BASIC AND TRANSLATIONAL—BILIARY

Altered Gut Microbial Metabolism of Essential Nutrients in Primary Sclerosing Cholangitis

Martin Kummen,[1,2,3,4,*] **Louise B. Thingholm**,[5,*] Malte C. Rühlemann,[5] Kristian Holm,[1,2,3]
Simen H. Hansen,[1,2,3] Lucas Moitinho-Silva,[5,6] Timur Liwinski,[7,8] Roman Zenouzi,[8]
Christopher Storm-Larsen,[1,2,3] Øyvind Midttun,[9] Adrian McCann,[9] Per M. Ueland,[9]
Marte L. Høivik,[2,10] Mette Vesterhus,[1,11,12] Marius Trøseid,[2,13] Matthias Laudes,[14]
Wolfgang Lieb,[15] Tom H. Karlsen,[1,2,3,16] Corinna Bang,[5] Christoph Schramm,[8,17,18]
Andre Franke,[5,§] and Johannes R. Hov[1,2,3,16,§]

[1]Norwegian Primary Sclerosing Cholangitis Research Center, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway; [2]Institute of Clinical Medicine, University of Oslo, Oslo, Norway; [3]Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway; [4]Department of Oncology, Oslo University Hospital, Oslo, Norway; [5]Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, Kiel, Germany; [6]Department of Dermatology, Venereology and Allergy, University Hospital Schleswig-Holstein, Kiel, Germany; [7]Institute of Medical Systems Biology, Center for Molecular Neurobiology, Hamburg, Germany; [8]I. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [9]Bevital, Bergen, Norway; [10]Department of Gastroenterology, Oslo University Hospital Ullevål, Oslo, Norway; [11]Department of Medicine, Haraldsplass Deaconess Hospital, Bergen, Norway; [12]Department of Clinical Science, University of Bergen, Bergen, Norway; [13]Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, Oslo, Norway; [14]Division of Endocrinology, Diabetes and Clinical Nutrition, Department of Medicine 1, University of Kiel, Kiel, Germany; [15]Institute of Epidemiology, Christian-Albrechts-University of Kiel, Kiel, Germany; [16]Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway; [17]Martin Zeitz Centre for Rare Diseases, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and [18]Hamburg Center for Translational Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

BACKGROUND & AIMS: To influence host and disease phenotype, compositional microbiome changes, which have been demonstrated in patients with primary sclerosing cholangitis (PSC), must be accompanied by functional changes. We therefore aimed to characterize the genetic potential of the gut microbiome in patients with PSC compared with healthy controls (HCs) and patients with inflammatory bowel disease (IBD). **METHODS:** Fecal DNA from 2 cohorts (1 Norwegian and 1 German), in total comprising 136 patients with PSC (58% with IBD), 158 HCs, and 93 patients with IBD without PSC, were subjected to metagenomic shotgun sequencing, generating 17 billion paired-end sequences, which were processed using HUMAnN2 and MetaPhlAn2, and analyzed using generalized linear models and random effects meta-analyses. **RESULTS:** Patients with PSC had fewer microbial genes compared with HCs ($P < .0001$). Compared with HCs, patients with PSC showed enrichment and increased prevalence of *Clostridium* species and a depletion of, for example, *Eubacterium* spp and *Ruminococcus obeum*. Patients with PSC showed marked differences in the abundance of genes related to vitamin B6 synthesis and branched-chain amino acid synthesis ($Q_{fdr} < .05$). Targeted metabolomics of plasma from an independent set of patients with PSC and controls found reduced concentrations of vitamin B6 and branched-chain amino acids in PSC ($P < .0001$), which strongly associated with reduced liver transplantation–free survival (log-rank $P < .001$). No taxonomic or functional differences were detected between patients with PSC with and without IBD. **CONCLUSIONS:** The gut microbiome in patients with PSC exhibits large functional differences compared with that in HCs, including microbial metabolism of essential nutrients. Alterations in related circulating metabolites associated with disease course, suggesting that microbial functions may be relevant for the disease process in PSC.

Keywords: Microbiome; Primary Sclerosing Cholangitis; Vitamin B; Branched Chain Amino Acids.

P rimary sclerosing cholangitis (PSC) is a chronic progressive cholestatic liver disease of unknown etiology, characterized by multifocal inflammation and fibrosis of the biliary tree.[1] Up to 80% of patients with PSC are diagnosed with concomitant inflammatory bowel disease (IBD), thus PSC is a prototypical disease of the gut–liver axis.

Compositional changes in the fecal bacterial microbiome of patients with PSC have been reported in several cross-sectional studies.[2–5] Changes are also present in pediatric patients,[6] but so far there are few established links between the gut microbiome and severe disease or late-stage disease like cirrhosis.[7] There are also disturbances in other

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Previous studies have identified large compositional differences in the gut microbiome of PSC patients and controls.

NEW FINDINGS

We identify several species-level differences in addition to large functional differences in the genetic composition of the gut microbiome between PSC patients and controls, especially genes related to microbial metabolism of essential nutrients, which could potentially be linked to disease progression.

LIMITATIONS

Cohort size, although the largest study of the microbiome in PSC utilizing full metagenomic sequencing published so far. No inclusion of samples from other liver diseases as additional disease controls, or matched blood and stool samples.

IMPACT

Altered functional status of the microbiome in PSC may be relevant for the disease course, and could thus also potentially be a new treatment target.

important members of the microbiome, like fungi, and the interplay between these microbial communities.[4] These data are all cross-sectional, limiting the possibility for conclusions on causality.

In mouse models, inducing a germ-free state can either improve or worsen the biliary disease, depending on disease mechanisms.[8,9] In humans, trials with antibiotics improved liver biochemistry in PSC,[10] but a pilot trial of fecal microbiota transplantation was too small to allow any firm conclusions.[11] Furthermore, specific bacteria found in human PSC stool can modify experimental biliary disease in mice.[12] Overall, both human and experimental evidence suggest that gut microbes can act as disease modifiers in PSC. The mechanisms underlying these effects are so far not known, but current data suggest that altered bile acid homeostasis, increased bacterial translocation, or uncharacterized immune alterations could be relevant.[7–10]

A major limitation of the available data in PSC is the lack of microbe identification on the species level when using 16S ribosomal RNA gene sequencing. Furthermore, one important possibility is that bacterial functions and the by-products of their activity, for example, metabolites, could be more important than the composition of the microbiome. The majority of compounds entering the body through the oral route may potentially be metabolized by gut microbes, resulting in a large number of microbial by-products entering the circulation of the host, potentially affecting human organs. One example for such a modified metabolite is the bacteria-dependent metabolite trimethylamine-N-oxide, which has been associated with reduced liver transplantation–free survival.[13] Both species-level identification and quantification of the bacterial functional potential can be analyzed using full shotgun metagenomic sequencing. The aim of our study was to apply large-scale

metagenomic sequencing in 2 independent cohorts of patients with PSC to define which species are associated with PSC, and whether specific microbial functions are altered, possibly pointing to pathways relevant for PSC pathogenesis and progression.

Materials and Methods

Participants

We collected fecal samples from nontransplanted patients with PSC from 2 independent cohorts published previously.[14] The Norwegian cohort was included in the Norwegian PSC Research Center Biobank at Oslo University Hospital, and patients with PSC in the German cohort were recruited at the University Medical Center Hamburg-Eppendorf. The diagnosis of PSC was made in accordance with clinical guidelines and typical findings on cholangiography or liver biopsy, and all patients with PSC had undergone screening for IBD.[15,16] Plasma samples from 191 Norwegian patients with PSC and 48 controls (collected 2008–2015) were included for analysis of metabolites and survival from sample date to liver transplantation or death. For an overview of the cohorts and the study design, see Supplementary Figure 1.

Norwegian healthy population controls were randomly selected from donors registered in the national Norwegian Bone Marrow Donor Registry (Oslo, Norway), and German healthy controls (HCs) were recruited through the PopGen Biobank[17] and the FoCus cohort.[18]

Norwegian patients with IBD without a medical history of liver disease, and in clinical remission were recruited in an outpatient setting from Oslo University Hospital (Oslo, Norway). German patients with IBD included as part of the KINDRED program were recruited through the PopGen Biobank. IBD diagnosis was based on colonoscopy and histology and accepted criteria.[19,20] Routine biochemical parameters for all participants were retrieved through hospital databases and the respective biobank databases. Mayo risk scores were calculated using the algorithm for the revised Mayo risk score,[21] and the Amsterdam-Oxford model for PSC was calculated according to the original formula.[22] Transient elastography measurements were obtained with Fibroscan (Echosens, Paris, France) and enhanced liver fibrosis score calculated as described previously.[23]

Ethics

The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. Ethical approval was obtained from the respective Local Ethics Committees (Norway, Regional Committee for Medical and Health Research Ethics in South-Eastern Norway, reference 2015/2140; Germany, Hamburg, reference MC-111/15; Kiel, reference A148/14, A117-13, A156-03). All authors had access to the study data and reviewed and approved the final manuscript.

Fecal Sample Collection, DNA Extraction, and Exclusion Criteria

Fecal samples were collected and DNA extracted as described previously (see Supplementary Material for details).[2,14] All participants with previous bowel resection, small-duct PSC, other chronic liver diseases, a gastrointestinal stoma,

or who were exposed to antibiotics in the 6 weeks preceding sampling were excluded.

Metagenome Library Preparation, Sequencing, and Quantitative Real-Time Polymerase Chain Reaction

Quality and quantity of DNA samples were determined using Qubit measurements and the Genomic DNA ScreenTape (Agilent, Santa Clara, CA). Subsequently, metagenomic library preparation was performed as described previously by using Illumina Nextera DNA Library Preparation Kit (Illumina, San Diego, CA).[24] Sequencing was performed with 2×125 bp on a HiSeq 2500 platform (Illumina; German HCs and all Norwegian samples) or with 2×150 bp on a HiSeq 4000 platform (Illumina; German patients with PSC and IBD). Primers for quantitative real-time polymerase chain reaction are given in Supplementary Table 1 and further details can be found in the Supplementary Methods.

Post-Sequencing Processing

Quality control and filtering of the raw reads was done with KneadData (version 0.7.0), and Bowtie2 (version 2.3.4.2). Taxonomic and functional profiling was performed using HUMAnN2 (version 0.11.2),[25] incorporating MetaPhlAn2[26] (version 2.7.8), and DIAMOND (version 0.8.38). Additional details on bioinformatic handling can be found in the Supplementary Material.

Plasma Metabolites

All analyses of metabolites in plasma were performed at Bevital (Bergen, Norway). Vitamers were analyzed by liquid chromatography-tandem mass spectroscopy,[27] whereas amino acids and related metabolites were analyzed by gas chromatography-tandem mass spectroscopy.[28]

Statistical Analysis

Comparison of categorical variables was performed using the χ^2 test, or Fisher exact test, where appropriate, and the Cochran–Mantel–Haenszel test across cohorts. Mann-Whitney U test was applied for continuous clinical variables, metabolomic data, and analysis of gene-family–based α-diversity measures. When adjusting for clinical covariates in analyses of gene richness, linear regression was used. Kaplan-Meier plots with log-rank test and Cox proportional hazards regression analyses were used to investigate associations with end points (death or liver transplantation); all analyses were performed in SPSS (version 25; IBM, Armonk, NY). Microbiome co-correlation networks were made using R (version 3.6.1) using the SparCC algorithm with correlations cutoffs set at $r >$ 0.40 and unadjusted $P < .05$. β-diversity analyses (nonconstrained ordination of genus-level Bray-Curtis dissimilarities) were performed via permutational multivariate analysis of variance using the "vegan" package in R with 10,000 permutations. For correlation analyses, Spearman's rank correlation test was used. Analyses of differential abundance of single taxa and functional features (enzyme families; level-4 Enzyme Commission [EC] categories), Kyoto Encyclopedia of Genes and Genomes ontology and MetaCyc pathways) were first performed within each country, including age, body mass index, and sex as covariates in the model unless otherwise stated, followed by a meta-analysis. Samples with missing information for covariates were excluded from the analyses. A generalized model with zero-inflated beta distribution was fitted using the R package "GAMLSS" (additional details in Supplementary Methods). Lastly, a random effects meta-analyses was then performed using the R package "metamicrobiomeR," allowing for variance in the effect sizes between the 2 cohorts, using the estimates and standard errors from the per-country analyses described above. An overview of the main health state and drug use comparisons analyzed (g1–g9) can be found in Supplementary Table 2. False discovery rate was calculated according to Benjamini-Hochberg, false discovery rate–corrected P values were denoted as Q_{fdr}. The machine-learning procedure random forest ("randomForest" package in R) was used to predict phenotypes based on the metagenome data. The cohorts were randomly split into training and prediction sets with a ratio of 8:2, or one country used for training and the other for prediction, and the procedure was repeated 100 times for each prediction task. Comparison of predicted outcomes was summarized with area under the receiver operating characteristic curve (AUC), Matthew's correlation coefficient, and F1 measure. For additional details see the Supplementary Material.

Results

After sequencing and quality control of 17 billion paired-end sequences, 5.5 terabytes of data were available for analysis from 387 individuals; in total 136 patients with PSC, 158 HCs, and 93 IBD controls (Table 1, Supplementary Tables 3 and 4).

Overall Microbial Taxonomic and Genetic Diversity

The overall microbiome composition showed significant shifts among phenotypes (PSC, HCs, and IBD) in the merged cohort (Figure 1A), which was also apparent when analyzing each cohort separately (Figure 1B and C). Geography was also an important factor for the overall microbiome composition for all phenotypes (Figure 1A), including HCs (Supplementary Figure 2).

Patients with PSC had markedly reduced microbial gene richness (observed genes) compared with HCs (Figure 1D), also when adjusting for age, sex, and body mass index (linear regression, $P < .016$, $P = .001$, and $P < .0001$ in the German, Norwegian, and merged cohort, respectively). Concomitant IBD had no effect on gene richness in patients with PSC (Supplementary Figure 3).

Individual Species

After quality control and filtering, a total of 413 taxa were identified in the full dataset (phylum to strain level). For each country, single taxa with <80% zeros across samples were analyzed both for a shift in distribution (mu) and a difference in zero abundance (nu). The analysis identified an enrichment of *Clostridium asparagiforme* and an unclassified *Escherichia* species in PSC compared with HCs, as well as a marked depletion of several species, for example, *Ruminococcus obeum*, *Eubacterium siraeum*, and *Eubacterium rectale* (meta-analyses $Q_{fdr} < .05$, Figure 2A). Furthermore, 9 species showed an increased prevalence,

Table 1. Summary of Primary Sclerosing Cholangitis and Healthy Control Characteristics in the Study Cohorts

Characteristics	German cohort			Norwegian cohort			PSC G vs N	HC G vs N
	PSC (n = 67)	HC (n = 120)	P value	PSC (n = 69)	HC (n = 38)	P value	P value	P value
Age, y	47.0 (17 to 74)	46.5 (21 to 72)	.353	48.0 (21 to 69)	47.0 (35 to 61)	.852	.880	.269
Sex, *male*	43 (64.2)	53 (44.2)	.010	45 (65.2)	24 (63.2)	.836	1.000	.064
BMI, kg/m^{2a}	23.7 (15.7 to 32.9)	23.6 (19.0 to 42.7)	.893	25.1 (17.7 to 36.9)	26.0 (19.4 to 39.4)	.654	.003	.013
Smoking, *yes*	11 (16.4)	48 (40.0)	<.001	1 (1.4)	6 (15.8)	.008	.002	.011
AB last 6 mo, *yes*[b]	16 (23.9)	NA	—	8 (11.6)	5 (13.2)	1.000	.098	—
IBD, *yes*	33 (49.3)	0	—	46 (66.7)	0	—	.060	—
Ulcerative colitis, *yes*	32 (47.8)	—		36 (52.2)	—		.730	
Crohn's disease, *yes*	1 (1.5)	—		10 (14.5)	—		.009	Available for n in G/N
Medication, *yes*		—			—		—	—
PPI	0	—		2 (2.9)	—		.500	67/69
Statins	NA	—		0	—		—	67/69
UDCA	63 (94.0)	—		23 (33.3)	—		<.001	60/69
Prednisolone/cortisone	13 (19.4)	—		9 (13.0)	—		.420	60/69
5-ASA	29 (43.3)	—		29 (42.0)	—		1.000	61/69
Infliximab	2 (3.0)	—		1 (1.4)	—		.614	60/69
Azathioprine	9 (13.4)	—		9 (13.0)	—		1.000	60/69
Budesonide	4 (6.0)	—		3 (4.3)	—		.709	57/69
PSC-specific variables		—			—		—	—
PSC duration, y	7.0 (1.0 to 35.0)	—		9.5 (1.6 to 31.7)	—		.057	61/69
IBD duration, y	15.0 (1.0 to 40.0)	—		14.2 (1.2 to 44.5)	—		.610	31/41
Other autoimmune disease, *yes*	NA	—		18 (26.1)	—			0/64
Mayo risk score	NA	—		–0.07 (–1.92 to 3.33)	—			0/51
Cirrhosis, *yes*	3 (4.5)	—		NA	—			67/0
P-ANCA–positive	NA	—		26 (37.7)	—			0/35
Creatinine, $\mu mol/L$	NA	—		68.0 (42.0 to 100.0)	—			0/63
Bilirubin, $\mu mol/L$	10.3 (3.42 to 34.2)	—		13.0 (5.0 to 114.0)	—		.007	43/62
Albumin, g/L	NA	—		43.0 (16.0 to 47.0)	—			0/58
AST, U/L	NA	—		42.0 (18.0 to 197.0)	—			0/58
ALT, U/L	37.5 (11.0 to 286.0)	—		51.0 (14.0 to 331.0)	—		.021	44/63
ALP, U/L	117.0 (44.0 to 590.0)	—		152.5 (30.0 to 598.0)	—		.004	44/62
GGT, U/L	NA	—		165.0 (12.0 to 1576.0)	—			0/61

NOTE. Demographics for IBD controls are provided in Supplementary Table 4. None of the HCs used any of the stated medications. Continuous data given as median (minimum to maximum), count data as n (%).

AB, antibiotics; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; G, German cohort; GGT, gamma-glutamyltransferase; N, Norwegian cohort; NA, not available; PPI, proton pump inhibitors; 5-ASA, 5-aminosalicylic acid.

[a]BMI data missing for 10 HCs in the German cohort, other data complete unless otherwise stated.

[b]None used AB <6 weeks before sample inclusion, data on AB use >6 weeks and <6 months NA for German HCs.
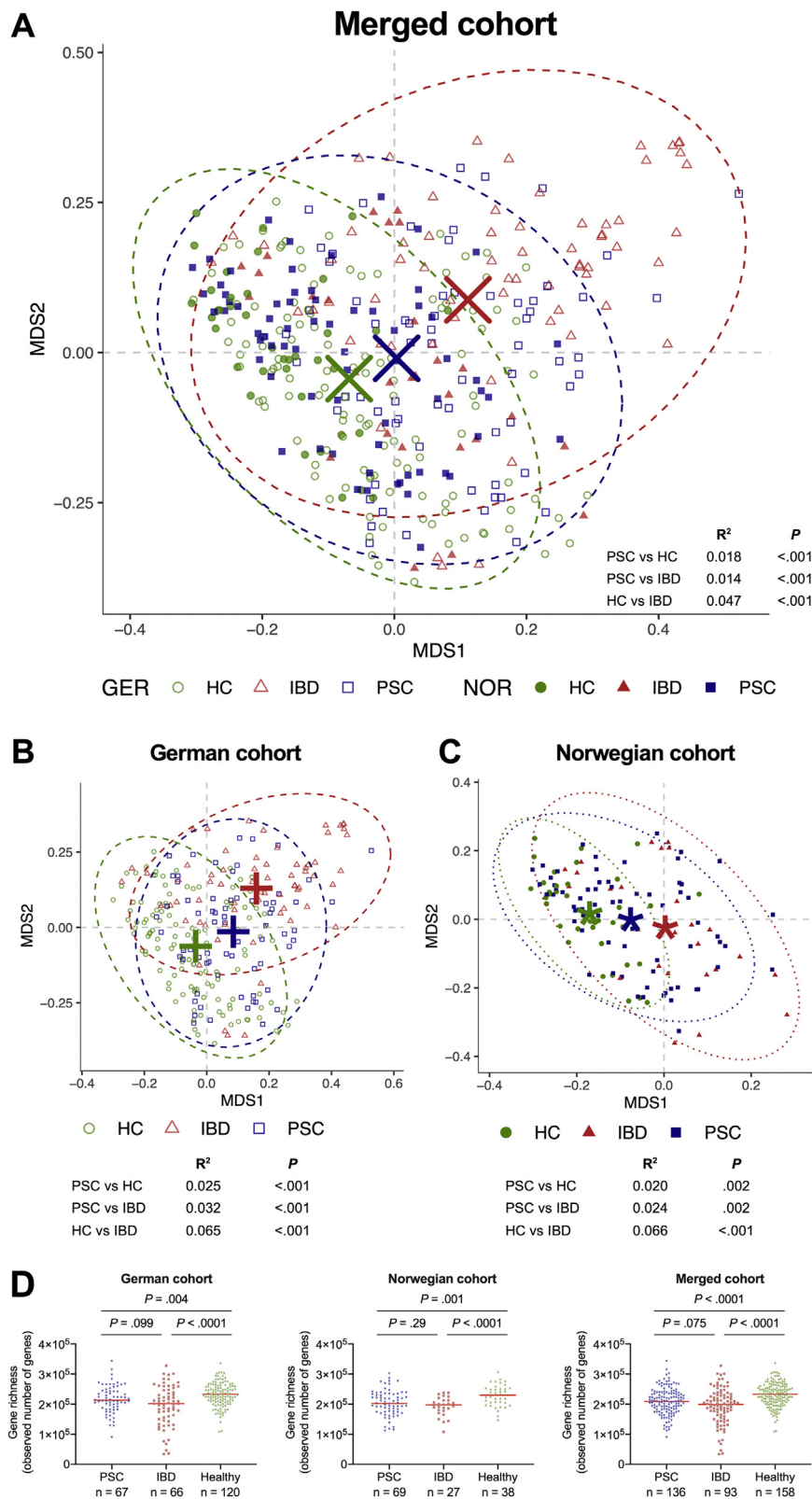
Figure 1. Overall bacterial taxonomic composition and gene richness in patients with PSC and controls. Non-constrained ordination of genus-level Bray-Curtis dissimilarities in the microbiome of (*A*) patients with PSC, as well as healthy and IBD controls, in the merged cohort, and in the (*B*) the German and (*C*) the Norwegian cohorts separately, showing shifts in the global microbiome composition. (*D*) Number of observed genes in PSC compared with controls. All group centroids in (*A*–*C*) represent the arithmetic mean of the groups' respective points in the ordination, ellipses represent 95% confidence level of the multivariate t-distributions. *Red horizontal bar* in (*D*) represents the median. GER, Germany; MDS, multiple dimension scale; NOR, Norway.

and we identified 5 species with lower prevalence in patients with PSC compared with HCs (Figure 2*B*). Although several species showed similar patterns in IBD, species like *Ruminococcus obeum, Bacteroides intestinalis,* and several *Clostridium species* did not differ between IBD and HCs (Supplementary File 1).

Previous works have found increased levels of the *Veillonella* genus in PSC.[7] *Veillonella atypica, Veillonella parvula,*
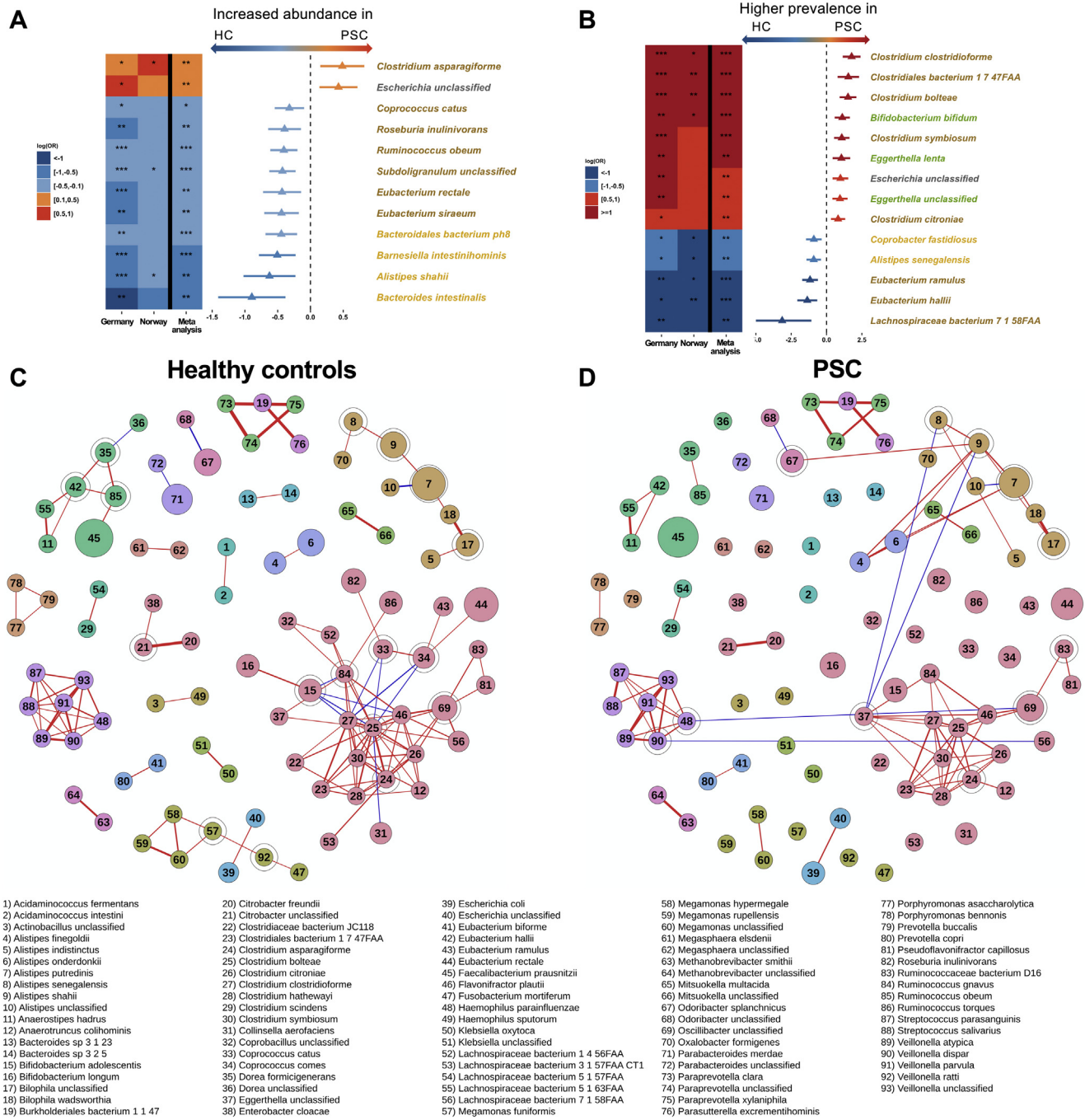
Figure 2. Differences in species composition in PSC compared with HCs. (*A*) Species that were differently abundant and (*B*) species with different prevalence in PSC and HCs. Species are colored by phyla affiliation. (*C*) Bacterial abundance co-correlation network in HCs and (*D*) patients with PSC. In (*A*) and (*B*) the *triangle/bar* in the forest plot indicate log(odds ratio)/SE. *Red* and *blue lines* in (*C*) and (*D*) indicate positive and negative correlations between species, respectively. Node size reflects the square-root–transformed median relative abundances in each phenotype, and *encircled nodes* mark articulations points. Only correlations with $r > 0.4$ are shown, and increasing correlation coefficient are reflected by increasing line thickness. *$Q_{fdr} < .05$; **$Q_{fdr} < .005$; ***$Q_{fdr} < .0005$.

and an unclassified *Veillonella* species showed trending increased prevalence in patients with PSC compared with HCs (meta-analyses $P = .0062$, $P = .026$, and $P = .025$, respectively, $Q_{fdr} = .058$, $Q_{fdr} = .14$, and $Q_{fdr} = .14$, respectively, Supplementary Figure 4 and Supplementary File 1). Other PSC-related species include *K pneumoniae*, in addition to *Proteus mirabilis* and *Enterococcus gallinarum*, previously linked to intestinal barrier function and immune responses in PSC.[12] *K pneumoniae* was left out from our initial analysis due to low abundance in the Norwegian cohort (see Materials and Methods). A targeted analysis detected *K pneumoniae* in the microbiome of 25% of patients with PSC, but

only in 4.4% of the HCs (Cochran–Mantel–Haenszel test, $P <$ .001). *K pneumoniae* was detected in the microbiome of 45.2% of IBD controls (Cochran–Mantel–Haenszel test, $P =$ .0057 vs PSC). *E gallinarum* and *P mirabilis* were detected in only a few patients (Supplementary Table 5). When applying a species-specific real-time polymerase chain reaction on a subset of the German samples (PSC n = 36, HCs n = 20, IBD n = 17), *K pneumoniae* showed similar prevalence in PSC, IBD, and HCs (77.8%, 94.1%, and 75.0%, respectively, Supplementary Figure 5A). *E gallinarum* and *P mirabilis* were detected in a minority of samples (Supplementary Figure 5B and C).

To further delineate structural microbiome differences between the phenotypes, we generated bacterial species abundance co-correlation networks using SparCC.[29] The network in HCs consisted of several large and small clusters (Figure 2C). When overlaying the co-correlations observed in PSC, there was a disruption of the network seen in the HCs in the patients with PSC, with loss of several clusters and fewer co-correlations in others (Figure 2D). IBD controls without PSC also showed a disrupted network compared with HCs, but markedly different from what was observed in PSC (Supplementary Figure 6).

Extensive Alterations in the Genetic Landscape of the Microbiome in Primary Sclerosing Cholangitis, Highlighting Changes in Biosynthesis of Amino Acids and B Vitamins

In total, after filtering we detected 121 metabolic pathways (MetaCyc pathways) and 424 enzyme families present in both cohorts. We identified 53 metabolic pathways differentially abundant in the microbiomes of patients with PSC compared with HCs, in addition to 106 enzyme families (all meta-analyses $Q_{fdr} <$ .05, Figure 3 and Supplementary File 1, respectively).

The microbiome of patients with PSC showed a marked depletion of the metabolic pathway related to the biosynthesis of the branched-chain amino acid (BCAA) isoleucine (Figure 3), as well as several enzyme families related to the BCAAs isoleucine, valine, and leucine compared with HCs (Figure 4A–C and Supplementary File 1), and levels in patients with IBD were similar to levels in PSC. There were also differences in metabolic pathways related to biosynthesis of aromatic amino acids, but less consistent, with some pathways enriched in PSC (PWY6628 superpathway of L-phenylalanine biosynthesis) and others depleted, for example, COMPLETE ARO-PWY superpathway of aromatic amino acid biosynthesis, as seen in Figure 3. Four of the 7 most highly enriched metabolic pathways in PSC compared with HCs were related to biosynthesis of 2 other amino acids, that is, arginine and ornithine (Figure 3). These 4 pathways had similar levels in PSC and IBD, but in direct comparisons only 2 of these pathways were significantly enriched in IBD compared with HCs (Supplementary File 1).

Patients with PSC showed higher levels of metabolic pathways related to biosynthesis of thiamine (vitamin B1) compared with HCs (eg, THISYN-PWY superpathway of thiamin diphosphate biosynthesis I, Figure 3). This pathway

showed similar levels in PSC and IBD, while patients with IBD showed higher levels compared with HCs (Supplementary File 1). However, 3 metabolic pathways for biosynthesis of 5'-phosphoribosyl-5-aminoimidazole (PWY6122, PWY6277, and PWY6121), a key intermediate in the biosynthesis of thiamine (vitamin B1), were all depleted in PSC (Figure 3). Furthermore, patients with PSC showed marked differences compared with HCs of several enzyme families related to vitamin B6 metabolism, most prominent was a marked depletion of EC4.3.3.6 pyridoxal-5-phosphate synthase (Figure 4D), an enzyme that alone constitutes the metabolic pathway pyridoxal 5'-phosphate biosynthesis II and the predominant biosynthetic route for the de novo production of the active form of vitamin B6, pyridoxal 5'-phosphate (PLP),[30] and EC2.6.1.52 phosphoserine transaminase (Supplementary File 1). The latter is 1 of 7 enzyme families comprising the metabolic pathway PYRIDOXSYN-PWY: pyridoxal 5'-phosphate biosynthesis I, which was increased in PSC compared with HCs (Figure 3). As for the last 6 enzyme families in this metabolic pathway, 1 (EC1.1.1.290) was enriched in PSC compared with HCs, 3 showed similar levels (EC2.6.99.2, 1.1.1.262 and 2.2.1.7), and the last 2 (EC1.2.1.72 and 1.4.3.5) did not pass our initial filtering (Supplementary File 1). A total of 84 species contributed to EC4.3.3.6 pyridoxal-5-phosphate synthase in the dataset (Supplementary File 2). *E rectale* and *Eubacterium siraeum*, which were both depleted in PSC compared with HCs in the meta-analyses (see Figure 2A), were among the top 5 species contributing to the abundance of this enzyme. Twenty-eight species contributed to PYRIDOXSYN-PWY: pyridoxal 5'-phosphate biosynthesis I (Supplementary File 2), but none of the contributing species detected in the metanalysis showed a difference between PSC and HCs (all *Bacteroides*, $Q_{fdr} >$ .30, Supplementary File 2).

Regarding other B vitamins, 2 enzymes, EC2.7.1.26 riboflavin kinase and EC2.7.7.2 flavin adenine dinucleotide synthetase, responsible for synthesis of the active forms of vitamin B2 (flavin mononucleotide and flavin adenine dinucleotide, respectively), were also reduced in PSC (Figure 4E and F), and riboflavin synthase (EC2.5.1.9), which is responsible for a reaction resulting in synthesis of riboflavin and 5-amino-6-D-ribitylaminouracil, was enriched in PSC (Figure 4G). Furthermore, the microbiome of patients with PSC showed increased abundance of enzyme families related to ammonia production (Figure 4H and I). Enzyme families related to antibiotic resistance like EC3.5.2.6 β-lactamase were also enriched in PSC compared with HCs (Figure 4J).

Metabolic Pathways Diminished in the Microbiome of Patients With Primary Sclerosing Cholangitis Show Low Levels of Related Metabolites in Plasma

As the initial microbiome analyses identified potential alterations in bacterial metabolism of both BCAAs and B vitamins, and the gut microbiome could be an important source of these metabolites,[31,32] we investigated selected vitamin B and BCAA metabolites in plasma from another
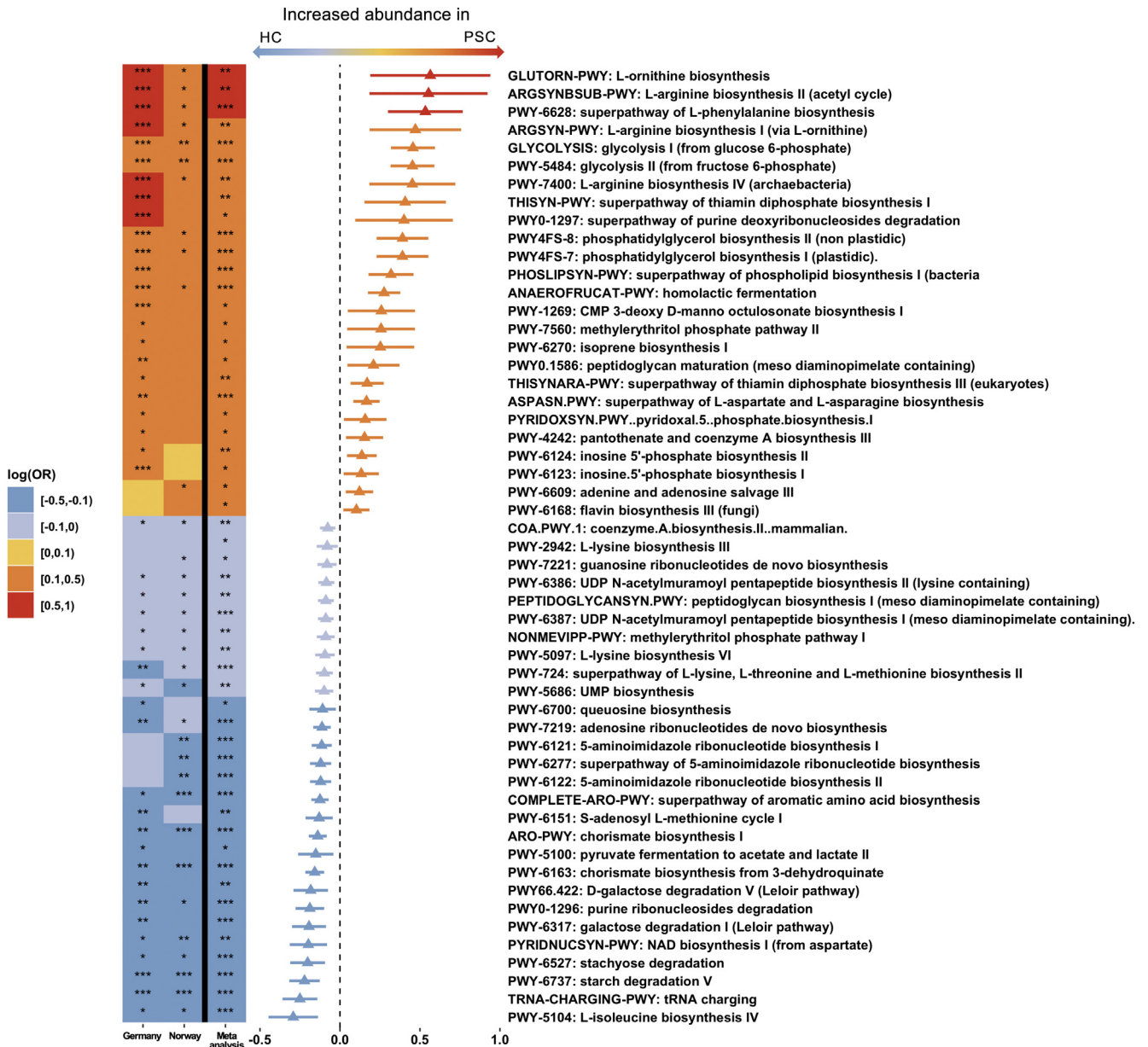
Figure 3. Alterations in metabolic pathways in the microbiome of PSC. *Heatmap* and *forest plot* showing significant differences in microbiome metabolic pathways (MetaCyc pathways, $Q_{fdr} < .05$) between PSC and HCs from the individual cohorts (*2 left columns* in the *heatmap*) and the meta-analysis (*right column* in the *heatmap* and the *forest plot*). *Triangle/bar* in *forest plot* indicate log(odds ratio)/SE. *$Q_{fdr} < .05$; **$Q_{fdr} < .005$; ***$Q_{fdr} < .0005$.

cohort of 191 patients with PSC (27 were also recruited in the metagenome study cohort) and 48 HCs (see Supplementary Figure 1). The main active form of vitamin B6, PLP, and the BCAAs isoleucine, leucine, and valine were clearly reduced in plasma of patients with PSC compared with HCs (Figure 5A–D). Other vitamin B6 metabolites (ie, pyridoxal and 4-pyridoxic acid) were also lower in PSC compared with controls (Supplementary Figure 7). Furthermore, the vitamin B2 (riboflavin) metabolite flavin mononucleotide showed reduced levels in PSC corresponding to the reduction in EC2.7.1.26, and there was no difference for vitamin B2 itself (Supplementary Figure 8A and B). For vitamin B1, thiamine was similar in patients

with PSC compared with controls, and thiamine monophosphate was reduced in PSC (Supplementary Figure 8C and D). Plasma levels of these B vitamins did not correlate with the relative abundance of associated metabolic pathways or enzyme families in the 27 patients with both microbiome and metabolomics data available (data not shown), possibly due to the long interval between sampling of stool and plasma (median interval, 2.9 years). The essential amino acid histidine was reduced in patients with PSC compared with HCs ($P = .005$, Supplementary Figure 9A). Among other measured amino acids (Supplementary Figure 9B–E), only ornithine was increased in PSC ($P = .016$).
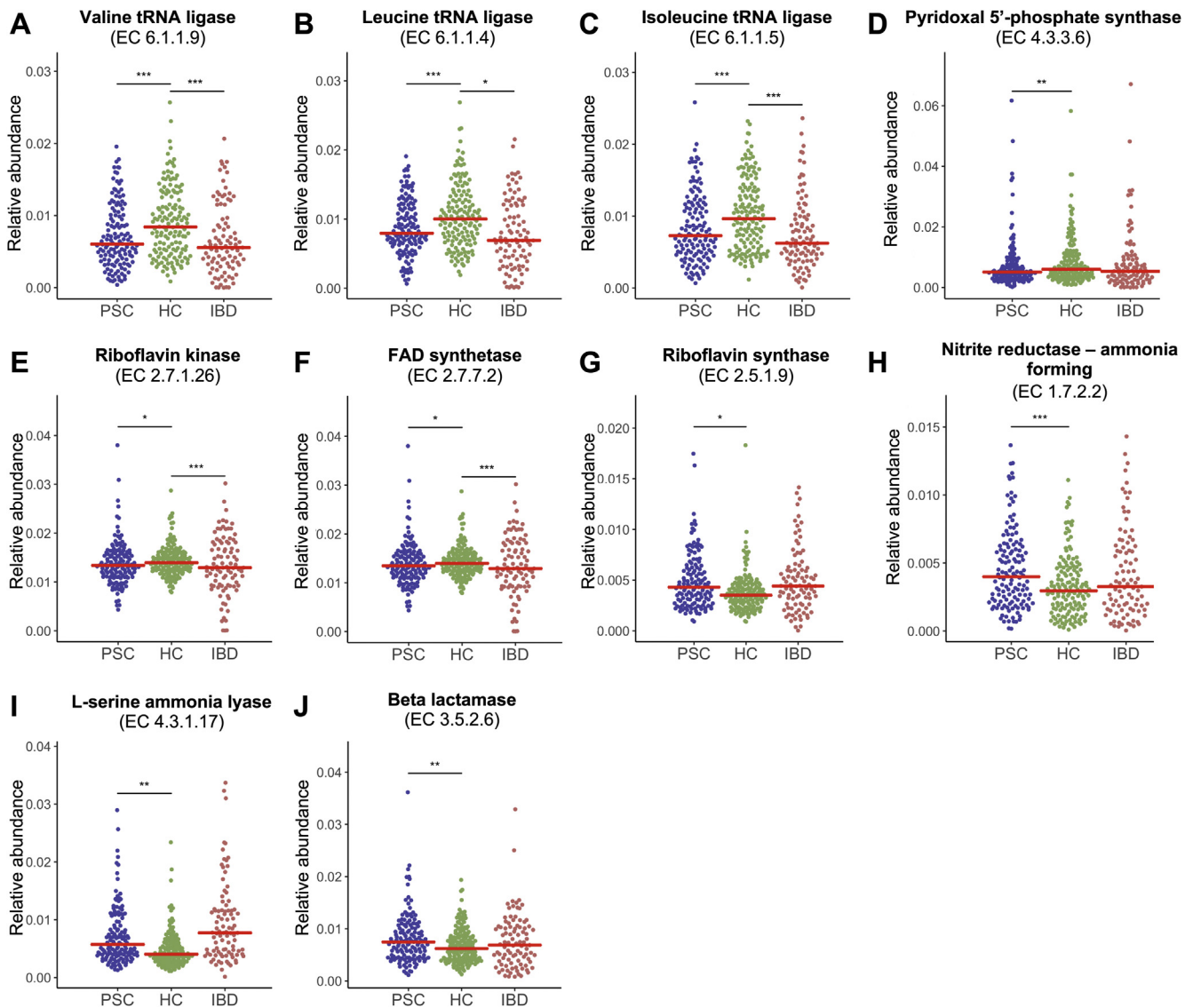
Figure 4. Genetic potential of the gut microbiome in patients with PSC. *Dot-plots* showing relative abundance of enzyme families (level-4 EC categories) related to microbial metabolism of BCAAs (*A*) valine, (*B*) leucine, and (*C*) isoleucine, (*D*) the active form of vitamin B6 (PLP) and (*E*) the active forms of vitamin B2, flavin mononucleotide (FMN), and (*F*) flavin adenine dinucleotide (FAD), and (*G*) synthesis of riboflavin (vitamin B2) and related metabolites, (*H*, *I*) ammonia production, and (*J*) antibiotic resistance. *Red bar* marks the median. *Q_{fdr} < .05; **Q_{fdr} < .01; ***Q_{fdr} < .001.

As circulating levels of BCAAs have been linked to the potential ability of the gut microbiome to transport these amino acids into bacteria,[32] we specifically investigated 5 Kyoto Encyclopedia of Genes and Genomes orthologue gene groups related to inward transportation of BCAAs into bacteria. These gene groups showed markedly higher abundance in PSC compared with HCs in the meta-analysis (K01995 Q_{fdr} = .078; K01996, K01999, K01997, and K01998 Q_{fdr} < .05), and similar levels in IBD compared with PSC (Supplementary Figure 10).

Microbial metabolites may modify the severity of diseases.[32] We therefore investigated the relationship between metabolites and liver transplantation–free survival in PSC. The plasma concentrations of PLP and all BCAAs (ie, isoleucine, leucine, and valine) were lower in the patients reaching an end point than in those who did not

(Supplementary Figure 11). When dividing the plasma concentrations into quartiles, low levels of these metabolites all strongly predicted reduced liver transplantation–free survival (Figure 5E–H). The effect seemed particularly strong for PLP (Figure 5A and E), which was associated with reduced risk of liver transplantation or death in a Cox regression model, independent of Mayo risk score and enhanced liver fibrosis score (hazard ratio, 0.66 per quartile increase in plasma PLP; 95% confidence interval, 0.51–0.87; P = .003) (Supplementary Table 6). PLP was also associated with reduced risk of liver transplantation or death independent of other risk scores, that is, Amsterdam-Oxford model for PSC (hazard ratio, 0.67 per quartile increase in plasma PLP; 95% confidence interval, 0.51–0.88; P = .003) (Supplementary Table 7). PLP may be metabolized by alkaline phosphatase,[33] and the 2 showed a moderate
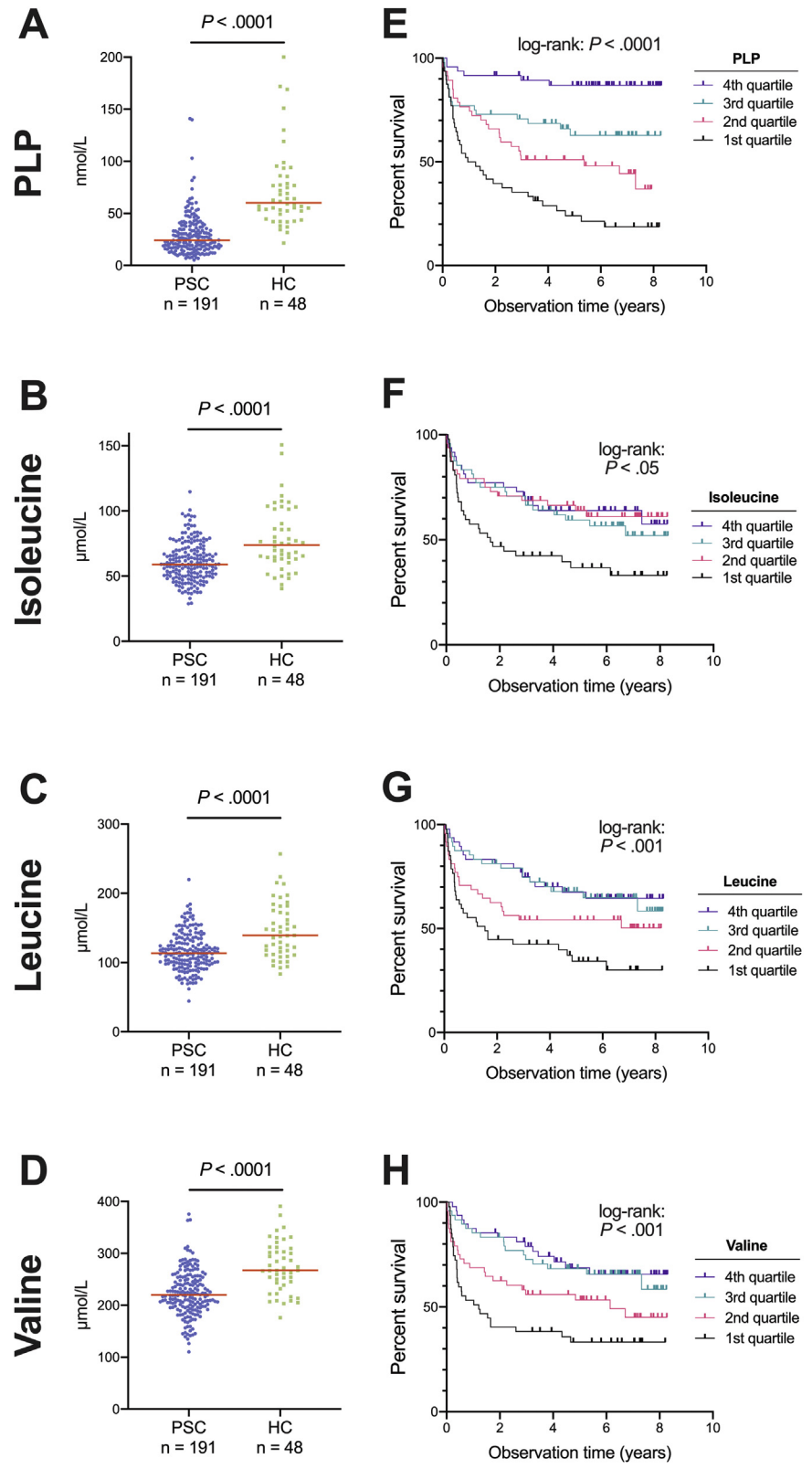
Figure 5. Microbiome-related plasma metabolites and liver transplantation–free survival. (*A*) The active vitamin B6 metabolite PLP, (*B*) isoleucine, (*C*) leucine, and (*D*) valine in plasma of patients with PSC compared with HCs. Kaplan-Meier plots showing that low levels (first quartile) of (*E*) PLP, (*F*) isoleucine, (*G*) leucine, and (*H*) valine were all associated with reduced liver transplantation–free survival in PSC.

inverse correlation ($\rho = -.38$, $P < .001$), but alkaline phosphatase was not independently associated with survival when added to the model (Supplementary Table 6).

Isoleucine, leucine, and valine were not independently associated with liver transplantation–free survival when corrected for Mayo risk score (data not shown).

Microbiome as Predictor of Disease Phenotypes and Clinical Characteristics in Primary Sclerosing Cholangitis

When applying a random forest model on the species data, we were able to separate PSC from HCs with a high predictive power (AUC = 0.88 ± 0.04; Figure 6A) and achieved similar results when separating PSC from IBD (AUC = 0.83 ± 0.07; Figure 6B). Separating PSC from HCs and IBD at the same time gave similar predictive power (Supplementary Figure 12). Adding anthropometric information (ie, age, sex, and body mass index) had minimal effect on the prediction of PSC vs HCs (AUC = 0.87 ± 0.05, Matthew's correlation coefficient = 0.54 ± 0.11; Supplementary Figure 12), and using species data generally performed better than enzyme families and metabolic pathways for separating the phenotypes (Supplementary Figure 12).

Finally, we merged the 2 microbiome cohorts for analyses of differences between clinical entities in patients with PSC (n = 136). Comparing patients with PSC with and without IBD, we were unable to detect any differences in gene richness (P = .39, Supplementary Figure 3), species, metabolic pathways, or enzyme families (all meta-analyses $Q_{fdr} > .1$; Supplementary File 1). Neither was there a significant shift in overall bacterial composition between patients with PSC with and without IBD (Supplementary Figure 13). There was no correlation between microbial gene richness and disease duration in PSC ($\rho = .007$, $P = .94$, n = 130) or the Mayo risk score ($\rho = -.07$, $P = .63$, n = 51). In a subset of the German patients with PSC (n = 41) with liver stiffness measurements available (measured by transient elastography), liver stiffness did not show significant correlation with gene richness ($\rho = -.21$, $P = .19$). When testing for associations of liver stiffness with enzyme families and metabolic pathways related to vitamin B6 or BCAA synthesis, there was a weak association between increased liver stiffness and reduced levels of EC6.1.1.4 leucine transfer RNA ligase ($\rho = -.39$, unadjusted $P = .012$, Supplementary Figure 14), but not for any other of the tested functional features.

Ursodeoxycholic acid (UDCA) use in patients with PSC was associated with higher levels of *E rectale* (estimate: 0.70, $P = .004$, $Q_{fdr} = .26$; Supplementary File 1), which was depleted in PSC compared with HCs in the meta-analyses (Figure 2A). UDCA use was also associated with reduced abundance of *Veillonella dispar* (estimate: −1.05, $P = .01$, $Q_{fdr} = .34$; Supplementary File 1). Furthermore, there was a shift in β-diversity (Bray-Curtis) between UDCA users and nonusers ($R^2 = 0.030$, $P = .005$; Supplementary Figure 13). We did not detect any significant effect by 5-aminosalicylic acid (5-ASA) on any species ($Q_{fdr} > .50$). Neither 5-ASA nor UDCA use was associated with differences in enzyme families or metabolic pathways (all, $Q_{fdr} > .19$). Notably, UDCA use in PSC was only analyzed in the Norwegian cohort, as 94% (n = 63) of the German patients with PSC were using UDCA at the time of sampling.

Discussion

In the present study, we analyzed the stool microbiome in 2 cohorts of Norwegian and German subjects comprising patients with PSC, patients with IBD, and HCs. Using high-resolution shotgun metagenomic sequencing and targeted metabolomics in a third independent cohort of Norwegian patients with PSC and HCs we uncovered a PSC-associated microbiome with a low gene count and multiple bacterial alterations unaffected by concurrent IBD, and which could now be defined at species level; marked alterations of gene functions related to, for example, vitamin B6 synthesis and BCAA synthesis in the microbiome of PSC; reduced plasma concentrations of vitamin B6 and BCAAs in PSC, and low concentrations of these metabolites strongly associated with reduced liver transplantation–free survival, suggesting that investigating altered microbial metabolism may help pinpoint biologic changes relevant for the disease course in PSC.

By using shotgun metagenomic sequencing, we make a major leap forward in microbiome studies investigating PSC, providing detailed functional analysis of microbial genes encoding enzymes and metabolic pathways. Genes related to bacterial synthesis of several essential nutrients like BCAAs and vitamin B6 (eg, PLP) showed clear changes in patients with PSC compared with HCs. PLP is the active form of vitamin B6, an essential cofactor of numerous metabolic reactions, and contributes to intestinal immune regulation and lymphocyte trafficking into the intestines.[34] PLP can be produced de novo via 2 independent pathways. The predominant route used by the majority of organisms capable of PLP production is via a single enzyme (EC4.3.3.6),[30,31,35] which was markedly depleted in PSC. The less abundant, multi-enzyme pathway PYRIDOXSYN-PWY: pyridoxal 5'-phosphate biosynthesis was increased in PSC. Overall, the data suggest a reduced genetic potential to synthesize PLP in PSC, and calls for further evaluations in an experimental model system. The capacity of the gut bacteria to synthesize vitamin B6 has been estimated to meet 86% of the daily recommended intake,[31] suggesting that alterations in the gut microbiome may dramatically influence dietary requirements. Targeted metabolite analyses in plasma showed reduced concentrations of both PLP and other B6 species (pyridoxal and 4-pyridoxic acid), suggesting a true vitamin B6 deficiency in PSC.[36] Notably, PLP is known to be reduced in end-stage liver disease of different etiologies,[37] linked not only to deficiency per se, but also due to increased catabolism of PLP to pyridoxal, by, for example, alkaline phosphatase or other mechanisms in the context of chronic inflammation.[38] Still, the predictive effect independent from Mayo risk score could point to a role of PLP in PSC beyond end-stage liver disease. Taken together, identification of altered bacterial functions pointed us to important biology that was altered also systemically. It should be emphasized that we do not show a direct link between the gut and blood, but additional studies should investigate such a link and whether PLP represents a mechanism or only a marker of disease activity and severity in PSC.
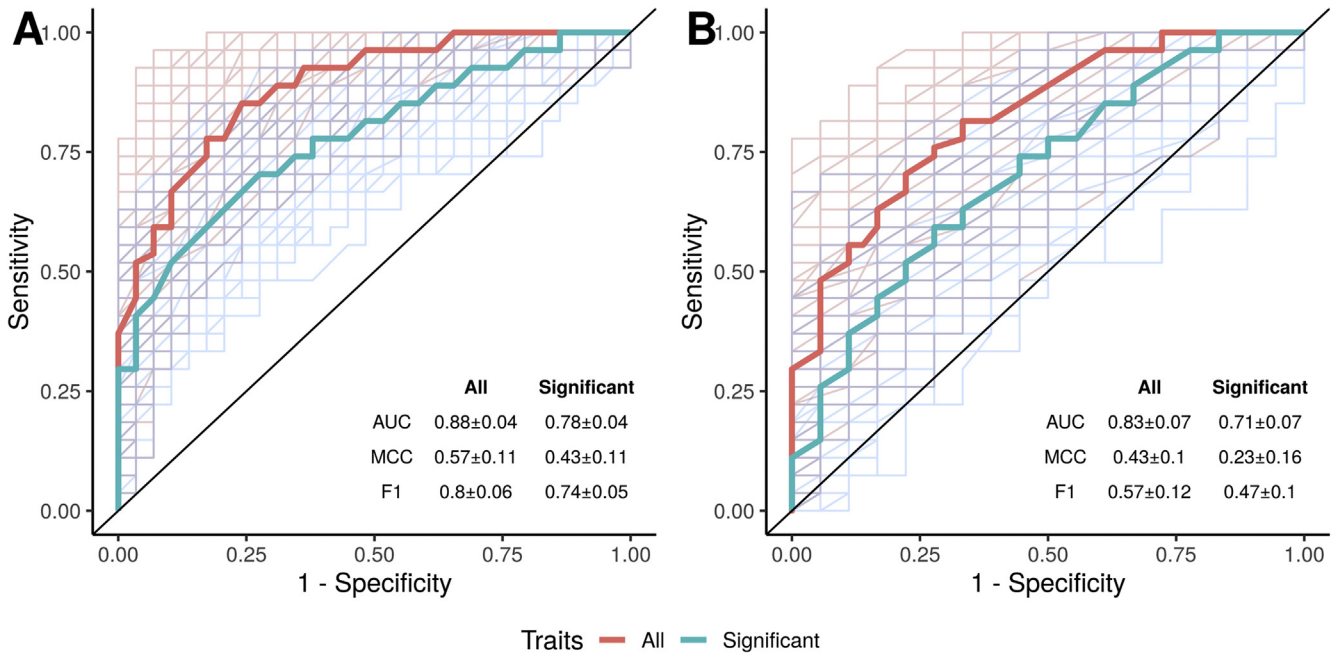
Figure 6. Separation of clinical phenotypes using random forest classification. (*A*) PSC vs HCs and (*B*) PSC vs IBD. Results using all available species (*red line*) or only the significant species hits (*blue line*). F1, F1 measure; MCC, Matthew's correlation coefficient (range, −1 to +1).

Similar considerations regarding microbial synthesis and dietary requirements are relevant for BCAAs. Circulating BCAAs are known to be reduced in patients with cirrhosis, but also less advanced liver diseases, including PSC.[39,40] In contrast, individuals with insulin resistance have been shown to have elevated circulating BCAA levels, correlating with increased synthesis capacity in the microbiome as well as reduced capacity to import these BCAAs into bacteria.[32] In PSC, we found the opposite, with reduced bacterial synthesis and increased import capacity into bacteria. This calls for consideration as to whether the gut microbiome contributes to the observed reduced plasma BCAA concentrations in PSC. Low plasma BCAA concentrations predicted reduced liver transplantation–free survival in our study, but not independently of the Mayo risk score, suggesting that alterations in BCAAs may relate to disease stage. Firm conclusions regarding the role of these metabolites in PSC in humans would require an interventional trial with, for example, supplementation,[41] while the potential clinical value of vitamin B6 or BCAAs as predictive biomarkers in PSC would require independent validation studies. Notably, because deficiencies of vitamin B6 and BCAAs have been observed in liver diseases of several etiologies, similar gut microbiome–related modifier effects may be seen also in other liver diseases.[37,42]

For other measured metabolites, the relationship between the gut microbiome and plasma levels was less obvious. For thiamine and riboflavin, this could perhaps be explained by the low potential contribution from the gut microbes, estimated to meet only 2%–3% of daily recommended intake.[31] However, the alterations in bacterial genes related to riboflavin metabolism in PSC may be of interest, because these are the source of bacterial riboflavin

metabolites that are essential in the activation of mucosal-associated invariant T cells, which are liver-abundant innate-like T cells relevant in PSC.[43–45]

Functional aspects of the gut microbiome are important, but the actions of specific bacterial species are also of interest. We observed an increase of the classical potential pathogen *Escherichia*, together with reductions in species typically classified as producers of the short-chain fatty acid butyrate. Previous work has highlighted an increased relative abundance of the *Veillonella* genus in PSC.[7] *Veillonella* was less prevalent when investigated with the current methodology, but we observed an increased prevalence of several *Veillonella* species in patients with PSC. In a study by Nakamoto et al,[12] *K pneumonia* strains isolated from patients with PSC contributed to increased susceptibility to hepatobiliary damage in mice and more bacterial translocation. In the shotgun sequencing data in the present study, *K pneumoniae* showed higher prevalence in patients with PSC compared with HCs, as reported by Nakamoto et al in their study. Using a more sensitive method (quantitative polymerase chain reaction) in a small subset of the German cohort, *K pneumoniae* sequences were identified in a much higher proportion of the patients with PSC compared with the shotgun sequencing data, but with similar prevalence in PSC and HCs. The clinical relevance of very low abundant taxa, such as *K pneumoniae*, could be questioned, and the role of *K pneumoniae* in human PSC is not well defined. As the experimental data from the article by Nakamoto et al suggest, it is possible that only specific strains of the *K pneumoniae* species are capable of inducing hepatobiliary damage, highlighting the need for increased resolution in further microbiome research. Interestingly, a similar concept was presented recently in patients with alcoholic

hepatitis, who had higher frequency of fecal *Enterococcus faecalis*.[46] The presence of *E faecalis* strains with the ability to produce the exotoxin cytolysin was associated with more severe disease.[46] Overall, it appears that both individual microbes and shifts in the collective metabolic capacity of the gut microbiome may modify human diseases without necessarily being the triggering events. Such mechanisms may point toward new treatment opportunities.

One important observation was the striking similarity between the microbiome in patients with PSC with and without IBD, both at species and functional level. In line with previous data,[2,3] the global microbiome composition in IBD without liver disease was different from patients with PSC. However, few species and functions were significantly different between patients with PSC and patients with IBD without PSC, suggesting that the differences separating PSC from IBD relate to smaller changes in multiple species or functions. Considering the microbiota literature, the differences between PSC and IBD are less consistent than between PSC and HCs.[2,3,7] It is possible that the challenge of appropriately matching patients with IBD with and without PSC for case–control studies makes these comparisons difficult.

Differences depending on geography impose another challenge but might also be an important tool in case–control studies. In addition, sampling and DNA extractions were performed differently in the Norwegian and German cohorts. Geography does, in some studies, constitute the strongest predictor of microbiome composition,[47] highlighting a need for identification and validation of core features in multi-cohort international studies to define true disease characteristics. However, in the present study, it is not possible to assign with certainty differences between Norway and Germany to geography or methodology.

The major strengths of this study are the use of 2 independent case–control cohorts from different countries and the use of state-of-the art sequencing methods with high taxonomic and functional resolution. Although this study, to our knowledge, is the largest published so far to investigate the microbiome in PSC using full metagenomic sequencing, it is likely that by including additional patients and also liver disease controls, the robustness of the results could further increase. Notably, the link between our findings of microbiome changes and differences in metabolites and prognosis are, as of now, purely associative. Time- and subject-matched blood and stool samples would be important in further studies, in addition to experimental studies, if these associations are to be further explored. Liver transplantation as an end point could vary in different countries and could make it less useful as an end point intended for clinical translation. Another limitation is that dietary data were only surveyed crudely through standard questionnaires, so it cannot be discounted that dietary factors could have influenced our results. In addition, due to the crosssectional design, we are unable to discern whether the changes we detect occur before or after disease onset, which limits the possibility of drawing conclusions about cause or effect. As PSC is a rare disease it is difficult, if not impossible, to profile the microbiome over the time of disease onset.

However, larger international cohorts with follow-up data should be established to help address these knowledge gaps.

In conclusion, the gut microbiome of patients with PSC exhibits large functional differences compared with HCs, including microbial metabolism of essential nutrients, irrespective of the presence of IBD. Alterations in related circulating metabolites associated with liver transplantation–free survival suggest that overall microbial functions may be relevant for the disease process in PSC.

Supplementary Material

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Author names in bold designate shared co-first authorship.

Correspondence
Address correspondence to: Johannes R. Hov, PhD, MD, Norwegian PSC Research Center, Division for Cancer Medicine, Surgery and Transplantation, Oslo University Hospital Rikshospitalet, Pb 4950 Nydalen, N-0424 Oslo. e-mail: j.e.r.hov@medisin.uio.no.

CRediT Authorship Contributions
Martin Kummen, MD, PhD (Conceptualization: Equal; Data curation: Equal; Formal analysis: Equal; Investigation: Equal; Methodology: Equal; Validation: Lead; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

Louise B. Thingholm, PhD (Data curation: Lead; Formal analysis: Lead; Methodology: Lead; Validation: Equal; Visualization: Equal; Writing – review & editing: Equal)

Malte C. Ruühlemann, PhD (Data curation: Equal; Formal analysis: Equal; Investigation: Equal; Methodology: Equal; Visualization: Equal; Writing – review & editing: Equal)

Kristian Holm, MSc (Data curation: Equal; Methodology: Lead; Writing – review & editing: Equal)

Simen H. Hansen, MSc (Formal analysis: Equal; Methodology: Equal; Visualization: Equal; Writing – review & editing: Equal)

Lucas Moitinho-Silva, PhD (Formal analysis: Equal; Methodology: Equal; Visualization: Equal; Writing – original draft: Supporting; Writing – review & editing: Equal)

Timur Liwinski, MD, PhD (Investigation: Supporting; Writing – review & editing: Supporting)

Roman Zenouzi, MD, PhD (Investigation: Supporting; Writing – review & editing: Supporting)

Christopher Storm-Larsen, MSc (Data curation: Equal; Methodology: Equal; Writing – review & editing: Supporting)

Øyvind Midttun, PhD (Methodology: Equal; Writing – review & editing: Equal)

Adrian McCann, PhD (Methodology: Equal; Writing – review & editing: Supporting)

Per M. Ueland, PhD (Methodology: Lead; Resources: Equal; Writing – review & editing: Equal)

Marte L. Høivik, PhD, MD (Investigation: Equal; Writing – review & editing: Supporting)

Mette Vesterhus, PhD, MD (Investigation: Equal; Writing – review & editing: Supporting)

Marius Trøseid, MD, PhD (Writing – review & editing: Supporting)

Matthias Laudes, MD (Investigation: Equal; Writing – review & editing: Supporting)

Wolfgang Lieb, MD (Investigation: Equal; Writing – review & editing: Supporting) Tom H. Karlsen, PhD, MD (Investigation: Equal; Writing – review & editing: Supporting)

Corinna Bang, PhD (Data curation: Equal; Investigation: Equal; Methodology: Supporting; Writing – review & editing: Equal)

Christoph Schramm, MD (Funding acquisition: Equal; Investigation: Equal; Writing – review & editing: Lead)

Andre Franke, PhD (Conceptualization: Lead; Funding acquisition: Lead; Investigation: Equal; Methodology: Equal; Project administration: Lead; Supervision: Lead; Writing – review & editing: Lead)

Johannes R. Hov, PhD, MD (Conceptualization: Lead; Data curation: Supporting; Funding acquisition: Lead; Investigation: Lead; Methodology: Supporting; Project administration: Lead; Supervision: Lead; Writing – original draft: Lead; Writing – review & editing: Lead).