Original research

Altered faecal microbiome and metabolome in IgG4-related sclerosing cholangitis and primary sclerosing cholangitis

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ABSTRACT

Objective Multiple clinical similarities exist between IgG4-related sclerosing cholangitis (IgG4-SC) and primary sclerosing cholangitis (PSC), and while gut dysbiosis has been extensively studied in PSC, the role of the gut microbiota in IgG4-SC remains unknown. Herein, we aimed to evaluate alterations of the gut microbiome and metabolome in IgG4-SC and PSC.

Design We performed 16S rRNA gene amplicon sequencing of faecal samples from 135 subjects with IgG4-SC (n=34), PSC (n=37) and healthy controls (n=64). A subset of the samples (31 IgG4-SC, 37 PSC and 45 controls) also underwent untargeted metabolomic profiling.

Results Compared with controls, reduced alpha-diversity and shifted microbial community were observed in IgG4-SC and PSC. These changes were accompanied by differences in stool metabolomes. Importantly, despite some common variations in the microbiota composition and metabolic activity, integrative analyses identified distinct host–microbe associations in IgG4-SC and PSC. The disease-associated genera and metabolites tended to associate with the transaminases in IgG4-SC. Notable depletion of *Blautia* and elevated succinic acid may underlie hepatic inflammation in IgG4-SC. In comparison, potential links between the microbial or metabolic signatures and cholestatic parameters were detected in PSC. Particularly, concordant decrease of *Eubacterium* and microbiota-derived metabolites, including secondary bile acids, implicated novel host–microbial metabolic pathways involving cholestasis of PSC. Interestingly, the predictive models based on metabolites were more effective in discriminating disease status than those based on microbes.

Conclusions Our data reveal that IgG4-SC and PSC possess divergent host–microbe interplays that may be involved in disease pathogenesis. These data emphasise the uniqueness of IgG4-SC.

Significance of the study

What is already known on this subject?
- IgG4-related sclerosing cholangitis (IgG4-SC) is a disease phenotype of IgG4-related disease with involvement of the hepatobiliary tract.
- IgG4-SC and primary sclerosing cholangitis (PSC) share similar clinical manifestations, while treatment and prognosis are different.
- Multiple studies have suggested a critical role of gut microbiota in the pathogenesis of PSC.

What are the new findings?
- The faecal microbiome of IgG4-SC was characterised by reduced intraindividual diversity and altered microbiome structure, compared with health controls (HCs).
- The faecal metabolomic profiles were significantly distinct across IgG4-SC, PSC and HCs, and were tightly coupled with microbiome compositions.
- Microbial and metabolic changes in IgG4-SC were associated with hepatic inflammation, while the dysbiosis in PSC was linked to cholestasis of the disease.
- Predictive models based on faecal metabolites performed better than those based on microbiota in discriminating disease status.

How might it impact on clinical practice in the foreseeable future?
- Our study reveals insights into host–microbiota interactions that differentially disturbed in IgG4-SC and PSC, and thus provide opportunities for developing microbiota-based diagnostic tools.

INTRODUCTION

IgG4-related sclerosing cholangitis (IgG4-SC) is the hepatobiliary phenotype of IgG4-related disease (IgG4-RD), a multisystem fibroinflammatory condition defined by elevated serum IgG4 level and lymphoplasmacytic infiltration with abundant IgG4-positive cells.[1 2] Primary sclerosing cholangitis (PSC) is a progressive chronic liver disease characterised by multifocal biliary strictures and frequent comorbidity of inflammatory bowel diseases (IBD).[3] The major clinical presentations of IgG4-SC, jaundice and pruritus, mimic that of PSC and are therefore misleading in some occasions.[4] Corticosteroids treatment is effective for the majority of patients with IgG4-SC, whereas therapeutic strategies are

limited for patients with PSC. Outcomes of the two diseases are different, with higher risk of liver cirrhosis, cholangiocarcinoma and colorectal cancer in PSC than IgG4-SC.[4 5]

The pathogenesis of these two sclerosing cholangitis is currently unknown. Nonetheless, environmental exposures, in particular the immense intestinal microbiota and its derivatives, have inspired recent aspects of research.[6] Indeed, patients with PSC exhibit compositional alterations in gut microbiota, which are characterised by decreased bacterial diversity and increased abundance of potential pathobionts including *Veillonella*, *Enterococcus* and *Fusobacterium*.[7–9] Mechanistic investigations found that *Klebsiella pneumonia* in the intestines of patients with PSC induced bacterial translocation and subsequent Th17-mediated liver inflammation.[10] In contrast, gut microbiota in IgG4-SC has not been elucidated. IgG4-RD is characterised by type II immune response and is often accompanied by a clinical history of allergy and elevated serum level of IgE.[11] Given that dysbiosis has been found to elicit type II immune response and allergic reaction,[12] whether gut microbiota plays a role in IgG4-SC needs further investigations.

The gut microbiota has a profound impact on host physiology, which can be mediated by small molecules produced by microbes. These microbial metabolites are able to induce host responses in the intestine and at the distant organs. Increasing evidence suggests that microbial metabolites, such as short-chain fatty acids (SCFAs), secondary bile acids and trimethylamine, are implicated in the pathogenesis of liver diseases.[13] The stool metabolome helps explain a high proportion of variance of gut microbiome composition and thus is considered as an intermediate phenotype that mediates host–microbiota crosstalk.[14 15] Therefore, integrated analysis of multiomics data from gut microbiome, metabolome and host physiology may provide a clue to the mechanistic links between gut microbiota and disease.[16 17]

In the current study, we performed 16S rRNA gene amplicon sequencing and untargeted liquid chromatography–tandem mass spectrometry metabolomic profiling of stool samples in a cohort comprised of IgG4-SC, PSC and healthy controls. The microbiome and metabolome data were then integrated to characterise IgG4-SC-associated and PSC-associated alterations in faecal microbial and metabolic profiles, as well as to assess potential functional links between gut microbiome, metabolome and host phenotypes in disease cohorts.

METHODS

Study group

There were three study groups: IgG4-SC (n=34), PSC (n=37) and health control (HC, n=64). Patients of IgG4-SC and PSC were recruited from the outpatient clinic of Renji Hospital, affiliated to the Shanghai Jiao Tong University School of Medicine, from September 2014 to May 2019. Thirty-four patients of IgG4-SC were diagnosed according to the Histology, Imaging, Serology, Other organ involvement and Response to steroid therapy (HISORt) criteria.[18] Thirty-seven patients with PSC were enrolled based on the 2009 European Association for the Study of the Liver Clinical Practice Guidelines 'Management of cholestatic liver diseases'.[19] The HC group was recruited from volunteers taking routine health examinations in health management centre of Renji Hospital. The inclusion criteria included (1) normal range of liver and kidney functional tests; (2) normal blood glucose/lipid, normal urine and stool; (3) free of hepatitis B/C virus antigen; and (4) not taking antibiotics within 8 weeks. Written informed consent of all patients and the HC group were obtained before sample collection. Patients or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

DNA extraction, 16S rRNA gene amplicon sequencing and data processing

Stool samples from all participants were freshly collected and frozen at −80°C within 3 hours after sampling. DNA was extracted using Hipure soil DNA kit (Magen, Guangzhou, China). The 16S rRNA V3–V4 region was amplified and sequenced using MiSeq platform (Illumina, San Diego, California, USA). The 16S rRNA data were processed using Quantitative Insights Into Microbial Ecology (QIIME2 V.2019.10) as described previously.[20] Sequencing reads filtering and feature table (amplicon sequence variants (ASVs)) construction were performed using DADA2 software. Taxonomic information was obtained according to the SILVA database V.138. ASVs with a number of sequences<0.005% of the total number of sequences were removed to reduce the effect of spurious sequences. We then built a phylogenic tree with Fast-Tree plugin after sequence alignment using MAFFT. Alpha and beta diversity analyses were conducted using q2-diversity. Taxa present in at least 10% of samples in either group were selected for further differential analysis.

Faecal metabolome profiling and data preprocessing

Faecal metabolome profiling was preformed using an ultrahigh-performance liquid chromatograph system (Vanquish, Thermo Fisher Scientific) along with Q Extractive HFX mass spectrometer (Orbitrap MS, Thermo). First, 25 mg of stool samples was added with 500 µL acetonitrile/methanol/water (2:2:1, v/v/v) containing isotopically labelled internal standard mixture. After 30 s vortex, samples were homogenised (35 Hz, 4 min) and sonicated (5 min) in ice-water bath three times. Then the samples were incubated for 1 hour at −20°C and centrifuged at 12 000 rpm for 15 min at 4°C. The supernatants (25 µL) were transferred to a fresh glass vial, from which 3 µL was injected for later liquid chromatography with tandem mass spectrometry analysis. An equal aliquot of each sample (10 µL) was mixed as the quality control (QC) sample.

The raw data were converted to mzXML format with ProteoWizard and then processed with an in-house program (developed using R for automatic data analysis) for peak detection, extraction, alignment and integration (Biotree, Shanghai). In total, 8203 and 5564 peaks were detected in positive and negative ionisation modes, respectively. Metabolite peaks with relative SD of >0.3 across QC samples and present in <50% of samples were removed from the following analysis. After that, 4513 (positive mode) and 3614 (negative mode) metabolite features were left and the remaining missing values were filled with half of minimum. The positive-mode and negative-mode features were then concatenated and a subset of 654 metabolites were annotated (MS2 score of >0.8) using an in-house reference data.

STATISTICAL ANALYSIS

All statistical analyses were performed using R V.4.0.2. For continuous variable comparison, a two-tailed Wilcoxon rank-sum test was used. Canonical correspondence analysis (CCA) was performed on ASV abundance profiles of all the samples to evaluate the effects of demographical variables and medication uses on microbiota community variation. Multivariate Association with Linear Models (MaAsLin) framework was used to adjust

the effects of antibiotics and ursodeoxycholic acid (UDCA) usage in taxonomic analysis.

Metabolome data were log2 transformed and scaled to unit variance in multivariate analysis. Principal component analysis was performed to filter out samples outside 95% CI prior to following faecal metabolome analyses. Orthogonal partial least squares discriminant analysis (OPLS-DA) and O2PLS-DA (two-way OPLS-DA) (for comparison of three groups) were performed in Simca-P V.14.0 (Umetrics AB), and subsequent models were further evaluated based on sevenfold cross-validation. Only confidently annotated metabolites were selected for differential analysis. Metabolites with (1) variable importance in the projection of >1, (2) fold change of >1.5 or<0.67 and (3) P_{fdr} of <0.05 (Wilcoxon rank-sum test) were log2 transformed and subjected to linear model analysis to control for confounding factors including antibiotics and UDCA medication. Finally, metabolites with P_{fdr} of <0.05 (linear model analysis) were regarded as differentially abundant.

Partial Spearman rank correlation test (PResiduals package) was used to evaluate associations between altered taxa (present in >20% of samples) and metabolites among all the subjects. The correlations between clinical indexes and taxa/metabolites were assessed in the IgG4-SC and PSC group, respectively.

Random forest models were built to differentiate disease status (randomForest package). For input features, IgG4-SC/HC and PSC/HC classifiers incorporated all differentially abundant taxa and/or metabolites, while IgG4-SC/PSC classifiers were based on features altered in IgG4-SC or PSC only (compared with HC). Fivefold cross-validation method (*rfcv* function) was used to determine the best number of discriminating features. The relative abundance of genus was arcsine squared-root transformed before random forest analysis.

Multiple hypothesis tests were adjusted using Benjamini and Hochberg false discovery rate (FDR), and FDR of <0.05 was considered significant.

RESULTS

Characteristics of the study population

We performed 16S rRNA gene amplicon sequencing of faecal samples in a total of 135 individuals and applied untargeted metabolomic profiling in a subset of 113 samples (figure 1A). Of the 34 patients with IgG4-SC, 18 were present with autoimmune pancreatitis and six progressed into liver cirrhosis stage. In the PSC group, 10 patients were concomitant with IBD, 15 with autoimmune hepatitis, and 7 had liver cirrhosis. The demographics and clinical characteristics of the participants are all presented in online supplemental table 1. In accordance with previously reported data,[3] patients with IgG4-SC were older compared with patients with PSC (median: 63 vs 33); male predominance in IgG-SC was more evident relative to PSC (85.3% vs 62.2%); and Body Mass Index (BMI) was balanced across all groups. Regarding prior medication history, 56% of patients with IgG4-SC had received corticosteroid therapy and 68% of patients with PSC had been administered UDCA.

Reduced alpha diversity and altered microbial composition in IgG4-SC and PSC

A reduced α-diversity was observed in both IgG4-SC (n=34) and PSC (n=37) in comparison with HC (n=64), measured by Simpson Index (p=0.033 and 0.0024, respectively, Wilcoxon rank-sum test) (figure 1B). Principal coordinated analysis (PCoA) based on Aitchison dissimilarities metrics was performed to evaluate the variation in community composition. Both of the disease groups significantly deviated in overall microbiome structure from HC (PERMANOVA test, IgG4-SC vs HC: pseudo-F: 1.59, p=0.001; PSC vs HC: pseudo-F: 2.24, p=0.001; figure 1C). However, there was no obvious difference in the global microbiome composition between IgG4-SC and PSC (figure 1C).

We next sought to identify the potential factors associated with the microbial profiles. CCA found that UDCA and antibiotics usage showed significant associations with overall taxonomic composition (permutation test, n=135; UDCA: pseudo-F: 1.15, p=0.017; antibiotics: pseudo-F: 1.38, p=0.003), among the variables including age, gender, BMI and medication uses (online supplemental figure 1). Accordingly, we deconfounded the effects of antibiotics and UDCA in the subsequent analyses.

Taxonomic signatures of IgG4-SC and PSC microbiota

The associations of individual genus with IgG4-SC or PSC were assessed by comparing the relative abundances between (1) IgG4-SC and HC, (2) PSC and HC, and (3) IgG4-SC and

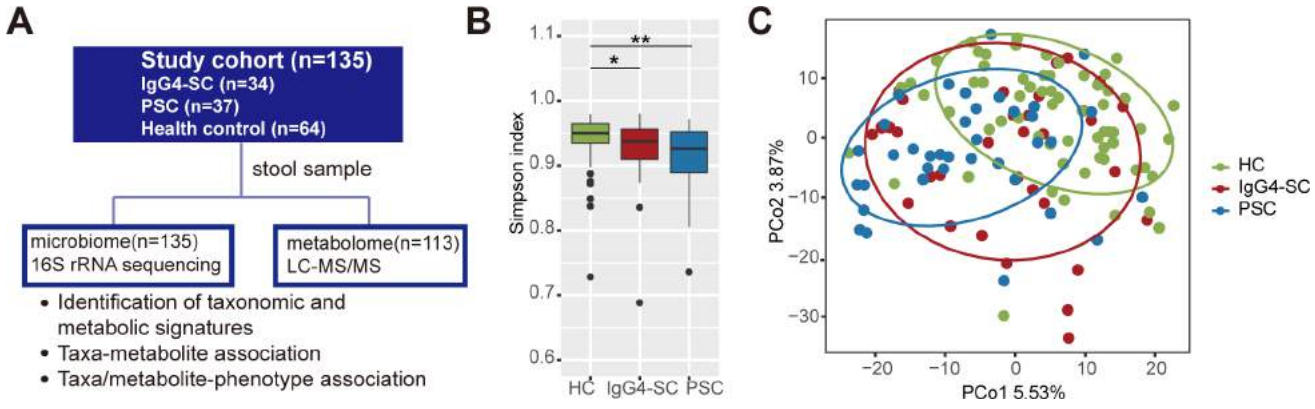

Figure 1 Faecal microbiome variations in IgG4-SC and PSC. (A) Overview of the study design. (B) Alpha diversity measured by Simpson Index was highest in HC (n=64) followed by IgG4-SC (n=34) and PSC (n=37) (Wilcoxon rank-sum test). (C) PCoA based on Aitchison distances at ASV level showed different taxonomic compositions between disease groups and HC. Boxes represent 25th–75th quartile range, and lines within boxes denote median values. Whisker denotes the highest and lowest values within 1.5 times IQR, and outliers are represented as dots. *P<0.05, **P<0.01. ASV, amplicon sequence variants; HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PCoA, principal coordinated analysis; PSC, primary sclerosing cholangitis.

PSC. Twenty-two genera reflected differential abundances between IgG4-SC and HC (P_{fdr}<0.05, Wilcoxon rank-sum test; online supplemental table 2). MaAsLin was then applied to control the potential confounding factors including antibiotics and UDCA uses, and 17 out of 22 genera still reached statistically significant associations (P_{fdr}<0.05, MaAsLin; online supplemental table 2). In PSC, the relative abundances of 37 genera were different from HC (P_{fdr}<0.05, Wilcoxon rank-sum test), 13 of which withstood correction for the confounders (P_{fdr}<0.05, MaAsLin; online supplemental table 3). Notably, fewer differences were observed between IgG4-SC and PSC, with four genera showing suggestive differences (p<0.05, Wilcoxon rank-sum test) and none of them remaining significant after multiple testing correction or covariate adjustments (online supplemental table 4).

Next, we focused on taxonomic signatures that significantly changed in one disease status (P_{fdr}<0.05, Wilcoxon rank-sum test) but not in the other (P_{fdr}>0.2, Wilcoxon rank-sum test), when compared with HC. Accordingly, three taxa were specific to IgG4-SC, including increased *Streptococcus* as well as reduced *Blautia* and *Lachnospiraceae ND3007 group* (figure 2A,E). In PSC, 11 taxa exclusively altered, among which *Ruminococcus gnavus* and *Turicibacter* were over-represented, while the others such as *Oscillospiraceae*

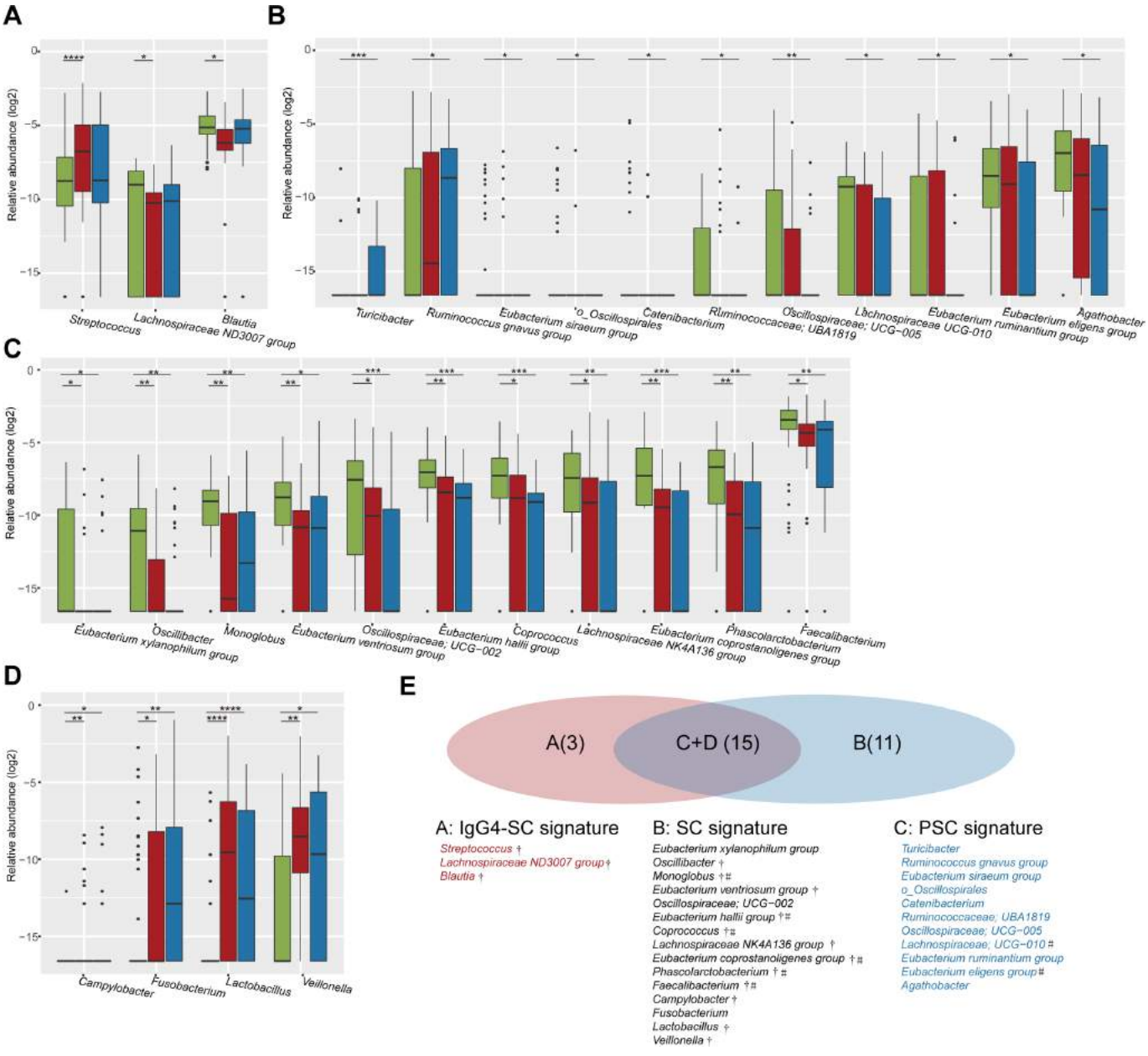

Figure 2 Gut microbiota signatures in patients with IgG4-SC and/or PSC. (A–D) Boxplots show the relative abundance of genera altered in (A) IgG4-SC (n=34), (B) PSC (n=37) and (C,D) both groups compared with HC (n=64) analysed by Wilcoxon rank-sum test (online supplemental tables 2 and 3). (E) Venn diagram outlined the genera associated with one disease status (P_{fdr}<0.05, Wilcoxon rank-sum test) but not with the other (P_{fdr}>0.2, Wilcoxon rank-sum test), as well as the genera consistently altered in both diseases (P_{fdr}<0.05, Wilcoxon rank-sum test). Relative abundances were logarithmic-transformed and 0 values were assigned 1e-05. † and # denote genera associated with IgG4-SC and PSC, respectively, after using MaAsLin adjusting for antibiotics and UDCA uses (P_{fdr}<0.05). Boxplot illustrations are described in figure 1. * P_{fdr}<0.05, ** P_{fdr}<0.01, *** P_{fdr}<0.001, **** P_{fdr}<0.0001. HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; PSC, primary sclerosing cholangitis; SC, sclerosing cholangitis.

UCG-005, *Lachnospiraceae UCG-010* and *Eubacterium eligens group* were under-represented (figure 2B,E).

Compared with HC, patients with IgG4-SC and PSC demonstrated substantial overlap in the changes of the individual taxa. In total, 15 taxa were associated with both IgG4-SC and PSC (P_{fdr}<0.05, Wilcoxon rank-sum test), including increased *Fusobacterium*, *Lactobacillus* and *Veillonella*, as well as decreased *Oscillospiraceae UCG−002* and *Faecalibacterium* (figure 2C–E).

Faecal metabolomic alterations in IgG4-SC and PSC

Considering the interplay between the gut microbiome and host metabolism, we performed untargeted metabolomics on faecal samples (IgG4-SC n=31, PSC n=37 and HC n=45). OPLS-DA revealed that the metabolic composition of IgG4-SC and PSC significantly deviated from controls as well as from each other (IgG4-SC vs HC: R^2Y=0.954, Q^2=0.508; PSC vs HC: R^2Y=0.971, Q^2=0.732; IgG4-SC vs PSC: R^2Y=0.893, Q^2=0.291; online supplemental figure 2). Next, we tested

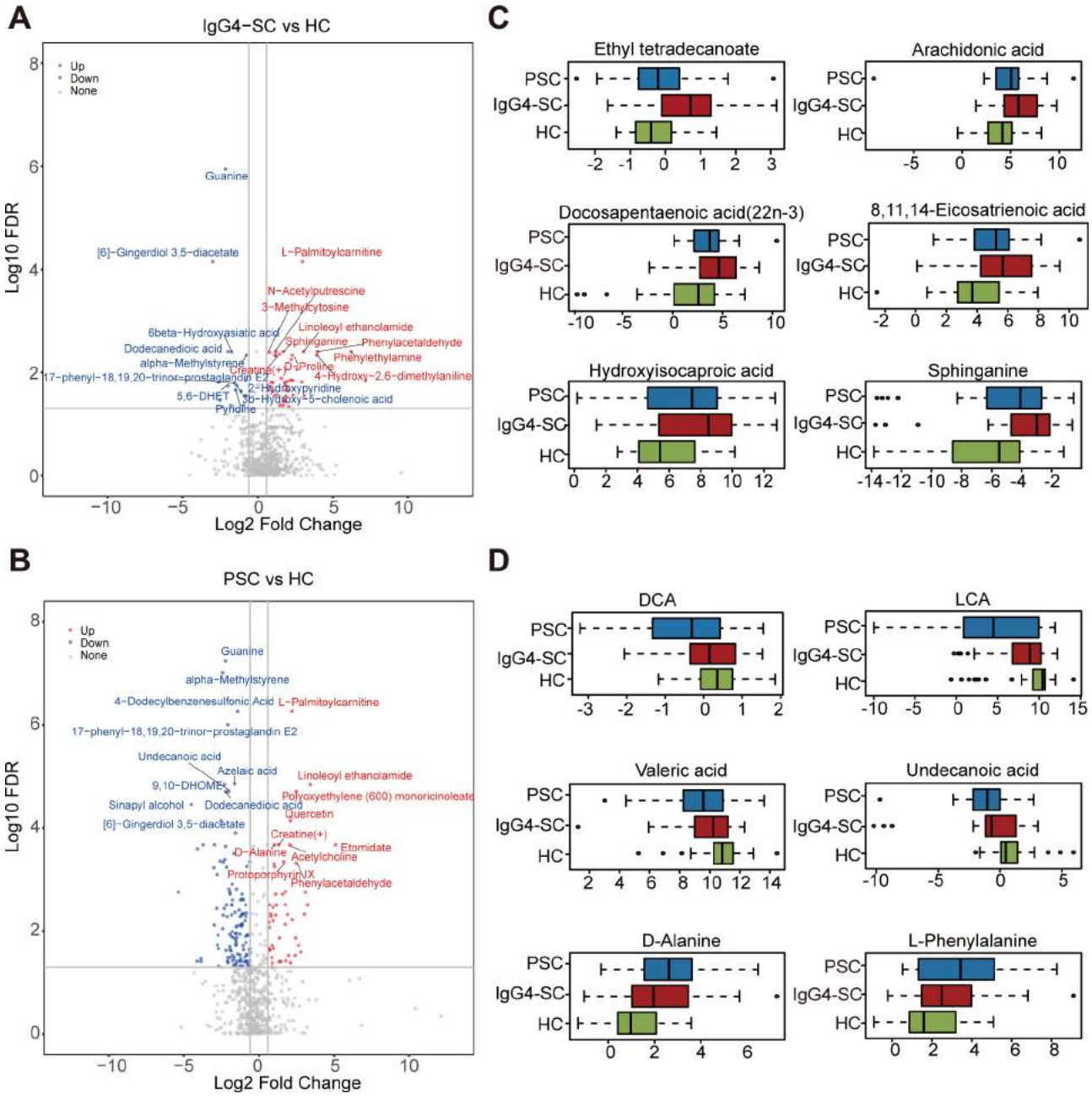

Figure 3 Faecal metabolome changes in disease groups versus HCs. (A,B) Volcano plot demonstrated metabolites changes in IgG4-SC (n=29) and PSC (n=36), compared with HC (n=43), respectively. The X axis indicates log2-transformed FC of faecal metabolite abundances, and Y axis denotes P_{fdr} analysed using Wilcoxon rank-sum test. The horizontal lines represent P_{fdr}<0.05 and vertical lines indicate FC of >1.5 or<0.67. Metabolites elevated or decreased are highlighted in red and blue, respectively, and top 10 metabolites with the lowest P_{fdr} are labelled with text. (C,D) Boxplot showed representative metabolites that were significantly changed in IgG4-SC or PSC after controlling for confounding factors using linear model on log-transformed abundance data (online supplemental table 5 and 6). Metabolite abundances are visualised after log2 transformation and scaled to UV . DCA, deoxycholic acid; FC, fold change; FDR, false discovery rate; HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; LCA, lithocholic acid; PSC, primary sclerosing cholangitis; UV, unit variance.

for associations of each annotated metabolite (n=654) with IgG4-SC and PSC (figure 3A,B). After adjusting for confounding factors including antibiotics and UDCA usage, 58 metabolites were differently abundant in IgG4-SC and 98 metabolites were different in PSC, compared with HC (P_{fdr}<0.05, linear model; online supplemental tables 5 and 6). Among them, 31 metabolites were concordantly altered in IgG4-SC and PSC. Particularly, succinic acid was remarkably increased in both diseases (online supplemental figure 3). Alterations in succinic acid signalling have been linked to activation and effector functions of immune cells, and a role of microbiota-produced succinic acid in intestinal inflammation has been demonstrated.[21 22] L-palmitoylcarnitine, a long-chain acylcarnitine, was also highly enriched in the disease groups (online supplemental figure 3). Previous studies have suggested the possible effects of long-chain acylcarnitines on activation of inflammation.[23] In addition, the microbiota-derived amino acid derivatives, including gamma−aminobutyric acid (GABA) and phenylethylamine were also upregulated in IgG4-SC and PSC (online supplemental figure 3).

Of the 27 metabolites uniquely altered in IgG4-SC, the majority (n=23, 85%) were increased in the disease group (figure 3A and online supplemental table 5). These included several potential proinflammatory mediators such as ethyl tetradecanoate, a long-chain ester,[24] and polyunsaturated fatty acids including docosapentaenoic acid (22 n-3), 8,11,14-eicosatrienoic acid and arachidonic acid (figure 3C). Hydroxyisocaproic acid, an end product of host leucine metabolism with broad-spectrum bactericidal activity, was also elevated in IgG4-SC (figure 3C).[25] Increased level of sphinganine was also found in IgG4-SC (figure 3C). Sphinganine is the precursor of ceramide and is involved in sphingolipid metabolism closely linked to cell signalling.[26]

A total of 67 metabolites were identified changed in PSC only. In contrast to IgG4-SC, most metabolites (n=49, 73%) manifested lower levels in the disease group (figure 3B and online supplemental table 6). Secondary bile acids including deoxycholic acid (DCA), lithocholic acid (LCA) and glycine-conjugated lithocholic acid (GLCA), metabolised by intestinal bacteria, were dramatically decreased in PSC (figure 3D and online supplemental table 6). Valeric acid, belonging to a group of SCFAs, was also decreased in PSC (figure 3D). In addition to the anti-inflammatory effect in intestinal and systemic immune diseases, a recent study noted that valerate induced Breg differentiation and suppressed Th17 cells, consequently protecting against colitis and multiple sclerosis.[27] Besides, reductions of medium chain fatty acids (MCFAs), including azelaic acid, undecanoic acid and dodecanedioic acid, were more pronounced in PSC compared with changes in IgG4-SC (figure 3D and online supplemental table 5 and 6). MCFAs have multiple effects including antifungal and anti-inflammatory.[28 29] In contrary, multiple amino acids and their derivatives were elevated in PSC (figure 3D).

When comparing patients with IgG4-SC and PSC, four metabolites including valproic acid, 4-dodecylbenzenesulfonic acid, 9,10-DHOME and pelargonic acid showed significant differences (P_{fdr}<0.05, linear model; online supplemental table 7).

Associations between the disease-linked microbiota and metabolites

Faecal metabolomic and gut microbial profiles were robustly correlated across all subjects (Procrustes analysis, n=108, M^2=0.946, p=0.004), confirming the strong link between faecal microbes and faecal metabolites. Subsequently, we performed correlation analysis to examine the associations between the differentially abundant genera and metabolites while adjusting for the covariates in the whole cohort (n=108, partial Spearman correlation; figure 4A). In general, we observed strong positive associations between taxa and metabolites that were both elevated in controls, as well as negative associations between control-enriched taxa and disease-enriched metabolites (figure 4A).

Notably, L-palmitoylcarnitine, a long-chain acylcarnitine elevated in both disease groups, was negatively associated with control-enriched genera, such as *Eubacterium coprostanoligenes group* and *Lachnospiraceae NK4A136 group* (P_{fdr}=2.18E-11, ρ=−0.61 and P_{fdr}=9.77E-08, ρ=−0.54, respectively; figure 4A,B). Overabundant *Streptococcus* (observed in IgG4-SC) was positively correlated with increased hydroxyisocaproic acid (P_{fdr}=0.008, ρ=0.28; figure 4B). *Blautia*, the genus decreased in IgG4-SC, negatively correlated with succinic acid (P_{fdr}=0.013, ρ=−0.26; figure 4B). The decreased levels of valeric acid and MCFAs (azelaic acid and undecanoic acid), observed in PSC were accompanied by reduced abundances of control-enriched genera including *Alistipes* and *Oscillospiraceae UCG-002* (P_{fdr}<0.05, figure 4A,B). In addition, LCA was positively associated with PSC-decreased taxa such as *E. eligens*, *E. hallii and E. coprostanoligenes* (P_{fdr}<0.05, figure 4A,B). *Eubacterium* spp. are involved in the metabolism of secondary bile acids.[30]

Associations of microbial taxa and metabolites with clinical phenotype

To further understand whether disease-associated taxa and metabolites contribute to disease severity, we tested for their correlations with clinical parameters using partial Spearman correlation. In IgG4-SC, the faecal microbes and metabolites differentially abundant in patients exhibited more associations with transaminases levels (figure 5A). Increased *Blautia* was linked to lower levels of alanine transaminase (ALT) (p=0.017, ρ=−0.46; figure 5A) while *Campylobacte*r positively correlated with AST (p=0.008, ρ=0.42; online supplemental figure 4A). However, these correlations did not withstand multiple testing correction. Interestingly, higher levels of succinic acid accompanied with increased ALT (P_{fdr}=0.027, ρ=0.61) and AST (P_{fdr}=0.006, ρ=0.63) (figure 5A,B and online supplemental figure 4A).

In PSC, in addition to validation of the correlations between *Veillonella* and clinical indices in previous studies,[9] more importantly, we identified novel relationships among microbes, metabolites and host phenotypes. Interestingly, many of the metabolic features altered in PSC were associated with cholestatic parameters (figure 5C). For example, DCA, depleted in PSC, was negatively associated with alkaline phosphatase (ALP) and TB (P_{fdr}=0.0002, ρ=−0.76 and P_{fdr}=0.003, ρ=−0.69), and LCA exhibited a tendency of negative association with TB (p=0.035, ρ=−0.38) (figure 5C,D and online supplemental figure 4B).

Although many of the associations were not significant after correction for multiple testing, possibly due to the small sample size of each disease group, the data still provided hints of potential links between microbial features and disease severity.

Integrative multiomics signatures of IgG4-SC and PSC

To investigate the potential utility of gut microbial and metabolic profiles in disease prediction, we built random forest models based on faecal taxonomic or metabolic features to discriminate IgG4-SC, PSC and HC from each other. Fivefold cross-validated random forest model was first employed to select key discriminatory bacterial taxa or metabolites (online supplemental figure

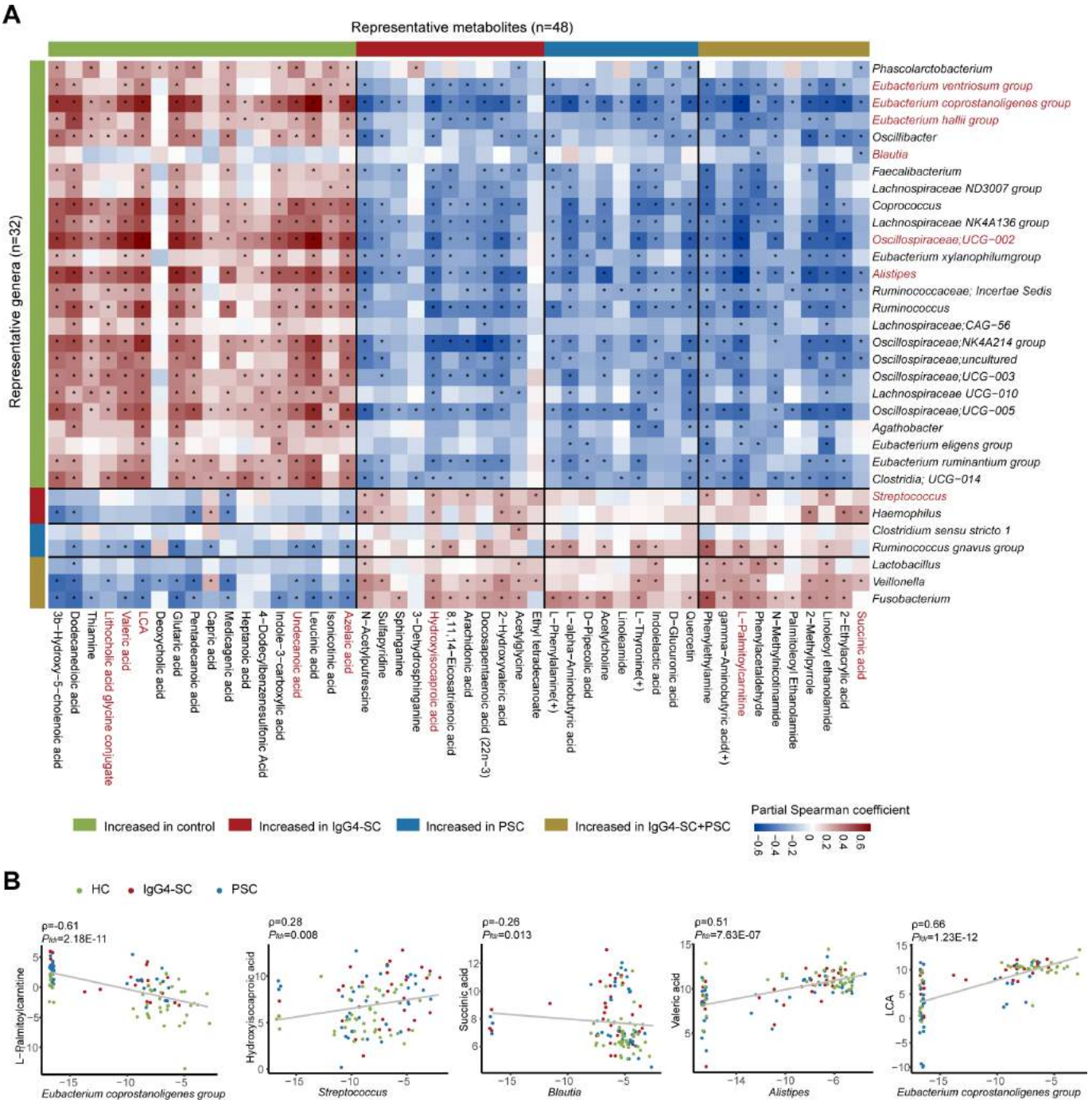

Figure 4 Associations of disease-related taxa and metabolites. (A) The heatmap depicts relationships between the taxa and metabolites changed in IgG4-SC and/or PSC (partial Spearman analysis, n=108). (B) Examples of individual taxa–metabolite associations. Abundances of taxa and metabolites are plotted after log2 transformation, and 0 values were assigned 1e-05. Each dot represents one sample. *P_{fdr}<0.05. HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; PSC, primary sclerosing cholangitis.

5). This method identified a gut microbiome signature composed of 10 genera that distinguished IgG4-SC from HC with an AUC of 0.81 (figure 6A and online supplemental table 8). The model based on seven metabolites performed better than microbial features with an AUC of 0.94 (figure 6A and online supplemental table 8). However, the integration of metabolic and taxonomic features did not improve the classification accuracy (AUC: 0.94) (figure 6A). Similarly, the metabolite-derived model was more accurate than the microbe-based model in discriminating between PSC and HC (AUC: 0.99 and 0.85, respectively) (figure 6B and online supplemental table 8). Comparatively, predicting IgG4-SC and PSC proved more challenging. The use of metabolomic data or both datasets combined showed similar accuracies in distinguishing IgG4-SC from PSC (AUC: 0.82 and 0.80, respectively), while a relatively poor performance (AUC: 0.64) was observed when using taxonomic data only (figure 6C and online supplemental table 8). The finding that combining microbes and metabolites did not lead to increased classifying accuracy was consistent with the high correlation between faecal microbiome and metabolome as aforementioned. Taken together,

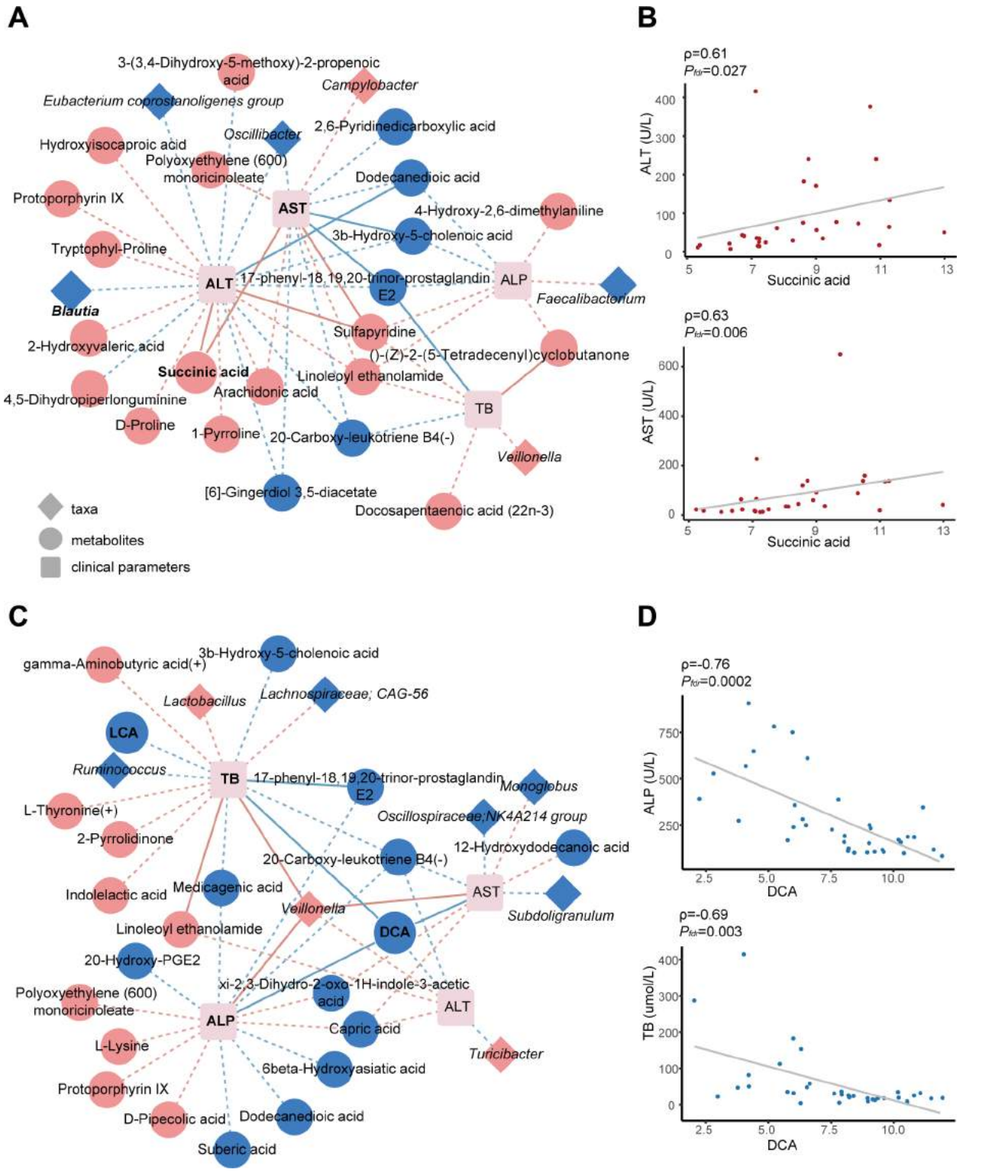

Figure 5 Integrative network of associations reflecting host–microbe interactions. (A,C) Network revealed both significant (P_{fdr}<0.05) and suggestive associations (p<0.05 and |ρ|>0.3, partial Spearman analysis) between differentially abundant taxa or metabolites and clinical indexes in IgG4-SC (n=29) and PSC (n=36), respectively. Nodes represent features increased (in red) or decreased (in blue) in disease groups compared with HC. Lines connecting nodes indicate positive (red) or negative (blue) correlations. The solid and dashed lines denote significant and suggestive correlations, respectively. (B,D) Examples of correlations between taxa/metabolites and clinical parameters in the IgG4-SC or the PSC group. Abundances of taxa and metabolites are plotted after log2 transformation, and each dot represents one sample. ALP, alkaline phosphatase; ALT, alanine transaminase; DCA, deoxycholic acid; HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; LCA, lithocholic acid; PSC, primary sclerosing cholangitis; TB, total bilirubin.

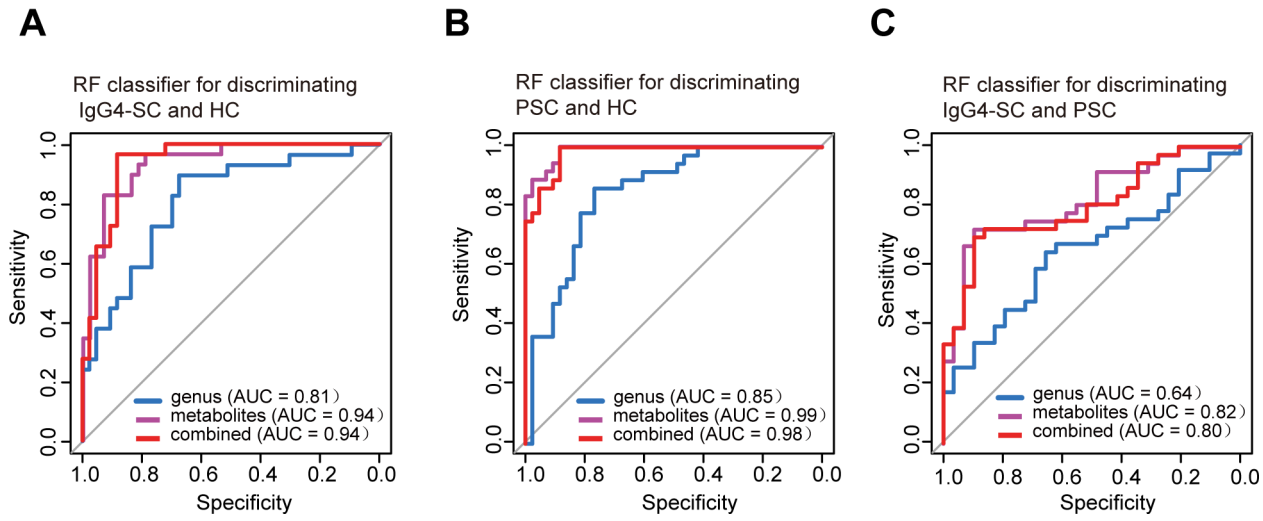

Figure 6 Disease status classification using disease-associated taxa and/or metabolites. (A–C) Random forest classifiers composed of genera, metabolites and their combination were respectively constructed to discriminate (A) IgG4-SC and HC, (B) PSC and HC, and (C) IgG4-SC and PSC. AUC, area under the curve; HC, health control; IgG4-SC, IgG4-related sclerosing cholangitis; PSC, primary sclerosing cholangitis; RF, random forest.

these data indicated that the predictive models based on faecal metabolites performed better than those based on microbiota in discriminating disease status.

DISCUSSION

In this study, we performed integrated multiomics analyses of gut microbiome, metabolome and host phenotypes in patients with IgG4-SC and PSC. In spite of the substantial overlap in the changes of faecal microbiome and metabolome between IgG4-SC and PSC, our study was able to identify disease-related signatures that can potentially be used to distinguish the patients with IgG4-SC from those with PSC. Furthermore, microbial and metabolomic profiles were found extensively correlated with each other and with disease severity in each condition, providing novel mechanistic insights into the disease pathogenesis.

The common characteristics of gut dysbiosis observed in IgG4-SC and PSC included reduced alpha-diversity, shifted microbial community as well as altered abundances of individual taxa. However, these changes in patients with PSC were more significant than those in patients with IgG4-SC compared with control individuals, suggesting a relatively modest dysbiosis in IgG4-SC. Similarly, greater changes in metabolomic profile were observed in the PSC cohort.

Many of the differentially abundant individual taxa and metabolites were consistently identified in IgG4-SC and PSC, which may indicate a general mechanism underlying these two conditions. A prominent increase in L-palmitoylcarnitine, a long-chain acylcarnitine, was detected in both disease groups, and was correlated with several disease-associated genera. A number of acylcarnitines were recently found enriched in faecal samples from a large IBD cohort.[31] Moreover, it has been reported that acylcarnitines may regulate T-cell differentiation and promote inflammation.[23 32] We also found an upregulation of GABA in stool samples of IgG4-SC and PSC. Although GABA has been shown to have anti-inflammatory and immunosuppressive effects in autoimmune diseases, a recent study found GABA signalling damaged the intestinal barrier and aggravated inflammation in a colitis model.[33 34] Intriguingly, the modules of GABA biosynthesis and metabolism were highly enriched in the gut microbiome of patients with liver cirrhosis.[35]

Regardless of the shared features between the gut microbiome and metabolome of the subjects with IgG4-SC and PSC compared with healthy controls, we identified distinct alterations of microbes and metabolites in each condition. It is noteworthy that when compared with healthy controls, most disease-associated metabolites were decreased in PSC, while IgG4-SC was marked by increased metabolites. These discrepancies may indicate yet unclear disease-specific mechanisms involving the gut microbiome. In particular, the depletion of *Blautia* in IgG4-SC is of interest, since this genus has been found associated with reduced risk of allergies,[36] and a history of allergy is commonly observed in patients with IgG4-SC.[1 11] Furthermore, *Blautia* inversely associated with ALT, and exhibited negative correlation with succinic acid. Although succinic acid was enriched in both disease groups, it demonstrated significant correlations with disease severity only in IgG4-SC. Succinic acid is an important immunoregulatory metabolite that can modulate immune response and inflammation in a variety of ways. Specifically, microbiota-derived succinic acid was shown to initiate a type II immune response,[37 38] coinciding with the type II immunity skewing in IgG4-SC. Collectively, the alterations of gut microbiota and metabolic activity in IgG4-SC are likely involved in the inflammatory process of the disease.

The dominant altered bacteria in PSC are in line with other studies, including *Veillonella*, *Lactobacillus*, *Fusobacterium* and *Ruminococcus*.[7–9 39–42] By integrating multiomics data, our study further revealed a range of host–microbiome interplays with potential mechanistic implications. The faecal secondary bile acids including DCA, LCA and GLCA were uniquely depleted in PSC, in agreement with previous results found in serum and bile of PSC.[43 44] It has been reported that secondary bile acids including LCA and DCA can exert protective effects on intestinal barrier both in vivo and in vitro.[45 46] Furthermore, depletion of LCA was associated with reduced abundance of *Eubacterium* spp, which contain bacteria capable of generating secondary bile acids.[30] Meanwhile, the inverse correlations between secondary bile acids and cholestatic parameters further supported the role of microbiota-mediated bile acid metabolism in disease pathogenesis. Taken together, these findings suggest gut microbial associations to metabolites linked to cholestasis in PSC.

Several limitations have to be acknowledged in our study. First, this is a single-centre study with relatively small sample size that may restrict generalisation of the results. Given that the prevalence of IgG4-SC and PSC is relatively low, future multicentre studies are needed to validate the data. Second, although we adjusted covariates including medication uses in the analysis, our results might be confounded by other host lifestyle and physiological characteristics. For example, recent studies have identified stool consistency as a strong source of gut microbiota variation which should be captured in the study design.[47] Additionally, a proportion of the patients were concomitant with other diseases and the effects were not evaluated in the current analysis. It will be important to increase the sample size and compare the gut microbial profiles between the individuals with and without the concomitant diseases in future studies. Third, use of 16S rRNA amplicon sequencing rather than metagenomics sequencing limited data interpretation such as functional inference. To overcome the shortage, we profiled the faecal metabolome, which is considered a functional readout of gut microbiome.[14] Finally, although our data revealed functional links between microbiome, metabolome and disease phenotype, it does not define cause-and-effect relationships. Functional studies are warranted to dissect the underlying mechanisms.

By leveraging multiomics data, our study represents the first endeavour to characterise the alterations of gut microbiome and metabolome in IgG4-SC, and to identify unique microbe–metabolite interactions in IgG4-SC and PSC, indicating plausible disease-specific mechanisms for future investigation.

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Contributors XM, RT, MEG and ML designed the study. RT, XM, ML, J-YF and QW obtained funding. ML, QM and XX performed clinical diagnosis. QL, BL, YL, YW, BH, JL, YL, ZY, ZL, RC, YC, JZ, RW, QQ and MS collected samples. QL, BL and YL contributed to the data collection. QL and BL analysed and interpreted the data. QL, RT, BL and ML drafted the manuscript. XM and MEG revised the manuscript. All the authors approved the final version of the manuscript.

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