Namisaki Tadashi (Orcid ID: 0000-0002-3158-5318)

Gut dysbiosis associated with clinical prognosis of patients with primary biliary cholangitis

Masanori Furukawa[1], Kei Moriya[1], Jiro Nakayama[2], Takako Inoue[3], Rie Momoda[2], Hideto Kawaratani[1], Tadashi Namisaki[1], Shinya Sato[1], Akitoshi Douhara[1], Kosuke Kaji[1], Mitsuteru Kitade[1], Naotaka Shimozato[1], Yasuhiko Sawada[1], Soichiro Saikawa[1], Hiroaki Takaya[1], Koh Kitagawa[1], Takemi Akahane[1], Akira Mitoro[1], Junichi Yamao[1], Yasuhito Tanaka[3,4], Hitoshi Yoshiji[1]

[1]Department of Gastroenterology, Nara Medical University; [2]Laboratory of Microbial Technology, Division of Systems Bioengineering, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, [3]Department of Clinical Laboratory Medicine, Nagoya City University Hospital, [4]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Japan

Corresponding author: Kei Moriya

Division of Gastroenterology and Hepatology, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

Phone: +81-744-22-3051; Fax.: +81-744-24-7122

E-mail: moriyak@naramed-u.ac.jp

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/hepr.13509

Electronic word count: 3855 words.

Number of figures and tables: 5 figures, 2 supplementary figures, 2 tables, and 7 supplementary tables.

Abbreviations for figuresBMI: body mass index

gamma-GT: gamma-glutamyl transpeptidase

M2BPGi: Mac-2 Binding Protein (M2BP) Glycosylation isomer

UDCA: ursodeoxycholic acid

PPI: proton pomp inhibitor

PT-INR: prothrombin time expressed as international normalized ratio

ULN: upper limit of normal range

Conflict of interest statement: The authors declare no conflicts of interest.

Financial support statement: The present study was supported, in part, by a grant-in-aid from Zeria Pharmaceutical Co. Ltd., Taisho Toyama Pharmaceutical Co. Ltd., Chugai Pharmaceutical Co. Ltd., and Yakult Honsha Co. Ltd, and a grant-in-aid for research from Nara Medical University.

Running title: Gut dysbiosis in primary biliary cholangitis

Availability of data and materials: The majority of data used to support the findings of the present study are included within the article; the rest were registered with the website of DNA Data Bank of Japan. Sequence reads are archived (DRA008224) under BioProject no.

PRJDB8027, which contains links and access to stool sampling data under BioSamples SAMD00163123 to SAMD00163195.

Authors' contributions: Concept and design: MF, KM, YT, and HY. Clinical data collection: MF, TI, HK, TN, S Sato, AD, K Kaji, MK, NS, YS, S Saikawa, HT, K Kitagawa, TA, AM, and JY. Writing of the article: KM. Editing of the article: JN. Data analysis: JN and RM. All authors have seen and approved the final version of this manuscript.

Abstract

Background&Aims: Although some relationships between gut microbiota and liver diseases have been reported, it remains uncertain whether changes in gut microbiota owing to differences in race, food, and living environment have similar effects. Response to ursodeoxycholic acid (UDCA) may predict the long-term prognosis of patients with primary biliary cholangitis (PBC); however, little is known about the significance of the gut microbiome in PBC patients. We elucidated the relationships among clinical profiles, biochemical response to UDCA, and gut microbiome composition in PBC patients.

Methods: Fecal samples from 76 PBC patients treated at our hospital were collected; patients whose UDCA intake period was <1 year were excluded. The patient microbiome structures of the patients were determined using 16S ribosomal RNA gene sequencing and were statistically compared with those of healthy subjects. The structures of patients in the UDCA responder (n=43) and non-responder (n=30) groups were compared according to the Nara criteria (reduction rate of gamma-glutamyl transpeptidase≥69% one year after).

Results: Compared with healthy subjects, bacterial diversity was lower in PBC patients, with a decreased abundance of the order *Clostridiales* and increased abundance of *Lactobacillales*. The UDCA non-responder group had a significantly lower population of the genus *Faecalibacterium*, known as butyrate-producing beneficial bacteria (p<0.05), although no significant differences in gender, body mass index, medicated drugs, or other serological data were indicated between these two groups.

Conclusions: Gut dysbiosis with loss of beneficial *Clostridiales* commensals was observed in PBC patients. Decrease in *Faecalibacterium* abundance might predict the long-term prognosis of PBC patients.

Keywords: autoimmune liver disease, bile acid, enteric bacterial microflora, *Faecalibacterium*, proton pump inhibition

Study highlights

This study demonstrates that the gut microbiome of primary biliary cholangitis (PBC) patients well treated with ursodeoxycholic acid (UDCA) reflectes reduced species richness compared with healthy individuals. Additionally, the microbial dysbiosis in the UDCA responders in patients with PBC was found to be significantly more ameliorated than that in the UDCA non-responders. Based on these data, the gut microbiome could be a novel biomarker for predicting the long-term prognosis of PBC patients.

Introduction

Recently, some associations between various diseases and the gut microbiome have been reported, with general citizens now recognizing this importance [1]. In chronic liver diseases, especially liver cirrhosis, many types of intestinal bacteria and inflammasomes spread to the liver through the portal vein and are greatly involved in the vicious progression of disease [2,3]. In the case of so-called leaky gut, the tight junctions of intestinal epithelium become injured, allowing transmittance of intestinal bacteria, endotoxins, lipopolysaccharides, pathogen-associated molecular pattern-like bacterial DNA fragments, and some inflammatory cytokines to the liver. These factors finally cause sustained organ inflammation [4-6].

Primary biliary cholangitis (PBC) is a female-dominant autoimmune hepatic disease that typically develops in individuals aged ≥40 years. Although the major disease type is slow progressive cholestasis [7], some patients with PBC show portal hypertension during the early disease stage, and others rapidly show liver failure. Although the number of patients with PBC in Japan has increased to a level three times that seen in the past 15 years and possible related causes such as genetic, environmental, and infectious factors have been suggested to be related to PBC occurrence, a definite etiology of PBC has not yet been elucidated [8,9].

Ursodeoxycholic acid (UDCA) has established itself as the first-line treatment for PBC because it improves the biochemical indicators related to cholestasis and suppresses the progression of hepatic fibrosis, liver failure, and pathological re-progression after liver transplantation [10-12]. Additionally, several trials speculating the long-term prognosis in patients with PBC based on their responses to UDCA have been undertaken [13-15]. We have also reported on the efficacy of the "Nara criteria" in predicting the long-term prognosis of PBC patients based on changes in serum gamma-glutamyl transpeptidase (gamma-GT) levels [16]. In brief, patients in whom serum gamma-GT levels decreased by ≥69% following a one-year intake of UDCA were defined as responders, whereas those in whom serum gamma-GT levels decreased by

<69% were defined as non-responders. According to the Nara criteria, the survival rate of the non-responders was significantly lower than that of the UDCA responders.

Although various approaches from many aspects have been directed toward the elucidation of the pathogenesis of this disease, little is known about the significance of the gut microbiome in patients with PBC. Moreover, fundamental treatments for PBC have not yet been established. Since identification analysis of the gut microbiome has made great technical strides in the last few decades, it has become popular and easily available, directing researchers' interest toward digestive tract diseases, such as colon cancer and inflammatory bowel disease, and chronic liver disease, including liver cirrhosis. Consequently, "gut–liver axis" correlations between liver disease and the gut microbiome have been noted [2,3]; however, there are limited reports demonstrating a relationship between the gut microbiome and hepatic pathological mechanisms of PBC [17,18], and no report that describes a relationship between the gut microbiome and clinical pathological factors in PBC patients who have achieved sustained disease remission by long-term administration of UDCA exists. Consequently, reproducible findings with respect to the gut microbiome composition of patients with PBC have not been reported yet, partly because it has been reported that the gut microbiome composition varies greatly owing to race and lifestyle. Therefore, we report, for the first time, a correlation between gut microbiome evaluation and the response to UDCA, which is important in the clinical prognosis of patients with PBC.

Patients and Methods

Patients

In the current study, 23 healthy subjects and 76 PBC patients were enrolled (Fig. 1). The sampling from healthy subjects was performed in Japan from April to May 2016. The inclusion criteria were as follows: (i) Age > 50 years and (ii) Japanese race. The exclusion criteria were as follows: (i) positive for hepatitis C virus (HCV), hepatitis B virus (HBV) and/or human immunodeficiency virus (HIV), or other secondary organism infections; (ii) with some liver disease and/or diabetes mellitus; (iii) under the administration of hepatotoxic drugs; (iv) past medical history of liver disease; (v) consumption of unhealthy commodities including drugs; (vi) hospitalization for >3 days; (vii) prescription of lactulose, antibiotics, histamine H2-receptor antagonists, beta2 adrenergic receptor agonists, UDCA, and probiotics within 6 months of the sampling; and (viii) with decompensated cirrhosis, severe cytopenia, renal failure, heart failure, and pregnant or lactating women. Finally, we screened and included 23 healthy subjects.

A total of 76 patients with PBC were enrolled at the Nagoya City University Hospital (Aichi, Japan) and Nara Medical University Hospital (Nara, Japan) from August 2015 to September 2016. Clinical diagnosis of PBC was determined by histopathological findings of the liver and serum positivity of anti-mitochondrial antibodies. The inclusion criteria were as follows: (i) Age > 20 years, (ii) Japanese race, (iii) positive for serum anti-mitochondrial antibodies. The exclusion criteria were as follows: (i) positive for HBV, HCV and/or HIV, or other secondary organism infections; (ii) evidence of liver disease because of other etiology; (iii) under the administration of hepatotoxic drugs; (iv) individuals diagnosed with malignancies, or who underwent prior anti-cancer treatment; (v) consumption of unhealthy commodities (including drug abuse); (vi) hospitalization for >3 days; (vii) prescription of lactulose, antibiotics, and beta2 adrenergic receptor agonists within 6 months of the sampling; (viii) medical history as

liver transplant recipients; (ix) individuals with severe cytopenia, renal failure, heart failure, and pregnant or lactating women. Finally, we screened and included 76 PBC patients.

Collection and storage of samples were performed according to method described by Inoue et al., [19]. Written informed consent was obtained from each individual, and the study was approved by the ethical committee of Nara Medical University, Nagoya City University Hospital, and Faculty of Agriculture, Kyushu University in accordance with the Declaration of Helsinki.

DNA extraction

DNA extraction, preparation of 16S rRNA samplicons, MiSeq sequencing, and sequence data analysis were performed as described in a study by Inoue et al., [19]. Total bacterial DNA was isolated from stool samples by the bead-beating method followed by phenol extraction. The variable V1–V2 region of the 16S rRNA gene was amplified via polymerase chain reaction and subsequently subjected to high-throughput sequencing using the MiSeq paired-end sequencing system (Illumina Inc., San Diego, California).

Data analysis

The obtained sequences were processed using the Uparse pipeline in Usearch v9.2 and v10.0 [20]. Briefly, 5,854,209 pairs of sequences were merged, subjected to quality filtering and denoizing, and subsequently clustered into operational taxonomic units (OTUs). Eventually, 1289 non-singleton OTUs were acquired. The sequences of OTUs were taxonomically annotated using the Sintax command in the Usearch pipeline [21]. The read counts of each OTU in each sample (37,061±12,706 reads/sample) were tabulated using the cluster_otus command in Usearch and subsequently subsampled for a sequence depth of 10,000 using the single_rarefaction.py command in QIIME 1.9.1. [22]. The duplicate data of healthy subjects

were averaged and subjected to further analyses (OTU table included in Supplementary Table S1). Bacterial compositions at phylum to the genus levels were determined based on data in the OTU tables using the summarize_taxa_through_plots.py command in QIIME 1.9.1. (data were included in Supplementary Tables S2–6).

The sequences of OTUs were aligned and subjected to phylogenetic tree calculations in the QIIME pipeline. The weighted UniFrac distances were calculated based on the OTU table and the phylogenetic tree, and the beta diversity was visualized by principal coordinate analysis (PCoA) using beta_diversity_through_plots.py in the QIIME pipeline and/or ggplot2 package of R program. Significance of the difference in beta diversity between subject groups was calculated by using compare_categories.py program with adonis function in the QIIME pipeline. The alpha diversity, observed_OTUs, PD_whole_tree, and Shannon Wiener indices were calculated based on data in the OTU table using the alpha_rarefaction.py command in the QIIME pipeline. The values of ten iterations at a depth of 10,000 sequences were averaged for each sample and used for statistical analysis. LEfSe (linear discriminant analysis effect size) analysis was performed with a pipeline of Galaxy at the following link: http://huttenhower.sph.harvard.edu/galaxy/ [23].

Statistics

Statistical differences in the microbiome data were analyzed by the non-parametric Wilcoxon rank-sum test using R version 3.5.2. For the analyses among >2 groups, a Benjamini–Hochberg adjustment was applied.

Pyrosequencing data registration

The raw sequence data were deposited in the DNA Data Bank of Japan sequence read archive (DRA008224) under BioProject no. PRJDB8027, which contains links and access to stool sampling data under BioSamples SAMD00163123 to SAMD00163195.

Results

Patient characteristics

In total, 76 patients with PBC, including 14 males and 62 females, had a mean age (SD) of 66.0 (8.3) years. Demographic features and characteristics of PBC patients and healthy individuals are summarized in Table 1.

Gut microbiome analysis

The beta diversity of the fecal microbiome of healthy individuals and PBC patients is displayed in the weighted UniFrac PCoA plot (Fig. 2a). Healthy samples are clustered in upper-left area, whereas PBC samples are widely spread from the position of the healthy cluster to the lower-right area, indicating that gut microbiotas of PBC patients varied substantially, whereas those of healthy subjects were more consistent. Bacterial loads of three genera, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, are directed to the lower-right area, suggesting that the variation in PBC samples is associated with the increase in the number of organisms belonging to these genera in the PBC group. The relative abundance of all taxonomic groups was statistically compared at each taxonomic level between the healthy and PBC groups using linear discriminant analysis, and statistically abundant taxa in any group are shown in the phylogenetic cladogram (Fig. 2b). LEfSe demonstrates that the abundance of the families *Lachnospiraceae* and *Ruminococcaceae* of class *Clostridia* was high in the healthy group. The class *Clostridia* is known to be a highly diversified taxonomic class, including a large number of beneficial commensal species, notably butyrate-producing bacterial genera, such as *Faecalibacterium, Roseburia*, and *Anaerostipes*. Conversely, *Bifidobacterium* and three *Lactobacillales* genera, *Streptococcus*, *Lactobacillus*, and *Enterococcus*, were more abundant in the PBC group than in the healthy group. The alpha diversity was also compared between

the healthy and PBC groups (Fig. 2c). Observed_OTUs and Shannon-Weiner indices were statistically lower in the PBC group than in the healthy group, and the PD_whole_tree index was not statistically different but close to significance (p=0.057). The reduction in the alpha diversity, in addition to the decreased diversity of commensal *Clostridia*, is known to be a typical feature of gut dysbiosis [24-26].

Effects of PPI on gut dysbiosis

The abundance of *Streptococcus* was most remarkably increased in the PBC group, in which >10% of the total population belonged to the genus *Streptococcus* in certain PBC patients (Fig. 3). OTU7 identified as *Streptococcus salivarius* with a 100% confidence score in the taxonomic annotation, accounted for a major part of the *Streptococcus* population. *S. salivarius* is usually a member of oral bacteria and exists as a subdominant species in the colon. *Lactobacillus* in the PBC samples was also largely accounted for by *Lactobacillus salivarius*, which is also generally recognized as an oral species. These facts may suggest a less efficient gastric juice barrier in PBC patients, which may be caused by the anti-peptic ulcer drugs that are sometimes prescribed to patients with hepatitis or cirrhosis. To address this possibility, we compared the fecal bacterial composition between healthy individuals and PBC patients with or without PPI intake. Overgrowth of *Lactobacillales* and decreased abundance of *Clostridiales* were observed even in the PBC patients without PPI, suggesting that dysbiosis occurs regardless of the PPI intake. However, these alterations were more remarkable in the PPI-prescribed group, although not all these genera showed a statistical difference.

Response to UDCA and the difference of gut microbiome in patients with PBC

In the current study, the numbers of UDCA responders and non-responders classified by the Nara criteria were 43 and 30, respectively. There were no significant differences in the gender,

body mass index (BMI), UDCA dose and duration, PPI/probiotics intake proportion, or other serological data between these two groups (Table 2). Pertaining to patient comorbidities, Sjogren's syndrome and thyroid disorders were the most frequent diseases in each group, and the coexisting rate of these diseases in the UDCA responders were 4.7% and 14.0%, respectively and 3.3% and 3.3% in the non-responders. No significant differences in the coexisting rate of the diseases between the two groups.

To examine whether microbiota of the UDCA responders was different from those of the UDCA non-responders and progressed toward healthy controls, the alpha diversity was compared between these groups. Both the PBC groups showed statistically lower Shannon-Weiner index, which represents a measure of the richness and evenness of microbial diversity in given samples, compared to the healthy group, whereas they showed marginal differences in the number of observed OTUs and phylogenetic diversity index (PD whole tree) (Fig. 4a). No difference was observed in these alpha diversity indices between the UDCA responder and non-responder groups.

The beta diversity of the fecal microbiome of healthy individuals and PBC patients is also displayed in the weighted UniFrac PCoA plot (Fig. 4b). The UDCA non-responders were conspicuously spread from the position of the healthy cluster to the lower-right area (p=0.002 in Adonis test), whereas the UDCA responders tended to be somewhat closer to the healthy cluster (p=0.006), compared to the non-responders. Subsequently, we compared the gut microbiome composition among the healthy, responder, and non-responder groups (Fig. 5a). As described above, an increased abundance of the genera *Streptococcus* and *Lactobacillus* and a decreased abundance of the series of *Clostridiales* genera were observed in the PBC groups. The abundance of the genus *Faecalibacterium* showed a statistically significant difference between the responder and non-responder groups, decreasing from healthy individuals to responders and from responders to non-responders, suggesting that the genus *Faecalibacterium*

contributes to remission of PBC (Fig. 5b). Additionally, we analyzed the abundance of *Faecalibacterium* as a function of UDCA dose and -duration. There was no significant difference in the abundance of *Faecalibacterium* (data not shown). Taken together, the signatures found in the beta diversities of PBC patients appeared to be markers for the UDCA responsiveness.

Moreover, we analyzed the gut microbiome composition of PBC patients sorted by the Paris-2 criteria, which has a worldwide consensus for predicting the long-term prognosis in PBC patients [15]. In this study, the criteria, defined as alkaline phosphatase≤1.5 times the upper limit of the normal range and bilirubin≤1 mg/dL after one year of UDCA treatment, could be successfully assessed in 69 of the 73 patients (94.5%); 51 patients were classified as responders and the rest as non-responders (Supplementary Table S7). Age, gender, and BMI did not significantly differ between the two groups, but the PPI or fibrate user population was markedly higher in the non-responders. The contrast in their genus composition was mostly similar to that found in the Nara criteria grouping, with some exceptions; when grouped according to the Paris-2 criteria, the statistical significance in the abundance of the genus *Faecalibacterium* disappeared, whereas the abundance of *Prevotella* was significantly higher in the non-responder group than in the responder group (Supplementary Figure S1).

Discussion

Bile acid, the main component of bile, is known to cause various effects on gut permeability involving intestinal epithelial and innate immune cells and to play an important role in deciding the composition and bacterial mechanism of the gut microbiome [27-29]. From a pathogenetic point of view, gut microbiota analysis in patients with PBC is thought to be meaningful because inflammation in PBC patients generally destroys interlobular bile ducts and results in a chronic cholestatic change. Additionally, UDCA, the main therapeutic drug for PBC, is largely responsible for bile acid metabolism.

Therefore, in the current clinical study targeting 76 Japanese patients with PBC who had continuously taken UDCA for at least one year since the primary diagnosis of PBC or had been UDCA naïve, we sampled their feces to analyze the gut microbiome and examined the relationship between the clinicopathological features and gut microbiome diversity. Although all PBC patients in this study had been administered UDCA treatment for >12 months except three UDCA naïve cases and their hepatic functions had been relatively stable, we found that the diversity of their gut microbiome was significantly diminished compared with that in healthy controls in every analysis. Even though Tang et al., reported that a partial improvement in the gut microbiome occurred following UDCA administration in patients with PBC [18], we found that the composition of the gut microbiome in Japanese PBC patients who had been treated according to the medical guidelines for PBC was disrupted compared with that of the healthy controls.

As noteworthy results of the present study, a significant reduction in the diversity of the order *Clostridiales*, including species known as butyric acid-producing symbiotic bacteria such as the genus *Faecalibacterium* or species in the *Clostridium* cluster XIVa, was observed in PBC patients. Short-chain fatty acids (SCFAs), including butyric acid, are thought to be nutrients for intestinal epithelial cells, keeping the gut microenvironment healthy by regulating the intestinal

pH [6]. Moreover, it is generally known that SCFAs induce regulatory T cells (Tregs) in colonic mucosa and behave as suppressors of intestinal inflammation [30,31]. Among SCFAs, butyric acid in particular plays a pivotal role in the improvement of intestinal inflammation via inhibition of the transcription factor NF-κB, suppression of inflammatory cytokines such as IFN-γ and IL-12, and production of the anti-inflammatory cytokine IL-10 [32-34]. In other words, when *Clostridia* is less abundant, which typically constitutes the highest percentage of SCFA-producing bacteria, intestinal immune disorders occur, increased levels of various inflammatory cytokines induce intestinal hyperpermeability, and the gut microenvironment deteriorates. Furthermore, in the experimental animal hepatitis model induced by bile acid synthesis inhibitors (so-called dysregulated bile acid synthesis-induced hepatitis), butyrate administration has been shown to generate hepatic pathohistological improvements [35].

By the way, all the patients with PBC in this study were Japanese; therefore, they would have many similarities in their food custom and living environment. However, there are still certain clinical factors such as age, gender, and drugs that could affect the composition of the gut microbiome. Among these, anti-peptic ulcer drugs, such as PPI, are regarded as strong confounding factors [36,37] because PPI could diminish the sterilizing effect of the stomach and allow oral bacteria to enter the intraductal space, inducing gut dysbiosis. Therefore, we classified the PBC patients according to their medication history of these anti-peptic ulcer drugs and compared their microbiome structures. Consequently, we found that gut dysbiosis represented by the decrease in the abundance of order *Clostridiales* and the increase of *Lactobacillales* was observed regardless of PPI administration. Although we cannot deny the possibility that these patients had taken PPIs until more than a year ago, which altered their gut microbial community, it is noticeable that the gut microbiota of these PBC patients was chronically suffering from dysbiosis independent of PPI administration. However, the data showing that the alteration of the microbial community was more remarkable in patients with

PPI use suggest that PPIs promote the recruitment of these oral commensals to the colon and may exacerbate the clinicopathological progression of PBC.

Subsequently, we conducted a study on the relationship between the clinical prognosis of PBC patients and their gut microbiome. First, the Nara criteria proposed in 2017 by our group were an excellent way to predict the long-term prognosis of PBC patients by assessing their treatment response prior to and following UDCA medication [16]. In the current study, we divided the PBC patients into two groups according to the Nara criteria, UDCA responder and non-responder groups, and compared their gut microbiomes. There were no significant between-group differences in clinically relevant parameters, such as UDCA dose and -duration or age, gender, or serum levels of hepatobiliary enzymes, all of which were recently reported implicated in the response to UDCA [38]; however, a definite decrease in the proportion of organisms belonging to the genus *Faecalibacterium* (one of the butyrate-producing bacteria) in the non-responder group was observed. Because *Faecalibacterium prausnitzii* is abundantly present in the gut and is very useful in maintaining a favorable gut microenvironment, this bacterium has been brought into the spotlight in recent years [39]. Particularly in patients with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, the proportion of *Faecalibacterium* is significantly decreased compared with healthy controls, and this bacterium is expected to be a potential biological indicator in the diagnosis of inflammatory bowel disease and judgment of the therapeutic effect [40,41]. It has previously been reported that *F. prausnitzii* enhances the expression of tight junction proteins, promotes the production of mucin-type O-glycans by affecting the mucus pathway, and further improves intestinal permeability and gut barrier functions [42-44]. Based on these definitive findings, the pathophysiology of PBC, including its clinical prognosis, is thought to be affected by the proportion of *Faecalibacterium* in the gut.

Recently, it has been reported that *F. prausnitzii* is significantly reduced in patients with type

B cirrhosis and is also associated with the pathology of NAFLD [45,46]. In a few reports on the relationship between this bacterium and PBC, Tang et al., have reported that the UDCA naive PBC patients have a lower proportion of *F. prausnitzii* in their gut microbiome compared with healthy volunteers, and the subgroup that is positive for anti-gp210 antibodies (reported as an index of poor prognosis) has a lower proportion of *F. prausnitzii* in their gut microbiome compared with the other subgroup, in which anti-gp210 antibodies are negative [18].

To increase the scope of this study, demonstration of longitudinal microbiotic changes following UDCA treatment in selected treatment-naïve PBC patients would have significantly helped in our understanding. It would clarify whether the abundance of genus *Faecalibacterium* before UDCA treatment or changes in the abundance by UDCA treatment determine the long-term prognosis of UDCA treatment. Concerning this issue, Tang et al [18] reported that the *Faecalibacterium* abundance actually decreased in UDCA-naïve Chinese patients with PBC, but they also demonstrated no longitudinal changes in the abundance of *Faecalibacterium* when comparing measurements before and after UDCA treatment. In contrast, Pearson et al [47] recently demonstrated using a microbial network analysis method that patients continuously treated with UDCA experienced compositional changes in their gut microbiome mainly with an overrepresentation of *Faecalibacterium prausnitzii*, and these occurred with no evidence of UDCA effect on microbial richness in their retrospective cohort study in which more than 400 participants with pervious history of colorectal adenoma were continuously observed. In this retrospective study, there was no significant difference of the abundance of genus *Faecalibacterium* in the UDCA-naïve patients compared with that in healthy controls (Supplementary Fig. S2). This result can likely be attributed to the insufficient number of available fecal samples of treatment-naïve PBC patients in this study. On the other hand, there was a distinct increase in the abundance of genus *Faecalibacterium* after UDCA treatment even in the patient classified as non-responder by the Nara criteria, (data not shown).

Based on these findings, the original abundance of *Faecalibacterium* in UDCA-naïve patients with PBC might determine not only the abundance of *Faecalibacterium* in UDCA-treated patients with PBC, but also the response to UDCA treatment in patients with PBC, potentially exerting an impact on the long-term prognosis of PBC patients. To establish much stronger evidence, longitudinal variation analyses of the gut microbial community, especially within individuals who were positive for anti-gp210 antibodies and generally regarded as a rapidly progressive type of PBC, are expected.

Prior to presenting our conclusion to this article, in which the relevance between the clinical prognosis of the PBC patients and their gut microbiome was investigated, we analyzed the data using both the Nara and Paris-2 criteria, and the latter has been widely accepted worldwide [15]. According to the results of the Paris-2 criteria, although the proportion of the genus *Prevotella* was significantly increased in the non-responder group compared with that in the responder group, the reduction in the *Faecalibacterium* population in the non-responders was not significant. Because some background bias of clinical factors, such as a medicated ratio with PPIs, may affect these results, assessing of more cases is necessary to deduce definite outcomes in the future.

Although we believe that this study accurately demonstrates novel and beneficial findings that the long-term prognosis of PBC patients treated with UDCA can be predicted by their gut microbiome composition, several limitations need to be raised. First, the present study included a relatively smaller population of advanced liver fibrotic cases smaller as well as healthy controls because majority of participants had been in a stable disease condition under appropriate UDCA administration, and validation by another study is needed, in which the population of not only patients with liver cirrhosis but also healthy controls is more abundant for obtaining an improved data set with increased statistical power. Second, other potential factors influencing the outcome, such as drugs other than anti-peptic ulcer drugs and probiotics,

current medical history, and past medical history, could not be eliminated from the analysis. Third, these results originated from only Japanese patients, and their diet, customs, and habitual circumstances were not assimilated.

Despite these limitations, we successfully demonstrated that the PBC patients administered with UDCA still have gut dysbiosis, which would affect their clinical prognosis. These novel findings will be useful in constructing a long-term treatment strategy for patients with PBC. To verify our results universally, a large-scale cohort study is desired in the future.

Acknowledgments

We thank Prof. Hiroshi Fukui for his kind and appropriate advice in the completion of this article.

References

1) Marchesi JR, Adams DH, Fava F, et al. The gut microbiota and host health: a new clinical frontier.

Gut 2016; 65: 330-39.

2) Qin N, Yang F, Li A, et al. Alterations of the human gut microbiome in liver cirrhosis.

Nature **2014**; 513:59-64.

3) Tripathi A, Debelius J, Brenner DA, et al. The gut-liver axis and the interaction with the microbiome.

Nat Rev Gastroenterol Hepatol. **2018**; 15: 397-411.

4) Fukui H. Gut-liver axis in liver cirrhosis: How to manage leaky gut and endotoxemia.

World J Hepatol. **2015**; 27:425-42.

5) Fukui H. Increased Intestinal Permeability and Decreased Barrier Function: Does It Really Influence the Risk of Inflammation?

Inflamm Intest Dis. **2016**; 1: 135-45.

6) Giordano DM, Pinto C, Maroni L, et al. Inflammation and the Gut-Liver Axis in the Pathophysiology of Cholangiopathies.

Int J Mol Sci. **2018** 1; 19: E3003.

7) Carey EJ, Ali AH, Lindor KD. Primary biliary cirrhosis.

Lancet **2015**; 386: 1565-75.

8) Corpechot C, Chretien Y, Chazouilleres O, et al. Demographic, lifestyle, medical and familial factors associated with primary biliary cirrhosis.

J Hepatol. **2010**; 53:162-9.

9) Julan BD, Lazaridis KN. Environmental factors in primary biliary cirrhosis.

Semin Liver Dis **2014**; 34:265-72.

10) Angulo P, Batts KP, Therneau TM. Long-term ursodeoxycholic acid delays histological

progression in primary biliary cirrhosis.

Hepatol. **1999**; 29: 644-7.

11) Pares A, Caballeria L, J Rodes. Excellent long-term survival in patients with primary biliary cirrhosis and biochemical response to ursodeoxycholic acid.

Gastroenterol. **2006**; 130: 715-20.

12) Bosch A, Dumortier J, Maucort BD, et al. Preventive administration of UDCA after liver transplantation for primary biliary cirrhosis is associated with a lower risk of disease recurrence.

J Hepatol. **2015**; 63: 1449-58.

13) Corpechot C, Abenavoli L, Rabahi N, et al. Biochemical response to ursodeoxycholic acid and long-term prognosis in primary biliary cirrhosis.

Hepatol. **2008**; 48: 871-7.

14) Kuiper EM, Hansen BE, de Vries RA, et al. Improved prognosis of patients with primary biliary cirrhosis that have a biochemical response to ursodeoxycholic acid.

Gastroenterol. **2009**; 136: 1281-7.

15) Corpechot C, Chazouilleres O, Poupon R. Early primary biliary cirrhosis: biochemical response to treatment and prediction of long-term outcome.

J Hepatol. **2011**; 55: 1361-7.

16) Namisaki T, Moriya K, Noguchi R, et al. Liver fibrosis progression predicts survival in patients with primary biliary cholangitis.

Hepatol Res. **2017**; 47: E178-86.

17) Lv LX, Fang DQ, Shi D, et al. Alterations and correlations of gut microbiome, metabolism and immunity in patients with primary biliary cirrhosis.

Environ Microbiol. **2016**; 18: 2272-86.

18) Tang R, Wei Y, Li Y, et al. Gut microbial profile is altered in primary biliary cholangitis

and partially restored after UDCA therapy.
Gut **2018**; 67: 534-41.
19) Inoue T, Nakayama J, Moriya K, et al. Gut Dysbiosis Associated With Hepatitis C Virus Infection. Clin Infect Dis. **2018**; 67:869-77. doi: 10.1093/cid/ciy205.
20) Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods **2013**; 10:996-8. [Pubmed: 23955772, dx.doi.org/10.1038/nmeth.2604].
21) Edgar, R.C. SINTAX, a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. Biorxiv **2016**; http://dx.doi.org/10.1101/074161.
22) Kuczynski J, Stombaugh J, Walters WA, et al. Using QIIME to analyze 16S rRNA gene sequences from microbial communities.
Curr Protoc Microbiol **2012**; Chapter 1: Unit 1E.5.
23) Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol **2011**; 12: R60.
24) Chen Y, Yang F, Lu H, et al. Characterization of fecal microbial communities in patients with liver cirrhosis.
Hepatology **2011**; 54:562-72.
25) Kakiyama G, Pandak WM, Gillevet PM, et al. Modulation of the fecal bile acid profile by gut microbiota in cirrhosis.
J Hepatol. **2013**; 58:949-55.
26) Wright G, Jalan R. Ammonia and inflammation in the pathogenesis of hepatic encephalopathy: Pandora's box?
Hepatology **2007**; 46:291-4.
27) Salonen A, de Vos WM. Impact of diet on human intestinal microbiota and health.
Annu Rev Food Sci Technol. **2014**; 5:239-62.
28) Vavassori P, Mencarelli A, Renga B, et al. The Bile acid receptor FXR is a modulator of intestinal innate immunity.

J Immunol. **2009**; 183: 6251-61.

29) Cipriani S, Mencarelli A, Chini MG, et al. The Bile acid receptor GPBAR-1(TGR5) modulate s integrity of intestinal barrier and immune response to experimental colitis.

PLoS One **2011**; 6: e25637.

30) Furusawa Y, Obata Y, Fukuda S, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells.

Nature **2013**; 504:446-50.

31) Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous Clostridium species.

Science **2011**; 331:337-41.

32) Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients.

Proc Natl Acad Sci USA **2008**; 105: 16731-6.

33) Inan MS, Rasoulpour RJ, Yin L, et al. The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line.

Gastroenterol. **2000**; 118: 724-34.

34) Qin J, Li R, Raes J, Arumugam M, et al. A human gut microbial gene catalogue established by metagenomic sequencing.

Nature **2010**; 464(7285): 59-65.

35) Sheng L, Jena PK, Hu Y, et al. Hepatic inflammation caused by dysregulated bile acid synthesis is reversible by butyrate supplementation.

J Pathol. **2017**; 243: 431-41.

36) Imhann F, Bonder MJ, Vich VA, et al. Proton pump inhibitors affect the gut microbiome.

Gut **2016**; 65:740-8.

37) Clooney AG, Bernstein CN, Leslie WD, et al. A comparison of the gut microbiome between

long-term users and non-users of proton pump inhibitors.

Aliment Pharmacol Ther. **2016**; 43: 974-84.

38) Chung SW, Lee JH, Kim MA, et al. Additional fibrate treatment in UDCA-refractory PBC patients.

Liv Int. **2019**; 00:1-10.

39) Lopez-Siles M, Duncan SH, Garcia-Gil LJ, et al. Faecalibacterium prausnitzii: from microbiology to diagnostics and prognostics.

ISME J. **2017**;11 :841-52.

40) Lopez-Siles M, Martinez-Medina M, Suris-Valls R, et al. Changes in the abundance of Faecalibacterium prausnitzii phylogroups I and II in the intestinal mucosa of inflammatory bowel disease and patients with colorectal cancer.

Inflamm Bowel Dis. **2016**; 22: 28-41.

41) Fujimoto T, Imaeda H, Takahashi K, et al. Decreased abundance of Faecalibacterium prausnitzii in the gut microbiota of Crohn's disease.

J Gastroenterol Hepatol. **2013**; 28: 613-19.

42) Carlsson AH, Yakymenko O, Olivier I, et al. Faecalibacterium prausnitzii supernatant improves intestinal barrier function in mice DSS colitis.

Scand J Gatroenterol. **2013**; 48: 1136-44.

43) Martin R, Miquel S, Chain F, et al. Faecalibacterium prausnitzii prevents physiological damages in a chronic low-grade inflammation murine model.

BMC microbiol. **2015**; 15: 67-78.

44) Wrzosek L, Miquel S, Noordine ML, et al. Bacteroides thetaiotaomicron and Faecalibacterium prausnitzii influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent.

BMC Biol. **2013**; 11: 61-73.

45) Lu H, Wu Z, Xu W, et al. Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. Intestinal microbiota of HBV cirrhotic patients.

Microb Ecol. **2011**; 61:693-703.

46) Schwenger KJP, Chen L, Chelliah A, et al. Markers of activated inflammatory cells are associated with disease severity and intestinal microbiota in adults with non-alcoholic fatty liver disease.

Int J Mol Med. **2018**; 42:2229-37.

47) Pearson T, Caporaso JG, Yellowhair M, et al., Effects of ursodeoxycholic acid on the gut microbiome and colorectal adenoma development.

Cancer Med. **2019**; 8: 617-28.

Table 1

Demographic features and clinical characteristics of patients with PBC and healthy controls

	PBC (n=76)	Healthy (n=23)
Age(years)	66.0±8.3	60.5±8.1*
Gender(M/F)	14/62	14/9*
BMI	23.1±3.6	23.1±3.4
UDCA dose (mg)	560±171	n.d.
Bezafibrate user (%)	14.5	n.d.
PPI user (%)	26.3	n.d.
H2 blocker user (%)	6.6	n.d.
Probiotics user (%)	9.2	n.d.
Platelet (×10000/mm2)	19.1±6.6	n.d.
PT-INR	1.05±0.08	n.d.
Albumin (g/dL)	4.2±0.4	n.d.
AST (IU/L)	36.5±34.5	n.d.
ALT (IU/L)	27.6±28.6	n.d.
gamma-GT (IU/L)	71.1±91.3	n.d.
Alkaline phosphatase (IU/L)	367.5±173.6	n.d.
Total bilirubin (mg/dL)	0.9±1.1	n.d.
Cholinesterase (IU/L)	308.4±90.6	n.d.
Total cholesterol (mg/dL)	192.6±33.8	n.d.
M2BPGi (C.O.I)	1.23±1.49	n.d.
ACA positive (%)	37.1	n.d.

Continuous data are expressed as the mean±standard deviation.

Asterisk indicates a significant difference ($p<0.01$), as analyzed using the Mann-Whitney U test.

Table 2 Demographic features and clinical characteristics of PBC patients classified by Nara criteria

	Responders (n=43)	Non-responders (n=30)	Naïve (n=3)	p
Age(years)	65.8±8.6	66.0±7.9	68.0±2.8	n.s.
Gender(M/F)	6/37	7/23	1/2	n.s.
BMI	23.2±3.4	23.3±3.7	18.9±1.7	n.s.
UDCA dose (mg)	593±121	570±142	0±0	n.s.
UDCA duration (days)	2206±2149	2051±1580	0±0	n.s.
Bezafibrate user (%)	11.6	20.0	0.0	n.s.
PPI user (%)	23.3	33.3	0.0	n.s.
H2 blocker user (%)	9.3	3.3	0.0	n.s.
Probiotics user (%)	7.0	13.3	0.0	n.s.
Platelet (×10000/mm2)	19.8±6.7	18.9±6.7	11.4±3.5	n.s.
PT-INR	1.05±0.10	1.07±0.10	1.00±0.02	n.s.
Albumin (g/dL)	4.1±0.4	4.2±0.4	4.4±0.1	n.s.
AST (IU/L)	28.8±11.6	47.8±50.9	33.0±11.3	n.s.
ALT (IU/L)	21.7±11.8	36.0±41.7	29.0±12.8	n.s.
gamma-GT (IU/L)	54.1±47.9	85.1±122.4	175.7±106.8	n.s.
Alkaline phosphatase (IU/L)	357.4±128.5	367.5±215.5	512.3±200.7	n.s.
Total bilirubin (mg/dL)	0.7±0.3	1.1±1.7	0.7±0.1	n.s.
Cholinesterase (IU/L)	304.6±92.9	315.0±115.1	294.3±30.1	n.s.
Total cholesterol (mg/dL)	194.8±34.8	189.9±32.3	189.3±32.3	n.s.
M2BPGi (C.O.I)	0.90±0.40	1.70±2.10	0.70±0.38	n.s.
ACA positive (%)	38.5	36.7	0.0	n.s.
Continuous data are expressed as the mean±standard deviation. n.s.: not significant				
No statistical significance was seen in the comparison between the Responders and the Non responders.				

Fig.1

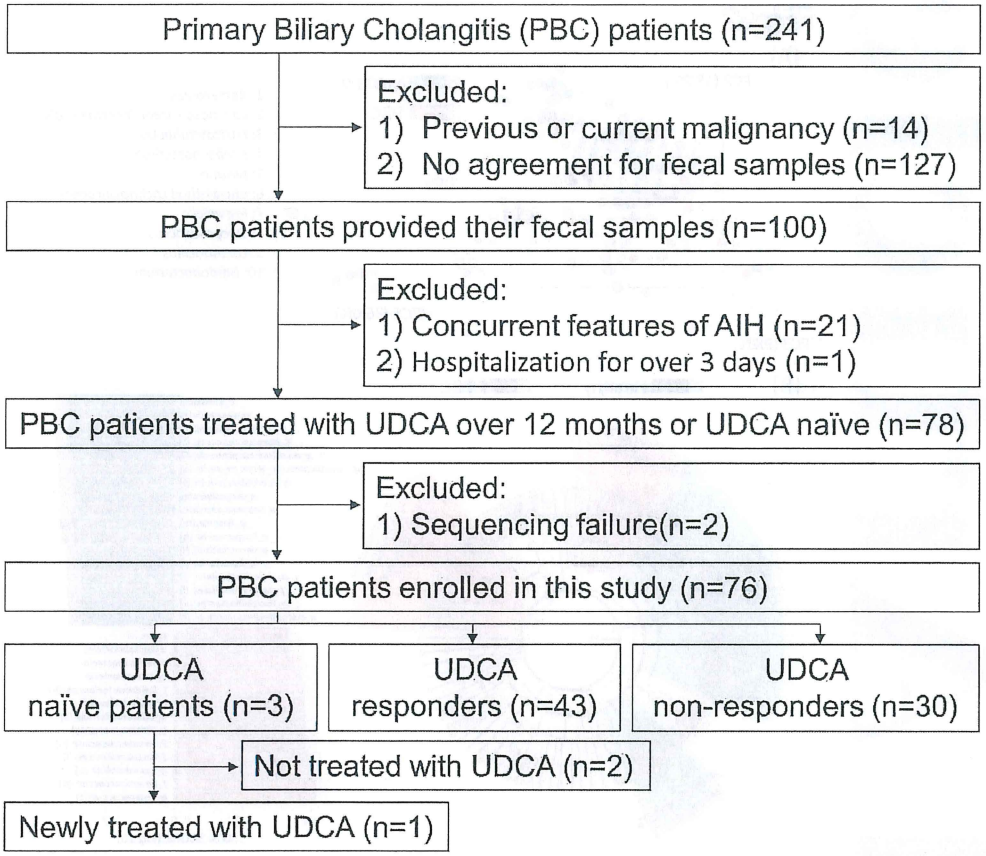

Fig. 1. Flowchart of PBC patient enrollment. PBC patients except UDCA naïve cases were divided into the two different subgroups according to the Nara criteria (reduction rate of gamma-GT ≥69% one year after).

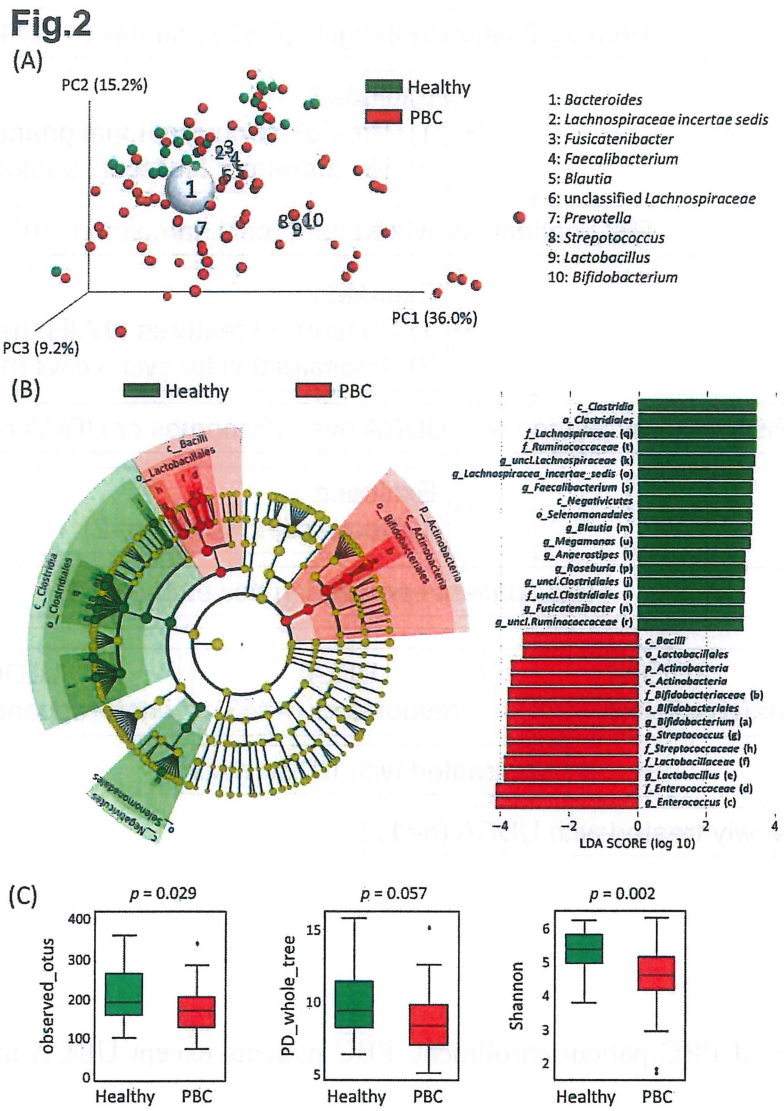

Fig. 2. Comparison of the fecal microbiome structures between healthy individuals and PBC patients. (A) Principal coordinate analysis based on weighted UniFrac distances of fecal 16S rRNA gene profiles in the PBC patients and healthy subjects. Position and size of spheres with the ten most abundant genera. representing their loading in the coordination and abundance in the total population, respectively. (B) LEfSe analysis showing the bacterial taxa that statistically differ in their abundance between the healthy and PBC groups. (C) Boxplot of the alpha diversity indices estimated for the healthy and PBC samples.

Fig.3

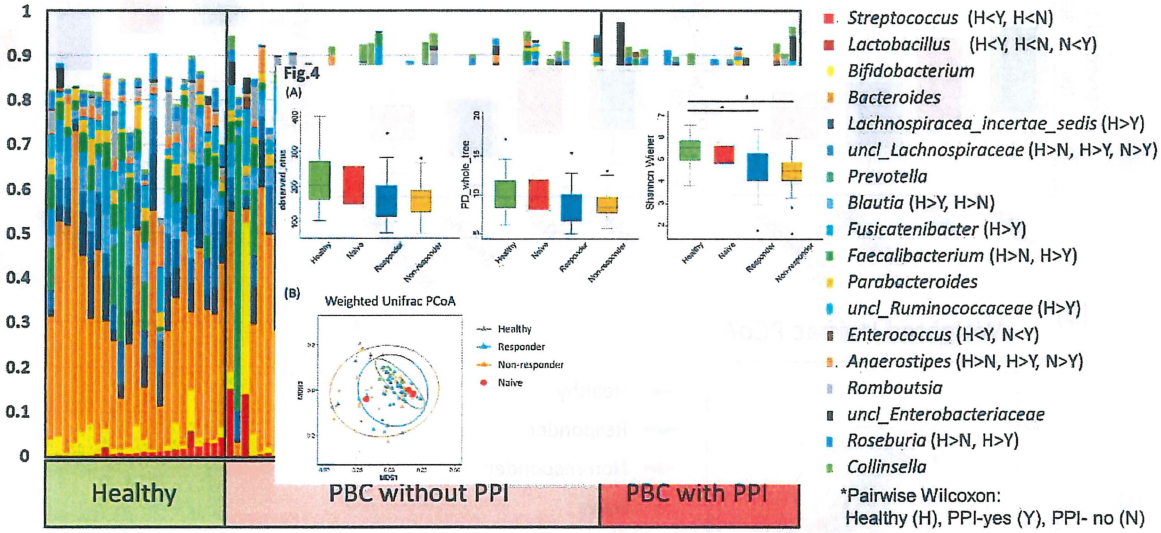

Fig. 3. Genus composition of fecal samples from healthy individuals and PBC patients with and without PPIs. Alphabet and sign of inequality in parentheses beside each genus name represent the pair of groups showing statistical significance in their abundance. The statistical differences were tested by the pairwise Wilcoxon test with a Benjamini–Hochberg adjustment.

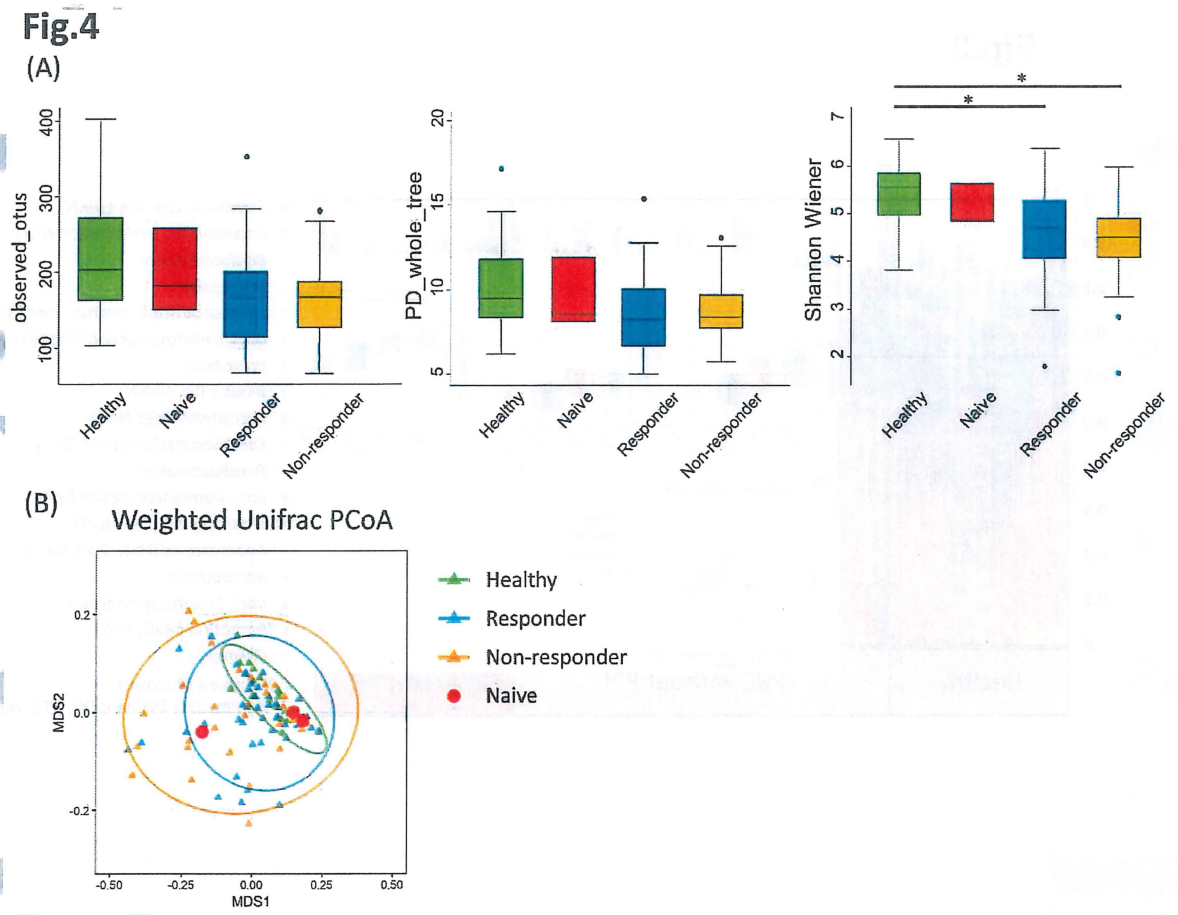

Fig. 4. Comparison of the fecal microbiome structures between UDCA responders (n=43), nonresponders (n=30), UDCA naïve patients (n=3), and healthy individuals (n=23). (A) Boxplot of the alpha diversity indices estimated for the healthy and PBC samples classified by the UDCA response. Statistical difference was tested by the pairwise Wilcoxon rank-sum test with a Benjamini–Hochberg adjustment, and the results are represented by a single asterisk for $p<0.05$. (B) Principal coordinate analysis based on weighted UniFrac distances of fecal 16S rRNA gene profiles in the UDCA responders, nonresponders, and healthy subjects. The UDCA nonresponders were conspicuously spread from the position of the healthy cluster to the lower-right area ($p=0.002$ in Adonis test), whereas the UDCA responders tended to be somewhat closer to the healthy cluster ($p=0.006$).

Fig.5

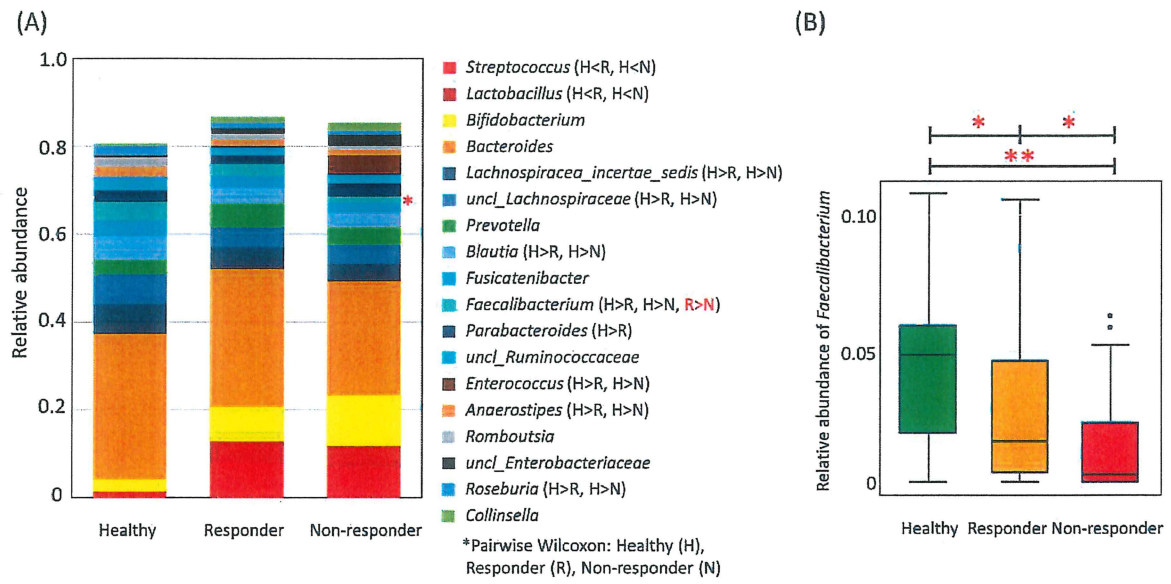

Fig. 5. Comparison of the microbiome structures among healthy, UDCA responder, and nonresponder groups in the Nara criteria. (A) Relative abundance of genera averaged in each group. Red asterisk shows genus with a statistical difference in the abundance between the responder and nonresponder groups. Alphabet and sign of inequality in parentheses beside each genus name represent the pair of groups showing statistical significance in their abundance. (B) Comparison of the relative abundance of *Faecalibacterium* among the three groups. Statistical difference was tested by the pairwise Wilcoxon rank-sum test with a Benjamini–Hochberg adjustment, and the results are represented by a single asterisk for $p<0.05$ and a double asterisk for $p<0.001$.