

# Open Label Vancomycin in Primary Sclerosing Cholangitis-Inflammatory Bowel Disease: Improved Colonic Disease Activity and Associations With Changes in Host–Microbiome–Metabolomic Signatures

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## Abstract

**Background:** We conducted a single-arm interventional study, to explore mucosal changes associated with clinical remission under oral vancomycin (OV) treatment, in primary sclerosing cholangitis-associated inflammatory bowel disease (PSC-IBD); NCT05376228.

**Methods:** Fifteen patients with PSC and active colitis (median fecal calprotectin 459 µg/g; median total Mayo score 5) were treated with OV (125 mg QID) for 4 weeks and followed-up for a further 4 weeks of treatment withdrawal (8 weeks, end-of-study). Colonic biopsies were obtained at baseline and Week 4. Clinical assessments, and serum and stool samples (metagenomics, metatranscriptomics, and metabolomics) were collected at Weeks 0, 2, 4, and 8. The primary efficacy outcome measure was the induction of clinical remission.

**Results:** Oral vancomycin resulted in clinical remission in 12/15 patients and significant reductions in fecal calprotectin. Oral vancomycin was associated with reduced abundances of *Lachnospiraceae*, genera *Blautia* and *Bacteroides*; and enrichment of *Enterobacteriaceae*, and genera *Veillonella*, *Akkermansia*, and *Escherichia*. Oral vancomycin treatment was associated with the downregulation of multiple metatranscriptomic pathways (including short-chain fatty acid [SCFA] metabolism and bile acid [BA] biotransformation), along with host genes and multiple pathways involved in inflammatory responses and antimicrobial defence; and an upregulation of genes associated with extracellular matrix repair. Oral vancomycin use resulted in the loss of specific fecal SCFAs and secondary BAs, including lithocholic acid derivatives. Colitis activity relapsed following OV withdrawal, with host mucosal and microbial changes trending toward baseline.

**Conclusions:** Four weeks of OV induces remission in PSC-IBD activity, associated with a reduction in gut bacterial diversity and compositional changes relating to BA and SCFA homeostasis.

**Key Words:** Primary sclerosing cholangitis; inflammatory bowel disease; vancomycin

## 1. Introduction

Between 2% and 14% of patients with inflammatory bowel disease (IBD) develop primary sclerosing cholangitis (PSC).<sup>1–5</sup> In turn, most individuals with PSC develop colonic IBD. Although rare, the development of PSC is a critical juncture for IBD patients, being associated with heightened risks of colectomy and colorectal cancer (necessitating annual colonoscopic surveillance<sup>6</sup>), hepatobiliary malignancy, need for liver transplantation, and all-cause mortality.<sup>7</sup> Liver transplantation is the only life-extending intervention for patients; however, recurrent disease develops among 1 in 3 patients, and post-transplant IBD can relapse/develop *de novo* in up to 40% of transplant recipients.<sup>8–10</sup> In turn, IBD activity and the timing of colectomy have been shown to impact liver-related outcomes, prior to and following liver transplantation.<sup>11–14</sup>

The etiology of PSC is unknown, but the association with IBD has generated several pathogenic hypotheses in which immune dysregulation, gut microbial changes, and alterations in bile acid (BA) homeostasis are proposed to contribute.<sup>15–17</sup> The mechanisms of classical IBD and PSC-IBD share some similarities. For instance, data from our group and others highlight the role of heightened IL-17 (interleukin-17) secretor cell responses to pathogen stimulation.<sup>18</sup> However, several differences are evident epidemiologically,<sup>5,7,19</sup> pathologically,<sup>16</sup> and phenotypically.<sup>20</sup> Moreover, genome-wide association studies suggest that the genetic architecture differs, with correlation modeling estimating an ulcerative colitis (UC) comorbidity rate of only 1.6% in patients with PSC, compared with the 70% rate seen in clinical practice.<sup>21</sup> This degree of ‘missing heritability’ indicates a role for other contributory factors.

We have previously shown that the composition and function of mucosa-adherent gut microbiota are also distinct in PSC patients compared with individuals with UC alone and healthy controls<sup>22</sup>; an observation that has been robustly and externally validated.<sup>23–26</sup> Furthermore, analysis of the colonic mucosal transcriptome shows clear disturbances in BA homeostasis, supporting a causative role of gut microbial alterations in disease pathogenesis.<sup>18</sup> Pilot studies using the orally administered, non-absorbable antibiotic vancomycin also demonstrate a reduction in serum alkaline phosphatase (ALP) values (the biochemical hallmark of PSC).<sup>27–29</sup> Moreover, the observational cohort series showed oral vancomycin (OV) was able to induce and maintain remission in IBD activity among children with PSC.<sup>30,31</sup>

In this interventional study, we used an intensive multi-omic approach to uncover the mechanisms by which OV attenuates colonic mucosal inflammation in patients with PSC and active IBD. In so doing, we explore biological changes in host pathophysiology, alongside shifts in gut microbiota, in an attempt to further understanding of this complex disease.

## 2. Methods

### 2.1. Participants

We conducted a single-arm interventional study of open-label OV treatment in patients with PSC and active colonic IBD. The overarching goal was to quantify the proportion

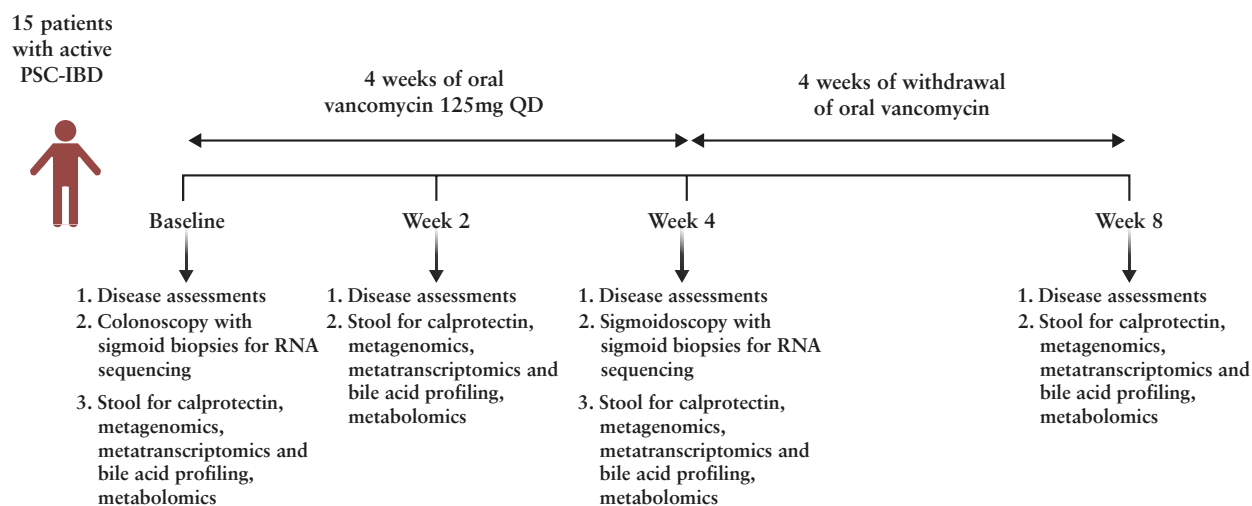
of patients who attained clinical remission from an IBD perspective, and identify the associated changes in host mucosal biology associated with OV treatment.

The study was conducted from February 2022 to November 2022 (NCT05376228). Patients aged  $\geq 18$  years with a diagnosis of PSC and concomitant pancolonic IBD were screened for eligibility at a single high-volume center. Inclusion criteria were mild to moderately active colitis based on a partial Mayo colitis score of  $\geq 3$  and  $\leq 6$  and commitment to participate in scheduled, standard-of-care lower gastrointestinal endoscopy (as part of disease assessment and annual colorectal cancer surveillance).<sup>6,32,33</sup> Exclusion criteria for study participation were any active infectious cause of diarrhea (including *Clostridioides difficile* toxin-positive stool); an isolated ileal, right-sided or rectal phenotype of PSC-IBD; use of antibiotics or probiotics in the 3 months prior to screening; presence of stricturing, fistulating, or perianal IBD; a history of small bowel or colonic resection; a change or initiation of corticosteroids in the 2 weeks prior to screening; commencement of an immunomodulator or advanced therapy regimen in the prior 3 months; a history of intolerance to vancomycin; and/or evidence of hepatic decompensation in the 3 months prior to screening. Liver transplant recipients with evidence of recurrent PSC were allowed to participate in the absence of other exclusion criteria, provided inclusion criteria were met. The sample size for this trial was determined empirically, based on available research grant funding. As the study was exploratory in nature, it was not powered to detect specific efficacy endpoints, but rather to evaluate mechanistic effects of OV in PSC-IBD, and the association with specific clinical outcomes.

### 2.2. Study design

Screening for the study and written consent were obtained from eligible patients up to 2 weeks prior to their scheduled annual surveillance colonoscopy (Week -2). At baseline (Week 0), the participant's annual surveillance colonoscopy was performed, the total Mayo score was recorded, and up to 8 biopsies from the sigmoid colon were collected for fulfilling translational research objectives. Individuals with evidence of active colitis (endoscopic Mayo score of 1 or 2 and a partial Mayo score of  $\geq 3$  and  $\leq 6$ ) were treated with 4 weeks of OV 125 mg 4 times a day (QID), followed by 4 weeks of treatment withdrawal (Week 8) (Figure 1).

Following completion of the treatment course (Week 4), a sigmoidoscopy was performed for a reassessment of endoscopic disease activity, and for collection of post-treatment sigmoid colon biopsies. Stool samples were collected at the baseline visit (prior to the patient taking bowel preparation for colonoscopy), Weeks 2, 4, and 8 to study metagenomics and metatranscriptomics (collected in DNAGenotek OMR-200 stool collection kits), short-chain fatty acids (SCFAs) and fecal BA profiling (collected in ME-200 collection kits), in addition to analysis of fecal calprotectin values. Serum was collected at the same timepoints for measurement of liver biochemistry (bilirubin, albumin, ALP, and alanine transaminase [ALT]), alongside clinical characteristics and the partial Mayo colitis score. Patients who experienced a



**Figure 1** Study schematic. Fifteen patients with active PSC-IBD participated in an 8-week study where they received open-label 125 mg QDS (quater die sumendum) of oral vancomycin for the first 4 weeks (Week 4), followed by withdrawal of OV for the subsequent 4 weeks (Week 8). Lower gastrointestinal endoscopies were performed at baseline and Week 4. Clinical assessments, serum, biopsies for transcriptomics, and stool for calprotectin, metagenomics, metatranscriptomics, and bile acid metabolomics were collected at Baseline, Weeks 2, 4, and 8. Abbreviation: OV, oral vancomycin.

significant increase in their partial Mayo score (defined as a >30% increase from baseline) during the study period, or based on clinician discretion, would exit the study and receive escalation in IBD treatment as per routine standard of care.

An unselected, random selection of participants ( $n = 6$ ) also provided stool samples for microbiological culture, to determine if OV treatment led to the selection of vancomycin-resistant enterococci (Slanetz and Bartley agar [Oxoid]; treated with 8 µg/ml vancomycin).

### 2.3. Clinical outcome analysis

The primary efficacy outcome was the induction of clinical remission at Week 4, the end of OV treatment, as defined in contemporary clinical trial practice.<sup>34</sup> Specifically, this was by the modified Mayo colitis score being <2, with a stool frequency subscore ≤1, rectal bleeding score (RBS) = 0, and an endoscopic score ≤1. Given the short duration of follow-up, the term herein references induction of 'short-term' clinical remission, and is used to reflect on-treatment changes at 4 weeks, the observed improvements, all while acknowledging that longer-term follow-up is required to assess more sustained or durable remission. Key secondary efficacy outcomes were assessed at Weeks 4 and 8, and included changes in fecal calprotectin, the partial Mayo colitis score, the total Mayo colitis score, and serum ALP, ALT, and bilirubin values.

#### 2.3.1. Data presentation and statistical analysis

Continuous variables are shown as mean values and SD unless otherwise specified. Categorical variables are presented as raw numbers and percentages. The paired sample Wilcoxon test and analysis of variance (ANOVA) were conducted between different study timepoints to assess changes in fecal calprotectin, Mayo colitis scores and subscores, and in liver biochemistry following the treatment and subsequent withdrawal phases of OV treatment. Differences between groups were considered significant at a value of  $p < 0.05$ .

### 2.4. Stool metagenomic and metatranscriptomic analysis

DNA and RNA were extracted from stool preserved in Omnigene tubes using Spin Column technology via a modified ZymoBIOMICS DNA/RNA Mini Kit protocol. The integrity of the isolated RNA was determined using the Bioanalyzer (Agilent) and only samples with RNA integrity number >7 were used. Libraries from extracted DNA for metagenomics were prepared using NEBNext® Ultra™ II FS DNA Library Prep Kit following the protocol recommended by the manufacturer. Single index, paired-end shotgun metagenomic sequencing was performed on the NextSeq2000 platform. For metatranscriptomics, the extracted total RNA was first ribo-depleted using the Illumina Ribo-Zero Plus Microbiome rRNA Depletion Kit prior to library preparation with the Illumina Stranded Total RNA prep following the protocol recommended by the manufacturer. Paired-end, dual-indexed run shotgun RNA sequencing was performed on the NextSeq2000 platform. The library preparations and sequencing were done as a single batch with appropriate negative controls to reduce potential confounders.

From the 60 stool samples collected at specified timepoints of the study, a total of 1.4 billion reads from shotgun metagenomic sequencing and of 950 million reads from shotgun metatranscriptomic sequencing were generated. Both metagenomic and metatranscriptomic sequence reads were processed with the KneadData v0.5.1 quality control pipeline (<http://huttenhower.sph.harvard.edu/kneaddata>), which uses the Trimmomatic to remove adapter sequences and low-quality bases and Bowtie2 for decontamination to remove host DNA/RNA and ribosomal RNA.<sup>35</sup> In addition, metatranscriptomic reads were filtered against the human transcriptome and the SILVA database. Following filtering and quality control (QC), an average of 18 million paired-end metagenomic reads per sample were processed for assembly and reference-based profiling and an average of 12 million paired-end metatranscriptomic reads per sample were processed for downstream analysis.

Bacterial taxonomic annotations from the metagenomic sequences were performed using MetaPhlAn4 which uses clade-specific markers that provide bacterial, archaeal, viral, and eukaryotic quantification at the species level using default parameters for reported relative abundances.<sup>36</sup> Species with relative abundance <0.1% in at least 5 samples were excluded from the study. Functional profiling was derived from metatranscriptomic (active) and metagenomes (potential) using HUMAnN 3.0.<sup>37</sup> For metatranscriptomic mapping the taxonomic profile of the corresponding metagenome was used as an additional input to HUMAnN 3.0 via the `--taxonomic-profile` flag. This guaranteed that RNA reads are mapped to any species' pangenomes detected in the metagenome rather than using derived species pangenomes from the RNA read. Briefly for both metagenomes and metatranscriptomes the translated search using DIAMOND was mapped against UniRef90 following which hits were counted per gene family and normalized for length and alignment quality. Gene families were regrouped into Kyoto Encyclopaedia of Genes and Genomes (KEGG) orthologs, enzyme classifications, and gene ontologies. Gene family abundances from both the nucleotide and the translated searches are then combined into structured pathways from MetaCyc and sum-normalized to relative abundances. All feature abundances that did not exceed 0.1% of the data with a minimum prevalence of 10% of the total samples were excluded from further analysis. Additionally, the ratio of transcript expression against gene abundance was measured to quantify changes in expression while controlling for gene copy number.

Alpha diversity analysis was performed using the Shannon diversity index, and Bray–Curtis dissimilarities between groups were calculated using the `Adonis` function from the `vegan` R package in PERMANOVA analyses. Principal Coordinates Analysis (PCoA), non-metric multidimensional scaling (NMDS) analysis dimensionality reduction analysis (species only), and sample cluster analysis were performed. Lastly, Microbiome Multivariable Associations with Linear Models (MaAsLin2) was used to quantify paired differences between the relative abundance of taxa, genes, and pathways between study timepoints and fecal calprotectin levels. Due to the hypothesis-generating and exploratory nature of this study, an FDR (false discovery rate)-corrected *p*-value threshold of ≤0.1 was used. The Spearman rank correlation coefficient ( $\rho$ ) was used to assess correlations between taxa, genes, fecal BA concentrations, and fecal calprotectin levels.

## 2.5. Mucosally adherent microbial 16S rRNA gene profiling (metataxonomics)

DNA and RNA were extracted from colonic mucosal biopsies taken at baseline and Week 4 of the study using a modified Qiagen AllPrep DNA/RNA Mini Kit protocol that included mechanical lysis and on-column DNase digestion. The RNA extracted was passed on for host mucosal transcriptomics as described later. The DNA extracted was subjected to 16S rRNA gene amplification and sequencing using the Earth Microbiome Project protocol.<sup>38</sup> Briefly, 16S rRNA genes were amplified in technical duplicates with primers targeting the 16S rRNA V4 region (515F–806R) using a 1-step, single-indexed PCR approach. As with DNA/RNA extraction, 16S rRNA gene PCR was done in a batch with appropriate negative controls. Paired-end sequencing (2 × 250 bp) was performed on the Illumina MiSeq platform (Illumina, San Diego, USA). A total of 7.2 million reads (240 324 reads/sample) and

1450 ASVs were obtained after QC using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) pipeline.<sup>39</sup> Taxonomy was assigned against the Silva-132-99% OTUs database. The functional profiles of microbial communities were inferred using PICRUSt2-derived relative MetaCyc and KEGG pathway analysis.<sup>40</sup> Differences in taxa and inferred functional profiles were assessed using MaAsLin2 as described previously.<sup>41</sup>

## 2.6. Host mucosal transcriptomic analysis

The RNA extracted from colonic mucosal biopsies using the protocol described earlier was subjected to Ribo-Zero Gold rRNA Removal Kit (Illumina, San Diego, USA) to remove contaminating ribosomal RNA, and SMARTer Stranded RNA-Seq kit (Takara, Japan) was used for library construction. Paired-end 75 bp sequencing was performed using NextSeq 500/550 v2 kit (Illumina, San Diego, USA). After the removal of low-quality and ambiguous reads an average of 15 million reads per sample were processed for downstream analysis. Reads obtained were quality controlled with FastQC and Trimmomatic.<sup>42,43</sup> Contaminating ribosomal RNA reads were removed using Bowtie2 and reads were mapped to the human genome sequence database (GRCh38) using STAR and quantified with featureCounts.<sup>35,44,45</sup> Genes were filtered and differential gene expression was analyzed based (FDR-corrected  $p \leq 0.05$ ) using edgeR.<sup>46</sup> Gene ontology and KEGG/Reactome pathway analysis was conducted using Camera for competitive gene set testing. ClueGo was used to functionally group gene ontology and pathway annotation networks.<sup>47</sup>

## 2.7. Fecal metabolomics

### 2.7.1. Bile acid profiling

Reversed-phase (RP) ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS) was performed on OmniMet stool-preservative mixture for BA profiling. The OmniMet Gut ME-200 tubes were shaken for 1 minute and centrifuged for 20 minutes at 3214 g, 4°C. The supernatant (500 µL) was transferred to a micro-centrifuge filter (0.22 µm, nylon, Costar) and centrifuged for 20 minutes at 16 000 g, 4°C. The samples were then prepared as previously described. Ultra-high performance liquid chromatography–mass spectrometry analyses were performed using an ACQUITY UPLC (Waters Corp., Milford, MA, USA) coupled to a Xevo G2-S TOF mass spectrometer (Waters Corp., Manchester, UK) via a Z-pray electrospray ionization (ESI) source. Chromatographic separation was conducted in an ACQUITY BEH C8 column (1.7 µm, 100 mm × 2.1 mm), thermostated at 60°C. The mobile phases consisted of ACN:H<sub>2</sub>O 1:10 (v/v) with 1 mM ammonium acetate and pH 4.15 adjusted with acetic acid (A) and ACN:IPA (acetonitrile:isopropyl alcohol) 1:1 (v/v) (B) with a flow rate of 0.6 ml/min. The chromatographic gradient elution program was conducted as previously described.<sup>48,49</sup> Two µl injections of prepared samples were made to the system. Mass spectrometry was performed in negative ESI mode using the following parameters: capillary voltage 1.5 kV, cone voltage 60 V, source temperature 150°C, desolvation temperature 600°C, desolvation gas flow 1000 L/h, and cone gas flow 150 L/h. To ensure mass accuracy, a lock-spray interface was used, with leucine enkephalin [*m/z* 554.2615 ([M–H]<sup>–</sup>)] solution used as the lock mass at a concentration of 200 ng/µl and a flow rate of 15 µL/min.

Quality control samples were prepared by pooling equal volumes of the fecal filtrates. Quality control samples were used as an assay performance monitor<sup>50</sup> and as a proxy to remove features with high variation. Quality control samples were also spiked with mixtures of BA standards (81 BA standards including 44 non-conjugated, 12 conjugated with taurine, 9 conjugated with glycine and 16 sulfated (Steraloids, Newport, RI, USA, Sigma-Aldrich, Gillingham, UK and QMX Laboratories, Thaxted, UK) and were analyzed along with the patient samples to determine the chromatographic retention times of BAs and to aid in metabolite identification.

Raw data were converted to the open-source format mzML and signals below 100 counts (absolute intensity threshold) were removed using the MSConvert tool in ProteoWizard.<sup>51</sup> Feature extraction was performed by XCMS<sup>52</sup> within R, and data filtering and correction for the elimination of potential run-order effects were performed using the nPYc-Toolbox.<sup>53</sup> Targeted extraction, and integration of annotated BA species were made using the R package peakPanther.<sup>54</sup> Probabilistic quotient normalization<sup>55</sup> was used to correct for dilution effects. Features below the limit of detection in more than 20% of study samples were discarded. Values were log-transformed and zeros were imputed using impute.QRILC from the imputeLCMD R package. For statistical analyses, features were also mean-centered.

### 2.7.2. Fecal SCFA/short-chain carboxylic acid profiling

A 300-μL aliquot of fecal extract collected in OMNIMET® tubes was centrifuged for 10 minutes and filtered through 0.45 μm nylon microcentrifuge tubes (Costar, Corning, New York, USA), then stored at -80°C. From this extract, a 5-μL aliquot was transferred into a 1.5-mL microcentrifuge tube (Eppendorf, Germany) and mixed with 45 μL of water. The preparation of the diluted fecal extract sample followed the protocol described by Valdivia-Garcia et al.,<sup>56</sup> with modifications made to the mass spectrometer parameters for acetate (cone voltage: 20 V; collision energy: 14 V). All other mass spectrometry and chromatographic parameters remained consistent with the original method. Additional calibrators were added to extend the original method up to 500 μmol/L for all 10 compounds.

### 2.7.3. Statistical analysis of metabolomic data

Statistical analyses were adapted from approaches described previously.<sup>57,58</sup> Multivariate analysis of UHPLC-MS BA profiling data was performed on log-transformed and Pareto-scaled data. Orthogonal partial least squares-discriminant analysis (OPLS-DA) models were validated using CV-ANOVA, which provides a significance test of the null hypothesis of equal residuals between the model under validation and a randomly fitted model that uses the same data.<sup>59</sup> S-plots were used to visualize the highly influential discriminatory features, and depict the covariance and the correlation structure between the X-variables and the predictive score  $t[1]$  of the model. Features at the far ends of the plot have a very high reliability while having a high model influence due to their high variance in the dataset.<sup>59</sup> Univariate statistics were performed using GraphPad Prism, v10.2.2; the Friedman test was used for BA data to compare pre- with post-vancomycin samples, while the Kruskal-Wallis test was used for SCFA data; in both cases, Dunn's statistical hypothesis testing was applied to account for multiple comparisons, with 2-tailed statistical tests being used in all cases.

## 2.8. Ethics statement

Ethical approval was obtained for this study from the West Midlands—South Birmingham Research Ethics Committee and HRA and Health and Care Research Wales (HCRW) (21/WM/0197). The study was registered on Clinicaltrials.gov (NCT05376228).

## 3. Results

### 3.1. Demographics of enrolled participants

We recruited individuals with a known history of PSC-IBD who had pan-colitis, without prior evidence of small bowel disease, stricturing/penetrating disease, or transmural involvement typically seen in Crohn's disease. This approach ensured that the study specifically targeted a well-defined subgroup with colonic disease, in keeping with the classical distribution of IBD seen in PSC.<sup>20,60</sup> Eighteen patients were

**Table 1** Baseline demographics.

| Characteristic               | N = 15 <sup>a</sup> |
|------------------------------|---------------------|
| Demographics                 |                     |
| Age                          | 33 (27, 45)         |
| Gender                       |                     |
| F                            | 4 (27%)             |
| M                            | 11 (73%)            |
| IBD characteristics          |                     |
| PSC-IBD phenotype            |                     |
| Isolated colonic disease     | 15 (100%)           |
| Fecal calprotectin (μg/g)    | 459 (287, 1,049)    |
| Mayo endoscopic subscore     |                     |
| 1                            | 4 (27%)             |
| 2                            | 10 (67%)            |
| 3                            | 1 (6.7%)            |
| Total Mayo score             | 5 (1)               |
| Liver characteristics        |                     |
| Previous liver transplant    | 4 (27%)             |
| ALP (IU/L)                   | 274 (157, 479)      |
| ALT (IU/L)                   | 70 (32, 113)        |
| Bilirubin (μmol/L)           | 22 (11, 31)         |
| Medications                  |                     |
| Mesalazine                   | 10 (67%)            |
| Current advanced therapy use |                     |
| Vedolizumab                  | 6 (40%)             |
| Adalimumab                   | 1 (6.7%)            |
| No                           | 8 (53%)             |
| Advanced therapy naive       | 6 (40%)             |
| Current immunomodulator use  |                     |
| Azathioprine                 | 2 (13%)             |
| Mycophenolate                | 1 (6.7%)            |
| None                         | 12 (80%)            |
| Tacrolimus                   | 5 (33%)             |
| Ursodeoxycholic acid (UDCA)  | 7 (47%)             |

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; IBD, inflammatory bowel disease; PSC-IBD, primary sclerosing cholangitis-associated inflammatory bowel disease.

<sup>a</sup>Median (IQR) for age and liver enzymes; mean (SD) for total Mayo.

formally screened for study participation, of whom 3 were excluded. This was due to positive stool microscopy and culture ( $n = 2$ ) and a new diagnosis of sigmoid cancer found on baseline colonoscopy ( $n = 1$ ). In all, 15 patients participated in the study (median age 33 years, 11 attributed male sex at birth). All participants had an isolated colonic disease phenotype, 6 were naive to advanced IBD therapies, while 7 were on advanced IBD therapies at the time of recruitment (Table 1). All patients had clinically and endoscopically active IBD at baseline with a median fecal calprotectin of 459  $\mu\text{g/g}$  (IQR 287, 1049) and a median total Mayo score of 5 (SD 1). Four participants had undergone a liver transplant previously (all with radiological evidence of recurrent PSC). Median ALP values were 274 IU/L (IQR 157, 479) at baseline. Among non-transplant patients, the median baseline transient elastography value was 7.3 kPa (IQR 4.3–11.1 kPa).

### 3.2. Oral vancomycin treatment is associated with improvements in colitis activity

Clinical remission at 4 weeks was achieved by 12 patients (80%) following OV therapy. A significant decrease in fecal calprotectin (mean  $\Delta -672$   $\mu\text{g/g}$ ; CI 337–1008;  $p < 0.001$ ), partial Mayo scores (mean  $\Delta -3$ ; CI 2.2–3.8;  $p < 0.001$ ); and endoscopic Mayo score (mean  $\Delta -1.4$ ; CI 1–1.8;  $p < 0.001$ ); was observed at Week 4 in comparison to baseline (Table 2; Figure 2, A), including on subgroup analysis of patients without prior history of liver transplantation (Supplementary Table 1), and excluding those taking ursodeoxycholic acid (Supplementary Table 2). Notably, all patients achieved mucosal healing at Week 4 (defined by Mayo endoscopy subscore of 0 or 1). At Week 8 (following 4 weeks withdrawal of OV) there was a significant increase in both fecal calprotectin (mean  $\Delta 223$   $\mu\text{g/g}$ ; CI 41.6–404;  $p < 0.05$ ) and partial Mayo scores (mean  $\Delta 1.3$ ; CI 0–2.5;  $p < 0.05$ ) compared with Week 4, although this remained lower compared with baseline readings.

No adverse events or tolerability concerns were observed or reported during the 4-week treatment period with OV. Stool culture in an unselected sample of participants ( $n = 6/15$ ) did not show evidence for the selection of vancomycin-resistant enterococci.

### 3.3. Oral vancomycin treatment is associated with changes in serum liver biochemistry

A reduction in serum ALP and ALT values was observed from baseline to Week 4 (mean  $\Delta -105$  IU/L; CI 25.4–185;  $p < 0.05$  and mean  $\Delta -19.3$  IU/L; CI 2.3–36.3;  $p < 0.001$ , respectively). At Week 8, following withdrawal of OV, a nonsignificant trend toward an increase in serum ALT and ALP values was observed compared with Week 4 (Figure 2, B). No change in total serum bilirubin was noted at any timepoint.

### 3.4. Changes in gut bacterial composition associated with OV treatment

A reduction in alpha diversity in stool was observed as early as Week 2 of OV treatment compared with baseline ( $\Delta -1.2$ ,  $p < 0.001$ ; Figure 3, A). At the end of treatment (Week 4), the alpha diversity index remained low compared with the baseline ( $\Delta -1.2$ ,  $p < 0.001$ ). On treatment withdrawal, diversity increased by Week 8 ( $\Delta 0.6$ ,  $p < 0.001$ ), but remained lower than baseline readout.

Beta diversity derived from Bray–Curtis analysis of fecal metagenomes showed significant differences between the microbial composition at different treatment timepoints ( $p = 0.001$ ; Figure 3, B). Bacterial composition clustered separately during the OV treatment phase at Weeks 2 and 4 compared with baseline (both FDR adjusted  $p < 0.01$ ). The clustering at Weeks 2 and 4 were similar to one another. Composition following vancomycin withdrawal (Week 8) clustered separately to that during treatment (both FDR adjusted  $p < 0.01$ ). This clustering at Week 8 returned closer to baseline values but remained significantly different (FDR adjusted  $p < 0.01$ ).

At the phylum level, a reduction in the relative abundance of *Bacteroidetes* and *Firmicutes*, along with an increase in *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, was observed during OV treatment compared with baseline (Figure 3, C). Marked differences were observed in 68 species (FDR adjusted  $p < 0.1$ ) following differential abundance analysis at species level at Week 4 compared with baseline (Figure 3, D). Notably, at Week 4 a significant decrease in *Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, *Bifidobacterium longum*, and multiple SCFA producing species belonging

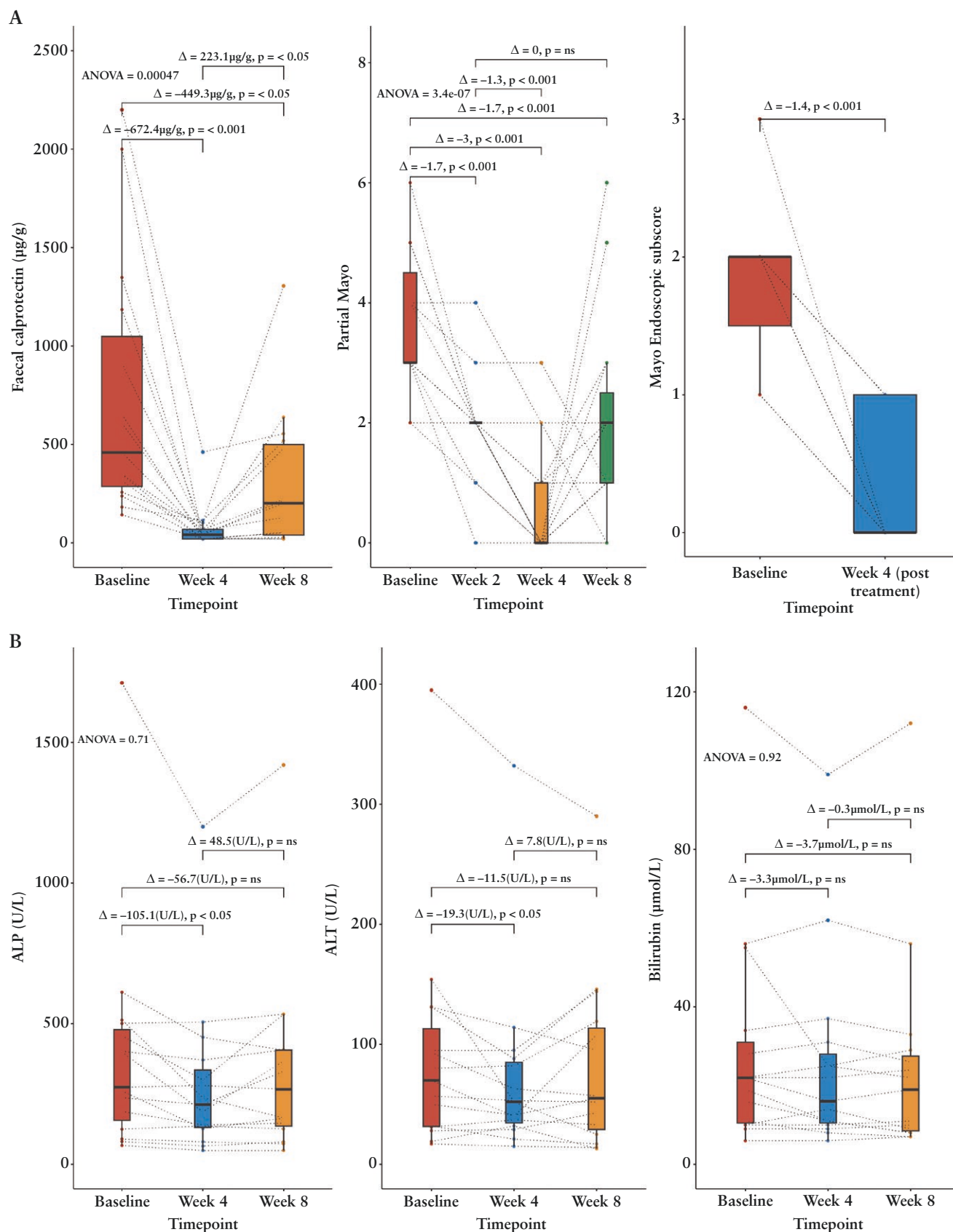
**Table 2** Clinical parameters following treatment and withdrawal of oral vancomycin.

| Clinical parameter                     | Baseline <sup>a</sup> | Week 4 <sup>a</sup> | Week 8 <sup>a</sup> | Overall $p$ -value <sup>b</sup> |
|----------------------------------------|-----------------------|---------------------|---------------------|---------------------------------|
| Fecal calprotectin ( $\mu\text{g/g}$ ) | 459 (287, 1,049)      | 41 (20, 69)         | 201 (40, 500)       | <0.001                          |
| Partial Mayo                           | 4 (1)                 | 1 (1)               | 2 (2)               | <0.001                          |
| Mayo endoscopic subscore               |                       |                     |                     | <0.001                          |
| 0                                      | 0 (0%)                | 9 (60%)             |                     |                                 |
| 1                                      | 4 (27%)               | 6 (40%)             |                     |                                 |
| 2                                      | 10 (67%)              | 0 (0%)              |                     |                                 |
| 3                                      | 1 (6.7%)              | 0 (0%)              |                     |                                 |
| Total Mayo score                       | 5 (1)                 | 1 (1)               |                     | <0.001                          |
| ALP (IU/L)                             | 274 (157, 479)        | 212 (132, 336)      | 267 (136, 407)      | 0.6                             |
| ALT (IU/L)                             | 70 (32, 113)          | 52 (35, 85)         | 55 (29, 114)        | 0.8                             |
| Bilirubin ( $\mu\text{mol/L}$ )        | 22 (11, 31)           | 16 (11, 28)         | 19 (9, 28)          | 0.8                             |

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; ANOVA, analysis of variance.

<sup>a</sup>Median (IQR).

<sup>b</sup>ANOVA.



**Figure 2** Changes in clinical parameters following treatment and withdrawal of oral vancomycin in patients with active PSC-IBD. A, Significant reductions in faecal calprotectin, and partial Mayo and Mayo endoscopic subscores were observed following 4 weeks of oral vancomycin therapy. Both faecal calprotectin and the partial Mayo score appeared to increase significantly following the subsequent 4 weeks of withdrawal of oral vancomycin. B, Both ALP and ALT appeared to significantly decrease following oral vancomycin, however, no changes in total serum bilirubin levels were observed. Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; PSC-IBD, primary sclerosing cholangitis-associated inflammatory bowel disease.

to the *Clostridium*, *Ruminococcus*, *Lachnospira*, and *Roseburia* genera was observed when compared with baseline. Conversely, a significant increase in *Fusobacterium nucleatum*, *Enterobacter hormaechei*, *Escherichia coli*, and multiple species belonging to the *Veillonella* and *Klebsiella* genera were increased at Week 4 compared with baseline. Of note, *Akkermansia muciniphila* appeared to increase following treatment with OV.

The relative abundance of the aforementioned phylae returned closer to baseline by Week 8 following vancomycin withdrawal. Importantly, the composition of 9 bacterial species at Week 8 showed differences when compared with Week 4 (FDR adjusted  $p < 0.1$ ). Specifically, an increase in *Roseburia hominis*, *Prevotella buccae*, *Lachnospiraceae bacterium*, *Blautia hansenii*, and *Bacteroides thetaiotaomicron*; alongside a decrease in taxa belonging to the *Proteobacteria* phylum was observed, suggesting a shift back toward baseline composition.

Comparing the composition of gut bacterial species in relation to fecal calprotectin in all patients at both Week 4 to baseline, and Weeks 8 to 4, revealed that fecal calprotectin levels were associated with a decrease in multiple species including *F. prausnitzii*, *Fusicatenibacter saccharivorans*, and those belonging to the *Clostridium*, *Ruminococcus*, *Lachnospira*, and *Roseburia* genera (FDR adjusted  $p < 0.1$ ). Reciprocally, a decrease in fecal calprotectin was associated with an increase in *F. nucleatum* and species belonging to the *Proteobacteria* phylum (FDR adjusted  $p < 0.1$ ). These observations were confirmed with correlation analysis at all available timepoints between these species and fecal calprotectin values (Figure 3, E).

### 3.5. Significant shifts in gut microbial function following OV therapy

Comparative metatranscriptomic analysis showed significant differences at both gene expression and pathway levels, during OV treatment and the withdrawal phase at different timepoints ( $p = 0.001$ ; Figure 4, A). The metatranscriptome during OV therapy at Weeks 2 and 4 clustered together on PCoA but were significantly different when compared with baseline (both  $p < 0.01$ ). Pathway analysis revealed that OV therapy was associated with significant changes in 159 MetaCyc pathways and 259 KEGG pathways (FDR adjusted  $p < 0.1$ ; Figure 4, B). The pathways that were increased included those implicated in the mannitol cycle, alanine biosynthesis, dodecenoate biosynthesis, lipid IVA biosynthesis, and fatty acids biosynthesis. Metatranscriptomic pathways that were decreased included metabolic processes involved in butyrate and propanoate production, mannan and rhamnose degradation, and bacterial chemotaxis. Similarly, KEGG orthology and enzyme classification analysis revealed significantly different expression patterns of 2011 genes (1276 increased and 735 decreased) and 827 genes (530 increased and 297 decreased), respectively, at Week 4 compared with baseline. Notably, shifts included a reduction of expression in genes associated with conversion of primary to secondary BAs (cholyglycine hydrolase, 3-oxo-5-beta-steroid 4-dehydrogenase), enzymes involved in butanoate metabolism (3-hydroxybutyryl CoA dehydrogenase, butyrate kinase, fucose transmembrane transporter activity) and an increase in expression of primary amine oxidase and enzymes involved in lipopolysaccharide production (lipopolysaccharide glucosyltransferase, lipopolysaccharide galactosyltransferase).

Metatranscriptome clustering at Week 8 (4 weeks of OV withdrawal), overlapped with baseline clustering suggesting restoration of pre-vancomycin gene expression. Comparative metatranscriptomic analysis of pathways, KEGG orthology, and enzyme classifications at Week 8 in relation to Week 4 demonstrated a reversal of these shifts in the majority of involved metabolic processes. Correlation analysis between selected microbial gene expression datasets and fecal calprotectin revealed a positive correlation between BA metabolism (cholyglycine hydrolase, 3-oxo-5-beta-steroid 4-dehydrogenase), butyrate kinase and primary amine oxidases.

Linking bacterial compositional analysis with metatranscriptomic datasets identified that these metabolic pathway changes following OV therapy were a result of increased gene expression from specific microbial species including *E. coli*, *F. nucleatum* species belonging to the *Klebsiella* and *Veillonella* genera, and a decrease in gene expression from species that included *A. hadrus*, *F. prausnitzii* and species belonging to the *Roseburia*, *Bacteroides*, and *Blautia* genera. These are likely to represent changes in the abundance of these species following OV treatment rather than a true inherent change in gene expression.

### 3.6. Colonic mucosal metataxonomics reveals similar changes in mucosally adherent bacterial diversity and composition following OV

Next, we proceeded to analyze the composition of mucosa-adherent gut bacteria. Alpha diversity analysis demonstrated a significant reduction in mucosal bacterial diversity following 4 weeks of OV treatment ( $\Delta -1.3$ ,  $p < 0.001$ ; Figure 5, A), mirroring findings from the stool. Beta diversity analysis (Bray–Curtis) confirmed that the mucosal bacteria clustered significantly differently between the baseline and Week 4 samples ( $p < 0.001$ ; Figure 5, B). Differential abundance analysis of mucosa-adherent bacterial composition showed a significant reduction in genera belonging to the families *Lachnospiraceae* (*Blautia*, *Ruminococcus*, *Faecalibacterium*, *Roseburia*), *Bacteroidaceae*, *Butyrivibrionaceae*, and the *Clostridia* class following OV (FDR  $p < 0.1$ ; Figure 5, C). In contrast, an increase in genera belong to the families *Fusobacteriaceae*, *Enterobacteriaceae*, *Veillonellaceae*, *Clostridia vadin*, and *Akkermansiaceae* was observed at Week 4 compared with baseline (FDR  $p < 0.1$ ). A significant positive correlation was observed between fecal calprotectin values and *Anaerostipes*, *Lachnospiraceae*, and *Blautia*, and negative correlation with *Fusobacterium* and *Enterobacteriaceae*.

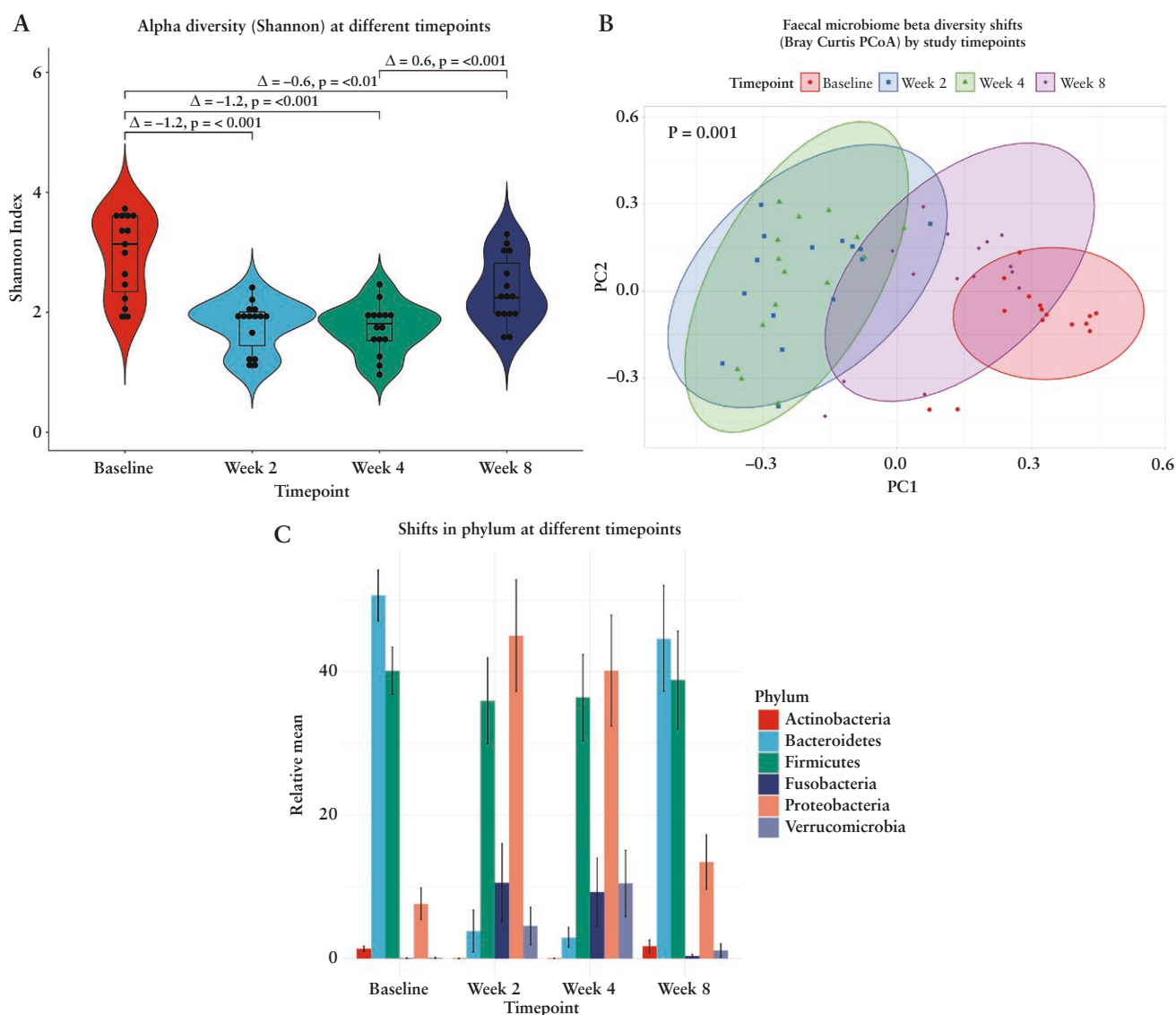
Metacyc pathway analysis of microbiota functions inferred from 16S rRNA gene sequence profiles revealed a decreased expression of genes within multiple pathways that included mannan and chondroitin sulfate degradation, sulfur oxidation, and pyruvate fermentation to butanoate following treatment with OV. In contrast, pathways that were increased include phenylacetate degradation, fatty acid beta-oxidation, and enterobactin biosynthesis. Kyoto Encyclopaedia of Genes and Genomes orthology analysis of predictive mucosal metagenomic pathways revealed that vancomycin treatment resulted in a significant reduction in the genes that include cholyglycine hydrolase, choline-sulfatase and enrichment of genes that include aromatic-amino-acid transaminase and peptidoglycan lipid II flippase.

### 3.7. Oral vancomycin is associated with marked alterations in the profiles of a diverse range of gut BA profiles and selected SCFAs

Stool BA profiling was first analyzed via multivariate analysis. A principal components analysis score plot of log-transformed, Pareto-scaled, stool BA profiling demonstrated clustering according to a week of collection of samples from patients (Figure 6, A). Specifically, stool samples collected prior to OV initiation (Week 0/baseline) and following withdrawal (Week 8) clustered together. Similarly, samples collected at Weeks 2 and 4 during OV treatment also clustered

together, but separately from the baseline and Week 8 samples. This was consistent with OV causing sustained changes in gut BA profile during its use, but rapid recovery back toward baseline profile after completion of its use.

Orthogonal partial least squares-discriminant analysis demonstrated strong models for the separation of stool BA profiles between baseline and Week 2 ( $R^2X = 0.4$ ,  $R^2Y = 0.736$ ,  $Q^2 = 0.496$ , CV-ANOVA:  $p = 0.0010$ ; Figure 6, B), as well as baseline and Week 4 ( $R^2X = 0.464$ ,  $R^2Y = 0.731$ ,  $Q^2 = 0.483$ , CV-ANOVA:  $p = 0.0018$ ), but not between baseline and Week 8. S-plots were used to interrogate the specific BA species that



**Figure 3** Metagenomic analysis of fecal microbiome at different study timepoints. A, A significant reduction in alpha diversity (Shannon Index) is observed following oral vancomycin by the second week of treatment (Week 2) compared with baseline. This diversity loss remains stable in the subsequent 2 weeks of treatment (Week 4) with some restoration of diversity following 4 weeks of vancomycin withdrawal (Week 8). B, Bray-Curtis PCoA analysis demonstrates distinct clustering of microbiota as early as Week 2 compared with baseline. The microbial compositional cluster appears to move back toward baseline at Week 8 following withdrawal of OV. C, Changes within the phylum are observed as early as Week 2 with depletion of *Bacteroidetes* and *Firmicutes* and enrichment of *Proteobacteria* and *Fusobacteria*. D, At the species level, a significant decrease in *Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, and multiple short-chain fatty acid (SCFA) producing species was observed when compared with baseline. Conversely, a significant increase in *Fusobacterium nucleatum*, *Enterobacter hormaechei*, *Escherichia coli*, and multiple species belonging to the *Veillonella* and *Klebsiella* genera were increased at Week 4 compared with baseline. Vancomycin withdrawal appeared to reverse some of these changes with an increase in *Roseburia hominis*, *Prevotella buccae*, *Lachnospiraceae bacterium*, *Blautia hansenii*, and *Bacteroides thetaiotaomicron* and a decrease in species belonging to the *Proteobacterium* phylum at Week 8 compared with baseline. E, Negative correlations were seen between fecal calprotectin levels and multiple species including those belonging to *Fusobacterium*, *Klebsiella*, *Veillonella*, and positive correlations with *Lachnospira*, *Roseburia*, and *Bacteriodes*. Abbreviations: OV, oral vancomycin; PCoA, Principal Coordinates Analysis.

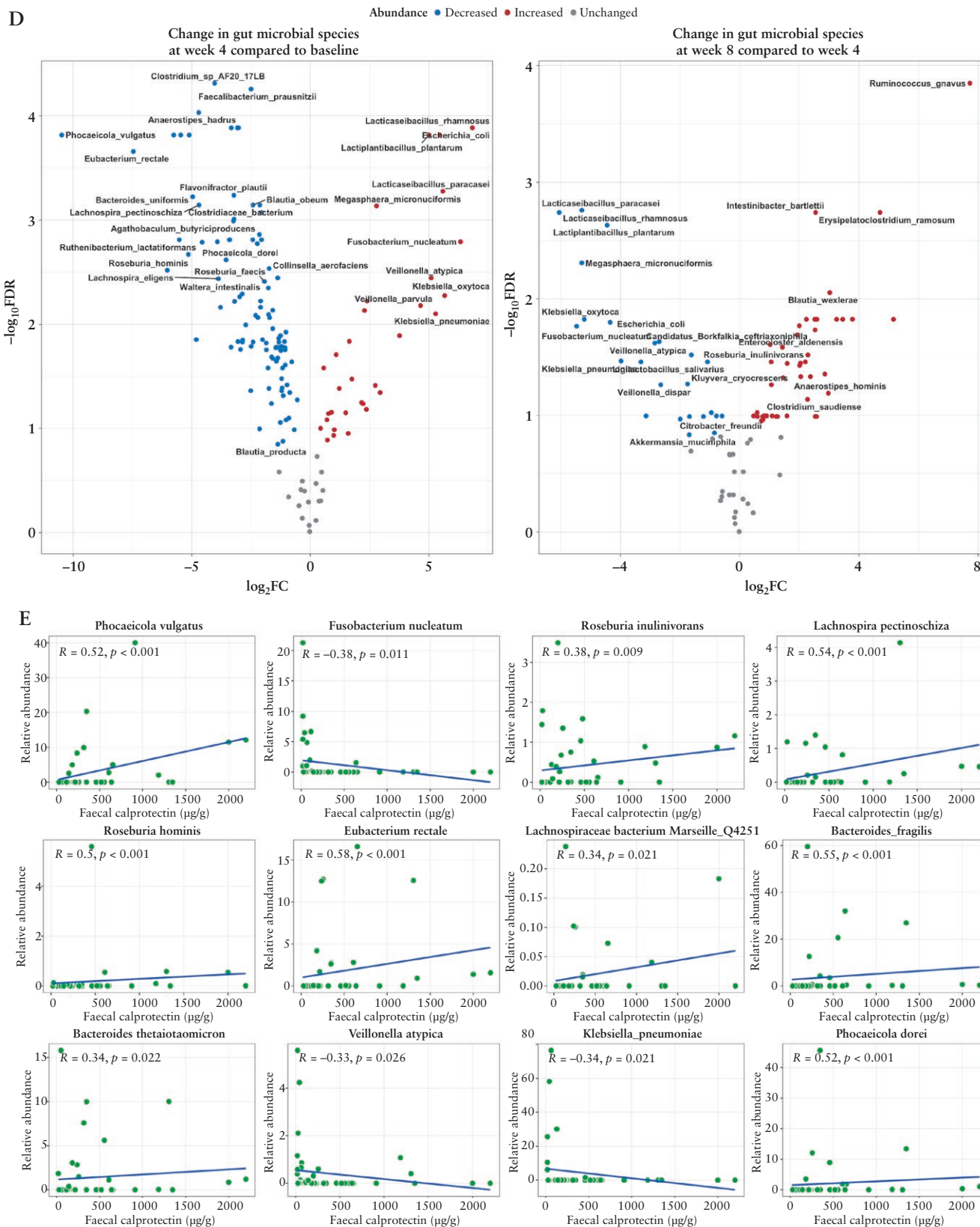
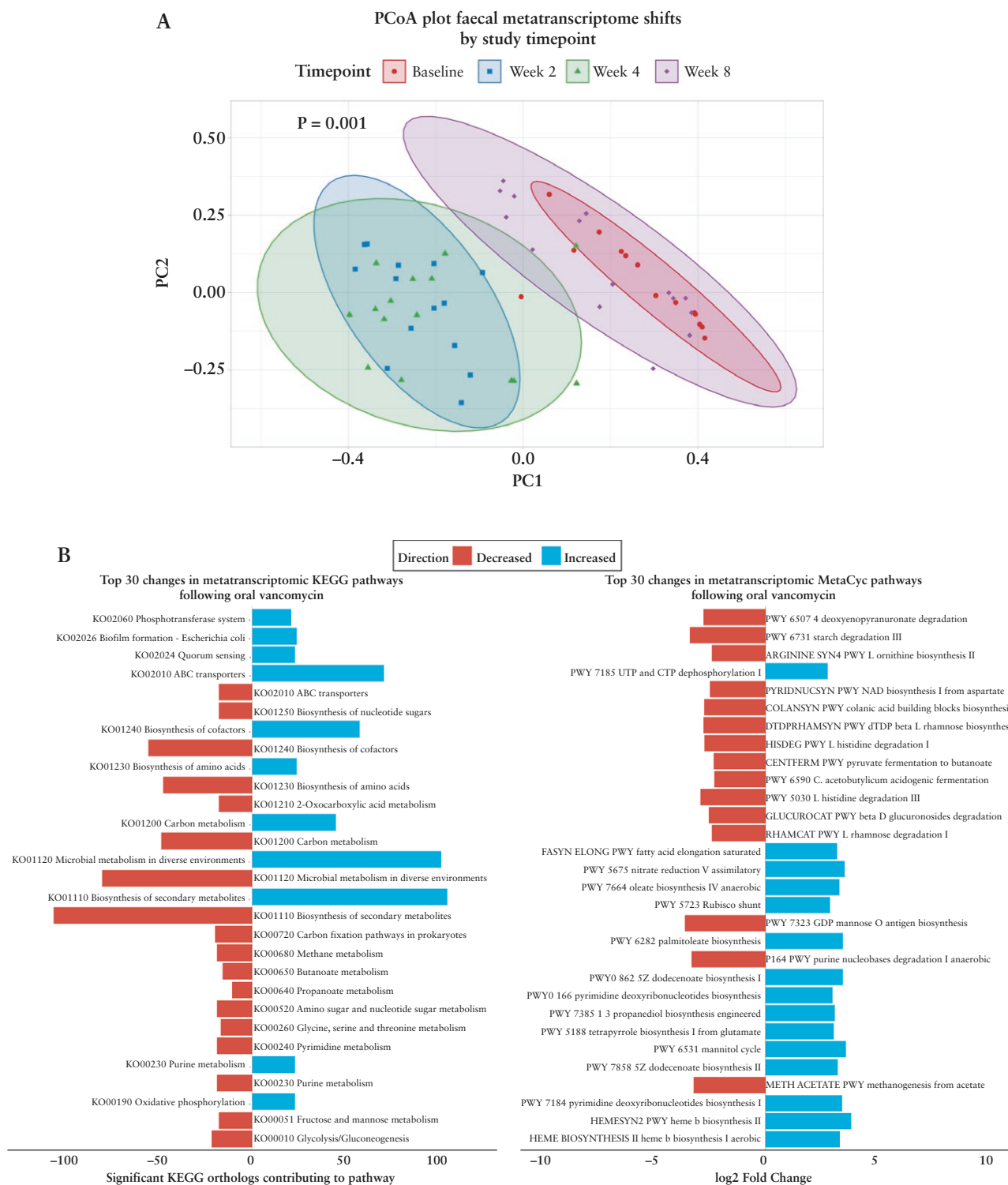


Figure 3 Continued

changed in association with vancomycin use in the OPLS-DA models (Figure 6, C); these were noteworthy for showing loss of secondary BAs (including derivatives of deoxycholic acid [DCA] and lithocholic acid [LCA]) in association with

OV treatment, and enrichment of primary BAs (particularly glycoconjugates, including glycocholic acid).

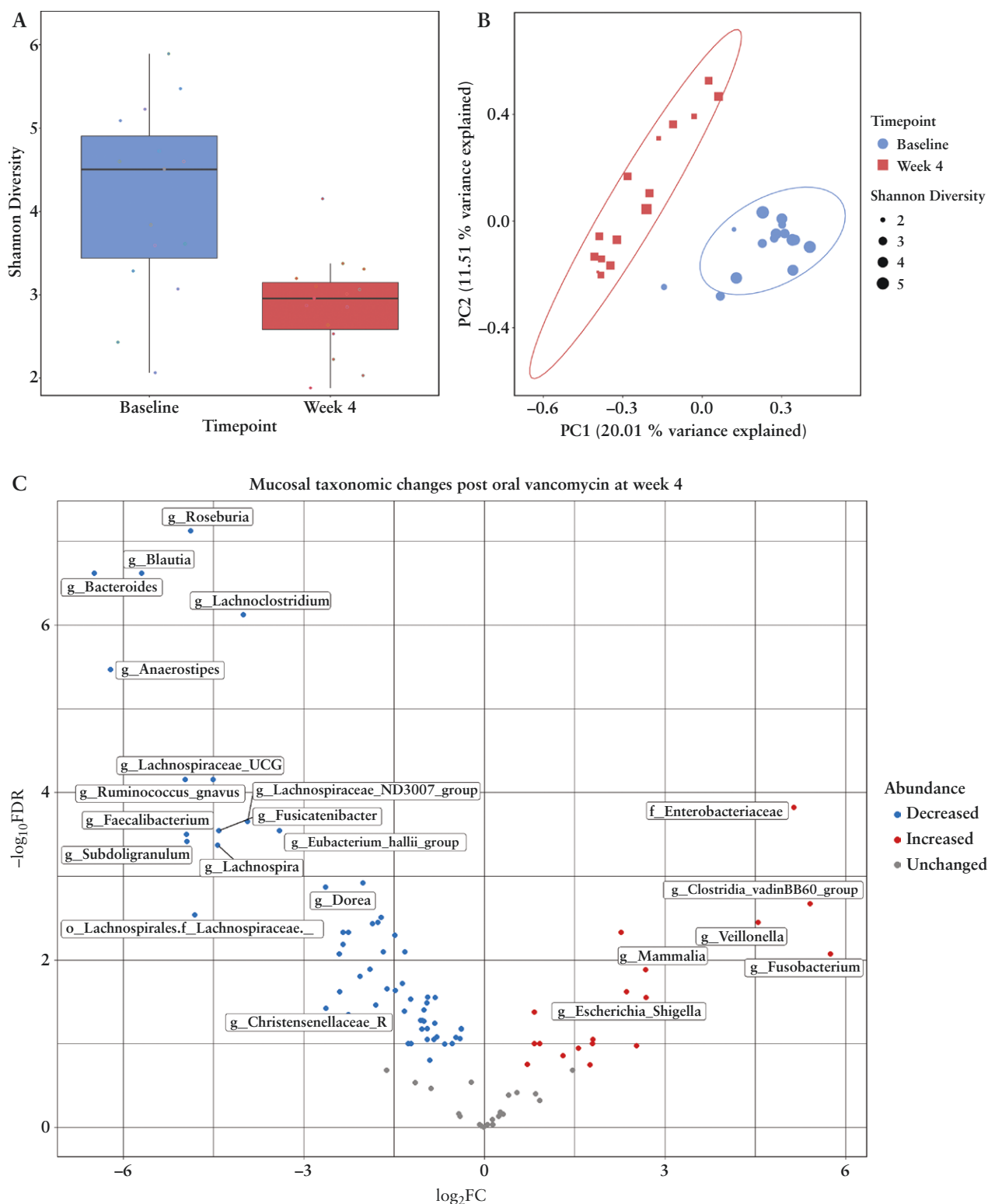
Univariate analysis of stool BA profiling supported these findings (Supplementary Figure 1). Stool secondary BAs



**Figure 4** Metatranscriptomic analysis of faecal microbiome at different study timepoints. A, Bray–Curtis PCoA analysis demonstrates distinct clustering of metatranscriptome following OV therapy at Weeks 2 and 4 compared with baseline (both  $p < 0.01$ ) with restoration of microbial function back to baseline at Week 8 following withdrawal of OV. B, Horizontal bar plot highlighting changes in the top 30 KEGG and MetaCyc metatranscriptomic pathways (FDR adjusted  $< 0.1$ ) following OV therapy (Week 4 compared with baseline). Abbreviations: KEGG, Kyoto Encyclopaedia of Genes and Genomes; OV, oral vancomycin; PCoA, Principal Coordinates Analysis.

(including DCA and LCA)—together with their microbially metabolized derivatives, including isoDCA and isoLCA—were found at significantly reduced stool levels at Weeks 2 and 4 compared with baseline (adjusted  $p$ -value  $< 0.01$ , Friedman's test with Dunn's statistical hypothesis testing).

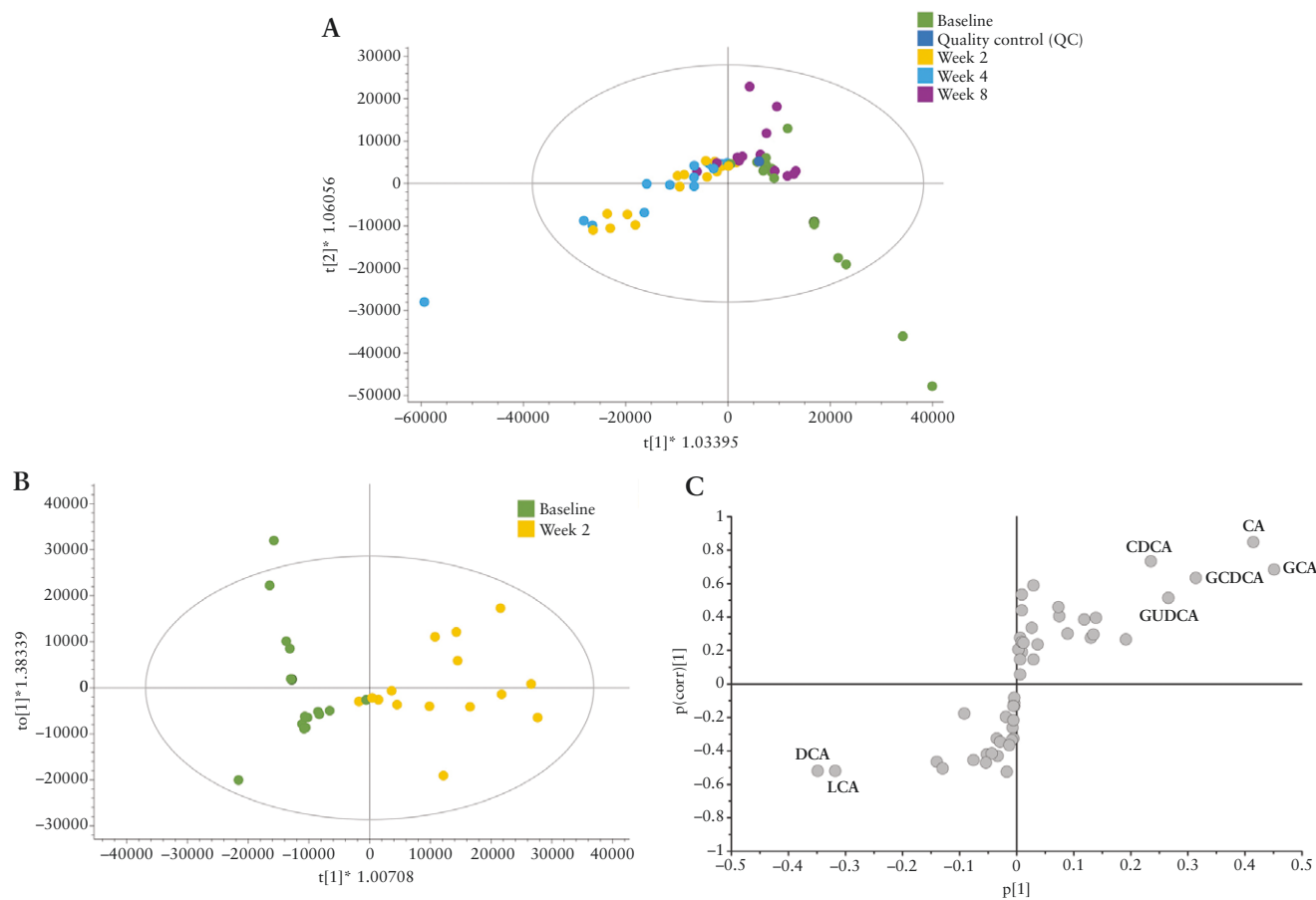
By Week 8 (after vancomycin washout), all secondary BAs showed an overall pattern of recovery toward baseline levels, although levels of stool DCA and LCA were still significantly reduced at this point compared with baseline (adjusted  $p < 0.05$ ), with wide inter-individual variability regarding



**Figure 5** Changes in mucosally adherent microbiota following treatment with oral vancomycin. A, Significant reduction in alpha diversity (Shannon Index) is observed following oral vancomycin at Week 4. B, Bray–Curtis PCoA analysis demonstrates distinct clustering of microbiota at Week 4 clusters compared with baseline ( $p < 0.001$ ). C, Oral vancomycin is associated with significant depletion of relative abundances of several genera including *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Bacteroides*, and an enrichment of *Fusobacterium*, *Enterobacteria*, and *Veillonella*. Abbreviation: PCoA, Principal Coordinates Analysis.

degree of post-vancomycin recovery (Supplementary Figure 1, A and B). Conversely, a range of primary BAs—including both unconjugated and glycol-conjugated variants of cholic acid and chenodeoxycholic acid—demonstrated significantly

increased levels within stool at Weeks 2 and 4 during vancomycin treatment (adjusted  $p < 0.05$ ), before recovery back to baseline levels by Week 8/after vancomycin washout (Supplementary Figure 1, C–F).



**Figure 6** Impact of vancomycin upon fecal bile acid profiles. Analysis of reversed-phase (RP) ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS), performed on OmniMet stool-preservative mixture, for bile acid profiling. A, Principal components analysis (PCA) scores plot, analyzing stool bile acid profiling data (with log-transformation and Pareto scaling), with samples annotated by a week of collection; B, orthogonal partial least squares-discriminant analysis (OPLS-DA) scores plot of baseline versus Week 2 samples; C, S-plot derived from OPLS-DA of (B); the further that bile acids are toward the bottom left of the plot then the higher they are in baseline samples, while those nearer to the top right of the plot are higher in Week 2 samples. Additional abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GUDCA, glyoursodeoxycholic acid.

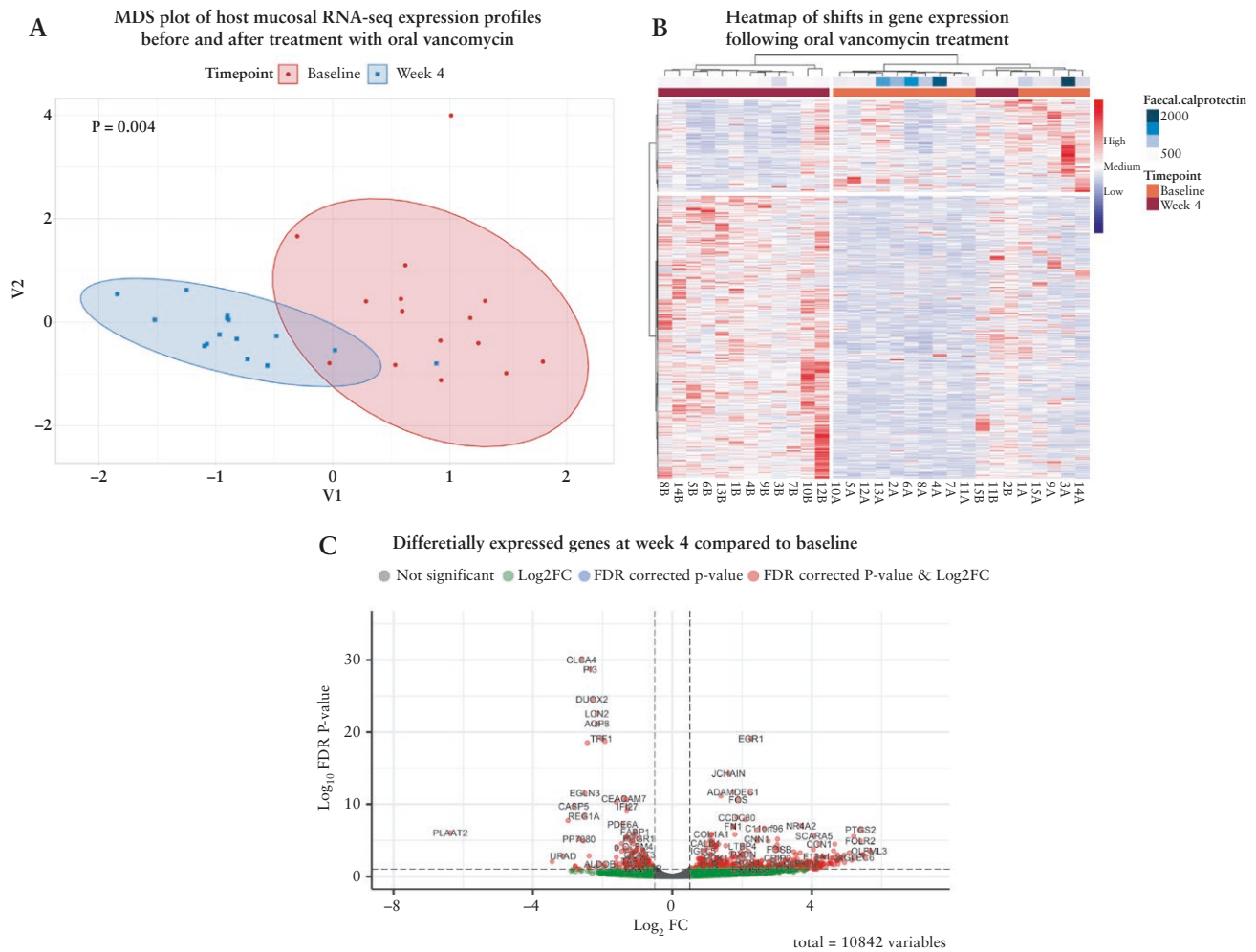
Univariate analysis was also performed for stool SCFA profiles, as well as a number of related short-chain carboxylic acids (Supplementary Figure 2). Oral vancomycin use was associated with significantly reduced fecal levels of 2 SCFAs, butyrate, and valerate, at Weeks 2 and 4 compared with baseline (adjusted  $p$ -value  $<0.01$ , Kruskal–Wallis test with Dunn’s statistical hypothesis testing; Supplementary Figure 2, F and I), but with recovery toward baseline levels by Week 8; no significant changes in levels of other stool SCFAs were observed associated with OV use. Additionally, OV use was associated with a significant increase in stool 2-hydroxybutyrate at Weeks 2 and 4 (adjusted  $p$ -value  $<0.01$ ; Supplementary Figure 2, D), which again recovered back to baseline level by Week 8.

### 3.8. Host transcriptomics reveals significant changes in mucosal gene expression profiles following OV treatment

Pairwise dissimilarity analysis of the mucosal transcriptome performed by NMDS revealed clear separation of clustering of patients at baseline and at Week 4 following treatment with OV ( $p = 0.004$ ; Figure 7). Heatmap

analysis of differentially expressed genes revealed clear clusters of downregulated and upregulated genes that appear to correlate with fecal calprotectin levels following OV therapy.

Differential gene expression analysis demonstrated a significant change in the expression of 843 genes with an increase in 629 genes and a decrease in 214 genes (FDR  $p < 0.1$ ). Notable genes that had a decreased expression included those associated with immune-mediated inflammatory responses such as TNF (tumour necrosis factor) receptor superfamily (*TNFRSF6B*, *TNFSF10*) and interleukin 1 receptor antagonist (*IL1RN*), cellular apoptosis such as caspase (*CASP5*, *CASP10*), tumor suppressor genes such as phospholipase and acyltransferase (*PLAAT2*), antimicrobial responses (*RSAD2*, *REG1A*), aquaporin (*AQP8*) and calprotectin (*S100A9*). Notable genes that demonstrated an increased expression included those involved in prostaglandin biosynthesis (*PTGS2*), extracellular matrix (*HAPLN1*), dendritic cell development (*FLT3LG*), lymphocyte trafficking (*ITGA5*, *ITGA9*) and copper-containing amine oxidase (*AOC3*). Heatmap analysis of the differentially expressed genes following OV treatment showed distinct mucosal gene clusters whose expression correlated with fecal calprotectin levels.



**Figure 7** Changes in host colonic mucosal transcriptomic profiles following treatment with oral vancomycin. A, MDS (multidimensional scaling) plot analysis demonstrates distinct clustering of colonic mucosal gene expression profiles following OV therapy at Week 4 compared with baseline ( $p = 0.004$ ). B, Heatmap plot of differentially expressed genes highlighting clear clusters of downregulated and upregulated genes that appear to correlate with fecal calprotectin levels following OV therapy. C, Volcano plot that demonstrating significant differential expression of 843 genes with an increase in 629 genes and a decrease in 214 genes (FDR adjusted  $p < 0.1$ ). Abbreviation: OV, oral vancomycin.

Gene ontology pathway analysis revealed significant downregulation of pathways that included antimicrobial humoral response, defence response, immune response, regulation of peptidase activity, oxoacid metabolic process, and bile salt transport (FDR  $p < 0.1$ ). Conversely, pathways that were significantly increased included extracellular matrix organization, cell surface receptor signaling pathway, response to wounding, signal transduction, and regulation of cell adhesion. Additionally, KEGG pathway analysis identified the downregulation of pathways involved in butanoate and tryptophan metabolism following treatment with OV.

#### 4. Discussion

This study is the first to comprehensively explore host-microbial mechanisms associated with a reduction in colitis activity, in patients with PSC-IBD treated with OV. Our data demonstrated that after 4 weeks, 80% of patients attained clinical remission, and 100% showed mucosal healing. A significant reduction in fecal calprotectin was observed, with nearly all patients achieving biochemical remission by Week 4.

Moreover, clinical response was rapid, with partial Mayo colitis scores dropping within just 2 weeks of treatment. In turn, cessation of OV was associated with an increase in partial Mayo colitis scores and fecal calprotectin values, indicating a lack of sustained response following short-term vancomycin therapy.

Another key finding was the rapid and significant changes observed in gut microbial diversity, composition, and function during OV therapy. These changes were evident as early as Week 2 and remained stable until the end of the treatment period. Compositional analysis indicated a significant reduction in species within the *Firmicutes* and *Bacteroidetes* phyla, coupled with an increase in *Proteobacteria* and *Fusobacteriia* phyla. Notably, the reduction in fecal calprotectin levels strongly correlated with a reduced relative abundance of key SCFA-producing species, including *Faecalibacterium*, *Lachnospiraceae*, and *Roseburia*. These metabolically active, immunomodulatory species are traditionally associated with a 'healthy' gut state and are consistently shown to be depleted in diseased states, including conventional IBD.<sup>61-64</sup> Despite the observed reduction in SCFAs, which are also known to regulate colonic Treg homeostasis, we hypothesize

that vancomycin's therapeutic effects may be independent of SCFA-mediated pathways and instead related to the modulation of BA metabolism. In parallel, a recent mouse model of PSC demonstrated that vancomycin exacerbated biliary inflammation, reduced SCFA production, and led to an expansion of pathogenic *Escherichia* and *Enterococcus* subsets.<sup>65</sup>

Given the observed reduction in SCFAs on metabolic analysis, we hypothesize that vancomycin's therapeutic effects on colitis activity are independent of SCFA-mediated pathways, and instead related to the modulation of BA metabolism. Indeed, the reported shifts in microbial communities challenge the long-held paradigm that colitis remission must be associated with an increase in SCFA concentration. However, it is important to consider that the increase in *Proteobacteria* and reduction in SCFA-producing species conceivably represent collateral changes, rather than direct contributors to the efficacy of OV.<sup>61,66</sup> As clinical and biochemical improvements occurred despite these changes, OV may target disease mechanisms in PSC-IBD, through pathways unrelated to SCFA production or *Proteobacteria* levels. For instance, vancomycin's effect on BA metabolism and microbial-host interactions, specifically its role in altering BA pathways and suppressing pro-inflammatory cytokine production, may account for putative therapeutic benefits. Pathways to colitis remission in PSC-IBD may differ under OV treatment compared with, for instance, currently available biologics and small molecules used to treat classical IBD. Indeed, we observed a significant increase in the relative abundance of *A. muciniphila* following vancomycin treatment, which diminished after treatment cessation. *A. muciniphila* has been implicated in improving gut barrier function by reducing plasma endotoxin levels and downregulating inflammatory responses.<sup>67,68</sup> While this observation warrants further investigation, it is possible that the increased abundance of *A. muciniphila* is a manifestation of its intrinsic resistance to vancomycin rather than a direct therapeutic effect.<sup>69,70</sup>

Our study also uncovered significant reductions in microbial pathways involved in BA conversion and mannan biosynthesis, with as little as 2 weeks of OV therapy. Reciprocally, we observed an upregulation of genes involved in heme biosynthesis, bacterial chemotaxis, and gut barrier regulation, such as the biosynthesis of multiple medium-chain fatty acids. These changes remained stable throughout the course of treatment and were partially restored after vancomycin withdrawal. Fecal SCFA profiling revealed an increase in 2-hydroxybutyrate levels, along with a reduction in butyrate and valerate, which returned to baseline after 4 weeks of therapy. The discordance between fecal calprotectin and the presence of specific pathogenic bacteria like *F. nucleatum* and *Klebsiella pneumoniae* warrants further exploration, as these shifts typically suggest increased inflammation. However, the reduction in calprotectin and observed clinical improvements suggest that the aforementioned microbial changes may not be the primary drivers of efficacy under OV treatment.

Previous studies have shown that OV was associated with changes in specific BA profiles, including a reduction in secondary BAs,<sup>71</sup> which may contribute to modulating the inflammatory response in PSC-IBD. Taken together, this suggests that the observed reduction in fecal calprotectin, partial Mayo colitis scores and endoscopic colitis activity are likely driven by these BA-related mechanisms. Further investigation is necessary to clarify these pathways; ideally via randomized

controlled trials, and with rigorous comparisons between calprotectin levels, microbial shifts, and serial colonoscopic assessment.

Vancomycin treatment resulted in a reduction in the expression of genes and pathways involved in pro-inflammatory immune responses, including TNF- $\alpha$ , IL-1 receptor, and anti-microbial genes such as REG1A and caspase-10. This was alongside downregulation in genes involved in intracellular BA transport and binding, such as FABP1 and SLC9A3, but not FXR or FGF19.<sup>72</sup> These findings were consistent with our fecal BA profiling, with a marked reduction in secondary BAs in as early 2 weeks, and a subsequent rise to baseline values on treatment withdrawal.<sup>27</sup> We also found a significant reduction in secondary BAs, including derivatives of DCA and LCA, with a commensurate increase in primary BAs. These findings are critically important, given the growing body of evidence to show how 3-oxoLCA and isoalloLCA inhibit the differentiation of effector Th17 cells, while isoDCA promotes the differentiation of regulatory T cells.<sup>73-75</sup>

The FDR threshold of  $p < 0.1$  used in this study was chosen to balance sensitivity and exploratory goals. While this allows for hypothesis generation, it may increase the risk of false positives, and future validation studies are needed to confirm these findings. With this caveat in mind, we observed a reduction in the expression of key microbial genes, such as cholesterylglucuronide hydrolase (bile salt hydrolase, BSH) and hydroxysteroid dehydrogenase (HSD), following vancomycin treatment. These enzymes play a critical role in the conversion of primary to secondary BAs and significantly correlate with fecal calprotectin levels. The loss of secondary BAs also correlated with a reduction in colonic inflammation, indicating that imbalances in homeostasis may drive gut inflammation.<sup>73,74,76,77</sup> Similar findings have been reported by others, with 1 study demonstrating a reduction in secondary BAs and improved insulin sensitivity following 1 week of OV therapy.<sup>71</sup>

While the trial provides important insights into host-microbial changes associated with vancomycin therapy, several limitations need to be addressed. Our study's cohort size was small, and the lack of a control group means that study findings need to be interpreted with caution. However, ours was designed as an exploratory open-label trial with mechanistic endpoints, to assess the short-term effects of OV in patients with PSC-IBD; specifically with regard to colitis activity. As such, liver transplant recipients were included by design, given that approximately 40% develop flares in colitis activity, and the fact that gut microbial changes persist when compared with individuals without PSC.<sup>78,79</sup> We nevertheless acknowledge that this introduces potential variability, especially related to BA changes, and the effects of post-transplant immunosuppression, which could influence readouts from omics' data. Secondly, a full colonoscopy is preferred in the assessment of PSC-IBD activity, due to some patients having a predominance of right-sided colitis. However, it was deemed inappropriate by our patient and public involvement group to perform 2 consecutive colonoscopies (baseline and 4 weeks post-intervention) so close together. Regardless, all study participants had evident recto-sigmoid inflammation at baseline, making sigmoidoscopy a pragmatic and less invasive option to assess colitis activity and perform gut mucosal sampling. The observed improvements in clinical and biochemical markers also suggest that sigmoidoscopy was sufficient for assessing treatment response in the short term. However,

future longer-term studies should consider a full colonoscopy for a more comprehensive evaluation, especially in PSC with right-sided colonic disease. It must also be stressed that the gene expression dataset, although modest in size, was used for exploratory hypothesis generation, focusing on known pathways of interest, such as immune regulation, inflammation, and BA metabolism. Larger datasets are typically preferred for pathway identification, but the selected genes were relevant to the study's objectives.

While we demonstrate on- versus off-treatment (washout) effects, ours was an open-label, uncontrolled study. Thus, any improvements in clinical, endoscopic, and biochemical markers should be viewed as associations with vancomycin therapy rather than definitive evidence of a treatment effect. Future randomized controlled trials (RCTs) are necessary to confirm the therapeutic effects, ideally over longer treatment periods. Moreover, the reductions in serum ALP and ALT (while encouraging) are by no means definitive evidence of benefit. Randomized controlled trials of BA therapy and anti-fibrotics have shown meaningful reductions in biochemistry, albeit with large intraindividual variability over time, and no real association with harder efficacy outcome measures.<sup>80–83</sup> This highlights the need to incorporate a broader range of biomarkers in future trials of OV therapy, including, for instance, the enhanced liver fibrosis score, transient elastography, and quantitative measures of ductal disease severity.<sup>82,84,85</sup>

No incidents of vancomycin-resistant *Enterococcus* were detected under OV treatment which is consistent with prior studies in the pediatric literature.<sup>27</sup> Nevertheless, the potential risk of antimicrobial resistance under long-term therapy warrants further study and is essential for later-phase trials of OV in PSC. This is particularly relevant given that biliary fibrosis is augmented by OV in animal models, in line with reduced SCFA production, and an expansion of pathogenic *Escherichia* and *Enterococcus* subsets.<sup>77</sup> Lastly, the depletion of BSH-producing species may have confounded BA findings, as their loss could affect BA composition independently of OV treatment.

Moving forward, the next phase would be to conduct a randomized trial of OV in patients with PSC-IBD, against an appropriately matched placebo-control group. It is important to dissect whether the therapeutic effects of vancomycin are PSC-IBD specific, or whether they can be generalized to IBD patients who do not have PSC. Of interest, previous double-blind RCTs of vancomycin in non-PSC IBD patients did not demonstrate significant benefit over placebo,<sup>86</sup> lending further support to the notion that PSC is associated with a unique form of colitis distinct from UC or CD alone.

In conclusion, OV appears to induce clinical remission in PSC-IBD, likely through modulation of BA metabolism and gut microbial function. While study findings offer insight into the effects of OV, this is but one part of understanding disease pathogenesis. Given the variability in treatment responses, and the heterogenous nature of PSC (including some patients who do not have IBD), further research is needed to explore mechanistic links between mucosal immunity, liver autoimmunity, and chronic biliary disease.

## Funding

This work at the National Phenome Centre was supported by the Medical Research Council and National Institute for Health Research [grant number MC\_PC\_12025], the

Medical Research Council UK Consortium for MetAbolic Phenotyping (MAP UK) [grant number MR/S010483/1], and infrastructure support was provided by the National Institute for Health Research (NIHR) Imperial Biomedical Research Centre (BRC). This article presents independent research supported by the Birmingham NIHR BRC based at the University Hospitals Birmingham National Health Service Foundation Trust and the University of Birmingham, UK. The views expressed are those of the author(s) and not necessarily those of the National Health Service, the NIHR, or the Department of Health.

## Conflict of Interest

MNQ has received speaker fees from Pfizer, Janssen, Takeda, Tillotts, Falk, and Nordic Pharma and consultancy fees from Nordic Pharma. BM has no conflicts of interest to declare. JRM has received consultancy fees from Cultech Ltd. and EnteroBiotix Ltd. PJT receives institutional funding from the NIHR Birmingham BRC. This article presents independent research supported by the Birmingham NIHR BRC, based at the University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health. PJT has received advisory board/consultancy fees from Chemomab, Cymabay, Albireo/Ipsen Pharma, Advanz Pharma/Intercept, Dr. Falk Pharma, GlaxoSmithKline, Gilead Sciences, Mirum, and Pliant Pharma. PJT has received independent grant funding from Albireo/Ipsen, Advanz Pharma/Intercept, Bristol Myers Squibb, GlaxoSmithKline, Gilead Sciences, Guts UK, the European Association for Study of the Liver (EASL), Innovate UK, LifeArc, the Medical Research Foundation, the NIHR, Mirum, PSC Support, Regeneron, and the Wellcome Trust.

## Acknowledgments

We thank the European Crohn's and Colitis Organisation for funding this study via the ECCO Grant (Recipient—MNQ). We would like to acknowledge our participating patients and the University Hospitals Birmingham Clinical Research Facility for their invaluable contribution to this study. BHM was the recipient of an NIHR Academic Clinical Lectureship (CL-2019-21-002) and is now the recipient of an MRC Clinician Scientist Fellowship (MR/Z504002/1). The Division of Digestive Diseases and MRC-NIHR National Phenome Centre at Imperial College London receive financial and infrastructure support from the NIHR Imperial Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London.

## Author Contributions

MNQ, PJT, THI, and ADB contributed to the study concept and design. MNQ, NS, TI, and PJT conducted the clinical delivery of the study. MNQ, JC, PJT, EE, BM, JRM, SC, KC, MAV-G AP, AA, and GG performed wet and dry lab analysis. MNQ wrote the first draft of the manuscript and all authors contributed to the interpretation of data and preparing the manuscript. MNQ, BHM, PJT, and THI finalized the submission.

## Data Availability

All sequencing data generated from this study (host RNA-seq, 16S rRNA sequences, metagenomics, and metatranscriptomics) have been submitted to the Sequence Read Archive (SRA) under accession reference PRJNA1138254.

## Supplementary Data

Supplementary data are available online at ECCO-JCC online.

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