The microbiome in primary sclerosing cholangitis: current evidence and potential concepts

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Abstract

The close relationship between primary sclerosing cholangitis (PSC) and inflammatory bowel disease has long inspired hypothetical models in which gut bacteria or bacterial products are key players in pathogenesis. Several studies using high-throughput sequencing technology to characterize the gut microbiota have been published over the last years. They all report reduced diversity and significant shifts in the overall composition of the gut microbiota. However, it remains unclear as to whether the observed changes are primary or secondary to PSC development and further studies are needed to assess the biological implications of the findings. In the present article, we review the published data in perspective of similar studies in other diseases. Furthermore, we propose that interpretation and further assessments of findings are structured into conceptual compartments, and elaborate three such possible concepts relating to immune function (the “immunobiome”), host metabolism (the “endobiome”) and dietary and xenobiotic factors (the “xenobiome”) in PSC.

Main Concepts and Learning Points

- The gut-liver axis is a bidirectional system whereby human and gut microbial physiology integrates, heavily influenced by dietary factors
- The gut microbiota in primary sclerosing cholangitis (PSC) is different from patients with inflammatory bowel disease without PSC and healthy controls
- Studies of metabolic factors related to the gut microbiota and antibiotic trials suggest that the gut microbiota exert pathogenic effects in PSC
- The biological implications of detected changes in the gut microbiota in PSC are unknown, and future studies need to implement functional assessments
- We propose a structured approach to interpretation and mechanistic studies along three conceptual compartments (i.e., the “immunobiome”, “endobiome” and “xenobiome”)

Primary sclerosing cholangitis (PSC) is a rare, chronic disease characterized by patchy inflammation and progressive fibrotic strictures throughout the entire biliary tree. In the majority of cases (60-80%) there is a concomitant diagnosis of colonic inflammatory bowel disease (IBD).¹ The etiology of PSC is unknown, and the potential link between biliary disease and intestinal inflammation has inspired several theories as to the pathogenesis of PSC (e.g. leakage of bacteria and bacterial products into the portal circulation and homing of intestinally activated lymphocyte to the liver).² The relationship between PSC and IBD also inspired the first treatment trials reported in PSC, which employed antibiotics.^{3,4} Although ursodeoxycholic acid (UDCA) is widely prescribed, there is no therapy consistently slowing disease progression,⁵ and PSC thus remains an important indication for liver transplantation in many countries.⁶

Registry studies have demonstrated a heritable component to PSC susceptibility as shown by an increased risk in siblings and other relatives.⁷ Large-scale genetic studies over the last 5-10 years have identified more than 20 robust genetic risk factors.⁸ The susceptibility genes in part overlap with typical autoimmune disease susceptibility and in part with IBD genes.⁹⁻¹¹ Importantly, a considerable risk fraction cannot be attributed to genetics,¹² leaving a large space (up to 80-90%) of PSC susceptibility on the environmental side. The identity of such environmental risk factors is poorly known beyond the potential protective effects of smoking and coffee.¹³⁻¹⁷ Among the many potential exogenous factors impacting PSC pathogenesis,¹⁸ the gut microbiota, the community of all microorganisms in the gastrointestinal tract, has more recently received the major attention (**Figure 1**).

All body surfaces are covered by microbes, which have important functions beneficial for the host. Situated on the interface between the environment and the host, these microbes convey and modulate external exposures, providing a strong rationale for the recent surge of research focusing on the human microbiota in health and disease far beyond the context of PSC. In the gut, the most recent estimates suggest that the microbiota comprises about 3.8×10^{13} bacteria with a total mass of about 0.2 kg, with the addition of an unknown number of fungi and virus.¹⁹ The number of bacteria is the same order of magnitude as cells in the human body ($\sim 3.0 \times 10^{13}$) and ten-fold the number of nucleated cells, highlighting the potential of this new organ. Multiple inflammatory and metabolic conditions are now linked to compositional or functional alterations of the gut microbiota.²⁰⁻²³ In the current article we aim to review the studies of the gut microbiota in PSC that have been published up to now. Findings are discussed in the context of those made in other diseases, and we propose a series of hypothetical concepts on how the detected alterations could be mechanistically linked to disease processes in PSC.

Methodological considerations in studies of the gut microbiome

The re-discovery of the gut microbiota as a major player in human health has in part been fuelled by methodological advances in genetics, sequencing methods and bioinformatics.²⁴ This has led to the use of the term microbiomics as a new omic, i.e. studies of quantification and characterization of structure and function and dynamics of the microbiome. In principle, however, microbiota refers to organisms in particular location (e.g. gut microbiota), while microbiome refers to the collective genomes (all genes) of one microbiota.

Laboratory methods

A state-of-the-art overview of any given microbiome compartment is now achieved by extracting relevant microbial DNA from a sample, perform DNA sequencing, and run relevant bioinformatics analyses. The methodology will be briefly described to provide a basis for the remainder of the review, but more extensive methodological considerations can be found elsewhere.²⁵ Materials utilized so far in studies of PSC are stool samples, perhaps largely reflecting luminal microbes of the colon, bile samples, as well as mucosal biopsies, reflecting adherent microbes at the segment of collection. Fecal and mucosal microbiomes differ, but show strong intraindividual correlations when comparing with other individuals.²⁶ Microbiome studies most often focus on bacteria. In community standard analysis, segments of the 16S rRNA marker gene are amplified with barcoded primers to allow multiplexing and submitted to next-generation sequencing.²⁷⁻²⁹ The rationale behind marker genes is that the variability of the marker gene makes it possible to determine the bacterial source of the gene when mapping the sequencing reads to databases. A similar strategy may be used for fungi, where the Internal Transcribed Spacer (ITS1) located in ribosomal RNA gene region in eukaryotes serves as one marker gene.³⁰ Critical confounding steps in these analyses include 1) choice of DNA extraction method, 2) choice of primers/amplification region 3) bioinformatic analysis including choice of databases. Major differences at these steps generally reduce the comparability of samples.

The gut microbiota composition can be described on different taxonomic levels. Considering the bacterial kingdom, a given bacterial species (e.g. *E. Coli*) can be classified broadly as part of a phylum (e.g. Proteobacteria), class (Gammaproteobacteria), order (Enterobacteriales), family (Enterobacteriaceae) or genus (*Escherichia*). The major phyla are Firmicutes and Bacteroidetes. Major alterations of the gut microbiota can in some conditions be observed as changes in Firmicutes/Bacteroidetes ratio,³¹ but the phyla comprise diverse sets of bacteria and it is highly

likely that the microbiota components of pathogenetic importance need to be identified at higher resolution than phylum level.

Microbiota profiling using 16S rRNA-based amplicon sequencing usually allows determination of the relative abundance of bacterial taxa on the genus level. Given the enormous genetic and functional variation of bacteria on species and strain level, this is a coarse measure of the composition of gut bacteria, although it provides reliable measures of microbial diversity. When using full metagenomic (shotgun) sequencing,³² all DNA in a sample is fragmented and sequenced preferably without amplification (see **Table 1** for a comparison of 16S rRNA vs. shotgun sequencing utility). The potential coverage of all microbial genes may be utilized to provide relative abundance on species level by identifying species-specific marker genes.²⁵ Furthermore, shotgun sequencing may be used to analyze the gene counts of the microbiome according to biological pathways, i.e. provide an overview of the functional content.²⁵ Since many bacterial genomes are known, multiple tools are available to predict the functional gene content from 16S rRNA-based data.³³⁻³⁵ However, these are biased by the inherent weaknesses of 16S rRNA-based profiling lack of species level resolution but may be useful for explorative analysis for further follow up.³⁶ A key lesson from our own experience with shotgun metagenomics is that a large number of reads can so far not be mapped to known organisms. Large collections of sequences with highly correlated abundances are therefore been binned into surrogates for uncultured microbes with bioinformatic language called metagenomic species or metagenomic linkage groups. These challenges have inspired classic microbiology, and with advances in culturomic techniques, identification of many such species will become reality.³⁷

There is increasing perception as to the gut microbiota as a virtual organ. Descriptions of the large number of physiological processes occurring in this organ is currently in its infancy, yet urgently warranted in order to understand interactions with human physiology and disease processes. Common approaches applied so far include metabolomics and metatranscriptomics, but largely provide arrays of correlative data and detailed methods for systematic assessments of biological pathways or individual microbes are urgently needed. The metabolomics methods applied to e.g. stool or peripheral blood samples may provide direct measures of microbial activity. Molecules produced by microbes can often cross epithelial barriers and act systemically,³⁸ and proof-of-concept for an involvement of such metabolites in human disease has been established.³⁹ The liver functions as a critical interface in this concept (**Figure 2**).⁴⁰ There is also a large potential in more accurately determining the functional status of the gut microbiome using metatranscriptomics,⁴¹ i.e.

gene expression analysis, or metaproteomics⁴² but these applications are so far less mature and more challenging in their applications.

Finally, the understanding of the importance of the microbiota as a confounder in animal studies,⁴³ and parallel expansion of gnotobiotic methods (initially developed decades ago⁴⁴) have led to groundbreaking observations of the impact of the microbiota in mammals.⁴⁵ In experimental studies it is therefore mandatory to take cage effects into account,⁴⁶ while addressing the effect of manipulating the gut microbes (e.g. germ-free re-derivation or antibiotic treatment) is of importance to establish mechanistic links between the gut microbes and disease.

Study designs

How should the gut microbiota be studied in PSC? In most human diseases, case-control studies have been the initial approach. These provide only associations and no demonstration of cause or effect. This is important since disease states per se induce changes in the gut microbiota, introducing a chicken-and-the-egg problem as to which changes may be important in causing disease and which are secondary changes. Nevertheless, case-control studies are popular as a first step and may give the first clues as to whether significant microbiota alterations are present. It is becoming increasingly clear that to make robust observations in case-control studies, numbers no less than that of genome-wide association studies (ideally $n > 1,000$ for each population) is required.⁴⁷⁻⁴⁹ This is due to noise introduced by a large number of known and unknown confounders that influence the gut microbiota. To control for these confounders to the extent feasible it is important to record and include details like stool consistency,⁵⁰ geography,⁵¹ body-mass index,⁵² and age⁵³, and exclude individuals recently exposed to antibiotics.⁵⁴

A particular emphasis must be made as to recording dietary data, since diet may both induce changes in the microbiota and provides substrates to microbial metabolism of relevance to e.g. associated metabolomics assessments (**Figure 1**). The literature suggest that there is a significant but moderate association between long-term diet and gut microbial composition,^{48,49,55} providing a rationale for baseline food frequency questionnaires as the pragmatic alternative. In addition, short term major dietary changes may have major effects on microbial composition,⁵⁶ suggesting that stable life-style participants should be the preference in gut microbiota studies.

Given the weaknesses of case-control studies, there is a general trend toward longitudinal and interventional studies. Confounders will also affect longitudinal studies with repeated sampling, but the strength of prospective studies is the opportunity to establish stronger links between disease

progression and the microbiota. Finally, interventional studies targeting the gut microbiota (e.g. drug trials, fecal transplantation, dietary interventions) may provide evidence for what microbiota changes associate with clinical disease parameters. To finally establish causality for observed changes, an array of experimental work is needed, typically including studies in germ-free animal models (e.g. transfer of microbiota⁵⁷ or monocolonization⁵⁸) and proof-of-concept human interventions.⁵⁹⁻⁶¹

The microbiome in PSC: Current evidence

Cross-sectional studies of gut microbiome in PSC

Cross-sectional studies comparing PSC patients with non-PSC controls published as full-length peer-reviewed papers are currently limited to five, three focusing on stool microbiota and two focusing on mucosal microbiota profile (**Table 2**). In the first description of the gut microbiota in PSC, Rossen et al. extracted DNA from colonic and ileal biopsies from 12 PSC patients, 11 UC patients and 9 non-inflammatory controls.⁶² PCR amplification of the 16S rRNA gene was performed, followed by hybridization to the Human Intestinal Tract chip, comprising >5000 probes targeting >1000 bacterial phylotypes. The main finding was of reduced diversity and richness of the PSC microbiota compared with UC patients and non-inflammatory controls, in addition to a reduction of uncultured Clostridiales II. In contrast, a study based on sequencing of the third and fourth variable region of the 16S rRNA gene in 20 PSC patients, 15 IBD patients and 9 healthy controls by Torres et al.,⁶³ no global differences in microbiota composition and similar diversities were observed. On taxonomic level, increased abundances of *Blautia* and a genus of the *Barnesiellaceae* family were observed. In a brief follow-up letter from the UK, additional non-overlapping results from the mucosal microbiota of 11 PSC patients was presented.⁶⁴ There are multiple possible explanations for the lack of overlap. Lack of statistical power and a mixture of type I and type II errors, different technical methods and sampling differences are among the most important ones. Regular colonoscopy performed in PSC-IBD simplifies the access to mucosal biopsies, whilst the choice of proper controls is challenging.

Fecal microbiota studies in PSC are more promising in terms of overlapping results (**Table 2**). In the so far largest study from Norway, 85 PSC patients were compared with 263 healthy controls and 36 UC patients in a two-stage design (discovery and validation panel).⁶⁵ The study reported global microbial differences between these phenotypes, characterized by reduced bacterial diversity in PSC. Multiple bacterial taxa had different abundances, most notably the *Veillonella* genus, which was elevated in PSC compared with both UC and healthy controls. In a similar study of 66 Belgian PSC patients, 66 healthy controls and 43 IBD patients, differences in global microbiota profiles were also observed, including reduced diversity.⁶⁶ A number of genera were significantly increased in PSC compared with IBD and controls, including *Enterococcus*, *Lactobacillus* and *Fusobacterium*. Increases in *Veillonella* and *Streptococcus* were also found, but these associations disappeared after excluding patients with cirrhosis and recent antibiotic treatment, respectively. In a study from the Czech Republic, 43 PSC patients, 31 healthy controls and 32 ulcerative colitis patients were included.⁶⁷ Further observations from studies of the fecal microbiota in PSC have

been reported in incomplete letter format from both Germany,⁶⁸ suggesting an increase in *Veillonella* in both PSC and UC, and Japan, suggesting an increase in both *Veillonella*, *Streptococcus* and *Enterococcus* in pediatric PSC patients.⁶⁹

Taken together, the reported microbiota profiling studies in PSC show extensive alterations compared with both IBD without PSC and healthy controls. Importantly, there is little evidence of microbiota differences in PSC patients with and without IBD, perhaps pointing to the liver disease as the primary basis for observed associations. The key question, as elaborated above, is whether the alterations are causing PSC or only appears secondary to disease already present. Reduced diversity dysbiosis is observed in multiple inflammatory and metabolic diseases, and it is tempting to propose that this aspect of the observations is secondary to disease and disease-related inflammation and other physiological disturbances. The relationship to specific bacteria is however of some interest and warrants further consideration. Notably, *Veillonella* is the genus most often reported in the PSC studies so far. *Veillonella* is a strict anaerobic gram negative coccus which has been associated with cystic fibrosis where it is seen in the lung microbiota,⁷⁰ and with recurrence of Crohn's disease after ileocecal resection. An increased prevalence of *Veillonella* is also observed in primary biliary cholangitis (PBC),^{71,72} and furthermore in a range of other liver diseases including hepatitis B cirrhosis and patients with hepatic encephalopathy showing that this genus is not related to etiology.^{73,74} Given the increase observed in PSC, Crohn's recurrence and lung- and liver fibrosis, it could be speculated that *Veillonella* directly contributes to fibrogenesis but there is so far no direct evidence supporting this hypothesis. In fact, it is difficult to state at this point whether *Veillonella* is a "good" bacterium, counter-acting the disease process, or a "bad" bacterium, causing increased progression. The relevance of single microorganisms in studies of the microbiota in complex diseases should be questioned, however may point to more complex niches of the microbiota that may ultimately serve pathogenic roles. Following up on such findings should thus be encouraged, accompanied by comprehensive microbiota assessments using shotgun sequencing. The latter type of data are eagerly awaited in PSC and may hopefully help guide further research to a deeper extent than what has become for the 16S rRNA data seen so far.

Gut microbiome profile and PSC severity and prognosis

The interpretation of an altered gut microbiota in PSC is elusive, but differences between phenotypes open opportunities for clinical translation. A "dysbiosis index" based on a limited number of bacteria altered between PSC and controls, did separate PSC and controls with an AUC of 0.78 in two independent panels in the Norwegian study, and an AUC of 0.86 in a German panel.^{65,68} Similar observations were made in the study from Belgium, where the abundances of *Enterococcus*,

Lactobacillus and *Fusobacterium* was able to predict correct phenotype of the PSC patients and controls in 95% and 71% in the discovery and validation panels, respectively. This means that there is a potential for clinical utility in diagnosing PSC in patients with IBD despite limited pathogenetic insight.

Ability to predict clinical course would also be relevant and also represent evidence of a causal pathogenic influence of the microbiota alterations observe. No data from PSC has yet been published, but in Crohn's disease mucosal microbiota profiles at diagnosis has been shown to predict clinical activity during follow-up.²³ There are no longitudinal data on gut microbiota profiles in PSC. The cross-sectional studies suggest that there is an association between disease severity and the microbiota, exemplified with a correlation between *Veillonella* and the revised Mayo risk score for PSC in the Norwegian study and increase of *Veillonella* in cirrhotics in the Belgian study,^{65,66} but no data on follow-up of these patients are available. Also, whether these are effects of disease stage or severity is difficult to discern.

As elaborated below, biomarkers of gut microbial activity may provide direct evidence for a physiological impact of the gut microbiome. One example exists in cardiovascular disease, where the metabolite trimethylamine-N-oxide (TMAO), which is generated in the liver from trimethylamine TMA, a bacterial product from metabolism of carnitine and choline, is strongly associated with hard endpoints.²¹ TMAO was lower in PSC patients (n=305) than in controls in a Norwegian study, but the difference was driven by patients with reduced liver function, suggesting that TMAO synthesis in the liver was impaired.⁷⁵ When exploring the subgroup with normal liver function, elevated TMAO was associated with reduced liver transplantation-free survival, suggesting that gut microbial function and/or related dietary factors may be relevant for disease progression. Based on these promising results, there is a strong rationale to include biobanking of relevant material for gut microbiome assessment in the prospective follow-up of PSC patients to be able to answer questions about the relationship between the gut microbiome and PSC behavior.

One important challenge when addressing the microbiota as biomarkers in PSC and other liver diseases is that the conditions progress towards a common end-stage state. Cirrhosis is by itself associated with alterations in the gut microbiota, both bacterial and fungal.^{76,77} One characteristic is an increase of buccal bacteria, i.e. an "oralization" of the microbiota.⁷⁷ It is evident from e.g. non-alcoholic fatty liver disease (NAFLD) that late stage disease can be separated from early disease based on the gut microbiota composition.⁷⁸ The key question is whether a specific disease profile of the microbiota can still be observed after cirrhosis development. The data in PSC are insufficient to

answer this, but in a study of 244 cirrhotics, patients with alcoholic liver disease could still be separated from NAFLD, suggesting that disease-specific gut microbiota characteristics persist.⁷⁹

Interventional studies targeting the gut microbiome in PSC

The strongest evidence of a potential cause-effect relationship between the gut microbiota and PSC may come from clinical trials, i.e. from interventional studies targeting the gut microbiota. Several prospective studies have explored the effects of anti-bacterial antibiotics on surrogate endpoints in PSC, employing metronidazol, minocycline, vancomycin or rifaximin (**Table 3**).⁸⁰⁻⁸⁴ In addition, case series of n=1-14 patients employing these the mentioned drugs, in addition to tetracyclin, sulfasalazine and azithromycin have been reported.^{3,85-89} The general observation has been that antibiotics do in part improve liver-related biomarkers. Metronidazole+UDCA over 36 months led to a significantly larger reduction in alkaline phosphatase (ALP) and Mayo risk score than UDCA alone, with trends towards effects on histology and cholangiographic findings, but with more side effects.⁸¹ In a pilot study including in total 35 patients comparing two different dosages of metronidazole and vancomycin over 12 weeks, both study drugs showed signs of favourable effects biochemically.⁸² However, only vancomycin reached the primary end-point (significant decrease in ALP from baseline) and there were more side effects reported in the metronidazole group.

A placebo-controlled clinical trial with vancomycin (added to previous UDCA treatment) was recently reported from Iran.⁸⁴ The 18 patients receiving vancomycin had a significant reduction in Mayo risk score and ALP at 12 weeks compared with baseline, while no changes was observed for the 11 placebo-receiving patients. There was also a reduction in symptom score. Furthermore, sporadic supporting evidence of vancomycin effects have been published, including case reports suggesting an effect against recurrent PSC,^{90,91} and a case series of 14 children on long-term vancomycin therapy associated with improved biochemistries and symptoms.⁹² The latter observations have later been linked to an increase in regulatory T cells and other immunomodulatory effects.⁹³ The findings demonstrate how gut microbiome changes may impact on systemic factors of relevance to PSC pathogenesis. Such effects may be even more pronounced for absorbable antibiotics like minocycline, which was shown to reduce ALP levels in one pilot study with 16 PSC patients treated for 12 months.⁸³ Like vancomycin, rifaximin is a non-absorbable antibiotic with well-documented effects against hepatic encephalopathy, although no signals of effects were observed in PSC.⁸⁰ The PSC associated *Veillonella* genus is generally regarded as resistant to vancomycin, while it is sensitive to metronidazole,⁹⁴ but the importance of such factors can so far only be speculated.

There has been an increasing interest in fecal microbiome transplantation (FMT) in PSC, following reports of potential effect from FMT at least in a fraction of the patients in the first randomized controlled trials in UC.⁹⁵⁻⁹⁸ No studies have so far been completed in PSC. Importantly, treatment mode, intensity and appropriate choice of donors are likely factors of major relevance to efficacy in UC,^{36,95} and second generation studies taking such factors into account hold the potential to show more consistent effects. Ultimately, a mechanistic understanding providing a rationale for the choice of microbial composition of the “graft” should be sought and would likely encompass personalized aspects. Studies with probiotics in PSC are so far scarce, with only one study including 14 patients in a randomized placebo-controlled cross-over design and a probiotic mixture of four Lactobacilli and two Bifidobacilli with no significant effects after three months.⁹⁹ In comparison, a recent meta-analysis suggest a potential role of probiotics in UC, at least in preventing relapse in quiescent disease.¹⁰⁰

Given the key role of gut microbes in bile acid transformation, an effect of UDCA on the microbiota profile could be suspected. However, neither of the fecal microbiota profiling studies observed a difference in patients with or without ongoing UDCA treatment.^{65,66} Still, it must be taken into account that these were cross-sectional study designs. In an interventional trial in PBC, six PBC-associated genera had their abundances reversed after half a year on UDCA treatment.⁷¹ Interestingly, it was also found that patients with an inadequate response to UDCA had increased abundance of *Veillonella* compared with those with adequate response.⁷¹ As mentioned above, the latter could either indicate that a positive impact on *Veillonella* improves liver disease, or that more severe liver disease favors *Veillonella* growth. From mouse models of colitis, it has also been shown that UDCA attenuated disease and normalized microbiota changes associated with human IBD.¹⁰¹ Besides UDCA, PSC patients are also commonly treated with 5-ASA against colitis, drugs also likely to influence the microbiota, but where very little data is available.⁶⁹

Microbes of the biliary tract in PSC

The biliary environment in PSC is often characterized by colonization.¹⁰² Conventional culture studies of bile reveal bacteriobilia and fungobilia in a large proportion of the patients,^{103,104} in line with findings in obstructive cholestatic disorders.¹⁰⁵ Modern culture independent profiling data of the biliary microbiota are scarce,^{106,107} but taxa from the most common phyla inhabiting the intestinal tract seem to regularly colonize the biliary tree. There are challenges when performing such studies, given the low prevalence of microbes and subsequent high risk of significant contamination either from the endoscope or the laboratory procedures and reagents.¹⁰⁸ There is also an ongoing discussion on whether even the healthy biliary tree is in fact devoid of microbes. In a

large recent study, the bile microbiota from 80 PSC patients (37 categorized as early disease, 32 as advanced disease and 11 as biliary dysplasia or carcinoma) was compared with 46 controls.¹⁰⁷ Bile was collected at endoscopic retrograde cholangiography (ERC). The controls underwent first time diagnostic ERCs and PSC was excluded. The bile microbiota in early disease was similar to controls. In contrast, there was a tendency to reduced microbial diversity in the group with neoplasia and increased relative frequency of certain streptococci in the both the advanced disease and the neoplasia group, which statistically was not dependent on number of ERCs. Overall, the results may implicate that the biliary microbiota does not have a role in the etiology of PSC but could influence disease progression. Positive cultures for bacteria and candida have been associated with worsened prognosis,^{103,109,110} also suggesting that colonization of the biliary tree has an impact on PSC outcomes. One study suggest that genetic variants increasing the risk of PSC influence the microbiome profiles in bile,¹⁰⁶ but whether this is a representative of the pathogenic effects from this genetic finding is unknown.

Microbiome in animal models of PSC

Early theories on PSC pathogenesis proposed that portal bacteremia or bacterial products from the inflamed gut in ulcerative colitis induce inflammation in the biliary tract (**Figure 1**).¹¹¹ This principle was proven in a rat model of small intestinal bacterial overgrowth.¹¹² In a series of papers Lichtman et al. in the early 1990s showed that the surgical creation of a jejunal blind loop in the susceptible Lewis and Wistar strains caused a cholangiographic picture of intra- and extrahepatic cholangitis with thickened, fibrotic and dilated ducts. The disease was most likely caused by gut bacterial cell components, and was attenuated by antibiotics and peptidoglycan-degrading enzymes.¹¹²⁻¹¹⁴

Studies of mouse models in gnotobiotic systems (i.e. germ-free animals or animals where the microbes present are known) have provided significant new insights into the influence of the gut microbiota on disease. It is well known that many mouse models of IBD depend on the presence of gut microbiome.¹¹⁵ This was further extended by Garret et al. in 2007, showing that the phenotype of a genetically induced model of colitis could be transferred to wild-type mice by microbiota transfer alone.¹¹⁶ The study also illustrates a classic approach to study the clinical and molecular effects of the gut microbiota in germ-free animals that has been applied in a broad range of disease states: the collection of gut microbiota in a disease states and transfer of this microbiota into germ-free mice with or without relevant genetic defects. Germ-free mice may also be utilized for monocolonization experiments and humanization of the gut microbiota. Spectacular effects of transfer of the gut microbiota to germ-free mice from human twins discordant for a phenotype like

obesity,²⁰ provide examples on how mechanistic links between the gut microbiome and human disease can be substantiated. An important challenge in the context of biliary diseases are the differences in bile acid profiles in mice and humans, which may complicate the interpretation of findings in experimental models.¹¹⁷

In biliary disease models animal data are so far limited. *Abcb4* (or *multidrug resistance gene 2*, *Mdr2*) knockout mice, which lack phospholipids in the bile, acquire intrahepatic sclerosing cholangitis characterized by fibrosis, and they are widely used as a model for biliary fibrosis resembling PSC. In a landmark study, Tabibian et al. rederived *Abcb4* ^{-/-} mice into a germ-free facility.¹¹⁸ This caused a dramatically worsened PSC phenotype, associated with increased ductular reaction and ductopenia, cholestasis and fibrosis. In addition, the mice exhibited increased cholangiocyte senescence, associated with release of profibroinflammatory cytokines.¹¹⁹ As expected in germ-free mice, secondary bile acids including UDCA were completely absent, since they are generated by microbial transformation. Based on the *in vitro* observation that UDCA abrogated cholangiocyte senescence, it was proposed that the protective effect of the gut microbiota in *Abcb4* ^{-/-} is mediated via secondary bile acids but this is not firmly proven.

In contrast to the observations in *Abcb4* ^{-/-} mice, the gut microbiota had a harmful effect on the immune-driven biliary disease in NOD.c3c4 mice.¹²⁰ This is the only mouse strain spontaneously developing both extrahepatic and intrahepatic bile duct affection. In a study by Schrumppf et al., the gut microbial composition in NOD.c3c4 was different from the NOD control strain, even after re-derivation into a new animal facility. When re-deriving the NOD.c3c4 mice into a germ-free facility, the biliary disease was attenuated, although not completely abrogated. A similar trend was observed in mice treated with antibiotics. Taken together, the observations in *Abcb4* ^{-/-} and NOD.c3c4 suggest that the gut microbiota influences biliary disease, but that effects depend on the disease mechanisms, which are so far unknown.

The gut virome and mycobiome

The main focus of gut microbiome research has so far been the bacterial compartment. However, viral components (the virome) and fungi (the mycobiome) are also present and have been so far largely uncharacterized. When performing full metagenomic (shotgun) sequencing only a very small fraction of the reads will be of viral origin, meaning that purification of a stool fraction enriched with virus-like particles (VLPs) is a prerequisite for assessments of the viral niche.¹²¹ The majority of VLPs are bacteriophages, most commonly the *Caudovirales* order and *Microviridea* family. In the first major report of gut virome in multiple IBD cohorts, sequencing of the virus-like particle fraction identified a disproportionate abundance of *Caudovirales* (increased richness and

relative abundance) and *Microviridea* (reduced) compared with controls.¹²² The expansion of *Caudovirales* was inversely related to the bacterial diversity in IBD, which was reduced as expected. Importantly, the *Caudovirales* expansion included many different viral taxa in UC and Crohn's disease, indicating disease-specific changes in the viral compartment.¹²² There are so far no similar data on PSC or other liver diseases.

The most prevalent fungi in the gut are *Candida* spp, *Saccharomyces cerevisiae* and *Malassezia* Spp.¹²³ In IBD, genetic variation in Dectin-1 which is a receptor for commensal fungi have been shown to influence colitis in mice and humans,¹²⁴ while mycobiome profiling have shown variable alterations in diversity and fungal abundance depending on IBD subtype and activity.¹²⁵ Little is known about fungi in PSC and most other liver diseases. Recently, intestinal fungi were linked to the development of alcoholic liver disease,³⁰ a potential mechanisms suggested to be fungal overgrowth and translocation due to chronic alcohol consumption. In a mouse model, chronic alcoholic consumption was associated with fungal overgrowth and translocation, while in human alcohol-dependence was associated with reduced fungal diversity and candida overgrowth. Also, patients with alcoholic cirrhosis had increased exposure and immune response to fungi, which correlated with mortality. PSC patients in general have a low level of alcohol consumption,¹³ and whether an altered gut mycobiome is a feature of cholestatic liver diseases remains to be elucidated.

The microbiome in PSC: Potential pathogenetic concepts

The diverse set of interactions between host physiology and a healthy or diseased microbiome makes up an almost unlimited number of hypothetical pathogenetic models in PSC. To guide the thinking, it is useful to compartmentalize the functional interactions that may be relevant into principle concepts. In the current review article, we will elaborate three such principle concepts, which we have denominated the immunobiome, the endobiome and the xenobiome. We herein define the *immunobiome* as a representative of interactions between the host immune system and the gut microbiome, with an emphasis on communication via conserved (e.g. toll-like receptors; TLR) or variable (e.g. TCRs) immune receptors (**Figure 3**). We also herein define the *endobiome* as biochemical pathways in the gut microbiome contributing to host physiology (**Figure 4**), either by synthesis of crucial compounds or by co-metabolizing molecules produced by the host. Finally, we herein define the *xenobiome* as biochemical pathways in the gut microbiome involved in the transformation of exogenous compounds, including nutrients, drugs and a broad array of poorly defined environmental exposures (**Figure 5**). The demarcations between the conceptual compartments are blurred, and likely there is an involvement of all three, and more, aspects throughout disease development in PSC.

The immunobiome in PSC

The relationship between PSC and IBD has long inspired theories as to the involvement of gut microbiome in PSC.² The classic hypothesis of PSC pathogenesis suggests that bacterial products translocate from the gut and causes biliary inflammation (**Figure 3**, left panel). The pathogenetic concept has been proven in animal models,¹¹² but there is limited evidence of increased bacterial translocation in PSC, the most striking being an accumulation of LPS as evaluated by immunohistochemistry in seven PSC patients.¹²⁶ In addition there is an increase in TLR4 expression and hyperresponsiveness to LPS in cholangiocytes isolated from PSC patients.^{127,128} In contrast, measurements of intestinal permeability in 22 PSC patients using the ratio of urinary excretion of lactulose/L-rhamnose did not differ from controls. Studies of circulating markers of bacterial translocation in PSC (e.g. LPS) are largely missing. Bile protein profiling also supports neutrophil and/or macrophage activation (e.g. interleukin 8, calprotectin, MMP9), but much work is needed to clarify whether the biliary LPS and innate immune activation is a result of secondary biliary bacterial colonization or due to gut derived microbial components.

Gut bacteria may serve as a source of microbial antigens relevant for adaptive immune responses in PSC (**Figure 3**, middle panel). There are three principle ways in which this may contribute to tissue injury in PSC: a) by directly eliciting immune reactions similar to the way gluten is driving gut pathology in celiac disease, b) by molecular mimicry, i.e. eliciting immune reactions that cross-reacts with endogenous peptides, c) by regulating responsiveness and/or tolerance of adaptive immune pathways. Adaptive immune reactions may also occur as bystander-effects, e.g. in the context of secondary biliary infections. The molecular mimicry model is still popular despite limited evidence. One important example is from pauci-immune glomerulonephritis,¹²⁹ where cross-reaction with fimbriated pathogens were proposed to explain autoantibodies with a protein target in neutrophils, explaining anti-neutrophil cytoplasmic antibodies (ANCA). Metagenomic methods have also been employed to search for mimicry in the gut, with proposed bacterial targets identified in shotgun sequence data, although these suggestions have not been validated experimentally.¹³⁰

In PSC, the most attention has been paid to the putative target of the atypical perinuclear (p)ANCA, which have been observed in between 26% and 94% of the patients in the studies performed.¹³¹ The most thorough work on the subject proposed tubulin beta 5 as a target of pANCA in PSC and autoimmune hepatitis, and proposed that these pANCA cross-react with the bacterial protein FtsZ.¹³² Atypical pANCA has also been observed in mouse models of colitis, but so far not

independently reproduced.¹³³ Irrespective of target, ANCA identify PSC patients diagnosed earlier and with increased frequency of the "autoimmune" HLA haplotype HLA-B*08-DRB1*03, associated with multiple autoimmune diseases.¹³⁴ In a prospective study of IBD patients, positive pANCA 10 years after diagnosis was associated with PSC-like pathology on MR cholangiography 20 years after diagnosis,¹³⁵ suggesting that pANCA are relevant in PSC. Whether they point to disease-causing adaptive immune responses towards intestinal antigens warrants further investigation.

A similar conclusion can be made regarding a broad array of other autoantibodies in PSC,¹³¹ the recently identified serum IgA autoantibodies against the pancreatic glycoprotein 2 (GP2) included. Anti-GP2 was present in about 50% of patients with PSC and associated increased cholangiocarcinoma risk and reduced survival.¹³⁶ The involvement of the GP2 protein in binding of bacterial proteins to facilitate transcytosis and anti-bacterial immune responses in the gut¹³⁷ may suggest that anti-GP2 IgA is generated as a bystander effect, alongside breach of normal gut epithelial barrier functions. This may be one explanation also for observations in IBD, where antibodies have been identified against multiple microbial antigens like OmpC as well as fungi (*Saccharomyces cerevisiae*), and associated with disease severity.¹³⁸ Along the same thinking, Palm et al. performed sorting of fecal bacteria from IBD patients according to levels of IgA coating, followed by sequencing-based microbiota profiling.¹³⁹ Culturing of IgA coated bacteria and transfer into a colitis model caused an aggravated phenotype, linking the profile of immune responses to intestinal microbes to disease severity.

The possibility that the gut microbiome could serve as source of specific antigens serving as primary drivers of adaptive immune responses in PSC is currently supported by circumstantial evidence only. Genetic studies in diverse autoimmune diseases, PSC included, have found a uniform picture with predominant effects from HLA gene associations and additional effect from numerous non-HLA genes.⁸ In some cases of HLA-associated autoimmunity, like coeliac disease, genetically determined HLA variants have been shown to determine the specificity of pathogenic T and B cells. In coeliac disease, exposure to gluten, as well as the resulting gluten-specific adaptive immunity, is required to maintain autoantibody production and immunopathology.¹⁴⁰ Similar observations have been made in drug-induced liver injury.¹⁴¹ Such studies challenge the concept that autoimmunity primarily results from immune activation toward self-antigens, and suggest a key involvement of exogenous antigens, microbial derived peptides possibly included. Further support for the presence of a specific adaptive immune response in PSC comes from studies of T cell receptors, in which clonality has been observed in PSC livers.¹⁴²⁻¹⁴⁴ The basis of these observations

remains as of yet speculative, but given the potential efficacy of antibiotics in PSC (**Table 2**), the lack of efficacy of immunosuppressive therapy,⁵ and even disease recurrence under immunosuppression after liver transplantation,¹⁴⁵ a model whereby microbial antigens play causative roles needs consideration.

Recent attention has also been directed to the possibility that unconventional (preset) T-cells such as mucosal invariant T (MAIT) cells and natural killer T (NKT) cells may be involved in biliary pathologies, PSC included (**Figure 3**, right panel).¹⁴⁶ These cells are enriched in compartments that are exposed to the microbiota, the liver and the gut in particular.¹⁴⁷ The MAIT cells react to bacterial B vitamins (riboflavin derivatives) presented on the MR1 molecules, and it was recently shown that biliary epithelium, liver B cells and macrophages activated MAIT cells causing proinflammatory cytokine release after exposure to bacteria.¹⁴⁸ Similar immune regulating effects may be associated with natural killer T cells, which react to lipids (some of which are of bacterial origin) presented by the CD1d molecule, which is also expressed in the bile ducts.¹⁴⁹ The importance of these cells in mouse models of colitis are well known.¹⁵⁰ The fact that both of these cell types can be activated by antigen presentation by the biliary epithelium makes them interesting actors at the interface between the microbiome and cholangiocytes in PSC.

The endobiome: co-metabolism by the gut microbiome

The close relationship between host and microbial physiology is the basis of the perception of the microbiota as a virtual organ (**Figure 4**). Important contributions from bacteria to host metabolism has been long known,¹⁵¹ for instance in the provision of short-chain fatty acids (SFCAs; e.g. butyrate and propionate), amino acid modifications (e.g. tryptophan), vitamin homeostasis (e.g. vitamin K production) and bile acid transformation. All these activities make the gut microbiome an extremely influential endocrine compartment, and many of the activities are closely dependent on liver function. It is beyond scope to go into all potentially relevant aspects of this compartment (which we will refer to as the “endobiome”), and the interested reader is guided elsewhere.¹⁵²⁻¹⁵⁵ The bidirectional aspect of this co-dependency has been documented by recent studies showing that human genetic variation in multiple genes of relevance to co-metabolism (e.g. *VDR*, *HNF4A*, *FDFT1* and *PLA2G3*) impacts on gut microbiota composition.⁴⁹

Although ALP levels may fluctuate and even be normal in patients with PSC, cholestasis is a key component of the normal clinical presentation. The exact mechanism is unknown, but cholestasis is thought to primarily arise through physical obstruction of obstructed segments of the biliary tree.

The involvement of the gut microbiome in bile acid homeostasis requires particular mentioning. End-stage livers in PSC thus has a more pronounced accumulation of bile acids than in other causes of cirrhosis.¹⁵⁶ This accumulation probably contributes to the development of fibrosis and cirrhosis. Upstream to these events, one set of hypotheses on initiating factors in PSC states that primary disturbances in bile composition (i.e. "toxic bile") or protective mechanisms against bile salts (i.e. the biliary "bicarbonate umbrella") may be responsible for disease development..^{157,158} There is so far little evidence that the composition of ductal bile differs between PSC patients and controls,^{159,160} but further and more detailed studies are needed to conclude this.

Microbes may perform multiple enzymatic transformations of bile acids.¹⁶¹ The most important processes are 7- α -dehydroxylation, generating secondary bile acids (deoxycholic acid and lithocholic acid) from primary bile acids (cholic acid and chenodeoxycholic acid, respectively), and deconjugation, generating free bile acids from glycine and taurine-conjugated bile salts. These transformations not only modify the bile acid pool but also alter bile acid homeostasis more broadly, since the secondary bile acids have a different affinity to bile acid receptors than primary bile acids.¹⁶² Several nuclear (e.g. farnesoid X receptor [FXR], constitutive androstane receptor [CAR], pregnane X receptor [PXR] and vitamin D receptor [VDR]) and membrane bound (e.g. TGR5) receptors mediate important biological functions upon bile acid binding. The interested reader should consult other sources for a comprehensive review,^{163,164} since bile acid signaling is an important axis for microbial influence on a broad range of human physiological functions. For PSC, current emphasis on these aspects resides much with FXR, for which agonists have shown clinical efficacy in PBC.¹⁶⁵ Amongst other functions, FXR inhibits the rate-limiting step of bile acid synthesis cholesterol 7 α -hydroxylase (CYP7A1), and studies suggest that bacterial enzymatic activity directly interferes with FXR signaling (e.g. in mice by reducing the levels of FXR agonists tauro-conjugated beta- and alpha-muricholic acids).^{117,166} Our understanding of how these pathways operate across the host-microbiome interface are still fragmented, but they are likely to play a role in PSC pathophysiology and may even host therapeutic targets.¹⁶⁷

In lack of properly powered clinical trials, the clinical benefit from UDCA in PSC is questionable.¹⁶⁸ In fact, the strongest observations regarding UDCA in PSC are that very high doses may be harmful.¹⁶⁹ In a trial of 28-30 mg/kg/day UDCA compared with placebo, 150 PSC patients were included but the study was stopped due to futility, since UDCA associated with 2.3 greater risk of clinical events (those related to portal hypertension in particular). High-dose UDCA was associated with increased circulating total bile acid levels. Also, lithocholic acid levels were markedly increased, probably because of microbial transformation of unabsorbed UDCA reaching

the colon.¹⁷⁰ Lithocholic is hydrophobic and toxic, and has been shown to cause cholangitis in murine models.¹⁷¹ Irrespective of cause, the reduced survival in the high-dose UDCA group was largely driven by patients with early stage or mild disease, as evaluated by early stage histology or normal bilirubin.¹⁷² To speculate from there that gut microbial transformation of bile acid may change PSC phenotype is a far shot, but should be investigated as one possibility. More recently, the modified bile acid *nor*UDCA has demonstrated promising results in a phase II trial in PSC with significant impact on liver biochemistries,¹⁷³ but the exact mechanism of action and whether this compound also may act on the gut microbiota is unknown.

The xenobiome: connecting with the exterior

The sum of all environmental exposures throughout the lifetime of an individual has recently been coined the “exposome”. Within this vast space, the gut microbiota is exposed to diet and all other oral environmental exposures. Alongside human provisions (e.g. mucus secretions¹⁷⁴), diet serves as a source of nutrients for the microbiota as much as the host, making it an environmental “sensor”.^{47-49,175} Through interfering with the oral exposures, the microbiome also makes major contributions to metabolism of basic nutrients (**Figure 5**), non-digestible fibres, proteins and lipids included.^{19,40} The SCFAs generated from non-digestible fibres warrant particular mentioning.¹⁷⁶ SCFAs include acetate, propionate and butyrate, and act on the G protein coupled receptors GPR41 and GPR43 in the gut,¹⁷⁷ influencing inflammatory and endocrine pathways. SCFAs serve as nutrients to the epithelial barrier and may also stimulate the accumulation of regulatory T cells in the gut mucosa, providing a potential link to theories of PSC pathogenesis. No data on SCFAs are available in PSC but it could be noted that increased abundance of butyrate-producing bacteria was a feature of successful donors in one of the FMT studies in UC.^{36,96}

In addition to the participation of the gut microbiota in general nutrition, the collective enzymatic capacity also engages in the transformation of molecular compounds encountered via the oral route. One example is the way the gut microbiome generates intestinal amines, e.g. the generation of trimethylamine (TMA) from choline. Phosphatidylcholine rich food (e.g. red meat and cheese) has been linked to atherosclerosis development due to hepatic transformation of the resulting TMA into trimethylamine-N-oxide (TMAO) by hepatocytes.^{21,39} TMAO has also been associated with PSC progression.⁷⁵ The mechanisms for the TMAO effect in PSC is unknown, but it could be noted that intestinal amines have been linked to cholangiocyte activation and fibrosis,¹⁷⁸ and also serves as a substrate for vascular adhesion protein-1 (VAP-1),¹⁷⁹ VAP-1 is an enzyme that has been shown to facilitate the adhesion of gut homing lymphocytes to hepatic endothelial cells in a substrate dependent manner.¹⁷⁹ The most potent inducer of VAP1 activity in this study was cysteamine,

which may be produced both by gut bacteria and the intestinal epithelium. Recently circulating levels of soluble VAP-1 was associated with PSC severity.¹⁷⁹ The TMAO and soluble VAP1 examples provides circumstantial evidence for pathogenic effects from the microbiome warranting further investigation.

Another example is the bioconversion of drugs and other xenobiotics.¹⁷⁵ It is becoming increasingly clear that the gut microbiome plays crucial role in both pharmacokinetic and pharmacodynamics aspects of many commonly prescribed drugs. The example of metformin is interesting, since investigations were initiated on the observation of gastrointestinal disturbances from the drug,^{57,180} a very common side-effect from drugs. Drug effects may be beneficial (e.g. drug activation as shown for sulfapyridine in **Figure 5**) or result in inactivation (e.g. digoxin) or toxicity.¹⁸¹ More complex examples of synergism are also observed (e.g. for CTLA-4 checkpoint blockade).¹⁸² The clinical implications are obvious, and “pharmacometagenomic” assessments along the same logic as pharmacogenomics may become reality in the near future. The drug examples also serve as prototypes of what in principle could happen to any compound via microbial transformation. In PSC, the possibility of microbial generation of bioactive compounds from xenobiotic substances thus opens for an almost endless list of potentially disease causing agents in the diet. Proof-of-principle for biliary disease due to oral exposures have been demonstrated in the animal setting,¹⁸³ and may hypothetically also involve the generation of antigenic compounds along the “immunobiome” concept of **Figure 3**. As our understanding of the metabolic machineries of the gut microbiome evolves, studies to elaborate their potential role in PSC pathogenesis will become feasible.

Conclusions

Studies of the gut microbiome are scientifically trendy, and the field is expected to continue to expand alongside rapid developments in sequencing technology and associated bioinformatics. PSC represents an important model condition for studies into the physiological and immunological implications of disturbances in the gut-liver axis. Together, the gut microbiome and the liver represents an integrated metabolic machinery, and based on the scarce data it should be hypothesized that disturbances at this double interface between the external environment and the interior serve pathogenic roles in PSC. Resolving the mechanisms for this pathogenic role will require substantial expansion of both human and murine/experimental work. Furthermore, there are important areas still unexplored, e.g. the relationship between the gut microbiota and the risk of cholangiocarcinoma in PSC. PSC patients exhibit a lifetime risk of cholangiocarcinoma of approximately 10%,¹⁸⁴ one of the highest rate of neoplastic transformation rates found in any

inflammatory condition. Cholangiocyte activation is likely a critical step in this development,¹¹⁹ and may interact with microbial metabolites at critical steps in carcinogenesis similar to what has been shown for hepatocellular carcinoma.^{185,186}

Besides resolving pathogenetic mechanisms and potentially even etiology in PSC, the gut microbiome holds great prospects for therapeutic intervention. In that regard we would want to pose a word of caution on hasty conclusions from preliminary data, e.g. as seen with the widespread off label prescription of vancomycin in children based on very limited data.^{82,93} Given the broad and pleiotropic effects from gut microbiome composition into host physiology (**Figure 2**), there is a reason to warn against long term and collateral consequences of long term antibiotic prescription.^{185,186} Similar considerations are also warranted for other interventions targeting the gut microbiota, and for some of them, e.g. fecal transplantation, even other safety considerations apply (i.e. inadvertent transfer of pathogenic microbes). However, such considerations should not hamper the enthusiasm to the fact that an entirely novel space of potential therapeutic targets is currently opening up in PSC. Patients and doctors are in desperate need of effective therapy, and perhaps the microbiome may prove the “unknown” that makes the equation finally add up in this challenging condition.

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Table 1. Features of culture-independent methods for gut bacterial profiling. As sequencing costs go down and bioinformatic tools evolve there is a shift towards increased usage of whole metagenome sequencing (shotgun sequencing) in studies of the gut microbiome. However 16S rRNA applications still hold benefits in studies of large sample collections and biopsy materials. *Emerging technologies may overcome this limitation.³²

Characteristic	16S rRNA profiling	Metagenome sequencing
Cost	Low	High
Taxonomic resolution	Low (genus level)	High (species level)
Gene count details	N/A (by imputation only)	High
PCR bias	Variable	Non-existent
File size	Small (~50Mb per sample)	Large (~4Gb per sample)
Computational complexity	Low	High
Throughput	High	Low
Applicable to stool/secretions	Yes	Yes
Applicable to biopsies	Yes	No (host DNA predominates*)

Table 2. Studies of full-length, peer-reviewed articles on the gut microbiota in primary sclerosing cholangitis (PSC). Letters and other communications are reviewed in the text. UDCA, ursodeoxycholic acid; IBD, inflammatory bowel disease; UC, ulcerative colitis; OTU, operational taxonomic unit.

	Rossen et al.⁶²	Torres et al.⁶³	Kummen et al.⁶⁵	Sabino et al.⁶⁶	Bajer et al.⁶⁷
PSC patients, n	12	20	85	66	43
Males, n (%)	10 (83)	16 (80)	53 (62)	48 (73)	34 (79)
Age (years), median	30	47	49	47 ^a	38 ^a
PSC-IBD, n (%)	12 (100)	19 (95)	55 (65)	48 (73)	32 (74)
Disease duration PSC (years), median	2	4	9	-	-
Age at diagnosis (years)	-	-	-	34 ^a	-
Cirrhosis, n (%)	0 (0)	-	-	13 (20)	-
Liver transplantation, n (%)	-	1 (5)	0 (0)	15 (23)	0 (0)
Antibiotics < 4 weeks, n (%)	0 (0)	0 (0)	0 (0)	11 (17)	0 (0)
UDCA treatment, n (%)	9 (75)	10 (50)	25 (29)	47 (71)	43 (100)
5-ASA treatment, n (%)	8 (67)	8 (40)	35 (41)	22 (33)	26 (60)
IBD controls, n	11	15	36	43	32
Males, n (%)	9 (82)	9 (60)	16 (44)	19 (44)	17 (53)
Age (years), median	50	48	40	51 ^a	40
UC, n (%)	11 (100)	13 (87)	13 (30)	36 (100)	32 (100)
Crohn's disease, n (%)	0 (0)	2 (13)	30 (70)	0 (0)	0 (0)
Healthy controls (HC)	9	9	263	66	31
Males, n (%)	7 (78)	0 (0)	108 (41)	49 (74)	13 (42)
Age (years), median	65	44 ^c	46	52	44
Material	Mucosa	Mucosa	Stool	Stool	Stool
Collection	Snap-frozen	Snap-frozen or RNAlater	Preservative PSP tube	Fresh frozen	Fresh frozen
Extraction kit:	-	Qiagen DNeasy	PSP spin stool DNA	MoBio	Masterpure
Extraction: bead beating?	-	yes	yes	yes	Yes
16S rRNA region amplified	27F-1369R	V4	V3-V4	V4	V3-V4

Profiling method	Microarray (HITchip)	Illumina Miseq	Illumina Miseq	Illumina Miseq	Illumina Miseq
Alpha diversity PSC vs. HC	Reduced (Shannon)	No difference	Reduced (all measures)	Reduced (observed OTUs)	Reduced ^b
Alpha diversity PSC vs. IBD	-	No difference	No difference	-	Reduced ^b
Taxa in PSC vs HC	Down: Uncultured Clostridiales II	Up: Barnesiellaceae, Blautia, Ruminococcus	Up: Veillonella Down: Coprococcus, Phascolarctobacterium, Lachnospiraceae, Christensenellaceae, S24.7, RF32, YS2	Up: Veillonella Streptococcus, Enterococcus, Lactobacillus, Fusobacterium	Up: Rothia, Enterococcus, Streptococcus, Clostridium, Veillonella, Haemophilus

^aCalculated from subgroup medians. ^bUC significantly lower diversity than HC, PSC inbetween

Table 3. Randomized controlled trials (RCTs) of antibiotics in primary sclerosing cholangitis.

For comparability, only results related to alkaline phosphatase (ALP) and Mayo risk score (MRS) are given in the table. UDCA, ursodeoxycholic acid; MTZ, metronidazole.

Study	Design	N	Drug	Dose	Duration	Primary endpoints	Results
Färkkilä et al. ⁸¹ (2004) Finland	RCT	80	MTZ or placebo (+UDCA)	600-800 mg/day	3 years	Histology, ERC, lab, MRS	ALP -52% (MTZ) vs -38% MRS -0.32 vs -0.06
Silveira et al. ⁸³ (2009) USA	Prospective, not controlled	16	Minocycline	100 mg x2	1 year	ALP, bilirubin, AST	ALP -20% MRS -0.53
Tabibian et al. ⁸² (2013) USA	RCT	35	Vancomycin or MTZ	125/250 mg x4/ or 250/500 mg x4	12 weeks	ALP	Vancomycin: ALP -40% & -46% MTZ: ALP -33% & +13%
Rahimpour et al. ⁸⁴ (2016) Iran	RCT	29	Vancomycin or placebo	125 mg x4	12 weeks	MRS, ALP	MRS -0.59 and ALP -53% (vancomycin) vs -+0.05 and -8%
Tabibian et al. ⁸⁰ (2017) USA	Prospective, not controlled	16	Rifaximin	550 mg x2	12 weeks	ALP	ALP +0.9% MRS +0.15

Figure legends

Figure 1. The gut-liver axis in primary sclerosing cholangitis (PSC). PSC is characterized by biliary strictures in the context of colonic inflammatory bowel disease. The relationship between the gut and the liver is bidirectional, with portal blood bringing a variety of gut derived factors to the liver and bile contributing to the luminal homeostasis in the gut. In this bilateral concept, the gut microbiome and the liver comprises an integrated metabolic machinery under the influence of endogenous as well as dietary factors. Disturbances in the gut-liver axis are currently being surveyed for disturbances relevant to PSC pathogenesis.

Figure 2. Schematic illustration of metabolic host-microbiome interactions. Microbial metabolites exert a broad range of host physiological effects in the liver and other organs. Examples include neurotransmitters such as 5-hydroxyindole, several amines, short-chain fatty acids, branched chain amino acids, phospholipid metabolites, triglycerides, microbiota-produced vitamins and metabolites of phenolics and aromatic acids such as 4-cresyl sulfate and hippurate. Figure reproduced from reference ⁴⁰ with permission from the authors and publisher.

Figure 3. The immunobiome concept. The gut microbiome interacts with the immune system via a variety of mechanisms. Conserved immune receptors with specificity against microbial components and products (pathogen-associated molecular patterns; e.g. lipopolysaccharide, LPS) are found in a variety of immune- and parenchymatous cells, cholangiocytes included (left panel). Conventional (middle panel) and non-conventional (right panel) T cells also express T cell receptors (TCRs) with microbial specificity. Under normal circumstances, there is homeostasis and tolerance at the interface between the host and the commensal microbial flora, but it may be hypothesized that in disease states, primary sclerosing cholangitis included, this balance is shifted towards loss of tolerance and inflammation. One conceptual compartment of research may thus be to understand which antigenic drivers are the most relevant at this interface.

Figure 4. The endobiome concept. Microbial metabolism is closely integrated with the host physiology. As exemplified to the right, a variety of endogenous compounds undergo co-metabolism within the microbial compartment (e.g bile acids). As exemplified to the left, several compounds of relevance to host physiology are also synthesized de novo by microbes (e.g. vitamins). Disturbances in this interaction between host and microbial metabolism may be hypothesized to involve in disease processes, either primarily, or downstream of initiating events (hepatic encephalopathy a classic example). Research to understand processes in this conceptual

compartment has been greatly facilitated by the advent of metagenome sequencing, whereby the complete gene counts of bacteria can be determined.

Figure 5. The xenobiome concept. The gut microbiome is interposed between the external environment and the host and is heavily influenced by dietary and other exogenous factors, in many ways serving as both an “environmental gatekeeper” and “environmental sensor”. The gut microbiome serves critical roles in nutrient metabolism (left) and also engages actively in the metabolism of drugs and other exogenous compounds (right). This transformation may lead to compounds of relevance to endogenous metabolism (Figure 4) or changes that lead to the eliciting of immune reactions (Figure 3). Collecting comprehensive information about environmental exposures is exceedingly difficult and good strategies to elaborate the role of this conceptual compartment are sorely needed. Based on the large role of environmental factors in primary sclerosing cholangitis and other complex genetic diseases (genetics typically accounting for no more than 10-20% of the overall disease liability), research in this area is urgently needed