. The bile is then reversed in the terminal ileum after a meal and the conjugated bile acids are reabsorbed through the gut epithelium and recycled in the liver after entering the portal circulation. Besides their function in micelle formation and absorption of fat and fat-soluble vitamins, bile acids have a key role in shaping the microbiota. The crosstalk between bile acids and gut microbiota can take place at several places, but importantly it is a two-way interaction as the microbiota affects bile acids metabolism [49] and bile acids affects microbiota composition [47]. Further, while bile acids were initially thought to simply recirculate from the gut to the liver now it is clear that after their transformation into secondary bile acids, these signal in the intestinal epithelium primarily via the farnesoid X receptor (FxR).

FXR engagement can enhance epithelial barrier properties [50], and repair damage of the GVB [21], but can also control the metabolic syndrome [51]. Contrasting results have been obtained when using mice that are full knock-out for FXR or only in epithelial cells, suggesting different roles of FXR engagement in NASH development. FXR deficient mice are resistant to diet-induced obesity [52] and this seems to be mediated by intestinal FXR [53] and by the microbiota [54].

THE GUT-LIVER AXIS IN DISEASE

Growing evidence indicates that cross-talk among the gut microbiome and its derived metabolites, immune system and liver plays a key role in the pathogenesis of ALD and NAFLD. In both diseases, gut barrier dysfunction in the form of increased intestinal permeability is the factor that facilitates portal influx of pathogen associated molecular patterns (PAMP), e.g. LPS (endotoxin), and

microbiome-derived metabolites to the liver, triggering a pro-inflammatory cascade that worsens hepatic inflammation [55]. In this setting of gut barrier dysfunction, the intestinal load of luminal bacteria determines the amount of PAMP that translocate to the portal and systemic circulation, and in consequence, the severity of liver inflammation (**Figure 1**). Progression from compensated to decompensated chronic liver disease is associated with damage at the different levels of intestinal defense, which results in further gut barrier function impairment.

Alcoholic liver disease

Current data indicate that besides the direct toxic effect of alcohol on liver parenchymal cells, abnormal microbiota, loss of intestinal barrier function and the resultant activation of toll-like receptors (TLRs) on liver immune cells contributes to the pathogenesis of ALD (**Figure 2A**). It is important to take into account that the contribution of an altered gut microbiota to ALD begins before there is evidence of liver disease.

Gut microbiome. Alcohol intake has direct effects on the gut microbiome, which appear long before fibrosis occurs [56]. Intestinal overgrowth of aerobic and anaerobic microorganisms has largely been recognized in jejunal aspirates of individuals featuring chronic alcohol abuse [57]. Recent metagenomic analysis of the intestinal microbiome of individuals with chronic alcohol abuse/feeding alcohol to mice has revealed reduced bacterial diversity and a shift in phyla towards a greater abundance of Proteobacteria and lower abundances of Bacteroidetes and Firmicutes, as well as of *Lactobacillus* species [58-60]. Interestingly, a specific microbiota pattern involving large amounts of Bifidobacteria and Streptococci has recently been identified in the gut of patients with severe alcoholic hepatitis [61].

Besides the microbiome, the gut mycobiome (i.e. yeast and fungi) is also altered in ALD, and systemic exposure to mycobiota correlates with the severity of liver damage. Chronic alcohol abuse in humans/feeding alcohol to mice causes reduced intestinal fungal diversity and *Candida* overgrowth, along with *Candida* β-glucan translocation to the systemic circulation with a subsequent immune

response [62]. In response to the treatment of mice with antifungals, intestinal fungal overgrowth is reduced, β-glucan translocation decreases and ALD improves.

Intestinal barrier dysfunction. The expression of gut barrier dysfunction, i.e. increased intestinal permeability, is a well-recognized feature of alcohol binging, chronic abuse, alcohol mouse models, and patients with ALD at pre- and -cirrhotic stages [63, 64]. Besides the toxic effects of alcohol and/or its metabolites on intestinal epithelial cells, more and more lines of evidence point to the contribution of dysbiosis-associated intestinal inflammation to gut barrier dysfunction and translocation of microbial products in ALD. Chronic alcohol abuse in humans/feeding alcohol to mice leads to subclinical intestinal inflammation and increased numbers of monocytes and macrophages activated to TNF-alpha production in the intestinal lamina propria [65]. In these circumstances, loss of epithelial tight junction proteins through tumor necrosis factor receptor-I-mediated activation of myosin light-chain kinase results in increased intestinal permeability, translocation of bacterial products and liver inflammation [65]. Interestingly, endotoxin and bacterial DNA increase in serum in both humans and mice after acute and chronic alcohol abuse, yet intestinal inflammation is severe after chronic but minimal after acute alcohol abuse [66, 67].

Alcohol also damages specific components of the intestinal barrier such as proteins involved in innate antibacterial defense. Chronic alcohol use in humans/feeding alcohol to mice suppresses the intestinal regeneration of islet-derived 3-beta (Reg3b) and 3-gamma (Reg3g) expression [59, 68], leading to increased bacteria adhesion to the mucosal surface, intestinal bacterial overgrowth, enhanced translocation of viable bacteria, and worsened liver inflammation. These consequences of Reg3 lectin suppression are reversed by Reg3g overexpression in intestinal epithelial cells or can be partially rescued with prebiotics, despite not modifying the increased intestinal permeability [59, 69]. A further example of the protective role of Reg3g and Reg3b in ALD is the fact that a compensatory increase in these lectins protects mice lacking mucin-2 from liver inflammation; this being the main mucin found in the intestinal mucous layer [68].

Bacterial products and metabolites. In a seminal study showing that LPS-induced release of TGF-beta was MYD-88-NF-kB mediated, LPS was linked to liver pro-inflammatory and pro-fibrogenic signals [70]. Besides immunological responses to barrier dysfunction, the course of ALD is also marked by system-wide changes in many bioactive compounds. Alcohol-induced abnormal microbiota in mice reduces the microbiome's capacity to synthesize saturated long-chain fatty acids (LCFA), thus reducing proportions of microbes whose growth depends on LCFA, such as *Lactobacillus* species [58]. In this last study, dietary supplementation with saturated LCFA was found to restore eubiosis, stabilize the intestinal gut barrier and improve ethanol-induced liver injury.

Bile acids. Alcohol-induced alterations in gut microbiota also results in changes in bile acid homeostasis, increasing the intestinal deconjugation of bile acids and exposure of hepatocytes to more toxic bile acids. In effect, intestinal microbiota abnormalities induced by alcohol leads to overrepresentation in the intestine of bacteria encoding cholylglycine hydrolase and disruption of the FXR activation pathway, whereby there is low FXR activity in enterocytes, low FGF15 plasma levels, and increased hepatic Cyp7a1 expression. In consequence, depleting commensal bacteria with non-absorbable antibiotics or improving bile acid-FXR-FGF15 signaling via hepatic Cyp7a1 modulation has been reported to improve ALD in mice [71].

Non-alcoholic fatty liver disease

NAFLD encompasses a spectrum of liver diseases that can be broadly classified into non-progressive and progressive phenotypes called NAFL and non-alcoholic steatohepatitis (NASH), respectively. NAFLD is strongly associated with obesity and shares mechanisms with insulin resistance, type 2 diabetes mellitus and cardiovascular risk factors. Data so far indicate a role of a high-fat diet in altering the microbiome, which in turn impairs the intestinal barrier and the gut vascular barrier [21], and facilitates the portal influx of bacterial products, worsening non-hepatic inflammation and metabolic abnormalities (**Figure 1 and 2B**). The liver, as a ‘first pass’ organ exposed to the highest concentration of portal system products such as PAMP, is the most vulnerable to their effects, particularly if pre-conditioned by a subclinical pathology such as lipid accumulation in hepatocytes.

Although ALD and NAFLD share the basic mechanisms of intestinal barrier dysfunction, subtle differences exist between them including alterations in intestinal microbial composition, gut permeability, and shifting levels of bile acids, ethanol and choline metabolites (**Table 1**).

Gut microbiome. Multiple preclinical and clinical studies have highlighted a role of the gut microbiome in NAFLD pathogenesis, although we are still far from finding a causal link. In agreement with preclinical models, the prevalence of intestinal bacterial overgrowth [72, 73] and changes in microbiota composition [74] are higher in patients with NAFLD than in healthy controls, in whom these two factors have been correlated with increased intestinal permeability and metabolic syndrome, but interestingly not with hepatic fibrosis or inflammation severity.

Recent research efforts have sought to correlate microbiome signatures with NAFLD phenotypes using culture independent techniques. Thus, shotgun metagenomics sequencing has identified an association between a microbiome signature characterized by an increased abundance of *Escherichia coli* and *Bacteriodes vulgatus* and advanced fibrosis in NAFLD patients [75]. Similarly, a greater abundance of the genus *Escherichia* has been observed in obese children with NASH [76]. In spite of these findings, it is unlikely that single microbiome signatures will explain the different NAFLD phenotypes, which probably result from varying impacts of the different microbiome signatures on the host according to its genetic predisposition or environmental factors.

Intestinal barrier dysfunction. Recently, a link has been established between intestinal microbiota abnormalities, barrier damage, and hepatic inflammation and metabolic abnormalities under high-fat diet conditions. Increased intestinal permeability has largely been identified in mice on high-fat or choline-deficient diets and in patients with NAFLD [72, 77-79] [21]. Mice fed high-fat diet or fiber-deprived diets feature an abnormal colon microbiome composition that causes increased bacterial penetrability and reduced thickness of the mucous layer, redistribution of tight junction proteins of the epithelial barrier and low-grade gut inflammation [15, 78, 79]. The altered microbiota is directly responsible to disrupt

the intestinal epithelial and vascular barriers as fecal microbial transplantation from high-fat diet fed mice to standard diet fed mice is sufficient to drive gut barrier damage, indicating that it is not the nutritional regimen, but its consequence on microbiota composition, that drives epithelial and GVB damage [21]. The altered microbiota acquires the ability to cross the epithelium which is disrupted as early as 48 h after high-fat diet as attested by the reduction in the expression of tight junction proteins [21]. Whether the capacity to cross the disrupted epithelium is an active invasive mechanism shared by the enriched pathobionts or is due to an increased leakiness of the epithelium caused by the downregulation of tight junction proteins remains to be established. Subclinical inflammation in these circumstances is characterized by reduced lamina propria Treg cells, increased IFN-gamma-producing Th1 and CD8+ T cells, and increased IL-17-producing gammadelta-T cells [78]. Similarly, patients with NAFLD show colon inflammation and reduced expression of the intestinal epithelial junction adhesion molecule Jam1 [80]. Mice genetically deficient in Jam1 on a high-fat and -fructose diet show increased intestinal permeability, endotoxinemia and hepatic inflammation, which emphasizes the importance of a healthy intestinal epithelial barrier to halt the portal entry of bacterial products under microbiota dysruption. Unlike in the case of ALD, loss of intestinal Reg3 lectins is insufficient to aggravate diet-induced obesity and NASH [81]

Bacterial products and metabolites. Changes in the functional capacity of the gut microbiome are probably more relevant than changes in its composition. Bacterial components (PAMP) and metabolites derived from the actions of the gut microbiome on exogenous (from diet and environmental exposure) and endogenous (bile acids and amino acids) substrates can reach the liver through the portal vein and there promote inflammation. The contribution of PAMPs to liver damage in NAFLD is supported by preclinical studies showing that hepatic steatosis, inflammation and fibrosis are attenuated in TLR-4 or TLR-9 deficient mice under a high-fat or choline-deficient diet [82-84]. Further, inflammasome deficiency-associated changes in gut microbiota in mice results in hepatic steatosis and inflammation through portal influx of TLR4 and TLR9 agonists, leading to enhanced hepatic TNF-alpha expression and inflammation, which are especially severe in mice models of hepatic steatosis [85]

Alterations in intestinal microbiota composition leads to a gut imbalance characterized by reduced bioavailability of choline and increased portal influx of trimethylamine, and both have been associated with hepatic steatosis in humans and experimental models. A high fiber diet in mice increases gut microbes that metabolize choline, which reduces its bioavailability and produces different metabolites such as trimethylamine [86, 87]. Additionally, microbial populations in children with NASH have shown an increased ability to produce ethanol [76, 88]. NAFLD has also been linked to gut microbiome-derived products of branched-chain and aromatic amino acid metabolism such as phenylacetic acid and 3-(4-hydroxyphenyl)lactate, both related to insulin resistance. In a cohort of obese, non-diabetic patients, those with hepatic steatosis and inflammation showed low microbial gene richness, increased microbial genetic potential for processing dietary lipids and endotoxin biosynthesis from Proteobacteria and dysregulated aromatic and branched-chain amino acid metabolism [89]. The pathogenic role of microbiome-derived metabolites is further shown by the facts that transplantation of fecal microbiota from human donors with hepatic steatosis or chronic administration of phenylacetic acid triggered steatosis in recipient mice.

The production of SCFA through bacterial fermentation of indigestible carbohydrates (e.g. dietary fiber) is a notable example of the mutualistic relationship between the gut microbiome and the host targeted at maintaining health. Diet provides non-digestible carbohydrates to support bacterial growth, and in return, these bacteria generate SCFA (e.g. butyrate) providing an energy substrate for colonocytes, mitigating intestinal inflammation and regulating satiety [90, 91]. In both obesity and NAFLD, incongruous evidence for the association between clinical phenotypes and SCFA is likely attributable to the differential abundance of individual SCFAs, each of which may have different effects on host metabolism. The microbiome of patients with type 2 diabetes mellitus shows a reduced abundance of butyrate-producing bacteria [92]. Supplementation with SCFAs improves diet-induced hepatic steatosis in mice. In agreement with these findings, a seminal randomized study has shown that a high-fiber diet in T2DM patients alters gut bacterial fermentation of carbohydrates promoting a greater

diversity and abundance of butyrate-producing bacteria, and improves hemoglobin A1c levels, partly via increased glucagon-like peptide-1 (GLP-1) production [93].

Bile acids. Impaired bile-acid signaling is another consequence of abnormalities in intestinal microbiota seen in mice on a high-fiber diet and in humans with NAFLD. In these settings, the microbiome is characterized by an abundance of bacteria that produce secondary bile acids, such as deoxycholic acid, a FXR antagonistic bile acid that suppresses FXR- and FGFR4-mediated signaling [94]. The consequence is augmented synthesis of bile acids with increased serum concentrations of primary and secondary bile acids [94, 95]. Therefore, the composition of the gut microbiome determines the production of the secondary bile acids, and influences FXR-mediated signaling in the intestine and the liver. In this regard, intestinal FXR expression is lowered in mice on a high-fiber diet, and obeticholic acid restores the integrity of the gut vascular barrier, reducing the portal influx of PAMP to the liver [21].

Cirrhosis

Cirrhosis is associated with marked gut barrier impairment paralleling the disease course. In compensated cirrhosis, features of barrier dysfunction are hardly different from those distinctive of each etiology of chronic liver disease. In contrast, gut barrier disruption in decompensated cirrhosis arises from damage at all levels of intestinal barrier defense, is independent of etiology and is associated with liver insufficiency, reduced bile flow and impaired immunity (**Figure 2C**). Gut microbiome and barrier dysfunction are directly involved in the pathogenesis of compensated cirrhosis, whereas both are related to the frequency and severity of complications in decompensated cirrhosis, namely bacterial infections and encephalopathy.

Gut microbiome. For decades, altered intestinal microbiota and bacterial overgrowth have been recognized in humans and experimental models of cirrhosis [96]. More recently, metagenomic techniques have characterized the fecal microbiome in cirrhosis as one of reduced diversity, increased relative overgrowth of potentially pathogenic taxa (such as Enterococcaceae,

Staphylococcaceae and especially Enterobacteriaceae), and decreased relative abundance of potentially beneficial autochthonous taxa (such as Lachnospiraceae and Ruminococcaceae) [58, 97, 98]. This microbiome profile in cirrhosis accompanies worsening disease, becomes more intense in the setting of decompensation and is associated with poor outcomes [99]

Alterations in gut microbiota set the stage for gut-liver axis impairment in cirrhosis. Changes in microbiota composition in cirrhosis arises from the disruption of most of the factors involved in microbiome control: i) reduced small bowel motility and transit time, mainly in the ascitic stage, as one the main contributors to dysbiosis [100-103]; ii) bile acid abnormalities, including reduced primary bile acid levels and increased secondary bile acid levels in the gut [104-106]; and iii) impaired intestinal immunity. Experimental cirrhosis with ascites is associated with a deficiency in Paneth cell alpha-defensins, and impaired function of dendritic cells [107, 108], both being especially severe in rats with ascites and pathological bacterial translocation. Hypochlorhydria present in cirrhosis, even in the absence of proton pump inhibition, is another factor that contributes to alter the microbiota [109-111]. Remarkably, the pattern of microbiota abnormalities in cirrhosis is independent of etiology [58, 106].

A distinctive feature of cirrhosis is the invasion of the intestine by bacteria from the mouth. Enrichment of patient stools in species taxonomically of buccal origin and Lactobacillaceae seems to be related to the change in salivary microbiota, proton pump inhibitors and relatively low gastric acid levels [97, 112]. An increase in Lactobacillaceae has been shown in prior studies of the gut microbiota in cirrhosis and could be related to lactulose use [113]

Intestinal barrier dysfunction. Cirrhosis is associated with damage to the physical and immunological layers that comprise the intestinal barrier. Increased gastroduodenal, small intestine, colon and whole intestine permeability, as an expression of gut barrier disruption, is a well-established feature of patients with and experimental models of cirrhosis, especially if ascites is present [103] [114]. Gut barrier disruption in cirrhosis leads not only to the increased passage to the systemic circulation of macromolecules, including bacterial components such as

LPS or bacterial DNA, but also of viable bacteria (i.e. bacterial translocation) which reach the systemic milieu [115]. Intestinal barrier damage parallels cirrhosis progression and is particularly severe when ascites and gut bacterial translocation have developed [103, 116, 117].

Controversy exists regarding the route by which gut bacteria gain access to the internal milieu in cirrhosis. Pathological translocation of viable bacteria from the gut lumen to the mesenteric lymph nodes and to the systemic circulation has been well established [103, 118, 119]. Recent evidence indicates that in cirrhosis the lymphatic route of translocation coexists with the portal-venous passage to the liver of bacteria and bacterial products due to disruption of the GVB [120]. Vascular hyperpermeability is independent of the lymphatic route as well as of portal hypertension, since it is only present in models incorporating liver insufficiency. Interestingly, obeticholic acid was able to restore reduced ileum FXR signaling, improve the mucus machinery and stabilize the GVB in rats with cirrhosis, which supports the notion that the nuclear receptor FXR partly modulates mucus- and GVB in cirrhosis [120]. Additionally, obeticholic acid and other FXR-agonists reconstitute microbiota composition, improve intestinal innate defense mechanisms, reduce intestinal inflammation and decrease bacterial translocation and endotoxinemia in experimental cirrhosis [108, 121-123]. In fact, reduced ileal FXR signaling has been described in experimental cirrhosis as a likely consequence of lumen reduction in primary bile acids and increase in secondary bile acids, as well as of intestinal inflammation [108, 120, 124].

The aforementioned intestinal barrier functional abnormalities in cirrhosis have been linked to structural changes in the intestine, including submucosal edema, minimal infiltration by immune system cells, and disorganization of interepithelial tight junction proteins in humans and experimental models of cirrhosis [108, 116, 118, 125-127]. According to a recent study, subclinical intestinal inflammation driven by modifications in microbiota composition contributes to worsen barrier dysfunction in advanced cirrhosis. As cirrhosis progresses to the ascitic stage, the intestinal immune system of cirrhotic rats is characterized by a switch to a Th1 regulatory pattern with expansion of lamina propria TNF-alpha and IFN-

gamma expressing lymphocytes, and concomitant Th17 depletion [116]. Bowel decontamination redistributes microbiota composition, reduces pro-inflammatory activation of mucosal immune cells, and diminishes intestinal permeability and bacterial translocation, supporting the key role of changes in microbiota in intestinal inflammation in cirrhosis.

Bacterial products, metabolites and bile acids. Reduced bile flow, decreased fecal bile acids, and increased serum bile acids are features of cirrhosis, which also worsen in parallel with cirrhosis severity [104, 106]. Liver insufficiency impairs the synthesis and excretion of bile acids, which results in deficient levels of total bile acids in the gut lumen and augmented levels in serum, respectively. Reduced fecal bile acids affects secondary rather than primary bile acids, and is due to collapse of 7α bile acid-dehydroxylating bacterial populations, eg, the Clostridium cluster XIVa that occurs in situations of reduced bile flow [104, 106, 113, 128, 129]. In alcoholic hepatitis, the extreme expression of these abnormalities is observed [61].

Since bile acids and the microbiome reciprocally influence each other, it is tempting to speculate that the reduced bile acids secreted into the intestine in cirrhosis contribute to severe dysbiosis with an abundance of pathobionts. As cirrhosis progresses, alterations in microbiota promote intestinal inflammation, intestinal barrier damage and liver inflammation, which in turn further suppress bile acid secretion by the liver. The decline in intestinal FXR-signaling that results from decreased bile flow disrupts intestinal barrier function by reducing mucous thickness and antibacterial protein synthesis and damaging GVB.

The gut liver axis and cirrhosis complications. Ample data support the contribution of enteric bacteria and PAMPs to the pathogenesis of immune cell activation and the inflammatory state of cirrhosis [115]. The causal link between systemic inflammation and gut microbiota has been recently reinforced in patients with cirrhosis undergoing TIPS by demonstrating compartment-specific patterns of circulating bacteria, i.e. different genera in central, hepatic, portal, and peripheral venous blood, and inflammatory cytokine clusters specific with the abundance of blood microbiome genera in each patient [130]. Similarly,

compartmentalization of microbiota composition and immune response is also observed between ascites and blood in patients with decompensated cirrhosis [131].

Beyond their contribution to systemic inflammation, upcoming evidence has linked the abnormal gut microbiome to cirrhosis complications and outcomes. The microbiome in decompensated cirrhosis is characterized by a relative abundance of potentially pathogenic taxa, mainly Enterobacteriaceae, but also Staphylococcaceae and Enterococcaceae, and a relative decrease in potentially beneficial commensal autochthonous taxa, particularly Lachnospiraceae, Ruminococcaceae and Clostridiales XIV [98, 113]. These changes can be found in the stools, sigmoid colon mucosa, and the saliva and serum of patients with cirrhosis, and mirror the severity of disease [98, 112, 113, 129]. While the gut microbiome remains unchanged in stable outpatients, the ratio of pathogenic to autochthonous taxa rises in decompensated cirrhosis, especially in patients with bacterial infection and encephalopathy [98, 113]. Interestingly, gut microbiome profiles differed in in- and out-patients, in non-infected and infected patients and in patients with poor outcomes (organ failure and death) [98]. Moreover, these studies have shown that the greater the abundance of pathogenic taxa, the greater the level of endotoxinemia, as an expression of gut barrier dysfunction.

Collectively, these data point to a direct influence of abnormalities in gut microbiota on cirrhosis complications and outcomes and this situation emerges as a target for therapy (**Figure 3**). Our rationale is reinforced by the fact that gut microbial diversity was independently associated with a lower risk of 90-day hospitalizations in patients with cirrhosis on a western-diet. Notably, a diet rich in fermented milk, vegetables, cereals, coffee, and tea has been found to increase microbial diversity and reduce hospitalizations [99]

NOVEL TOOLS TO TARGET THE GUT LIVER AXIS

The Leuven EASL conference was planned as a platform to facilitate interaction and research networking among academia and industry. In this regard, we will describe novel tools to target the gut-liver axis that were presented by industry

partners and academic researchers during the conference. Interventions can be systematized as those addressing intestinal content and mucus, microbiome, and intestinal mucosa, and those acting outside the intestine (**Table 2**)**.** A thorough review of the armamentarium to target the gut-liver axis is beyond the scope of this Seminar and this topic has been the subject of a recent Clinical Trial Watch article in Journal of Hepatology [132]

Interventions on intestinal content and mucus

Hydrogel technology. Fibers and high-viscosity polysaccharides have been employed as a strategy to improve glycemic control, suppress appetite, and facilitate weight loss in patients with increased metabolic risk. Hydrogel is made from two naturally derived building blocks, modified cellulose (carboxymethylcellulose) cross-linked with citric acid, that create a three-dimensional matrix, which mimics that of natural dietary fibers in vegetables. Orally administered in capsules with water before a meal, hydrogel particles rapidly absorb water in the stomach and homogeneously mix with ingested foods, but without caloric value [133]. Once hydrated, the ingested dose of hydrogel occupies about one-fourth of the average stomach, mimicking food in terms of volume and thereby improving satiety. It maintains its three-dimensional structure and mechanical properties during transit through the small intestine and once in the large intestine, the hydrogel is partially digested by enzymes and loses its three-dimensional structure along with most of its absorption capacity. The released water is then reabsorbed, and the remaining cellulosic material is expelled in the feces. Hydrogel is considered a medical device because it achieves its primary intended purpose through mechanical modes of action.

In an *in vitro* model of gastrointestinal digestion, hydrogels demonstrated viscoelastic profiles that were orders of magnitude superior to that of common processed functional fiber supplements (psyllium, guar gum and glucomannan). Results from the GLOW study demonstrated that hydrogel was an effective weight loss therapy, safe and well tolerated. A significant association was observed between baseline fasting plasma glucose levels and the effectiveness of the treatment suggesting a benefit for a higher-risk population otherwise known to be less responsive to therapy [133]. Hydrogel is currently being tested in

preclinical models [134] and ongoing clinical trials in obese patients with and without type 2 diabetes (NCT03622424 and NCT03058029)(**Table 3**). Currently, no data are available on the effects of this technology on the liver, although it can prevent or improve NAFLD through weight loss and relieve of insulin resistance.

Gut-restricted polymers. Polymers have a wide range of applications as therapeutic agents due to their intrinsic properties, such as avidity and multiple binding sites, to bind and retain target entities [135]. Insoluble crosslinked polymers are typically administered orally and act in the gastrointestinal tract and the bound substance is then cleared from the body in the feces, along with the polymer. Polymer sequestrants for binding of inorganic ions including potassium, phosphate, or bile acids have been used clinically for several years. For example, therapy with colesevelam has proved efficacy in dyslipidemia and type 2 diabetes [136].

Next generation polymer sequestrant development has sought to extend the application of polymeric drugs to achieve more defined physical properties with better moiety binding efficacy and selectivity. Soluble polymers have been in investigation as antimicrobial agents, due to potential high-avidity interactions between repeating polymer units and multivalent surface features on bacteria or viruses. Tolevamer, a soluble anionic polystyrene sulfonate, was developed for the treatment of Clostridium difficile associated diarrhea, due to its binding to toxins A and B which produced by the bacteria [137]. An example of a polymeric binder to sequester proteins, such as gliadin, is currently tested in phase I/II clinical studies on celiac disease [138]

Carbon nanoparticles. Yaq-001, a new synthetic non-absorbable carbon, has been shown to exhibit a high adsorptive capacity for bacterial toxins and thus represents a novel strategy to counteract gut microbiota alterations and translocation of bacterial-derived products in patients with advanced chronic liver disease. Experimental evidence in a rodent model of secondary biliary cirrhosis showed that Yaq-001 was associated with a significant increase in Firmicutes and a decrease in Bacteroidetes in stool samples [139]. Moreover, this treatment significantly attenuated the monocyte LPS-induced reactive oxygen species

production in bile duct ligated rats. These results showed that gut microbial products are one important target of this therapeutic approach.

The first-in-human clinical investigation with Yaq-001 is a currently ongoing multicentre, randomized, double blinded, placebo-controlled trial intended to evaluate safety and tolerability of oral administration of Yaq-001 therapy in cirrhotic patients with diuretic-responsive ascites and Child-Pugh 7-11 (CARBALIVE-SAFETY, NCT 03202498).

Interventions on microbiome

Bacteriophages. Bacteriophages, viruses that specifically infect and kill bacteria, have been continually evolving to overcome the defense mechanisms developed by bacteria. As phages work through a completely orthogonal mode of action as compared to antibiotics, antibiotic resistance does not convey resistance to phages and highly antibiotic resistant bacteria may still be efficiently eliminated by phages. The therapeutic administration of phages under compassionate protocols has shown recent success in terminally ill patients with multi-drug resistant infections of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [140, 141]. Furthermore, there are an increasing number of controlled and EMA/FDA-regulated clinical trials that are being conducted by multiple biotech companies to bring this modality into mainstream medicine [142].

While most clinical development activities employing phages presently are directed at infectious diseases, a novel program of phage therapy for patients with primary sclerosing cholangitis is currently being developed. Recent discoveries show that specific pathogenic gut bacteria infect and penetrate the epithelial lining of the GI tract, allowing bacterial translocation and a liver immune response [143]. Ongoing research projects aim at identifying specific bacterial targets and at creating ‘phage cocktails’ that are designed to eliminate these particular bacterial strains while leaving the healthy microbiome intact.

Synthetic live bacterial therapeutics. Engineered probiotics may be uniquely suited to consume toxic metabolites in the intestine and convert them into nontoxic forms. SYNB1020, a E. coli Nissle 1917 strain engineered by deleting a

negative regulator of L-arginine biosynthesis and by inserting a feedback-resistant L-arginine biosynthetic enzyme, has been demonstrated to improve hyperammonemia and survival in mice and was well tolerated in a phase I clinical study in hyperammonemia disorders [144]. SYNB1020 has been recently tested in a phase 1b/2a randomized, placebo-controlled study to evaluate the safety, tolerability and pharmacodynamics of SYNB1020 in patients with cirrhosis (NCT03447730). However, its development has been discontinued in favor of other synthetic live bacterial therapeutics due to negative data from an interim analysis (https://investor.synlogictx.com/news-releases/news-release-details/synlogic-discontinues-development-synb1020-treat-hyperammonemia). Using the same technique, bacteria expressing genes encoding specific enzymes have been tested to treat phenylketonuria [145]. The same principle was applied to develop an engineered Lactococcus lactis strain producing interleukin-10 that has been evaluated for the treatment of inflammatory bowel disease [146].

Fecal microbial transplantation. Fecal microbial transplantation as a method to replenish a healthy gut microbial environment and restore physiological colonization along different ways of administration, is currently an accepted treatment for recurrent or refractory Clostridium difficile infection and data are arising about its efficacy in improving inflammatory bowel disease, metabolic syndrome and hepatic encephalopathy (18,19). Confirming the findings of a previous report, a phase 1 study has shown that fecal microbial transplantation with oral capsules after pretreatment with antibiotics in patients with cirrhosis and recurrent hepatic encephalopathy is well tolerated and safe in the long-term [147-149]. In this study, microbiota transplantation restored antibiotic-associated disruption in microbial diversity and function, led to sustained improvement in cognitive function parameters, and reduced hepatic encephalopathy recurrence and liver-related hospitalizations [147-149]. It is likely that the beneficial effects on encephalopathy recurrence could be in part due to amelioration of neuroinflammation and microglial activation, which have been shown to be reduced by stools from human donors in germ-free mice colonized with stools from patients with cirrhosis [150]. Moreover, evidence is arising that suggests fecal microbial transplantation may reverse early portal hypertension, intrahepatic endothelial dysfunction and insulin resistance in rats on high-fat and -fructose diet

[151]. Despite these promising advances, only few controlled trials of interventions that alter the microbiome have been performed so far and a number of important questions remain to be answered including the safety and the duration of the effect of this intervention.

Non-absorbable antibiotics. Rifaximin, as the non-absorbable antibiotic with the most favorable safety profile, is currently tested to target the microbiome in different clinical trials in chronic liver disease (recently reviewed in [132]). Rifaximin is a broad-spectrum compound, which exerts endotoxin-lowering and anti-inflammatory effects largely independent from their bactericidal action. The efficacy of the combination of rifaximin and simvastatin, targeting the key mechanisms of cirrhosis progression, i.e. the gut-liver axis and the systemic inflammatory response, is currently being tested to prevent ACLF development in patients with decompensated cirrhosis in a phase 3, multicentre, double-blind placebo-controlled, randomized clinical trial (LIVERHOPE EFFICACY, NCT03780673). This study has been preceded by the LIVERHOPE_SAFETY trial, which has concluded that the dose of simvastatin 20 mg per day plus rifaximin is not associated to a higher risk of liver or muscle toxicity in patients with decompensated cirrhosis (NCT03150459)

Interventions on intestinal mucosa

The duodenum is increasingly recognized as a metabolic signaling center playing a role in regulating insulin action and, consequently, insulin resistance. Specialized enteroendocrine cells play a sensing and signaling role throughout the intestine, and the diversity of derived peptides reflects the nuanced manner by which the gut orchestrates many aspects of ingestion, digestion and assimilation of nutrients. Enterocytes have more recently been included in these complex processes with the discovery of fibroblast growth factor 19 (FGF19). The pharmacological modulation of gut peptide axis offers opportunities that have converged on metabolic homeostasis in obesity, diabetes and NAFLD.

Pharmacological modulation of gut peptides. In the gut, glucagon like peptide-1 (GLP-1) is produced and stored by L-cells and released into the hepatic portal system in response to luminal sugars, fatty acids and amino acids. Binding of

GLP-1 to its cognate G-protein coupled receptor (GLP-1R) increases insulin and decreases glucagon secretion by the pancreas, decreases gastric emptying and elicits a decrease in food intake among many other beneficial effects. The LEAN trial (Liraglutide Efficacy and Action in NASH) was the first randomized controlled trial to report efficacy of a GLP-1RA in patients with biopsy-proven NASH over a 48-week treatment period. Biomarker effects of daily injections of semaglutide have been reported in sub-analyses of patients treated in obesity studies and long-term efficacy studies in patients with biopsy proven NASH are currently ongoing with daily semaglutide over 72 weeks (NCT02970942) and planned with weekly dulaglutide over 52 weeks (NCT03648554) (**Table 3**).

FGF19 is produced and released by enterocytes of the terminal ileum in response to FXR activation by bile acids. FGF19 flows to the liver through the portal circulation where it binds to a cell surface receptor complex including FGFR4 and β-klotho to elicit pleiotropic effects, including a decrease in bile acid production. Approved FXR agonists drugs such as the bile acid obeticholic acid and investigational medicinal products tropifexor or cilofexor moderately increase circulating FGF19 levels. FXR agonists mediate pleiotropic effects in the liver both directly on the hepatocyte and indirectly via FGF19. Phase 3 data have shown a beneficial effect of OCA on fibrosis in NASH patients [152]. Elucidating how much of this effect is due to FXR agonism in the liver versus an effect of FGF19 from the gut poses a clinical challenge. A FGF19 mimetic compound, NGM282, is also in clinical trials and in proof of concept studies offers a high effect size on biomarker endpoints [153, 154]- One aspect of the FGF19 axis that also requires attention is the association of FGFR4 mutations with hepatocellular carcinoma and transgenic overexpression of FGF19 inducing HCC in mouse models [155]. Indeed, blockade of FGFR4 has been investigated in specific forms of hepatocellular carcinoma in humans [156]. Nevertheless, if histological effects reported from an open label study are confirmed, a FGF19 mimetic could offer an attractive therapy for NASH.

Duodenal mucosal resurfacing. Duodenal mucosal resurfacing is a technique that targets the duodenal surface mediating an abnormal signal to endogenous insulin-sensitive tissues based on the fact that limiting nutrient exposure or

contact with the duodenal mucosa exerts powerful metabolic effects. Morphologic changes in the small intestine of patients and rats with diabetes included hyperplasia of enteroendocrine cells and proliferation of endocrine cells differentiating toward K cells and oversecreting gastric inhibitory polypeptide [157, 158].

Duodenal mucosal resurfacing can be performed using different techniques including a catheter system that delivers a hydrothermal exchange at the mucosal surface. The resultant superficial mucosal ablation is followed by a re-epithelialization of the treated duodenal lumen starting within days following the procedure. In the first human study, hydrothermal ablation was safely administered with no evidence of perforation, pancreatitis, or hemorrhage. Duodenal biopsy specimens obtained 3-6 months post-procedure demonstrated full mucosal regrowth, without inflammation and fibrotic scarring, and improvement in glycemic and hepatic measures [159]

Postbiotics. As mentioned above, recent data suggest that the underlying mechanism of microbiota-based control of intestinal homeostasis relies on their metabolic products also called postbiotics [160]. Postbiotics are the new frontier in microbiome science acting as key factors in maintaining long-term health benefit. Their composition is variable and depends on bacteria strains and their metabolic status. Examples include SCFAs, secondary bile acids, proteins (e.g. p40 molecule, HM0539), enzymes, peptides, bacteriocins, endo- and exo-polysaccharides, vitamins and organic acids [161]. In general, postbiotic components have been described to possess immunomodulatory and protective roles on intestinal barrier function. In particular, postbiotics can act on immune cells protecting the gut tissue from immunopathology by increasing the secretion of anti-inflammatory cytokines such as IL-10 [162, 163]. Moreover, postbiotics potentiate epithelial tight junction structure by increasing the expression of tight junction proteins (zonula occludens, ZO-1) and intestinal mucin level thus, favoring the restoration of the gut barrier function [164]. In addition, postbiotics behave also as microbiota resilience-inducing factors. They can function as pathogenic bacteria inhibitors and possibly as quorum sensing signaling molecules to regulate bacterial cell density and biofilm formation maintaining

microbial composition [165]. Postbiotic mechanisms of action are still not yet fully identified and rigorous clinical trials are needed to support their health claim and their possible application in gut-barrier dysfunction diseases.

Interventions outside the intestine

Ammonia uptake particles. VS-01 is an innovative liposomal fluid, that consists of a suspension of large transmembrane pH-gradient liposomes, containing citric acid and designed to rapidly capture ammonia [166]. VS-01 can be delivered temporarily into the peritoneal cavity via a paracentesis catheter routinely used in decompensated cirrhotic patients with ascites, thereby allowing its application at the same time of paracentesis. Following administration of VS-01 into the peritoneal cavity, uncharged ammonia diffuses from the blood into the peritoneal space and across the liposomal bilayer membrane, where it remains trapped due to its positive charge (**Figure 4**). VS-01 simultaneously removes uremic and toxins that accumulate in these patients, by promoting their passive diffusion into the liposomes' supporting fluid [167]. At the end of the treatment, the fluid containing ammonia-loaded liposomes is withdrawn from the peritoneal cavity by gravity, in the same way ascitic fluid is drained in a paracentesis procedure.

In experimental models of cirrhosis and hepatic encephalopathy, VS-01 has demonstrated a fast and efficient clearance of ammonia, up to twenty times superior to conventional peritoneal dialysis solutions, significantly reducing plasma ammonia levels and attenuating the associated brain edema [167]. Additionally, untargeted metabolomic studies revealed that the vast majority of toxins known to be present in excess in patients with liver dysfunction were removed by VS-01 concomitantly with ammonia. Hence, VS-01 can provide prompt detoxification and thereby support the 3 primary organs (brain, liver and kidney) that can fail in advanced cirrhosis. VS-01 is currently being evaluated in a first-in-human phase 1b clinical trial in patients with mild hepatic encephalopathy and ascites requiring paracentesis.

CONCLUSIONS AND FUTURE TRENDS

The gut-liver axis refers to the reciprocal interaction that takes place between the gut and its microbiota on the one hand, and the liver on the other. Although independent, the key players, namely diet, microbiota and intestinal mucosa, are interrelated and are connected to the host through the bile and portal blood. Effectively, bile acids produced in the liver regulate microbiota composition and intestinal barrier function, and gut products regulate bile acid synthesis and glucose and lipid metabolism in the liver. Further, there is growing evidence that gut-liver axis disruption leads to the progression of most forms of chronic liver disease, including cirrhosis. The main features of a disrupted gut-liver axis are shared by ALD, NAFLD and cirrhosis itself. These features include an altered intestinal microbiota, gut barrier damage with the consequence of its increased permeability, and changes in luminal levels of bile acids. In turn, shifting levels of bile acids lead to reduced intestinal FXR-signaling, which compromises intestinal mucous and antimicrobial peptide synthesis along with gut-vascular barrier integrity. The relative contribution of each of these abnormalities to gut-liver axis disruption depends on the etiology and stage of liver disease. Hence it is during advanced end-stage liver disease, i.e., decompensated cirrhosis, that the greater severity of barrier damage is observed. The functional consequences of a disrupted gut-liver axis are also shared by different chronic liver diseases and involve repetitive exposure of liver innate immune cells to gut derived bacterial products such as endotoxin, and metabolites like ethanol and trimethylamine, resulting in liver inflammation.

Advances in knowledge of the gut-liver axis are driving the development of diagnostic, prognostic and therapeutic tools based on microbiota to manage liver disease. An active area of research is the search for a set of microorganisms that will serve as indicators of liver damage and disease progression in ALD and NAFLD, and also help predict hospitalization, bacterial infection and liver-related complications in cirrhosis. Microbiome analysis via multi-omic profiling could provide a mechanistic picture to understand and identify the different microbiota phenotypes observed in ALD and NAFLD. In effect, this type of analysis in serum could facilitate testing. A promising therapeutic approach is the modification of bile acid signaling to strengthen intestinal barrier function and modulate the gut-liver axis. For this purpose, FXR agonists could reduce intestinal FXR-signaling

and improve antibacterial protein synthesis rescuing GVB damage. Modifying intestinal contents through the use of targeted fecal microbial transplantation or specific probiotic consortia isolated from human feces is another therapeutic modality worth exploring. Indeed, the increasingly recognized role of gut microbiota in the pathogenesis and progression of liver diseases opens up a wide gateway for microbiome-based tools to effectively diagnose and treat liver disease.

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Table 1. Differences in the components of the intestinal barrier in alcoholic liver disease, non-alcoholic fatty liver disease and cirrhosis

Component	**Alcoholic liver disease**	**Non-alcoholic fatty liver disease**	**Cirrhosis**
Gut microbiome	**Proteobacteria**↑: Enterobacteriaceae↑***** **Bacteroidetes**↓: Bacteroidaceae ↓ **Firmicutes**↓: Streptococacceae↑, Lactobacillaceae↓, Lachnospiraceae↓, Veillococcaceae↑ **Candida**↑	**Proteobacteria**↑: Enterobacteriaceae↑ **Bacteroidetes**↑: Prevotellaceae↑**,** Rikenellaceae ↑ **Firmicutes**↓: Lactobacillaceae↑, Lachnospiraceae↓**,** Ruminococcaceae↓	**Proteobacteria**↑: Enterobacteriaceae↑ **Bacteroidetes**↓: Bacteroidaceae ↓ **Firmicutes**↓: Streptococaceae↑, Clostridiacceae↑, Lachnospiraceae↓, Veillococcaceae↑**,** Ruminococcaceae↓ **Fusobacteria↑:** Fusobacteriaceae↑
Intestinal bacterial overgrowth	Present	Present**	Present
Intestinal permeability	Increased	Increased***	Increased (ascites)
Intestinal inflammation	Intestinal TNF-alpha ↑	Intestinal TNF-alpha↑, IFN-gamma↑, Treg↓	Intestinal TNF-alpha↑, IFN-gamma ↑, Treg↓

Intestinal antimicrobial proteins	Intestinal Reg3 lectins↓	-	Intestinal alpha-defensins
Bile acids pool	Hepatic synthesis and fecal bile acids↑ Fecal secondary bile acids ↑ Intestinal FXR signaling↓	Fecal secondary bile acids ↑ Intestinal FXR signaling↓	Hepatic synthesis and fecal bile acids↓ Fecal secondary bile acids ↑ Intestinal FXR signaling↓
Intestinal bacterial metabolites	Intestinal LCFA ↓ Blood ethanol and acetaldehyde↑	Intestinal trimethylamine↑ Intestinal SCFA↓ Intestinal BCCA and AAA↑ Blood ethanol↑ Blood choline↓	
Translocation of bacteria and bacterial products (PAMP)	Blood PAMP↑	Blood PAMP↑	Blood PAMP↑ Viable bacteria in blood/lymph nodes

***Phylum**: Family. ** Only in NASH vs. NAFLD, obese or healthy controls *** Unrelated to the presence of NAFLD or NASH

BCCA, branched-chain aminoacids; AAA, aromatic aminoacids. PAMP, pathogen associated molecular patterns: LPS, DNA

Table 2. Selected therapeutic interventions along the gut-liver axis

Place of action	Denomination	Principle of action
Intestinal content	Hydrogel technology	Modified cellulose cross-linked with citric acid that mimics natural dietary fibers in vegetables. Hydrogel particles rapidly absorb water in the stomach and increase in volumen, thereby improving satiety. Once in the large intestine, the hydrogel is partially digested and releases water, which is then reabsorbed.
	Gut-restricted polymers	Insoluble crosslinked polymeric drugs selectively bind with high-avidity multivalent surface features on bacteria or viruses, toxins, inorganic ions including potassium, phosphate, or bile acids.
	Carbon nanoparticles	Non-absorbable carbon particles exhibit a high adsorptive capacity for bacterial toxins and represent a novel strategy to counteract dysbiosis and translocation of bacterial-derived products.
	Non-selective beta-blockers	Beta-blockers reduce the load of enteric bacteria and inhibit intestinal bacterial overgrowth by fastening intestinal transit time and reducing intestinal permeability
Intestinal microbiome	Non-absorbable antibiotics	Selectively reduce the burden of enteric bacteria that mostly contribute to translocation, e.g. gram-negative bacteria. Rifaximin is a broad-spectrum compound, which exerts endotoxin-lowering and anti-inflammatory effects largely independent from their bactericidal action.
	Bacteriophages	Bacteriophages are viruses that specifically infect and kill intestinal bacterial pathogens. In contrast to antibiotics, phages do not induce resistance.
	Synthetic live bacterial therapeutics	These are engineered probiotics that can selectively consume toxic metabolites in the intestine and convert them into nontoxic forms.

	Fecal microbial transplantation	Fecal microbial transplantation is a method to replenish a healthy gut microbial environment and restore physiological colonization by recolonizing the intestine with microbial flora from a healthy donor.
Intestinal mucosa	Pharmacological modulation of gut peptides.	Specific agonists of mucosal gut receptors may elicit responses including the release of regulators of glucose or bile acid metabolism.
	Duodenal mucosal resurfacing	Superficial duodenal mucosal ablation mediates an abnormal signal to endogenous insulin-sensitive tissues by limiting nutrient exposure or contact with the duodenal mucosa.
	Postbiotics	Postbiotics are metabolic products from intestinal bacteria that include short-chain fatty acids, secondary bile acids, proteins polysaccharides, vitamins and organic acids acting as metabolic regulators.
	FXR agonists	FXR-agonists reconstitute microbiota composition, restore epithelial and vascular intestinal barrier function, improve intestinal innate defense mechanisms, reduce intestinal inflammation and decrease bacterial translocation and endotoxinemia
Peritoneal cavity	Ammonia uptake particles	This treatment consists in a suspension of large transmembrane pH-gradient liposomes, containing citric acid and designed to rapidly capture ammonia from ascites of cirrhotic patients.

Table 3. Selected ongoing studies on novel targets of therapy along the gut-liver axis

Disease	Title	Reference
Overweight and type 2 diabetes	Effect of Gelesis200 on Body Weight in Overweight and Obese Subjects With Prediabetes and With and Without Type 2 Diabetes (LIGHT-UP)	NCT03058029 NCT03622424
Irritable bowel disease with bile acid malabsorption	Trial to Understand Efficacy of Colesevelam in Diarrhea Predominant IBS Patients With Bile Acid Malabsorption	NCT03270085
Non-alcoholic steatohepatitis	Safety and Tolerability of Yaq-001 in Patients With Non-Alcoholic Steatohepatitis	NCT03962608
Cirrhosis	Safety and Tolerability of Yaq-001 in Patients With Cirrhosis	NCT03202498
Cirrhosis	Safety, Tolerability and Pharmacodynamics of SYNB1020	NCT03447730
Decompensated cirrhosis	Efficacy of the Combination of Simvastatin Plus Rifaximin in Patients With Decompensated Cirrhosis to Prevent ACLF Development	NCT03780673
Metabolic syndrome	Fecal microbiota transplantation and fiber in Patients With Metabolic Syndrome	NCT03727321
Alcoholic cirrhosis	Fecal Microbial Transplant for Alcohol Misuse in Cirrhosis	NCT03416751
Type 2 diabetes	Safety & Effectiveness of Duodenal Mucosal Resurfacing Using the Revita™ System in Treatment of Type 2 Diabetes	NCT03653091
Non-alcoholic steatohepatitis	Effect of Duodenal Mucosal Resurfacing in the Treatment of NASH (DMR_NASH_001)	NCT03536650

Non-alcoholic steatohepatitis	Investigation of Efficacy and Safety of Three Dose Levels of Subcutaneous Semaglutide Once Daily Versus Placebo in Subjects With Non-alcoholic Steatohepatitis.	NCT02970942
Non-alcoholic steatohepatitis	Researching an Effect of GLP-1 Agonist on Liver STeatosis (REALIST)	NCT03648554
Non-alcoholic steatohepatitis	Randomized Global Phase 3 Study to Evaluate the Impact on NASH With Fibrosis of Obeticholic Acid Treatment (REGENERATE)	NCT02548351
Type 2 diabetes	Safety & Effectiveness of Duodenal Mucosal Resurfacing Using the Revita™ System in Treatment of Type 2 Diabetes	NCT03653091
Non-alcoholic steatohepatitis	Effect of Duodenal Mucosal Resurfacing in the Treatment of NASH (DMR_NASH_001)	NCT03536650

FIGURE LEGENDS

Figure 1. The intestinal barrier of the colon is composed of several layers of defence. Left: Under healthy/homeostatic conditions, the most external layer of defense is the mucus which is composed of an outer, microbiota-colonized layer, and an inner sterile layer. Just below, one can find the epithelium which is a monolayer of cells sealed one to the other by tight junctions. A further layer of defence is provided by the gut vascular barrier (GVB), which controls the systemic dissemination of microbial metabolites and the microbiota through the portal circulation. Right: Under inflammation, the intestinal barrier can be disrupted at several places, when the GVB is also damaged as demonstrated by increased detection of the fenestrated marker PV1, then the translocation of inflammatory microbial metabolites or microbes can occur to systemic sites, including the liver where they can induce local inflammation and promote liver disorders, such as non-alcoholic fatty liver disease (NAFLD)

Figure 2. Disruption of the gut-liver axis in alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) and cirrhosis. An altered gut microbiota is the cornerstone of gut-liver axis disruption in chronic liver diseases. Ethanol and a high-fat diet have direct effects on gut microbiota composition in ALD and NAFLD, respectively (a, b). In cirrhosis (c), abnormalities in intestinal microbiota primarily results from reduced bile acid flow with deficient luminal levels of bile acids and intestinal hypomotility. Levels of secondary bile acids are increased in the gut lumen as a consequence of alterations microbiota and an abundance of 7-alpha-dehydroxylating bacteria. These elevated secondary bile acids lead to reduced intestinal FXR-signaling, which compromises mucous and antimicrobial peptide synthesis and gut-vascular barrier integrity. Ethanol also directly impairs the synthesis of antimicrobial peptides. Abnormalities in intestinal microbiota jeopardize the availability of energy substrates for epithelial cells such as short- and long-chain fatty acids (SCFA and LCFA). Consequences are loosening of epithelial cell intercellular junctions, mucous layer thinning and reduced synthesis of antimicrobial peptides all of which facilitate bacterial penetrability and the interaction of pathobionts with mucosal immune system cells. Ultimately, the result is intestinal inflammation featuring a Th1 regulatory

pattern of immune cell activation along with the increased synthesis of IFN-gamma and TNF-alpha, which further contribute to increased intestinal permeability. Alterations in microbiota composition and bacterial overgrowth challenge a hyperpermeable intestinal barrier with an overload of pathogen associated molecular patterns (PAMP) or metabolites such as trimethylamine (TMA) in NAFLD that elicit liver inflammation. An extreme severity of intestinal barrier disruption is reached in advanced end-stage liver disease, i.e., decompensated cirrhosis. At this stage, the concurrence of profound abnormalities in microbiota with an overabundance of Enterobacteriaceae on top of markedly damaged physical, immune and vascular intestinal barriers leads to the massive passage of not only PAMP but also of viable bacteria, causing systemic and liver inflammation as well as spontaneous bacterial infections.

Figure 3. Targeting the gut-liver axis in cirrhosis. In cirrhosis, abnormalities in microbiota composition and bacterial overgrowth are the hallmark of gut-liver axis disruption and are the consequence of intestinal hypomotility, changes in bile flow and composition, and impaired intestinal immunity. Gastric hypoacidity favored by proton pump inhibitors use or environmental factors such as a Western-diet will also contribute. In this setting of marked disruption of intestinal microbiota composition, concurrent damage to the epithelial and vascular intestinal barriers enables passage to the systemic circulation of pathogen associated molecular patterns (PAMP) and viable bacteria through the vascular or lymphatic routes. A topic of active research is the search for alternatives to antibiotics for bowel decontamination designed to halt PAMPs and bacterial translocation in cirrhosis. Intestinal transit time can be sped up by beta-blockers or prokinetics, e.g., cisapride, to reduce bacterial overgrowth and translocation. Exogenous conjugated bile acids increase bile acid secretion and reduce bacterial overgrowth, endotoxinemia and translocation. Obeticholic acid and other FXR-agonists reconstitute microbiota composition, restore epithelial and vascular intestinal barrier function, improve intestinal innate defense mechanisms, reduce intestinal inflammation and decrease bacterial translocation and endotoxinemia in experimental cirrhosis. A diet rich in fermented milk, cereals, coffee and tea increases microbial richness and reduces hospitalizations in patients with cirrhosis. Adsorbent carbon nanoparticles absorb gut-derived

toxins and bacterial products reducing liver injury in experimental cirrhosis and are a target of on-going proof of concept studies. Fecal microbial transplantation in patients with recurrent hepatic encephalopathy corrects abnormalities in intestinal microbiota, increases antimicrobial peptide expression, and improves hepatic encephalopathy performance.

Figure 4. Mechanism of action of VS-01. VS-01 is a liposomal fluid designed to rapidly capture ammonia once delivered into the peritoneal cavity. Ammonia (NH3) from the blood passively diffuses to the peritoneal cavity (1) and into the liposomes (2). NH3 is then protonated due to acidic conditions in the liposomes and charged molecules ($NH4^{+}$) remain trapped (3). Uremic and hepatic toxins in the fluid also passively diffuse and are trapped in the liposomes (4)

Figure 1

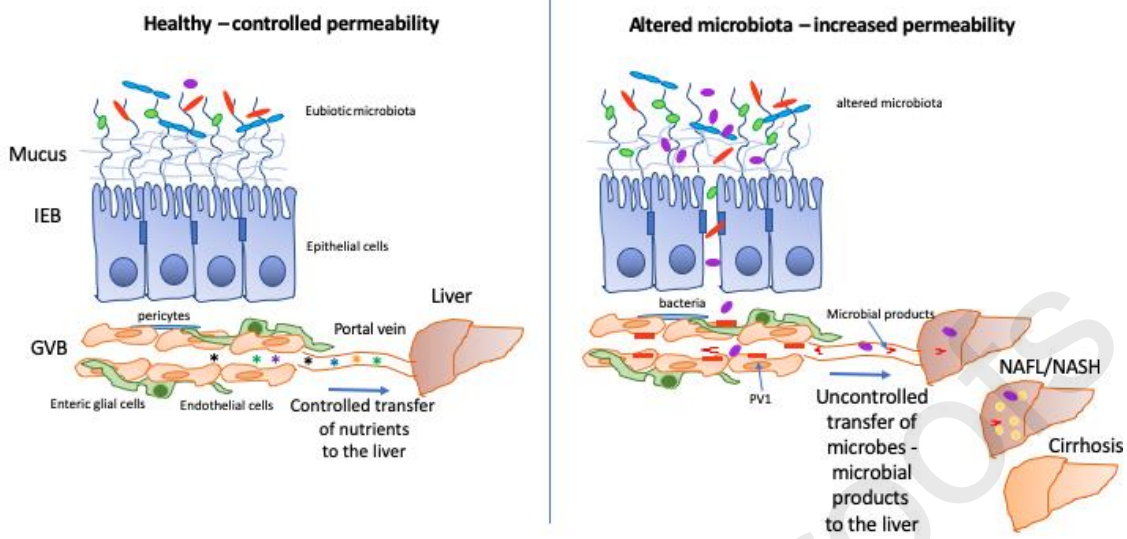


Figure 2-A

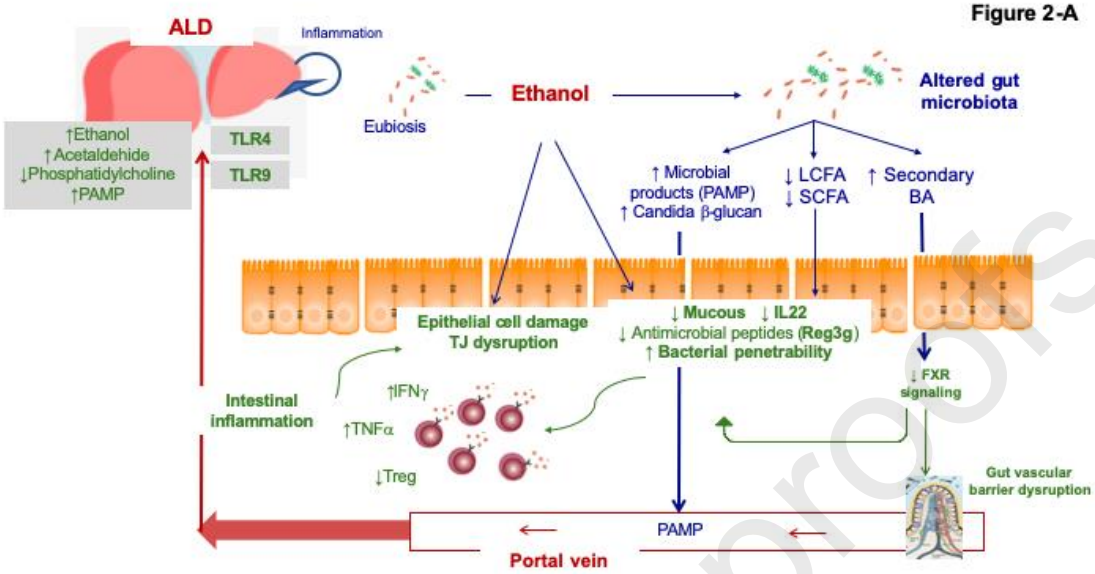
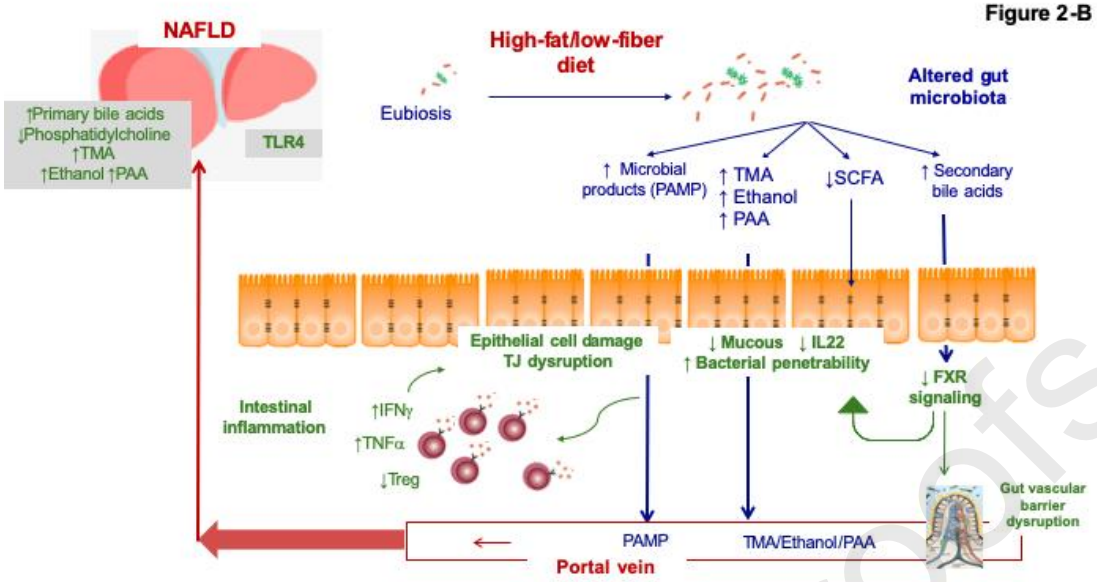


Figure 2-B



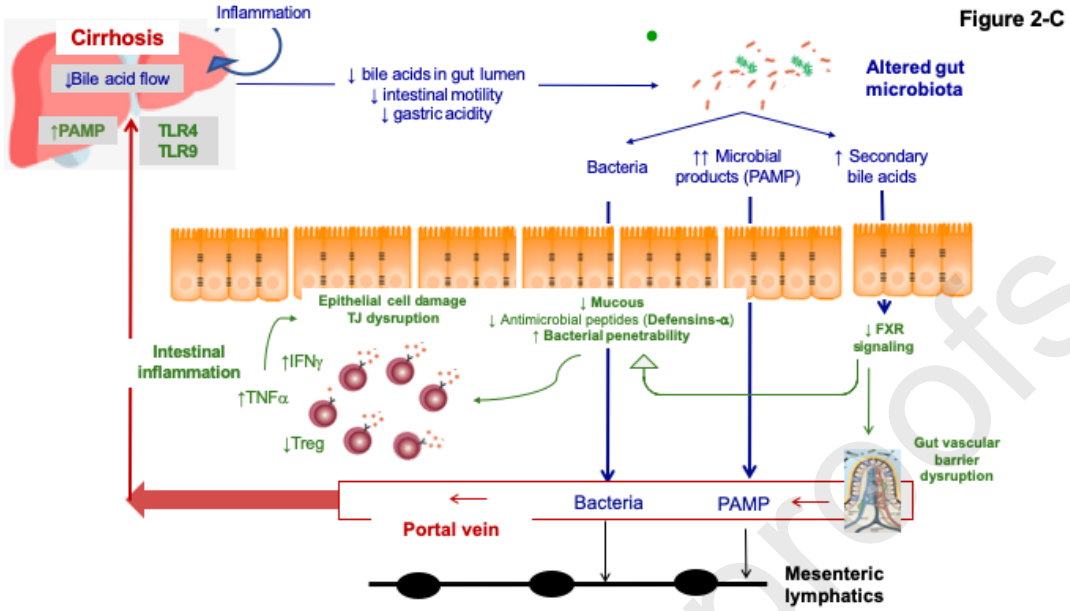


Figure 3

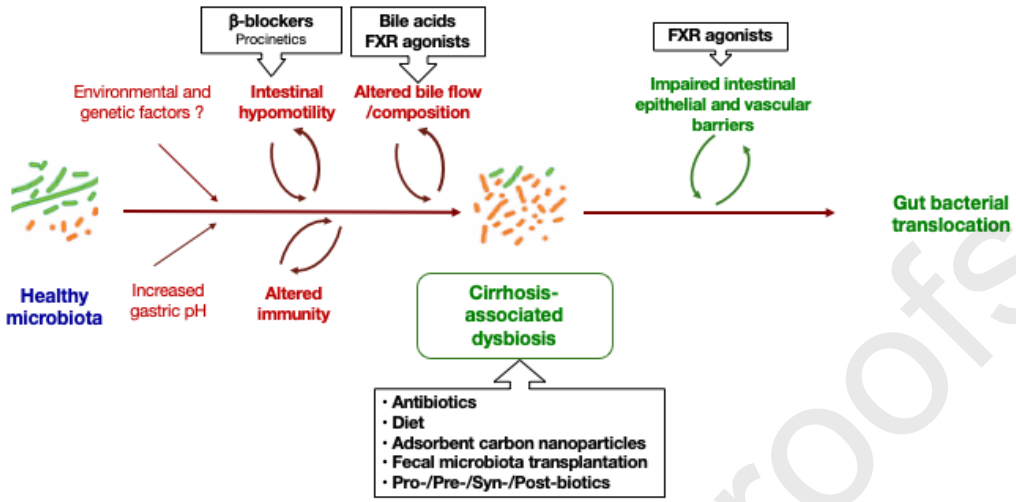


Figure 4