Original Article

The Mucosa-associated Microbiota of PSC Patients is Characterized by Low Diversity and Low Abundance of Uncultured Clostridiales II

Noortje G. Rossen1, Susana Fuentes2, Kirsten Boonstra1, Geert R. D’Haens1, Hans G. Heilig2, Erwin G. Zoetendal2, Willem M. de Vos2,3, Cyriel Y. Ponsioen1,∗

1Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands 2Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands 3Departments of Bacteriology & Immunology and Veterinary Biosciences, University of Helsinki, Finland

∗Corresponding author: Cyriel Y. Ponsioen, Departement of Gastroenterology & Hepatology, Academic Medical Center Amsterdam, room C2-328, 1105 AZ Amsterdam, The Netherlands. Tel: +31205666012; email: c.y.ponsioen@amc.uva.nl

Abstract

Background: Primary sclerosing cholangitis (PSC) is a cholestatic liver disease that is strongly associated with a particular phenotype of inflammatory bowel disease (IBD) with right-sided colonic involvement. In IBD, several studies demonstrated significant aberrancies in the intestinal microbiota in comparison with healthy controls. We aimed to explore the link between IBD and PSC by studying the intestinal mucosa-adherent microbiota in PSC and ulcerative colitis (UC) patients and noninflammatory controls.

Methods: We included 12 PSC patients, 11 UC patients, and nine noninflammatory controls. The microbiota composition was determined in ileocecal biopsies from each patient by 16S rRNA-based analyses using the human intestinal tract chip.

Results: Profiling of the mucosa-adherent microbiota of PSC patients, UC patients, and noninflammatory controls revealed that these groups did not cluster separately based on microbiota composition. At the genus-like level, the relative abundance of uncultured Clostridiales II was significantly lower (almost 2-fold) in PSC (0.26 ± 0.10%) compared with UC (0.41 ± 0.29%) and controls (0.49 ± 0.25%) (p = 0.02). Diversity and richness in the microbiota composition differed across the groups and were significantly lower in PSC patients compared with noninflammatory controls (p = 0.04 and p = 0.02, respectively). No significant differences were found in evenness.

Conclusions: Reduced amounts of uncultured Clostridiales II in PSC biopsies in comparison with UC and healthy controls can be considered a signature of a compromised gut, as we have recently observed that this group of as yet uncultured Firmicutes correlates significantly with health.

Keywords: Primary sclerosing cholangitis; inflammatory bowel disease; intestinal microbiota

1. Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease of unknown cause characterized by inflammation of the intra- and extrahepatic bile ducts, leading to obstruction, fibrosis, and liver cirrhosis. The estimated median survival time until death after liver transplantation is 21.3 years, with cholangiocarcinoma as the most common cause of death.1,2 PSC is highly associated with inflammatory bowel disease (IBD), with prevalence rates ranging from 67% to 73%.3 PSC–IBD patients represent a distinct phenotype of IBD, with a preponderance of pancolitis in PSC–ulcerative colitis (UC) patients and almost invariably colonic involvement in PSC–Crohn’s disease (CD) patients.4,5 Hence, PSC is mainly associated with right-sided colonic involvement.
Several studies demonstrated significant aberrancies in the intestinal microbiota of IBD patients in comparison with healthy controls. These included strong reductions in specific signature microbial species (notably *Faecalibacterium prausnitzii* in CD and *Akkermansia muciniphila* in UC) as well as lower overall species richness and lower diversity of members of the *Clostridium* cluster IV in mucosal biopsies and fecal samples from IBD patients. In PSC, bacterial translocation from the gut to the liver is hypothesized to play a major role in disease development, and from animal studies it is known that bacterial translocation in rats induces PSC-like changes. Multi-tagged pyrosequencing analyses of stool samples from patients with liver cirrhosis showed progressive changes in the gut microbiome characterized by a significant increase in *Clostridiales XIV* (Ruminococcaceae and Lachnospiraceae) and a decrease in Enterococcaceae, Staphylococcaceae, and Enterobacteriaceae compared with controls. To date, there are no data available on the intestinal microbiota in cholestatic liver diseases, apart from cirrhotic liver disease. Conflicting data exist with regard to the uniform intestinal microbiota in cholestatic liver diseases, apart from cirrhotic liver disease. The interplay of microbe and host occurs along the mucosa of the gastrointestinal tract. Therefore, if an aberrant microbiota plays a role in the link between the gut and the liver in PSC–IBD, the right side of the colon seems the most appropriate target for investigation of the mucosal microbiota in PSC patients.

In this study we explored the link between IBD and PSC by studying the intestinal mucosa-adherent microbiota in PSC patients, UC patients, and noninflammatory controls. We expected to see specific microbes in PSC patients that differed from those in IBD patients and noninflammatory controls.

### 2. Materials and methods

#### 2.1 Subjects and sampling of mucosal biopsies

Patients with PSC, patients with UC, and noninflammatory controls were included between January 2011 and June 2013. The study had a cross-sectional design. We included PSC patients with concomitant IBD and UC patients scheduled for surveillance colonoscopy. Subjects who were scheduled for colonoscopy for diagnostic purposes were included as noninflammatory controls. Diagnosis of PSC was based on the following: (1) clinical presentation (i.e., pruritus), pain in the right upper abdominal quadrant, fatigue, weight loss, and episodes of fever; and/or (2) elevated alkaline phosphatase and γ-glutamyl transferase, not otherwise explained; (3) presence of characteristic bile duct changes with multifocal strictures and segmental dilations on endoscopic retrograde cholangiography or magnetic resonance cholangiography; and/or (4) liver histology; and (5) no evidence of secondary sclerosing cholangitis. Criteria 2 and 3 were considered mandatory for PSC, and criterion 4, where available, confirmed the diagnosis. When criteria 2, 4, and 5 were fulfilled in the absence of cholangiographic abnormalities on magnetic resonance cholangiography or endoscopic retrograde cholangiography, cases were diagnosed as small-duct PSC. To assess the prognosis of PSC, the Child–Pugh classification was determined at the time of endoscopy. Diagnosis of IBD was confirmed by previous pathology and endoscopy reports compatible with IBD based on the Lennard-Jones criteria. Subjects included as noninflammatory controls were asked to participate in the study if they had a normal alkaline phosphatase level and did not have any form of gastro-intestinal inflammatory disease. Endoscopy was performed for diagnostic purposes in these subjects, such as suspicion of colorectal carcinoma due to rectal bleeding or surveillance of polyposid lesions.

According to routine, all patients received bowel lavage with polyethylene glycol solution before colonoscopy. Mucosal biopsies were taken from the ascending colon and from the terminal ileum. Samples were collected in sterile tubes and snap-frozen in liquid nitrogen directly, stored at −80°C and transferred to −80°C within 4 months.

A flowchart of inclusion of subjects, biopsy sampling and statistics is shown in Figure 1.

#### 2.2 Microbiota analyses

DNA from the biopsy specimens was isolated as previously described and used for phylogenetic profiling using the Human Intestinal Tract Chip (HITChip), a phylogenetic microarray containing over 5000 probes based on 16S rRNA gene sequences of 1140 bacterial phylotypes from the human intestine. Analyses were performed as previously described with minor modifications in the 16S rRNA gene PCR to obtain optimal amplification. In short, 16S rRNA genes were amplified using the *T7prom-Bact-27-for* and *Prok-1369-rev* primers. The latter primer was used for better performance.
due to the overabundant human DNA in the biopsy samples. The 16S rRNA gene amplicons were subsequently transcribed into RNA in vitro, labeled with Cy3 and Cy5, then fragmented and hybridized in duplicate. Data were extracted from the microarray-scanned images using Agilent Feature Extraction software version 10.7.3.1 (http://www.agilent.com) and array normalization was performed using a set of R scripts (http://r-project.org) in combination with a custom-designed relational MySQL database (http://www.mysql.com). Duplicate hybridizations with a Pearson correlation >98% were considered for further analysis.

2.3 Sample size calculation

Relevant differences in the abundance of bacterial groups and microbiota composition can be demonstrated in relatively small numbers of subjects; changes in the microbiota in time were determined in a group of five subjects in a study of the long-term monitoring of fecal microbiota composition.19 By applying adequate statistical methods with correction for the false discovery rate, inclusion of approximately 10 subjects per study group was considered to be sufficient to demonstrate relevant differences in the composition of the mucosal microbiota.

2.4 Statistical analysis

Data were expressed as the mean and 95% confidence interval (CI) of the mean or, when they were not normally distributed, as the median and interquartile range (IQR). To test whether ileal and colonic samples from the same patient differed in microbiota composition, a similarity index was calculated for each patient using the Pearson correlation. The Shannon index was used to calculate the diversity of mucosal biopsies. The Kruskal–Wallis test was used to establish significant differences for nonparametric data, such as comparisons of relative abundance at the genus-like level, and analysis of variance was used for parametric data, such as the diversity index across groups. Pairwise comparisons were used to identify (when possible) the origins of differences, such as the Mann–Whitney U test or the T test. We corrected p values for the false discovery rate (the expected proportion of false discoveries amongst the rejected hypotheses), which was calculated on the 130 genus-like comparisons over the three study groups using R, applying the Benjamini–Hochberg–Yekutieli method, which controls the false discovery rate.20 Statistical significance was accepted at p < 0.05. Statistical analyses were performed using SPSS v. 19.0 software (Chicago, IL) and R (package DTComPair).

2.5 Ethical considerations

The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center Amsterdam prior to the start of the study (METC registry number 2009_059). All patients signed an informed consent form.

3. Results

3.1 Characteristics of the study group

Bacterial DNA was successfully extracted from 32 colonic mucosal biopsies and 29 available terminal ileum biopsies from 12 PSC patients, 11 UC patients and nine noninflammatory controls. Clinical characteristics of PSC and UC patients are shown in Table 1. As mentioned in the inclusion criteria, all PSC patients had concomitant IBD, mostly classified as UC in eight patients and as CD in four patients. Seven PSC–UC patients (88%) had a pancolitis and one patient (12%) had a left-sided colitis. Two PSC–CD patients had colonic disease localization and two had localization of disease in both the terminal ileum and colon. The Child–Pugh class was low in the majority of patients; none of the included PSC patients had overt cirrhosis. Two patients were jaundiced, which was confirmed by an elevated serum bilirubin level. All IBD patients included in the study had UC; 10 of these patients (91%) had pancolitis and one patient had left-sided colitis (9%). Non-inflammatory controls had a medical history of polyposis coli (n = 2), diabetes mellitus (n = 1), Parkinson’s disease (n = 1), neurofibromatosis (n = 1), or prostate cancer (n = 1); the remaining three patients had a blank medical

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the study population.</th>
<th>PSC patients</th>
<th>UC patients</th>
<th>Noninflammatory controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 12)</td>
<td>(N = 11)</td>
<td>(N = 9)</td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>10 (83)</td>
<td>9 (82)</td>
<td>7 (78)</td>
<td>1.0</td>
</tr>
<tr>
<td>Age, years (median, IQR)</td>
<td>29.5 (23–56)</td>
<td>50.0 (37–67)</td>
<td>65.0 (50–70)</td>
<td>0.017</td>
</tr>
<tr>
<td>IBD, n (%)</td>
<td>12 (100)</td>
<td>11 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD, n</td>
<td>4</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UC, n</td>
<td>8</td>
<td>11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Disease duration, years (median, range)</td>
<td>10 (2–38)</td>
<td>18 (1–27)</td>
<td>–</td>
<td>0.06</td>
</tr>
<tr>
<td>PSC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small duct, n (%)</td>
<td>1 (7)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Large duct, n (%)</td>
<td>11 (92)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Disease duration, years (median, range)</td>
<td>5 (2–12)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Child–Pugh class, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10 (83)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>2 (17)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>0 (0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDCA</td>
<td>9 (75)</td>
<td>0 (0)</td>
<td>–</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-Aminosalicylate, n (%)</td>
<td>8 (67)</td>
<td>8 (73)</td>
<td>–</td>
<td>0.53</td>
</tr>
<tr>
<td>Thiopurine, n (%)</td>
<td>3 (25)</td>
<td>5 (45)</td>
<td>–</td>
<td>0.40</td>
</tr>
<tr>
<td>Steroids, n (%)</td>
<td>2 (17)</td>
<td>0 (0)</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>Infliximab, n (%)</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>–</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Bold p-values indicate statistically significant differences.
history. None of these noninflammatory controls used immunosuppressive therapy. Patients and noninflammatory controls were not on antibiotic treatment within 4 weeks before endoscopy or at the time of endoscopy. The three study groups were not comparable according to age; PSC patients were significantly younger than noninflammatory controls ($p = 0.008$).

3.2 Macroscopic appearance of the ascending colon and terminal ileum

In the group of PSC patients, 21% had endoscopic disease activity. In three patients (one classified as PSC-CD and two as PSC-UC) inflammation in the ascending colon was found at endoscopy; two of them also had inflammation in the terminal ileum, which was considered as backwash ileitis in the patient diagnosed with PSC-UC. In the UC group, 18% of the patients had endoscopic disease activity in the ascending colon. Patients included in the control group were scoped for diagnostic purposes and did not have any signs of inflammation or suspicion of IBD at the time of endoscopy. No gastrointestinal adenomas or cancers were found in the noninflammatory controls. None of the PSC or UC patients was diagnosed with dysplasia before or at the time of surveillance endoscopy.

3.3 Microbiota analyses

The microarray datasets of ileal and colonic biopsies were acquired and hierarchically clustered based on the signal intensity of the distinct HITChip oligonucleotide probes. Ileal and colonic biopsy samples clustered together according to the subject they were sampled from Figure 2, with an average $r$ value of 0.89 ± 0.09. Results did not change after exclusion of two PSC non-IBD patients (all subjects: Pearson $r = 0.89$, SD 0.09), indicating a high correlation or similarity between samples. With a cutoff value of $r > 0.8$ (corresponding to a Pearson distance of 0.2; dotted line in Figure 2), all samples clustered by individual with the exceptions of PSC patients 1 and 32, UC patients 21, 27, and 35, and noninflammatory control 19, which was explained by the absence of ileal biopsy sampling in patients 21 and 35. Samples did not separate according to disease. Hierarchical clustering of the microbiota profiles was not influenced by type of IBD (CD or UC) or the presence of active macroscopic inflammation at the time of endoscopy (data not shown). In the subgroup of noninflammatory subjects ($n = 8$), in whom the potential effect of chronic inflammation due to IBD on the microbiota composition was absent, the average Pearson correlation for intra-individual comparisons was $r = 0.88 ± 0.13$. The correlation was very low ($r = 0.64$) in subject 19 in this group, an 82-year-old male known to have hyper-tension and hyperlipidemia. Similarly, intra-individual comparisons in the PSC and UC groups revealed high correlations, with average $r = 0.9$ ± 0.08 and 0.89 ± 0.08 for PSC and UC, respectively. These findings justified using pooled ileocecal biopsies for further analyses.

3.4 Diversity of microbiota in PSC, UC, and noninflammatory controls

The diversity of the microbiota was significantly lower in PSC patients compared with noninflammatory controls ($p = 0.04$). The Shannon index of diversity was 5.52 ± 0.41 in PSC, 5.66 ± 0.41 in UC, and 5.78 ± 0.33 in noninflammatory controls Figure 3A. Zooming in on the two components contributing to diversity (richness Figure 3B and evenness Figure 3C, representing the number and distribution of different species within study groups), evenness was 0.80 ± 0.04 in PSC patients, 0.80 ± 0.04 in UC patients, and 0.81 ± 0.04 in noninflammatory controls, and did not differ between groups. Richness was low in PSC patients (673 ± 187 phylotypes) compared with UC (746 ± 210) and noninflammatory controls (821 ± 175), and was significantly lower in PSC patients compared with noninflammatory controls ($p = 0.01$).

3.5 Abundance of different phyla

The relative abundances of the major phyla were assessed by calculating the percentages of the phylum/order taxa in the biopsies in each study group. In PSC, UC, and noninflammatory controls the main phyla were equally distributed and comparable to the fecal profiles observed in previous studies.23 Firmicutes was the most abundant phylum in ileocecal mucosa, accounting for approximately 69.42 ± 3.08, 68.45 ± 3.08, and 67.43 ± 2.80% of the probe signals in PSC, UC, and noninflammatory controls, respectively, followed by Bacteroidetes, accounting for 25.69 ± 3.38, 25.04 ± 3.47, and 26.27 ± 3.58%; Proteobacteria, accounting for 2.81 ± 0.18, 3.05 ± 0.29, and 3.92 ± 0.27%; and Actinobacteria, accounting for 1.50 ± 0.36, 3.01 ± 0.90, and 1.94 ± 0.53%. The relative abundances in ileocecal biopsies at phylum and genus-like levels are shown in the Supplemental Digital Content.

3.6 Abundance of genus-like bacterial groups

Comparison of the 130 hybridization signals corresponding to the genus-like bacterial groups (data shown in Supplemental Digital Content) showed that the relative abundance of uncultured Clostridiales II differed significantly between the study groups. This bacterial group was almost 2-fold less abundant in PSC patients (0.26 ± 0.10%) than in UC (0.41 ± 0.29%) and controls (0.49 ± 0.25%) ($p = 0.02$). Other genus-like level groups, including members of Clostridium clusters IV and XIVa, as well as Akkermansia sp. (Verrucomicrobia), frequently found to be more abundant in healthy controls in previous studies, did not differ significantly between our study groups.

4. Discussion

This study describes the intestinal microbiota in PSC patients compared with patients with UC, a type of IBD strongly related to the disease of interest, and a noninflammatory control group. At a genus-like level, the relative abundance of uncultured Clostridiales II was significantly lower in PSC patients compared with UC patients and noninflammatory controls. Furthermore, the mucosa-adherent microbiota at the ileocecal level in PSC patients showed significantly reduced diversity and richness. The HITChip is a well-established platform for probe-based microbiota profiling and has high reproducibility and accuracy. This method was applied successfully in many previous clinical studies.24 Chatelier et al. observed associations between Prevotella, uncultured Clostridiales I, and uncultured Clostridiales II and a high gene count, which has been linked to a potentially healthy metabolic phenotype.25 In this respect, reduced richness in our PSC cohort may be an indicator of a downgrading effect of chronic cholestatic liver disease on the microbiota. Reduced amounts of the uncultured Clostridiales II in PSC biopsies could be considered a signature for a compromised gut, which is further supported by recent observations that this group of as yet uncultured Firmicutes correlates significantly with health.26 Patients with IBD are at increased risk of developing colorectal carcinoma. Guidelines for surveillance state that UC patients undergo surveillance at 1- to 5-year intervals after a disease duration of >10 years or from the time of confirmed dysplasia.27 The risk of developing colorectal cancer in PSC-IBD patients is
10-fold higher than that in patients with UC only and surveillance is recommended annually from diagnosis in this group of patients.\(^{2,25}\) As pre-defined in our study protocol, IBD and PSC–IBD patients scheduled for surveillance colonoscopy were included in our study, which resulted in a younger PSC population compared with the UC control group, which may constitute a confounder.

No medicinal treatment with proven efficacy for PSC is available. Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, ameliorates symptoms of bile obstruction and the formation of bile stones by decreasing the cholesterol component of bile salts and is often prescribed in PSC patients (it was prescribed in 79% of our PSC study group). Several studies have been published on the effect of UDCA on clinical symptoms, liver function tests, and serum bile acid levels in PSC.\(^{26,27}\) However, bile acid composition in the gut of PSC patients remains uninvestigated, as does the effect of UDCA on fecal and mucosal bile acid composition.

Figure 2. Hierarchical cluster of samples from PSC patients, UC patients, and noninflammatory controls. Hierarchical cluster of ileal and colonic biopsies. Samples clustered together according to the subject they were sampled from. Samples did not separate according to disease.
Regarding the effect of bile acid on the microbiota, it has been recently reported that increased bile production (as a result of a high-fat diet) has a significant impact on the composition and activity of the intestinal microbiota. Duboc et al. suggested that altered bile acid transformation in the gut lumen resulting from the altered enzymatic activity of a disturbed microbiota in IBD can impair the anti-inflammatory effects of some forms of bile acids on the gut epithelial cells and could participate in the chronic inflammation loop of IBD. In PSC–IBD, chronic cholestasis may have an additional effect on the altered transformation of bile acids by dysbiosis of the microbiota. In addition, the composition of the microbiota itself was found to influence fecal bile acid concentrations. Vrieze et al. showed that reducing fecal microbial diversity in male subjects with metabolic syndrome by a course of a high-fat diet has a significant impact on the composition and activity of the intestinal microbiota.

Vrieze et al. showed that reducing fecal microbial diversity in male subjects with metabolic syndrome by a course of a high-fat diet has a significant impact on the composition and activity of the intestinal microbiota. This is the first observation of reduced amounts of uncultured Clostridiales II in PSC patients and external validation is now warranted. Results of microbiota profiling are dependent on several factors, such as sampling location, which may account for the differences in their findings compared with the present study. Patient material was retrieved according to a strict, predefined standardized operation procedure for biopsy sampling, preservation of the biopsies, DNA extraction, and HfChip analyses. We should be cautious when comparing our microbiota profiles with microbiota profiles of sigmoid biopsies preserved by a different method.

In conclusion, the mucosa-adherent ileocecal microbiota in PSC patients showed significantly reduced diversity and relative abundance of uncultured Clostridiales II compared with values in UC patients and noninflammatory controls. Recently, we observed a correlation with health and these Firmicutes, which could explain our findings in biopsies from PSC patients.

**Acknowledgments**

NGR, CYP, EGZ, and WMdV designed the study. NGR, KB, CYP, and GRD performed the research, HGH performed the laboratory work, and SF performed the statistical analyses. NGR, CYP, EGZ, WMdV, and SF drafted the paper. All authors critically reviewed the results and structured and reviewed the manuscript. The authors would like to thank all patients who participated in this study. Sources of funding: NGR, MLDS Grant 2011 (WO 11–17); WMdV, NWOSpineoza Grant 2008.

**Conflict of interest**

The funding sources did not have any role in the study. The authors declare that they have no conflict of interest with respect to this manuscript.

**References**


31. Kevans D, Tyler AD, Silverberg MS, et al. Characterization of the intestinal microbiome in ulcerative colitis patients with and without primary sclerosing cholangitis. In: Abstracts accepted to 2014 CCDWCASL Winter Meeting, A145. An abstract of this work was presented at Digestive Disease Week 2014, Chicago, USA.