# Alterations of the bile microbiome in primary sclerosing cholangitis

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Abbreviations: ANOVA: analysis of variance; ALP: alkaline phosphatase; BMI: body mass index; ERC: endoscopic retrograde cholangiography; FDR: false discovery rate; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; KEGG: Kyoto Encyclopaedia of Genes and Genomes; NGS: next-generation sequencing; PSC: primary sclerosing cholangitis; TE: transient elastography; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TLCA: taurolithocholic acid; UDCA: ursodeoxycholic acid; VAP-1: vascular adhesion protein 1.

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## Abstract

**Background**: Patients with primary sclerosing cholangitis (PSC) display an altered colonic microbiome compared to healthy controls. However, little is known on the bile duct microbiome and its interplay with bile acid metabolism in PSC.

**Methods**: PSC patients (n=43) and controls without sclerosing cholangitis (n=22) requiring endoscopic retrograde cholangiography were included prospectively. Leading indications in controls were sporadic choledocholithiasis and papillary adenoma. A total of 260 biospecimens were collected from the oral cavity, duodenal fluid and mucosa and ductal bile. Microbiomes of the upper alimentary tract and ductal bile were profiled by sequencing the 16S-rRNA-encoding gene (V1-V2). Bile fluid bile acid composition was measured by high-performance liquid chromatography mass-spectrometry and validated in an external cohort (n=20).

**Results**: The bile fluid harboured a diverse microbiome that was distinct from the oral cavity, the duodenal fluid and duodenal mucosa communities. The upper alimentary tract microbiome differed between PSC patients and controls. However, the strongest differences between PSC patients and controls were observed in the ductal bile fluid, including reduced biodiversity (Shannon entropy, $P$=0.0127) and increase of pathogen *Enterococcus faecalis* ($P_{FDR}$=4.18×10$^{-5}$) in PSC. *Enterococcus* abundance in ductal bile was strongly correlated with concentration of the noxious secondary bile acid taurolithocholic acid (r=0.60, $P$=0.0021).

**Conclusion**: PSC is characterised by an altered microbiome of the upper alimentary tract and bile ducts. Biliary dysbiosis is linked with increased concentrations of the pro-inflammatory and potentially cancerogenic agent taurolithocholic acid.

## Significance of this study

### What is already known on this subject?

- Primary sclerosing cholangitis (PSC) is associated with alterations of the colonic microbiome
- Opposed to traditional understanding, human bile is a non-sterile environment (even in healthy humans).

### What are the new findings?

- Composition of bile duct microbiome is different from other upper digestive sites such as the oral cavity and duodenum.
- PSC patients display ecologic alterations of ductal bile, including reduced biodiversity and expansion of pathogenic bacteria.
- Microbial dysbiosis in PSC is associated with an increase of the proinflammatory and potentially cancerogenic bile acid taurolithocholic acid.

### How might it impact on clinical practice in the foreseeable future?

- Microbial dysbiosis of the ductal bile fluid highlights the potential pathophysiologic importance of the biliary microbiome in PSC.
- This finding encourages precise modulation of biliary microbial colonisation to reduce the risk of adverse health outcomes associated with PSC.

## Introduction

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown origin which is characterised by progressive fibrotic strictures of bile ducts and ulcerative lesions of the bile duct mucosa.[1-3] PSC is strongly associated with a unique phenotype of inflammatory bowel disease. Patients with PSC suffer from an increased mortality, mainly due to increased risk of cholangiocarcinoma and cancers of the gallbladder and colon.[1,2] Liver transplantation is the only curative treatment option available.

The pathogenesis of PSC remains obscure. Genetic studies support the hypothesis that PSC is an autoimmune disorder, but male preponderance and poor response to immunosuppression render it different from typical autoimmune diseases.[1,2] Multiple lines of evidence point at commensal bacterial communities as key players in the pathophysiology of PSC.[2,3] Recent next-generation sequencing (NGS) studies revealed an altered gut bacterial microbiome in patients with PSC, both at the faecal and mucosal level, which was different from both healthy individuals and patients with ulcerative colitis.[4-10] Until the advent of NGS, healthy human bile has been widely considered sterile. Nevertheless, earlier culture-based studies implicated bacteria inhabiting the bile fluid in T helper cell type 17 immune response and clinical outcomes in PSC.[11,12] A recent NGS study showed that both PSC patients and controls without sclerosing cholangitis harbour a diverse bile microbiome.[13]

Bile acids are the major organic solutes of human bile.[14] Bile acids are believed to play a crucial role in pathogenesis of PSC, although evidence of a “toxic” bile composition per se in PSC patients is lacking.[15,16] Since conversion of the primary bile acids cholic acid and chenodeoxycholic acid into the secondary and potentially noxious bile acids deoxycholic acid and lithocholic acid is thought to be primarily driven by the bacterial gut microbiome, microbial dysbiosis is expected to exert a profound influence on the bile acid pool and in turn mucosal homeostasis in PSC.[15] However, to the best of our knowledge, the link between bile acids and microbiome of the bile fluid has not been investigated so far.

In the present study, we aimed to investigate the bacterial ecology of the upper alimentary tract as well as ductal bile fluid in selected cohorts of PSC patients and controls undergoing endoscopic retrograde cholangiography (ERC). Furthermore, we assayed the entire bile fluid bile acid profiles of the respective cohorts in order to analyse potential interactions between bile fluid bacteria and bile acids in PSC.

## Methods

*Patient recruitment and biospecimen acquisition*

All patients with PSC and controls were recruited at the University Medical Center Hamburg-Eppendorf. The diagnosis of PSC was established based on presence of typical biliary lesions on cholangiography, liver biopsy (if available) and exclusion of secondary causes of sclerosing cholangitis, according to most recent guidelines.[17,18]

Exclusion criteria were acute bacterial cholangitis on index ERC, previous ERC within the last 12 months, patient age <18 years, severe medical comorbidity, small duct PSC and any evidence of secondary sclerosing cholangitis. Patients were required not having received any antibiotic treatment during 6 months before ERC as this time interval is expected to be sufficient for broad microbiome recovery.[19,20] In order to reduce the influence of geography and diet all recruited participants were residents of Northern Germany for years and consumed a mixed Western style diet.

In total, 65 patients were eligible for the analysis (PSC n=43, controls n=22). A detailed patient description is provided in Table 1. Indications for ERC in PSC patients included cholestasis or suspicion of dominant strictures on magnetic resonance imaging. In controls, PSC was excluded by cholangiography and clinical follow-up. Detailed indications for ERC for both cohorts and diagnoses of the control cohort can be found in **Supplementary Table 1**. Intervention and specimen acquisition are described in the **Supplementary Methods** in detail. During ERC, bile fluid aspiration was performed before application of contrast media or periinterventional antibiotic prophylaxis, which is standard in our unit. There were no patients with biopsy proven bile duct dysplasia or cholangiocarcinoma.

All participants provided written informed consent. The protocol was reviewed by the appropriate ethics committee (PV4114). The study was conducted in compliance with the 1975 Declaration of Helsinki.

*Sequencing, bioinformatics and bile acid assay*

A detailed description is provided in the **Supplementary Methods**. In brief, DNA extraction and sequencing of the variable regions V1-V2 of the 16S rRNA gene were performed on Illumina MiSeq (Illumina Inc., San Diego, California, USA), as described previously.[21] `DADA2` was used for meta-taxonomic bioinformatics, a method which retrieves unique ribosomal sequence variants. Sequences abundance was normalised according to the `GMPR` method.[22] `SILVA` was chosen as the taxonomic reference database (v132; https://www.arb-silva.de). `Tax4Fun` was employed for inferred metagenome profiling against canonical pathways of the Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://tax4fun.gobics.de).

High-performance liquid chromatography mass spectrometry was performed, essentially as described previously (**Supplementary Methods**).[23]

*Data analysis*

All analyses were carried out with `R` (v3.4.3, R Foundation for Statistical Computing, Vienna, Austria). A detailed account is given in the **Supplementary Methods** section. To summarise, standard community ecology analyses were carried out mainly using `vegan`. Single bacteria differential abundance testing was conducted with negative-binomial generalised linear models from the `MASS` package, and negative-binomial hurdle models, which account for zero-inflation in count data, implemented in the `pscl` library. To exclude that contamination via the endoscopic route accounted for the abundance patterns observed, normalised distributions of each taxon at the respective proximal sampling sites were included as covariables in the respective models, alongside clinical variables with significant differences between cohorts (Table 1), where possible. An $\alpha$-level $<0.05$ was set as the threshold for statistical significance. P-values were adjusted for the false discovery rate (FDR), where necessary.

## Results

*Ductal bile fluid harbours a unique and diverse microbiome*

Since it is unknown whether microbial ecology of any of the upper alimentary sites investigated resembles the bile fluid microbiome, we first examined differences of microbiome structure between oral cavity, duodenal fluid, duodenal mucosa and bile fluid by constrained analysis of principal coordinates using Bray-Curtis distance (Figure 1A, 1B). Microbial communities obtained from duodenal mucosal biopsies clustered separately from the other sites (ANOVA-like permutation test, $P<0.01$, respectively). While in both cohorts oral and duodenal fluid communities were similar ($P>0.1$), there was a clear separation between bile fluid microbiome and all other communities ($P\leq0.01$, respectively).

We next explored the community structures on the phylum level (highest taxonomic hierarchy level). Only five phyla accounted for virtually the entire microbiome in all sites, which are Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. Comparing PSC patients and controls, we observed no significant differences in phyla abundances in the oral cavity and duodenal sites (Welch's t-test, $P>0.05$). Regarding the ductal bile fluid, Proteobacteria showed a significant increase in PSC patients compared to controls (Mean relative abundance = 25% vs 12%; $P=0.0384$; Figure 1C). Other biliary phyla in PSC patients showed an abundance similar to controls ($P>0.1$, respectively).

Since the baseline structure of the healthy bile fluid core microbiome is unclear, we investigated the biliary core microbial profiles of the control cohort and PSC patients on the genus taxonomic level.[22] Both groups harboured a diverse core microbiome with *Streptococcus* as the predominant genus (Figure 1D). However, patients with PSC displayed decreased richness of the core microbiome evidenced by decreased prevalence of genera with >0.1% relative abundance. These results highlight the deviation in PSC from a stable core bile microbial composition in a healthy state (Figure 1D).

*Altered upper alimentary and bile duct fluid microbiome in PSC*

We compared the within-sample diversity (α-diversity) between PSC patients and controls in the different body sites. The bile fluid microbial communities of PSC patients displayed a reduced average α-diversity compared to controls (Shannon entropy, Welch's t-test, $P=0.0127$; Figure 2A). Differences of α-diversity were not observed in the other body sites, which indicates that the biliary microbiome in PSC is more severely altered than the other upper digestive communities tested. We observed significant differences in the overall community structure (β-diversity) between PSC patients and controls in the oral cavity, duodenal fluid and

bile duct fluid (ANOVA-like permutation test, $P \leq 0.01$, respectively; Figure 2B), and a trend in the duodenal mucosal communities ($P=0.064$).

*Expansion of pathogens in bile fluid of PSC patients*

We tried to identify differentially abundant bacteria on the lower taxonomic hierarchy levels (genus and species) between PSC patients and controls.

We first investigated differential abundance patterns in the oral cavity, duodenal fluid and duodenal mucosa. (**Supplementary Results**, **Supplementary Figure 1**). Notably, we found a pronounced overrepresentation of the species *Escherichia coli* (log2FC=4.19, $P_{FDR}=6.02\times10^{-29}$) and *Veillonella dispar* (log2FC=1.7, $P_{FDR}=0.0034$) in duodenal mucosal biopsies of PSC patients.

The biliary communities of PSC patients showed the most extensive differences on the single taxa level compared to controls (Figure 2C). The strongest increase was observed for *Enterococcus* (log2FC=8.13, $P_{FDR}=4.18\times10^{-5}$), followed by *Staphylococcus* (log2FC=6.71, $P_{FDR}=4.07\times10^{-6}$). Amongst other bacteria, *Neisseria* (log2FC=5.97, $P_{FDR}=7.63\times10^{-5}$) was also overabundant in PSC patients.

Regarding the differential abundance of bile fluid species (Figure 2C), *Enterococcus faecalis* showed the strongest increase in PSC patients (log2FC=10.01, $P_{FDR}=0.0054$), followed by *Veillonella dispar* (log2FC=3.03, $P_{FDR}=0.0256$).

Bile samples obtained within our study were sent for standard culture. We compared the results obtained by NGS with bile culture results in PSC patients and controls. There were no differences in the rate of positive bacterial bile cultures (46% vs 47% with positive culture, $\chi^2$ test, $P>0.1$). Patients with PSC showed a trend towards an increased rate of culturally detected known pathogenic bacteria in bile fluid (*Enterococcus spp., Klebsiella spp., Enterobacter cloacae, Citrobacter freundii* or *Staphylococcus spp.*; 23% versus 5%, Fisher's test, $P=0.082$).

*Previous ERC has minor influence on bile duct bacterial composition in PSC*

We aimed to identify clinical factors that may influence bile fluid bacterial communities in PSC patients.

We first investigated potential association with overall ecologic indices in a multivariate approach, including the variables sex, body mass index (BMI), presence of PSC-associated colitis, bilirubin, transaminase, alkaline phosphatase (ALP) and leukocytes levels, liver stiffness measured by transient elastography (TE), previous bacterial cholangitis and previous ERC.

These variables were neither significantly associated with α-diversity using best subset selection by leaps algorithm ($P$>0.05, respectively), nor were they significant predictors of β-diversity using stepwise forward selection for constrained ordination ($P$>0.05, respectively; **Supplementary Methods**).

Most single bacteria identified overrepresented in PSC patients in the previous section did not show any significant differential abundance between PSC patients with or without previous ERC ($P_{FDR}$>0.05, respectively). However, amongst bacteria overrepresented in PSC *Staphylococcus* (log2FC=7.47, $P$=0.004) and *Streptococcus sanguinis* (log2FC=9.15, $P$=5.33×10$^{-4}$) were overrepresented in PSC patients who formerly received ERC.

*Altered metabolic functional profiles of bile fluid microbiome in PSC*

Since metagenomic shotgun sequencing was hindered by the relatively large amount of human sequences in the bile fluid, we inferred the functional profiles from the 16S profiles (**Supplementary Methods**).

Of the 321 pathways recovered in the bile duct fluid, 52 showed an altered expression (16.2%, 34 under-expressed, 18 over-expressed; Figure 3A). Corresponding the reduced alpha diversity, we observed an extensive loss of basic microbiome functions such as “tryptophan metabolism” ($P_{FDR}$=1.72×10$^{-4}$) or “biosynthesis of amino acids” ($P_{FDR}$=2.24×10$^{-3}$). We observed an increase in several potentially pathogenic bacterial pathways, including “shigellosis” ($P_{FDR}$=5.12×10$^{-5}$), “*Salmonella* infection” ($P_{FDR}$=3.01×10$^{-3}$) and “pathogenic *Escherichia coli* infection” ($P_{FDR}$=5.11×10$^{-3}$).

Metabolic pathways were differently distributed in PSC patients and controls also in the other upper digestive tract sites, e.g., an increase of “biofilm formation by *Escherichia coli*” in the oral cavity ($P_{FDR}$=0.0190) and the duodenal fluid ($P_{FDR}$=1.17×10$^{-10}$), as well as an increase of

"lipopolysaccharide biosynthesis" ($P_{FDR}$=1.89×10$^{-4}$) and "bacterial invasion of epithelial cells" ($P_{FDR}$=0.0087) in the duodenal fluid.

As we were interested in bacterially triggered dysregulated biliary mucosal immunity associated with inflammation-driven carcinogenesis we additionally applied targeted Bayesian generalised linear modelling to inferred bacterial genes involved in nuclear factor (NF-)kappaB signalling (**Supplementary Methods**). "NF-kappa B signalling pathway" was overrepresented in PSC patients (**Supplementary Figure 2**).

*Altered bile acid concentrations and noxious lithocholic acid levels associated with bile dysbiosis in PSC*

We aimed to investigate if microbiome composition was associated with bile acid composition in the bile fluid.

Relative bile acid concentrations in controls were as expected from the literature with little difference in PSC patients (**Supplementary Figure 3**). As expected from the treatment of PSC patients, the absolute concentrations of ursodeoxycholic acid (UDCA) conjugates were greatly increased in PSC patients (measured in µg/l; Welch's t-test, $P$<0.0001, respectively). Most other bile acids showed reduced absolute concentrations in PSC samples ($P$<0.05, respectively). However, taurolithocholic acid (TLCA), a potentially noxious agent, was the only bile acid with similar concentrations between PSC patients and controls ($P$>0.05, Figure 3B).

To exclude that the reduced absolute bile acid concentrations in PSC patients could be explained by UDCA treatment, we analysed the biliary bile acid profiles in an independent cohort of 20 patients with PSC without UDCA treatment from the Norwegian PSC Research Center, Oslo. Here, we observed the same trend towards reduced single bile acid concentrations (**Supplementary Figure 4**), suggesting that this observation relates, at least partly, to PSC pathophysiology itself.

We tried to establish relationships between bile fluid microbiome and bile acid concentrations in the PSC cohort. To reduce the burden of multiple testing, we first identified variables which maximised the correlation between bile fluid microbial genera abundances and bile acid concentrations by sparse canonical correlation analysis (`PMA` library, **Supplementary Methods**). Next, we tested partial correlations between genera and bile acids adjusting for sex, BMI, ALP and TE levels (Figure 3C). The strongest correlation was observed between *Enterococcus* and TLCA (r=0.60, test for zero partial association, $P$=0.0021).

299 Thus, the biliary genus with the strongest increase in PSC, *Enterococcus*, was associated with
300 an increase in the noxious and potentially carcinogenic bile acid TLCA.

## Discussion

PSC is a disease that mainly affects the bile ducts, which represent a large mucosal barrier within the body.[1] An altered microbiome may significantly contribute to the non-genetic risk associated with PSC.[2,15] In the present study, we detected differences in the microbial composition between PSC patients and controls without sclerosing cholangitis in the oral cavity, the duodenum, and the ductal bile fluid. Adding to previously reported changes of the faecal and colonic mucosal microbiome in PSC, our study shows that the upper alimentary tract and bile ducts of PSC patients are likewise affected by microbial dysbiosis. The biliary microbiome in PSC patients exhibited the most extensive alterations which were evident on both the taxonomic and inferred functional levels. *Enterococcus*, the genus with the strongest increase in bile ducts of PSC patients, was associated with lithocholic acid, a noxious and potentially carcinogenic bile acid.

In the single previous study on the biliary microbiome in PSC, the authors detected only slight microbial alterations in patients with either biliary dysplasia or cholangiocarcinoma, while patients without disease complications showed virtually no microbial differences compared to controls.[13] In contrast, we observed significant biliary microbial alterations with considerable effect sizes in well-characterized PSC patients without dysplasia or carcinoma. The significant results may have been facilitated by a more refined statistical approach, and a well-controlled design with an extended period after last antibiotic or ERC treatment. Furthermore, additionally sequencing the microbiome from proximal upper digestive sites allowed us to control for the effect of bile fluid contamination via the endoscopic route, which may have been a shortcoming of the previous study.[13] Clearly, the biliary microbiome was found to be distinct from the duodenal mucosal or luminal microbiome, demonstrating that duodenal fluid cannot be used as a proxy in studies aiming to address the biliary microbiome.

We observed a significant increase of the facultative anaerobic phylum Proteobacteria in the bile fluid of patients with PSC. The abnormal bloom of Proteobacteria, which comprises many known human pathogens, such as members of the Enterobacteriaceae family, typically occurs in association with increased epithelial oxygen availability and is therefore believed to be a hallmark of inflammation, epithelial dysfunction and disease.[25] Furthermore, we observed a reduced average biodiversity of the bile fluid microbiome in PSC patients. From an ecological standpoint, a decreased biodiversity is a critical event that leads to a loss of ecosystem resilience and a loss of favourable ecosystem functions.[26]

Regarding the results of bile fluid cultures, known pathogens of cholangitis were detected more frequently in samples of patients with PSC. This trend was confirmed on the 16S rRNA gene level, where we observed an overrepresentation of potential pathogens such as *Enterococcus*

*spp.*, *Prevotella spp.*, *Staphylococcus spp.*, *Lawsonella spp.*[27] and *Cutibacterium*. Here, *Enterococcus faecalis* showed the most marked increase in PSC patients. *Enterococcus* has previously been shown to be more abundant in faeces of patients with PSC.[6] *Enterococcus faecalis* has been associated with epithelial barrier damage and mucosal inflammation due to its production of matrix metalloproteinases.[28] In addition, biliary isolates of *Enterococcus faecalis* have been shown to induce T helper type 17 immune responses in peripheral blood of patients with PSC.[12] In a recent report, Enterococcus gallinarum was among the gut pathobionts translocating into mesenteric lymph nodes and driving T helper 17 cells mediated hepatobiliary injury in a model of PSC.[29]

We detected an increased abundance of *Veillonella dispar* on the duodenal mucosa as well as the bile duct fluid of PSC patients. This pathogen has previously been repeatedly detected as overrepresented in faecal communities of patients with PSC.[9,10] In previous studies on Crohn's disease, a disease which shares considerable overlap with PSC in clinical phenotype, *Veillonella spp.* alongside *Enterococcus spp.* were associated with an increased risk of recurrent disease after surgical resection and predisposition to penetrating complications in paediatric patients.[30,31] Thus, we believe that the potential functional and prognostic role of *Veillonella* and *Enterococcus* in PSC should be studied not only in the intestine but also within the bile ducts in future follow-up studies.

Interestingly, we found a marked increase of *Escherichia coli* on the duodenal mucosal surfaces of PSC patients. An increased prevalence of mucosa-adherent *Escherichia coli* is well-recognised in inflammatory bowel diseases, where it is believed to instigate mucosal injury.[32] Furthermore, *Escherichia* has been shown to produce cysteamine which is the most potent inducer of vascular adhesion protein (VAP)-1. Elevated levels of soluble VAP-1 have been linked to poor prognosis in PSC patients, therefore, providing a link between overgrowth of *Escherichia coli* and clinical outcomes in PSC.[33]

The expansion of bacterial pathogens was reflected on the level of the upper alimentary tract and biliary microbiome functional profiles, in which an increase of invasive and proinflammatory bacterial metabolic capacity was observed. Amongst increased microbial pathways, we found epithelial cell adhesion and invasion as well as synthesis of lipopolysaccharides, molecules which may drive biliary epithelial inflammation in PSC.[34] Since the bile duct mucosa is the primary site of inflammation in PSC, these results point towards a possible contribution of altered biliary microbiome to cholangiocyte and bile duct mucosal damage. Furthermore, the extensive loss of function in the PSC bile fluid communities might reflect a decline of beneficial microbial contribution to bile duct mucosal homeostasis. This result may relate to the recent discovery of an altered bile metabolome in PSC.[35] Furthermore,

the dysbiotic bile fluid microbiome in PSC was associated with increased NF-kappaB signalling. Bacterial activation of NF-kappaB is an important factor in the immunopathology of the bile ducts.[36] Moreover, the NF-kappaB family of transcription factors are linked to inflammation driven carcinogenesis.[37] It is therefore tempting to speculate on a link between microbially triggered bile duct inflammation and development of cholangiocarcinoma in PSC.

Examining the bile acid profiles, we found a reduced bile acid pool in patients with PSC, except for the secondary bile acid TLCA. Our observation is in accordance with previously reported globally reduced bile acid concentrations in patients with obstructive cholestasis due to PSC.[16] The causes of this alteration are unknown, but reabsorption of bile acids, reduced synthesis and dilution of stagnant bile are obvious explanations,[16] and may represent protective mechanisms in cholestatic liver disease. Lithocholic acid is a rare example of a noxious endobiotic, which together with its conjugates is considered the most harmful bile acid.[38] Lithocholic acid causes segmental bile duct obstruction, destructive cholangitis and periductal fibrosis[39] and exerts cancerogenic effects.[40] For PSC it was proposed that bile duct injury induced by bile acids is caused by more vulnerable bile ducts rather than by absolute excess of noxious bile components.[3,16] Therefore, physiologic TLCA concentrations may already cause bile duct injury in PSC patients with cholestasis and a damaged mucosal barrier. Interestingly, TLCA levels were strongly correlated with *Enterococcus* abundance in PSC samples. *Enterococcus faecalis* expresses higher bile salt hydrolase activity than other human commensal microbes.[41] Bile salt hydrolases catalyse the crucial step of deconjugation in the process of converting primary to secondary bile acids. Hence, the dysbiotic excess of *Enterococcus spp.* may be causally linked to secondary bile acid levels with both proinflammatory and cancerogenic impact on PSC patients. Previously, the increased malignancy rate and disease progression in PSC patients imposed by high-dose UDCA treatment has been linked to increased lithocholic acid levels resulting from conversion of UDCA.[42] As both the abundance of Enterococcus and concentration of TLCA were heterogenous amongst PSC patients, it is an interesting question to be addressed in follow-up studies whether patients with increased TLCA levels represent a subgroup at risk for adverse outcomes such as cholangiocarcinoma.

Our study has strengths and limitations. It is difficult to study human ductal bile and almost impossible to obtain samples either in a sterile way or without prior perioperative antibiotic prophylaxis. By controlling for the microbiome in the oral cavity as well as duodenum we tried to circumvent this technical problem. Selecting proper controls is challenging, since ERC is a method associated with health risks for the examined individual and thus cannot be performed on healthy volunteers for ethical reasons. Although we required no antibiotic treatment during 6 months before biliary sampling during ERC, an effect of prior antibiotic treatment on the biliary microbiome cannot be excluded entirely. In addition, species level and functional

information obtained through 16S rRNA gene sequencing are not as reliable as by employing shotgun metagenomic sequencing of the entire DNA. However, due to the low abundance of bacterial DNA as compared to human DNA in bile fluid, metagenomic shotgun sequencing could not be performed successfully on our samples at reasonable sequencing depths. Our microbiome analysis is confined to an ethnically, geographically and dietary homogenous single centre cohort. Future studies should assess the robustness of the alterations observed by including international multicentric cohorts and by analysing potential impact of differing diets. While a comparison of the biliary microbiome between patients with PSC and other chronic liver diseases may add to the understanding of disease-specific pathophysiological implications of microbial alterations, obtaining bile via ERC from patients with parenchymal liver diseases is not feasible for ethical reasons. Therefore, caution is warranted regarding the specificity of our result for PSC. Many microorganisms cannot be grown using routine cultivation methods. The present results show that an NGS approach surpasses the constraints of standard bile culture, as has been previously demonstrated for numerous human and environmental habitats.[43]

In summary, the present study demonstrates a dysbiosis in the microbial communities of the upper alimentary tract and bile ducts of patients with PSC, with the most significant alterations found in the bile fluid. We hypothesise that changes in the biliary microbiome may contribute to PSC pathogenesis by enhancing the damage of bile duct mucosa and potentially by effects on the concentration of the noxious bile acid lithocholic acid. As our study is cross-sectional and therefore cannot prove causality, this hypothesis should be further investigated in future follow-up and more functional experimental studies. Multiple lines of evidence point towards microbial factors influencing clinical outcomes in PSC.[11,33,44-46] As advances in microbiome research are spurring the development of precision medicine interventions, such as techniques of finely tuned control of bacterial strain abundance[47] and selective inhibition of pathogen expansion in inflammation,[48] our results may provide a starting-point for clinical studies on the bile microbiome in PSC.

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575 **Table 1:** Clinical patient characteristics

| | **PSC** | **Controls** | **P-value** |
|---|---|---|---|
| Patients, n | 43 | 22 | NA |
| Female, n | 16 (37%) | 11 (50%) | > 0.1 |
| Age, years | 39 (20, 55) | 55 (22, 89) | < 0.01 |
| BMI, kg/m² | 23.38 (15.9, 35.4) | 25 (18.7, 41.3) | > 0.1 |
| Liver cirrhosis [a], n | 7 (16.3%) | 1 (5%) | > 0.1 |
| Previous ERC, n | 29 (67.4%) | 1 (5%) | < 0.001 |
| Previous bacterial cholangitis, n | 2 (5%) | 0 | > 0.1 |
| Transient elastography, kPa | 8.7 (3.5, 66.4) | NA | NA |
| Disease duration, years | 8 (0, 28) | # | NA |
| IBD, n | 29 (67.4%) | 0 | < 0.001 |
| Bilirubin, mg/dl | 1 (0.2, 5.8) | 0.8 (0.2, 7.0) | > 0.1 |
| ALT, U/l | 76.5 (9, 274) | 49.0 (15, 580) | > 0.1 |
| ALP, U/l | 244 (49, 961) | 129 (53, 539) | < 0.05 |
| CRP, g/dl | < 5 (< 5, 39) | < 5 (< 5, 61) | > 0.1 |
| WBC, $10^3$/µl | 6.1 (3.3, 17.2) | 7.9 (3, 16.6) | < 0.01 |
| UDCA [b], n | 40 (93%) | 2 (9%) | < 0.001 |
| Azathioprine, n | 8 (18.6%) | 0 | < 0.05 |
| Mesalazine, n | 16 (37.2%) | 0 | < 0.001 |
| Corticosteroids, n | 3 (7%) | 2 (9%) | > 0.1 |
| Proton pump inhibitors, n | 2 (5%) | 2 (9%) | > 0.1 |

576

577 All data are provided for the time of index ERC. Median and range or counts and percentages
578 are reported, respectively. Continuous variables were tested by Wilcoxon rank-sum test.
579 Nominal variables were tested either with $\chi^2$ test or Fisher's exact test. BMI: body mass index;
580 ERC: endoscopic retrograde cholangiography; IBD: inflammatory bowel disease; ALT: alanine
581 aminotransferase; ALP: alkaline phosphatase; CRP: C-reactive protein; WBC: white blood
582 count; UDCA: ursodeoxycholic acid. NA: not available/applicable. #: All control subjects
583 received first diagnosis of biliary obstruction. [a] Liver cirrhosis was diagnosed based on criteria
584 of clinical signs, imaging, transient elastography and biopsy (if available).[b] All PSC patients
585 treated with UDCA received a daily dose of 15-20 mg/kg.