Characterization of the gut microbiota in patients with primary sclerosing cholangitis compared to inflammatory bowel disease and healthy controls

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Abstract
Background Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease. Its etiology remains largely unknown, although frequent concomitant inflammatory bowel disease (IBD) hints towards common factors underlying intestinal and bile duct inflammation. Herein, we aimed to explore the relative abundance of fecal microbiota in PSC-IBD patients compared to IBD-only subjects and controls.

Methods and results We included 14 PSC-IBD patients, 12 IBD-only patients, and 8 healthy controls (HCs). A quantitative real-time PCR (qPCR) assay was used to determine a selection of bacterial phyla, families, and genera. Relative abundance of taxa showed that Bacteroidetes was the most abundant phylum among the patients with PSC-IBD (29.46%) and also HCs (39.34%), whereas the bacterial species belonging to the phylum Firmicutes were the most frequent group in IBD-only subjects (37.61%). The relative abundance of the Enterobacteriaceae family in fecal samples of PSC-IBD patients was similar to those with IBD-only, which was significantly higher than HCs (p value = 0.031), and thus, could be used as a PSC-IBD or IBD-only associated microbial signature.

Conclusions Our findings showed that intestinal microbiota composition in PSC-IBD patients was completely different from that of IBD-only patients. Further studies using large-scale cohorts should be performed to better describe the contribution of the gut microbiota to PSC pathogenesis with underlying IBD.

Keywords Gut microbiota · Primary sclerosing cholangitis · Inflammatory bowel disease · Ulcerative colitis · Real-time PCR

Introduction

Primary sclerosing cholangitis (PSC) is an immune-mediated, chronic cholestatic liver disease of unknown etiology, characterized by inflammation and fibrosis of the intra- and extrahepatic bile ducts [1]. It is a progressive disorder without effective medical treatment, which eventually leads to cirrhotic end-stage liver disease and represents a major risk factor for cholangiocarcinoma, which indicates the need for liver transplantation. However, PSC recurrence occurs in up to 20% of patients after liver transplantation, and the median survival time of a patient is estimated to be about 20 years [2, 3]. The pathogenesis of PSC has long served as a controversial point of debate, with current evidence suggesting that the exposure of genetically predisposed individuals to environmental antigens elicits an aberrant immune response, leading to the development of the disease [4].
PSC is strongly associated with inflammatory bowel disease (IBD), in which 60–80% of the subjects with PSC have concomitant IBD, predominantly ulcerative colitis (UC) [5, 6]. PSC patients with underlying IBD display a distinct phenotype as compared to IBD alone, with a predominance of pancolitis in PSC-UC patients and invariably colonic involvement in PSC-Crohn’s disease (CD) patients [7, 8]. Hence, these data suggest that an inflamed colon, but not small bowel, is of importance in PSC development, and the disease is mainly associated with right-sided colonic involvement [7].

The association between PSC and IBD could in part be explained by shared immune-related genetic susceptibility [9]. Accordingly, immune-related gene polymorphisms, particularly genetic variations in inflammatory pathways, associated with both PSC and IBD, may increase the abundance of the deleterious intestinal microbiota capable of translocating from the gut to the liver. Moreover, some immune-related variants enhance the sensitivity of the biliary epithelium to bacterial antigens, leading to chronic inflammation and fibrosis [10]. However, genetic predisposition has only clarified a fraction of disease risk, signifying that environmental factors could also play a major role in the disease development. Among potential contributing environmental factors, alternations in the gut microbiota composition, i.e., intestinal dysbiosis, are hypothesized to have an important role in the disease process as they mediate intestinal inflammatory responses by local effects and hepatic inflammation through the enterohepatic circulation of bacterial antigens [11]. In addition, a few small clinical trials of antibiotics on PSC have indicated that both vancomycin and metronidazole can reduce alkaline phosphatase in patients with PSC, however, so far not proven clinically effective [12, 13]. Moreover, some evidence previously revealed that biliary histological and cholangiographical abnormalities found in animal models of small intestinal bacterial overgrowth (SIBO) resemble features of PSC [11]. Collectively, these data suggest that manipulation of the gut microbes could be directly involved in the pathogenesis of PSC as well as PSC-IBD.

Alterations in the diversity and composition of the gut microbiota have lately been implicated in the pathogenesis of several inflammatory and metabolic gut disorders, particularly IBD and PSC. However, to the best of our knowledge, no data are available on the gut microbiota composition among Iranian patients with PSC-IBD so far. Therefore, the current study aimed to compare the structure of the gut microbiota of Iranian patients with PSC-IBD compared to patients with IBD-only without liver disease as well as healthy controls (HCs).

### Materials and methods

#### Study population

PSC patients with concomitant IBD, IBD-only patients, and non-IBD HCs were involved from February 2018 to July 2019. The Institutional Ethical Review Committee approved the study’s protocol at the Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU.RIGLD.REC.1395.140). All experiments were performed following the relevant guidelines and regulations recommended by the institution and informed consents were obtained from all subjects and/or their legal guardians before sample collection. Patients with non-transplanted PSC referred to the Research Institute for Gastroenterology and Liver Diseases at Taleghani Hospital in Tehran, Iran, were eligible for the current study. The diagnosis of PSC was made according to accepted clinical, radiological, and pathological criteria including chronically elevated alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT); standard radiological evidence of PSC on endoscopic retrograde cholangiopancreatography (ERCP); and/or typical findings on liver histology consistent with PSC; as well as no evidence of secondary cholangitis [14]. All patients with PSC had also undergone screening for IBD. The IBD diagnosis was made by clinical, endoscopic, and pathological findings compatible with IBD based on the Lennard Jones criteria [15]. Patients who had been receiving antibiotics, ursodeoxycholic acid, corticosteroid, or immunosuppressant medications were excluded. Moreover, subjects with a previous orthotopic liver transplant, bowel resection, or on specific diets (e.g., vegan, vegetarian, gluten-free, and milk-free diets) were also excluded from this study. Additionally, patients with UC without a medical history of liver disorders and in clinical remission were recruited. HC subjects were randomly selected from individuals who were scheduled for surveillance colonoscopy for diagnostic purposes. Demographic data, medical history, clinical presentation, and medication use of all subjects enrolled in this study were recorded using a validated questionnaire. Laboratory biochemical parameters were retrieved from hospital databases, including total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, GGT, albumin, creatinine, platelets, international normalized ratio, as well as, perinuclear anti-neutrophil cytoplasmic antibody (P-ANCA) status. Mayo risk scores were calculated as a measure of disease severity using repeated measures longitudinal model [16]. A flowchart of inclusion of subjects and study design is shown in Fig. 1.
Sample collection and DNA extraction

Fresh stool samples were collected using Stool Collection Tubes via a standardized collection device from all participants after voiding. All stool samples were homogenized by vortexing and divided into aliquots within 3 h of defecation. The aliquots were immediately frozen and stored at −80 °C in screw-capped cryovial tubes until used for DNA extraction. Total DNA content was extracted from stool samples using the QIAamp DNA Stool Mini Kit (Qiagen Retsch GmbH, Hannover, Germany) according to the manufacturer’s instructions with some minor modifications. The concentration and purity of extracted DNA were assessed by NanoDrop ND-2000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA). Extracted DNA samples were stored at −20 °C until further analysis.

Taxonomic profiling of gut microbiota by quantitative real-time PCR

In the current study, a quantitative real-time PCR (qPCR) assay was performed for determining a selection of bacterial taxa (phyla, families, and genera) including Eubacteria, Firmicutes, Bacteroidetes, Actinobacteria, Gamma-proteobacteria, Epsilon-proteobacteria, Verrucomicrobia, Enterobacteriaceae, Clostridium cocoides group, Clostridium cluster I, Bifidobacterium, Lactobacillus, Enterococcus, and Staphylococcus. The qPCR was performed using universal and group-specific primers based on the bacterial 16S rRNA sequences and reaction conditions as described previously with minor modifications (Table 1). Each PCR reaction was carried out in a final volume of 25 μL, comprising of 12.5 μL of SYBR green PCR master mix (Ampliqon, Odense, Denmark), 1 μL of 10 pmol of forward and reverse primers, and 100 ng of the DNA template. The temperature profile for the amplification was 10 min at 95 °C, followed by 40 cycles of the 20 s at 95 °C, 30 s of annealing at optimal temperature for each primer pair as indicated in Table 1, and 20 s at 72 °C. All PCR amplifications were performed in triplicate by using a Rotor-Gene® Q (Qiagen, Germany) real-time PCR system. The specificity of amplification was determined by the melting curve analysis with increasing temperature from 60 to 95 °C (at a regular increment of 0.5 °C for 5 s). The relative abundance of each taxon among PSC-IBD patients as well as IBD-only and HC subjects was calculated according to the method described previously by Bacchetti et al. [17]. Accordingly,
the average Ct value obtained from each primer pair was transformed into a percentage using the following formula:

\[ X = \left( \frac{\text{Eff. Univ}}{\text{Eff. Spec}} \right)^{\text{Ct spec}} \times 100, \]

where the Eff. Univ refers to the calculated efficiency of the universal primers for Eubacteria (2 = 100% and 1 = 0%) and Eff. Spec indicates the efficiency of the taxon-specific primers. Ct uni and Ct spec represent the threshold cycles registered by the thermocycler. “X” addresses the percentage (%) of taxon-specific 16S rRNA gene copy number in an individual fecal sample.

## Results

### Baseline demographics and clinical characteristics of the subjects

In total, 14 PSC patients with concomitant IBD, 12 age, gender, and BMI-matched IBD-only (UC) patients without PSC, and 8 age, gender, and BMI-matched non-IBD HC
subjects were included in this study after exclusions and data quality control. PSC-IBD group in our study classified as UC in 12 patients (85.7%) and as CD in two patients (14.3%). None of the included PSC-IBD patients had overt cirrhosis. All PSC-UC patients had pancolitis, one PSC-CD patient had colonic disease localization, and one had localization of disease in both the terminal ileum and colon. Ten UC patients (83.3%) had pancolitis and two patients had left-sided colitis (16.7%). Non-IBD HC subjects had a blank medical history and none of them used immunosuppressive therapy. PSC-IBD patients, UC patients, and non-IBD HCs were not exposed to therapeutic antibiotic treatment during the last 4 weeks before fecal sample collection. Baseline demographics and clinical characteristics of the cohorts are presented in Table 2.

Relative abundance of analyzed microbiota

Analysis of the relative abundance of taxa using the 16S rRNA qPCR assay showed that Bacteroidetes was the most abundant phylum among the patients with PSC-IBD (29.46%) and HCs (39.34%), whereas the bacterial strains belonging to the phylum Firmicutes were the most frequent group detected in IBD-only patients (37.61%). Our findings also showed that the relative abundance of the Enterobacteriaceae family in fecal samples of PSC-IBD patients was similar to those with IBD-only, which was significantly higher than HCs (p value = 0.031). Interestingly, when performing a relative comparison of the investigated taxa between PSC-IBD and IBD-only cohorts, three genera including Enterococcus, Lactobacillus, and Bifidobacterium had different abundances in PSC-IBD and IBD-only and were observed to be enriched in IBD-only patients.

The relative abundance and mean percentage of each bacterial group among PSC-IBD and IBD-only patients, as well as HCs, are shown in Figs. 2 and S1. The distribution of the explored bacterial phyla and genera among PSC-IBD and IBD-only patients, as well as HCs, is illustrated in Fig. 3. The PCA also revealed that taxonomic profiles were notably different in the microbial communities of IBD-only patients compared to PSC-IBD patients and HCs, as schematically described in Fig. 4.

Determination of Firmicutes/Bacteroidetes ratio

Our measurements of the Firmicutes/Bacteroidetes ratio achieved by qPCR indicated that this ratio was significantly higher in IBD-only subjects compared to both PSC-IBD patients (p value = 0.0013) and HCs (p value = 0.01). Moreover, this ratio was lower, albeit not significantly, in PSC-IBD patients compared to HCs (p value = 0.56). Our analysis also showed that the Firmicutes/Bacteroidetes ratio was significantly higher among IBD-only patients than both PSC-IBD and HCs (Fig. 5).

Discussion

In this study, fecal samples of 14 patients with PSC-IBD, 12 subjects with IBD-only, and 8 HCs from an academic medical center in Tehran, Iran, were analyzed to compare the relative abundance of the gut microbiota. As main results, we could not demonstrate a consistently significant difference in the microbial abundance, at the phylum level, in PSC-IBD cohorts compared to HCs. However, our results showed that family Enterobacteriaceae was significantly more abundant in patients with PSC-IBD and IBD-only compared to HCs.

One of the important observations in this report was that the estimated relative abundances of fecal microbiota were found to be similar between the PSC-IBD cohorts and HCs, which were significantly different from the IBD-only patients. These results are in contrast with earlier findings showing a significant difference in the gut microbiota of patients with PSC compared with controls [10, 18], however, they are in accordance with those indicating gut microbiota differences between PSC-IBD and IBD-only patients [18–20]. These data suggest a strong effect of other factors such as host genetic determinants, ethnic background, and diet on the fecal microbiome composition. Additionally, as the enrolled patients in this investigation were from different geographical districts and ethnicities, it is suggested that the geographical distance of patients may remarkably influence the gut microbiota composition [21, 22].

In our study, altered abundances of few bacterial groups contributed to the unique gut microbial signature found in PSC-IBD patients compared to HCs, including a marked enrichment of the species belonging to the Enterobacteriaceae family and a reduction in phylum Verrucomicrobia and Clostridium cocoides group. Recently, Nakamoto et al. have described that the pathobiont community of the Enterobacteriaceae family is associated with intestinal barrier dysfunction, liver inflammation, and progressive fibrotic conditions in PSC patients [23]. Several authors previously described that the members of the Enterobacteriaceae family are often observed in the microbiota of individuals with hepatobiliary diseases, such as primary biliary cholangitis (PBC) and liver cirrhosis [24, 25]. Recently, Nakamoto et al. identified Klebsiella pneumoniae and Proteus mirabilis in the microbiota of patients with PSC and demonstrated that these bacterial strains disrupt the intestinal epithelial barrier to increase the permeability and bacterial translocation, inducing strong hepatobiliary inflammatory responses [23]. Moreover, it has been long demonstrated that most biliary infections...
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in patients with obstructive biliary disorders are caused by the members of Enterobacteriaceae such as Escherichia coli, K. pneumoniae, and P. mirabilis [26–28]. In a study conducted by Pohl et al., it was found that members of the Enterobacteriaceae family were detected in the bile specimens of 40% of PSC patients with dominant stenosis, suggesting an important role of these bacteria in the progression of PSC [29]. Although such data suggest that the human gut Enterobacteriaceae pathobionts have a significant role in the IBD pathogenesis [30], the evident

Table 2 Baseline demographic and clinical characteristics of subjects enrolled in this study

| Patients characteristics | PSC-IBD (n = 14) | IBD-only (UC) (n = 12) | HC (n = 8) |
|--------------------------|------------------|-----------------------|-----------|
| Gender                   |                  |                       |           |
| Female (%)               | 5 (35.7)         | 3 (25)                | 3 (37.5)  |
| Male (%)                 | 9 (64.3)         | 9 (75)                | 5 (62.5)  |
| Age (years)              |                  |                       |           |
| Median age ± SD          | 41.07 ± 12.3     | 43 ± 11.9             | 35.8 ± 7.53 |
| Median age at diagnosis of PSC | 34 ± 11.9 | NA                    | NA        |
| Median BMI (kg/m²)       | 23.45            | 24.1                  | 21        |
| Smoker status (%)        |                  |                       |           |
| Never                    | 13 (92.9)        | 12 (100)              | 7 (87.5)  |
| Ever                     | 1 (7.1)          | 0                     | 1 (12.5)  |
| Unknown                  | 0                | 0                     | 0         |
| Type of IBD              |                  |                       |           |
| UC/IBD                   | 12 (85.7)        | 12 (100)              | 0         |
| CD/IBD                   | 2 (14.3)         | 0                     | 0         |
| Disease-specific variables|                |                       |           |
| IBD duration, median years | 15.5 (4–31)    | 8 (2–12)              | NA        |
| PSC disease duration (years ± SD) | 7 (1–10) | NA                    | NA        |
| PSC mayo score, median years (IQR) | 0.5 (1.6–2.1) | NA                    | NA        |
| Medication at the time of colonoscopy | | | |
| Unknown                  | 0                | 0                     | 0         |
| 5-ASA (%)                | 2 (14.3)         | 3 (25)                | NA        |
| UDCA (%)                 | 4 (28.6)         | 0                     | NA        |
| Anti-TNF (%)             | 1 (7.1)          | 0                     | NA        |
| Corticosteroids (%)      | 2 (14.3)         | 4 (33.3)              | NA        |
| Immunosuppression (%)    | 2 (14.3)         | 1 (8.3)               | NA        |
| PPI                      | 0                | 3 (25)                | NA        |
| Underlying conditions    |                  |                       |           |
| Cirrhosis                | 0                | 0                     | NA        |
| Pouch                    | 0                | 0                     | NA        |
| Liver transplantation    | 0                | 0                     | NA        |
| Laboratory parameters    |                  |                       |           |
| Platelet count, 10⁹/L, median (min–max) | 220 (57–590) | 242 (189–472) | 250 (195–320) |
| Creatinine, mmol/L, median (min–max) | 0.8 (0.7–1.1) | 0.7 (0.5–1.8) | 0.67 (0.4–0.8) |
| Albumin, g/L, median (min–max) | 3.9 (3.2–4.3) | NA                    |          |
| CA 19–9                  | 4.9 (0.6–35.7)   | 1.1 (0.9–4.2)         | NA        |
| AST (UI/L) median (min–max) | 24 (16–74)   | 17 (10–46)            | NA        |
| ALP (UI/L) median (min–max) | 23 (12–227)   | 20 (8–171)            | NA        |
| Alkaline phosphatase (UI/L) | 208 (132–363) | 177 (61–335)          | NA        |
| Total bilirubin, mmol/L, median (min–max) | 0.1 (0.1–13) | 0.5 (0.2–1.8) | NA        |
| Direct bilirubin, mmol/L, median (min–max) | 0.3 (0.1–1.1) | 0.2 (0.1–7) | NA        |

PSC primary sclerosing cholangitis, IBD inflammatory bowel disease, BMI body mass index, UC ulcerative colitis, CD Crohn’s disease, 5-ASA 5-aminosalicylic acid, UDCA ursodeoxycholic acid, PPI proton pump inhibitor, CA-19–9 carbohydrate antigen 19–9, AST aspartate aminotransferase, ALT alanine aminotransferase, NA not applicable
association between overgrowth of the Enterobacteriaceae pathobionts and PSC warrants further investigations.

Our qPCR analysis revealed that while the relative abundance of the fecal microbiota of patients with IBD without PSC was different from HCs in our study, as reported by others [31], the IBD-only cohort also showed a different microbial abundance from PSC patients with concomitant IBD. Kummen et al. previously described that the occurrence of IBD could not influence the gut microbiota composition in PSC-IBD patients, signifying that patients with PSC exhibit a gut microbial signature distinct from IBD [18].

In our study, the Firmicutes/Bacteroidetes ratio was analyzed among cohorts and it was significantly higher among IBD-only patients than both PSC-IBD and HC cohorts. Interestingly, there was no significant difference in the Firmicutes/Bacteroidetes ratio between PSC-IBD patients and HCs. On the contrary, some previous studies indicated...
a relative depletion of *Firmicutes*, such as *Faecalibacterium* and *Coprococcus* in PSC patients [32]. Moreover, the *Firmicutes*/Bacteroidetes ratio could describe the degree of dysbiosis in IBD. Some authors previously reported that this ratio is significantly reduced in both forms of IBD, in both inflamed and normal mucosa, and regardless of treatment [33, 34]. Contrastingly, our analysis revealed that the *Firmicutes*/Bacteroidetes ratio was significantly increased among IBD patients compared to both PSC-IBD and HC cohorts. These controversial findings might be explained by differences in the characteristics of study participants (especially host genetic determinants, ethnic background, and diet), employing of varied microbiome assessment approaches, and the small number of patients enrolled in this study resulting in reduced statistical power.

The close association between PSC and IBD could propose a common microbial landscape or inflammatory pathway that initiates and develops intestinal and hepatic inflammation. Accumulating evidence generated from animal model studies has suggested that alteration of gut microbiota is associated with PSC development [35]. However, our study indicated no significant microbial differences between PSC-IBD patients and HCs. The data is in accordance with those reported by Kevans et al., in which the absence of a strong microbial association with PSC-IBD was found [10]. Although it might reflect a true lack of differential microbial contributions in PSC-IBD, however, could also be due to the limited sample size and technical factors. Recruiting an adequate number of homogeneous PSC and control cohorts is commonly a challenging issue due to the low prevalence of PSC worldwide. On the contrary, Sabino et al. reported a unique microbial signature of three bacterial genera in patients with PSC, independent from that of healthy controls and IBD patients, suggesting the intestinal microbiota could be a contributing factor in PSC pathogenesis [20]. Altogether, these data suggest the need for further investigations to assess the potential role of the gut microbiota in PSC-IBD.

**Conclusion**

In conclusion, this study characterized fecal microbiota in PSC-IBD patients compared to IBD-only patients and also HCs using a qPCR-based approach. Overall, our study showed no significant alterations in the gut microbiome composition of PSC-IBD compared to the HC cohort. However, the relative abundance of the *Enterobacteriaceae* family was higher than HCs and thus, could be used as a PSC-IBD or IBD-only associated microbial signature. Furthermore,
our microbial analysis indicated that the fecal microbiota composition in IBD-only patients was completely different from that of PSC-IBD patients and HCs. Importantly, some limitations could be noted in our study. For instance, this study was limited by small sample size. Another important limitation in our study is that we used qPCR analysis to investigate the relative abundance of the gut microbiota, in which several microbial taxa were not explored, and also, we were not able to identify diversity in the gut microbiota of cohorts. Further large-scale metagenome prospective studies with a more refined characterization of the gut microbiota should be performed to describe the contribution of the gut microbiota to PSC pathogenesis.

**Fig. 4** Bacterial community clustering and variations in microbial community composition represented in a principal component analysis (PCoA). Patients with PSC-IBD and IBD-only are significantly different, while PSC-IBD and healthy control subjects are almost similar. Percentage values in parentheses next to Dim1 and Dim2 represent the percentage of variance explained by each component. Arrows show the contribution of each type of microbiota on Dim1 and Dim2. Each data point denotes an individual patient, colored based on their group. (Color figure online)
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Author contributions  SO, MA participated in experimental work, qPCR assays, and data collection. EJ extracted the DNA from fecal samples. KN and AY participated in data analysis and interpretation. HH and AY drafted the manuscript. AY and HM critically revised the manuscript. AS, HAA, and MRZ participated in intellectual input and clinical consultation. All authors approved the final version of the manuscript and the authorship list.

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Data availability  The datasets supporting the conclusions of this work are included within the article.

Declarations

Conflict of interest  The authors declare no conflicts of interest.

Ethical approval  Ethical clearance for conducting this study was obtained from the Institutional Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences (Project No. IR.SBMU. RIGLD.REC.1395.140).

Informed consent  Informed consent was obtained from all individual participants included in the study before sample collection.

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