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1 **The relationship between mucosal microbiota, colitis and systemic inflammation in**
2 **Chronic Granulomatous Disorder**

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25 **Funding**

26 Rare Disease Foundation / BC Children's Hospital Foundation (Grants 1915 and 1920 to D.M.L.) and
27 UCLH Biomedical Research Centre (BRC299/III/DL/101350 to D.M.L.).

28

29 **Competing interests**

30 D.M.L. has received travel and subsistence costs for consultancy work for CSL Behring and fees for
31 roundtable discussion from Merck. Other authors declare no conflicts of interest.

32

33 **Abstract**

34 **Background:** Chronic granulomatous disorder (CGD) is a primary immunodeficiency which is
35 frequently complicated by an inflammatory colitis and is associated with systemic inflammation.

36 **Objective:** To investigate the role of the microbiome in the pathogenesis of colitis and systemic
37 inflammation.

38 **Methods:** We performed 16S rDNA sequencing on mucosal biopsy samples from each segment of 10
39 CGD patients' colons, and conducted compositional and functional pathway prediction analyses.

40 **Results:** The microbiota in samples from colitis patients demonstrated reduced taxonomic alpha
41 diversity compared to unaffected patients, even in apparently normal bowel segments. Functional
42 pathway richness was similar between the colitic and non-colitic mucosa, although metabolic
43 pathways involved in butyrate biosynthesis or utilisation were enriched in patients with colitis and
44 correlated positively with faecal calprotectin levels. One patient with very severe colitis was
45 dominated by *Enterococcus* spp., while among other patients *Bacteroides* spp. abundance correlated
46 with colitis severity measured by faecal calprotectin and an endoscopic severity score. In contrast,
47 *Blautia* abundance associated with low severity scores and mucosal health. Several taxa and
48 functional pathways correlated with concentrations of inflammatory cytokines in blood but not with
49 colitis severity. Notably, dividing patients into 'High' and 'Low' systemic inflammation groups
50 demonstrated clearer separation than on the basis of colitis status in beta diversity analyses.

51 **Conclusion:** The microbiome is abnormal in CGD-associated colitis and altered functional
52 characteristics probably contribute to pathogenesis. Furthermore, the relationship between the
53 mucosal microbiome and systemic inflammation, independent of colitis status, implies that the
54 microbiome in CGD can influence the inflammatory phenotype of the condition.

55

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56 **Key Messages:**

57 The colonic mucosal microbiome and bacterial metabolic pathways in patients with CGD colitis differ
58 from patients without colitis, even in macroscopically normal bowel segments.

59 The mucosal microbiome and bacterial metabolic pathways in patients with CGD also differ
60 according to the extent of systemic inflammation, independently from the presence of colitis,
61 suggesting a role for the gut microbiota in the inflammatory phenotype of this condition.

62

63 **Capsule summary:**

64 The pathogenesis of chronic granulomatous disorder (CGD)-associated colitis and other inflammatory
65 complications is unclear. We demonstrate potentially treatable alterations in the mucosa-associated
66 microbiome in CGD colitis and microbial differences which associate with systemic inflammation
67 independently of colitis status.

68

69 **Key words:** CGD; chronic granulomatous disorder; colitis; microbiome; Bacteroides; Blautia

70

71 **Abbreviations**

72 **AC:** Active colitis

73 **ANCOM:** Analysis of composition of microbes

74 **ASVs:** Amplicon sequence variants

75 **CGD:** Chronic granulomatous disorder

76 **CVID:** Common variable immune deficiency

77 **FCP:** Faecal calprotectin

78 **HoC:** History of colitis

79 **IBD:** Inflammatory bowel disease

80 **ImS:** Immunosuppressants

81 **nAC:** no Active colitis

82 **PCA:** Principal component analysis

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83 **PCoA:** Principal coordinate analysis

84 **PERMANOVA:** Permutational multivariate analysis of variance

85 **PLP:** Pyridoxal 5'-phosphate

86 **SCFAs:** Short chain fatty acids

87 **UCEIS:** Ulcerative colitis endoscopic index of severity

88

89 **Introduction**

90 Chronic granulomatous disorder (CGD) is a primary immunodeficiency characterised by failure of
91 phagocyte oxidative burst [1]. In addition to life-threatening infection, affected patients frequently
92 suffer inflammatory colitis [2], characterised by cryptitis, crypt abscesses and crypt architectural
93 distortion as well as granulomas. It is known that the microbiome is altered in other inflammatory
94 bowel diseases and that this may have a causative role in the pathogenesis [3]; this may be of especial
95 importance in CGD where poor innate control of bacteria is a core feature. An important role for the
96 microbiome has been suggested in a mouse model of CGD [4] and the stool microbiota has been
97 described as abnormal in patients with CGD [5].

98 Microbial dysbiosis is also strongly associated with systemic inflammation of any cause, even in the
99 absence of overt gut disease [6–8]. In a recent study, we demonstrated that CGD patients have
100 elevated blood inflammatory markers and cytokines which do not necessarily correlate with the extent
101 of colitis [9].

102 We therefore first hypothesised that the mucosa-associated microbiome and microbial metabolic
103 pathways would differ between CGD patients with and without colitis, supporting a causative role in
104 the development of colitis as seen in other inflammatory bowel diseases. Next, we hypothesised that
105 the microbiome and microbial pathways would differ according to the extent of systemic
106 inflammation regardless of the extent of colitis, implicating the microbiota in the wider inflammatory
107 phenotype of CGD.

108 In our recent study, where we demonstrated that CGD-associated colitis can be monitored non-
109 invasively [9], ten participants underwent colonoscopy with biopsies taken from each segment of the
110 large bowel. Blood was also assayed for markers of systemic inflammation. We have here
111 investigated the microbiota in each of these bowel segments and correlated this with the severity of
112 the patients' colitis and systemic inflammation.

113

114

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115 **Methods**

116 Patient characteristics have been described previously [9] and are provided in Table 1 and
117 Supplementary Table E1. All patients were receiving antibiotic and antifungal prophylaxis.
118 Colonoscopy and biopsies were performed as part of the study, although there was an urgent clinical
119 indication in one patient with new onset (several weeks) of colitis symptoms; blood was taken
120 contemporaneously for serum cytokine analysis.

121

122 **Table 1: Clinical characteristics of participants in the study.**

Patient	Age (years)	Sex	CGD Type	Mutation	Total UCEIS	History of Colitis (onset)	Antibiotics	ImS
P01 (nAC)	35	F	AR (p47)	NA	0	Yes (2014 - adulthood)	Co-trimoxazole	+
P02 (nAC)	26	M	AR (p40)	NA	0	No	Co-trimoxazole	-
P03 (nAC)	37	M	XL	CYBB c.943_945 delAAG, p.Lys 315del	0	No	Co-trimoxazole	-
P04 (nAC)	39	M	XL	CYBB c.252G>A, splice defect	0	Yes (unknown onset)	Co-trimoxazole	+
P05 (AC)	37	M	XL	CYBB total gene deletion	7	No*	Ciprofloxacin, Doxycycline	-
P06 (AC)	27	M	XL	CYBB c.665A>G, p.His222Arg	32	Yes (2006, childhood)	Co-trimoxazole	-
P07 (AC)	24	F	AR (p67)	NA	41	Yes (1997, childhood)	Co-trimoxazole	+
P08 (AC)	25	M	XL	CYBB c.388C>T, p.Arg130X	9	Yes (2006, childhood)	Co-trimoxazole	-
P09 (AC)	24	M	XL	CYBB deletion exon 6_13	28	Yes (2006, childhood)	Co-trimoxazole	+
P10 (AC)	22	M	XL	CYBB c.676C>T, p.Arg226X	24	Yes (2015, adulthood)	Ciprofloxacin, Metronidazole	+

CGD type: XL, X-linked; AR, autosomal recessive.
ImS: Immunosuppressants.
NA: Not available.
AC, active colitis; nAC, no active colitis
* No prior history of colitis but diagnosed with these investigations

123

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124 Based on cytokine profiles, a rank scoring (1-9) was used to divide patients into two groups, (1) high
125 level of systemic inflammation (High - below median, ranks 13-21) and (2) low level of systemic
126 inflammation (Low - above median, ranks 29-38). Rank scores and serum IL-1β, IL-6, TNFα, IL-12
127 and sCD14 measurements are given in Supplementary Table E1. Serum cytokine measurements were
128 not completed for patient P04. Colonic biopsy specimens were obtained from each bowel segment
129 reached (rectum, sigmoid, splenic flexure, hepatic flexure, caecum, terminal ileum), and the presence
130 of colitis in each segment was assessed by the endoscopist and scored according to the Ulcerative
131 Colitis Endoscopic Index of Severity (UCEIS) score. Samples were stored at -80°C in RNALater
132 (ThermoFisher).

133 Metagenomic DNA was extracted from approximately 1 x 1 mm biopsy sections or a blank sample
134 (extraction control) using DNeasy PowerLyzer PowerSoil kit [11]. For 16S rDNA sequencing, V3-V4
135 hypervariable region of 16S rRNA gene was amplified by PCR using universal 341F and 805R
136 primers fused with Nextera XT index and MiSeq adapter sequences. Molecular grade water was used
137 as a negative control and Mock Community B (HM-783D, www.beiresources.org) used as a positive
138 control, and amplicons were confirmed on a 1% agarose gel. The extraction kit control and PCR
139 negative controls did not generate any amplicons, therefore were not included in library pooling.
140 Subsequently, 62 samples including the mock community were pooled at equimolar concentrations
141 and the library was sequenced using a MiSeq sequencer with 2 x 250 bp paired-end run (Illumina
142 MiSeq, v2 kit). The resulting sequence data was processed using 'Quantitative insights into microbial
143 ecology 2' (QIIME2 version 2020.2, https://qiime2.org/) [52]. The raw sequences were de-
144 multiplexed, and de-noised using the DADA2 algorithm with default parameters to create amplicon
145 sequence variants (ASVs). The mean sequencing depth was 63,509 (range of 9,476 – 124,026), and
146 the resulting ASVs were assigned taxonomy using the SILVA v132 16S database. Functional
147 metabolic predictions were calculated on ASVs using the PICRUST2 (v2.3.0-b) software with default
148 parameters [53], and the resulting pathway functional profiles were imported into QIIME2
149 environment. Taxonomic profiles were generated using the 20 most abundant genera across all
150 samples. Alpha diversity was calculated on ASVs and predicted functional pathways using the
151 observed ASV index (number of unique features) and Shannon index. For group-wise comparisons at
152 community level, Principal Coordinate Analysis (PCoA) was performed using the Aitchison distance
153 [54]. The effect of active colitis, history of colitis, immunosuppression, bowel segment, age, CGD
154 type and individuality was tested by Adonis test. The ASVs and functional pathway data sets were
155 further standardised by analysing just two segments (sigmoid and rectum) that were available for all
156 patients. Mann-Whitney test was used to compare the alpha diversity metrics between active colitis
157 (AC) and no active colitis (nAC) groups as well as between high and low systemic inflammation
158 groups. Similarly, Aitchison distances were tested for the same groupings using PERMANOVA with
159 999 permutations for changes in the community composition. Genus level associations for colitis

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160 status were investigated using q2-geneiss and q2-ANCOM plugins [55]. Functional pathway data
161 were further analysed using the DEICODE plugin [56]. The resulting robust Aitchison distances were
162 visualised using PCoA limited to 2-axes and statistical significance was tested using PERMANOVA
163 on the basis of colitis status and systemic inflammation group (High versus Low).

164 Correlations between faecal calprotectin and genus level taxonomy and functional pathways were
165 calculated using Spearman correlation on standardised data sets (i.e. two bowel segments per patient).
166 To explore associations between gut microbiome and inflammatory markers a correlation analysis
167 was performed between the top 20 genera and cytokine concentrations using Spearman's rank
168 correlation. The top 40 pathways that were associated with the separation of the high and low
169 systemic inflammation groups on axis-1 in the PCoA were further investigated using correlation
170 analyses with cytokine concentrations. Each of the resulting correlation matrices was clustered via
171 hierarchical clustering using Ward's minimum variance method (Ward.D) and Rho (r^2) was reported.
172 Patient P10 was excluded from some analyses, as indicated in the relevant sections, due to the
173 extreme difference in microbial composition from other patients.

174

175 Results

176 The microbiota of CGD patients with and without colitis differs in terms of dominant taxa,
177 alpha-diversity and beta-diversity

178 Consistent with existing studies, the mucosal microbiome composition showed strong inter-
179 individuality and the differences along the bowel segments within individuals were less than the
180 differences between individuals. Examining the dominant taxa (Figure 1A), a patient with severe
181 acute colitis (rapid onset of symptoms over several weeks with no prior history of bowel disease) and
182 extremely elevated faecal calprotectin (P10) had a microbiota dominated almost exclusively by
183 *Enterococcus*. Other patients with colitis exhibited predominantly *Bacteroides* species, and in total
184 there were nine bacterial genera which distinguished colitis patients from those without colitis
185 (Supplementary Figure E1A). Analysis using the q2-gneiss tool revealed that increased proportions of
186 *Bacteroides*, *Clostridium innocuum* group, *Escherichia – Shigella* and *Lachnoclostridium* were
187 associated with active colitis, while greater abundance of *Blautia*, *Alistipes*, *Bifidobacterium*, *Dorea*
188 and *Subdoligranulum* were associated with non-colitic gut. Notably, in colitis patients there was no
189 clear difference between segments affected or unaffected by disease, unlike some reports in Crohn's
190 disease [10]. We used a secondary approach (ANCOM test) to identify differentiating genera, and the
191 difference in abundance of *Subdoligranulum* was the only statistically significant result. The
192 discovery of this genus - in agreement with prior studies - despite the small size of this cohort, may
193 indicate a functional protective role against colitis development and progression [11,12].

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194 To further investigate the drivers of the non-colitis and colitis-associated mucosal microbiome, we
195 performed an exploratory multivariate analysis using Aitchison distances on the 16S data. The results
196 showed significant effects of active colitis, history of colitis (HoC), age, CGD type and use of
197 immunosuppressants (ImS), although the largest explanatory factor was patient individuality
198 accounting for 33% of the variance (Figure 2C). The effect of active colitis was evident in 16S alpha-
199 diversity measures in which patients with colitis (n = 5, excluding P10 with almost exclusively
200 *Enterococcus*) demonstrated reduced taxonomic alpha-diversity compared to those without colitis (n =
201 4; Figure 2A). A difference in Shannon index was also seen between those with a history of colitis
202 versus those with no prior colitis (Supplementary Figure E2) which is in agreement with trends
203 reported previously [5]. Thus, from both alpha- and beta-diversity results, it appears that having a
204 history of colitis is a significant underlying factor which appears to have a long-lasting effect on the
205 composition of the gut microbiome. The use of immunosuppressants did not impact the richness and
206 diversity of the mucosal microbiome in this cohort but did make some contribution to beta-diversity.
207 Eight patients were receiving co-trimoxazole prophylaxis and two were on different antibiotic
208 regimes; however, the outlier patient P10 was in the latter group and thus – although they may be an
209 important contributing factor – analysis on the basis of antibiotics would not be informative. All
210 patients received itraconazole as antifungal prophylaxis.

211

212 ***Bacteroides* abundance positively correlates while *Blautia* abundance negatively correlates with**
213 **colitis severity**

214 We proceeded to investigate correlations between the abundance of bacterial genera and colitis
215 severity. Genus *Blautia* showed a strong negative correlation (r^2 = -0.81, p = 0.008) with the
216 endoscopic score of disease severity (UCEIS), while the genus *Bacteroides* showed a positive
217 correlation (r^2 = 0.70, p = 0.037) with the same measure (Figure 1B-ii,iii). Disturbances in some of
218 these taxa have been implicated in other inflammatory bowel diseases suggesting similarities in
219 pathogenesis, and the strong associations with these genera suggest they may be useful as indicators
220 of colitis activity or severity in this cohort [3, 7].

221 In addition to the UCEIS scoring, elevated levels of faecal calprotectin are associated with intestinal
222 inflammation which can be caused by colitis. In our patients, a level of faecal calprotectin (FCP)
223 above 50 ug/g was indicative of active colitis, and the levels showed some correlation with the
224 *Bacteroides* genus (r^2 = 0.50, $p < 0.001$; Figure 1B-i).

225

226

227

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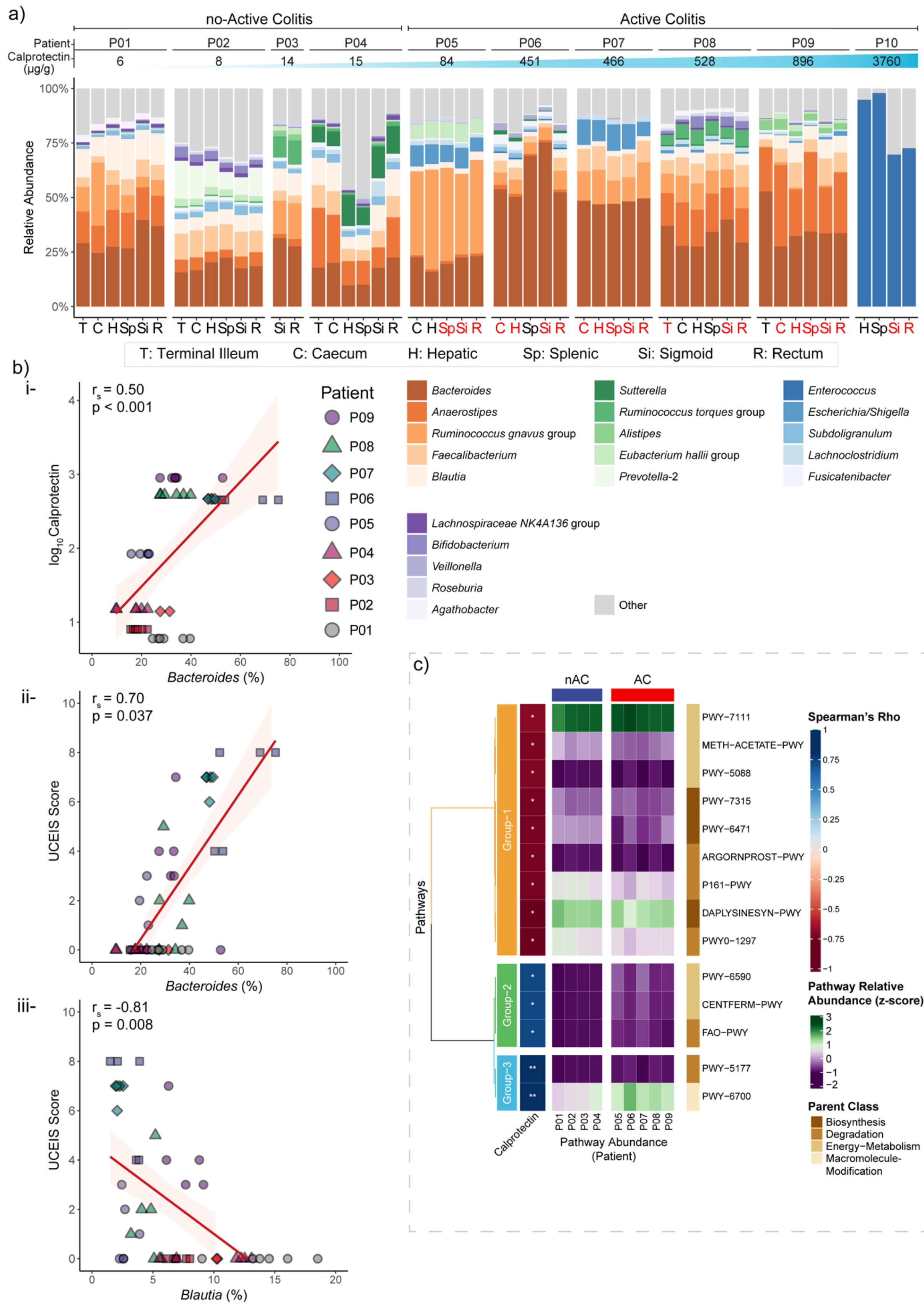

228

229 **Figure 2: Genus level gut microbiome profiles of CGD patients with non-colitic or colitic colon.** (a)

230 Taxonomic profiles of microbiota along the gut for each patient are shown, arranged according to increasing

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231 concentrations of faecal calprotectin. In addition to overall colitis status, bowel segments with active colitis are
232 highlighted in red. (b) Significant correlations between the faecal calprotectin level (measured per patient) and
233 UCEIS score (measured per bowel segment) and relative abundance of *Bacteroides* and *Blautia* genus. (c)
234 Correlation between functional pathways and faecal calprotectin level. For correlation analyses, Spearman's
235 correlation coefficient(r_s) is reported. P10 was excluded from the correlation analyses because of the extreme
236 difference in microbiome composition.

237

238 **Certain microbial functional pathways correlate with colitis severity as measured by faecal**
239 **calprotectin**

240 Calprotectin is known to chelate metallic ions such as Zn, Mn, Fe and Cu, and it can therefore act as
241 an inhibitor for metalloenzymes. This could be a potential underlying reason for the enrichment or
242 reduction of certain metabolic capabilities which cannot be directly inferred by taxonomic
243 assignments. To investigate the relations between the level of FCP and functional characteristics of
244 the mucosal microbiome a correlation analysis was completed. 14 functional pathways were found to
245 significantly correlate ($p < 0.05$) with the FCP level (Figure 1C). The first cluster (Group-1) contained
246 negatively correlated pathways. Among these, DAPLYSINESYN-PWY (L-lysine biosynthesis I)
247 pathway contains the zinc-dependent dapE metalloenzyme, which plausibly could be limited due to
248 calprotectin mediated sequestration [13]. Moreover, this pathway has been previously identified as
249 one of the differentially abundant pathways between healthy and ulcerative colitis patients [11].

250 The second cluster (Group-2) contained three functional pathways that positively correlated with the
251 FCP levels. (1) PWY-6590, the superpathway of *Clostridium acetobutylicum* acidogenic fermentation
252 pathway; (2) CENTFERM-PWY, the pyruvate fermentation to butanoate pathway; (3) FAO-PWY,
253 the fatty acid β-oxidation I pathway. Notably, all three are either involved in butyrate biosynthesis or
254 utilisation of fatty acids such as butyrate. Compared to the non-colitis patients, the butyrate producing
255 pathways as well as butyrate utilising pathways were enriched in patients with colitis, particularly in
256 patients P05 and P07. This suggests that reduced butyrate levels in IBD patients could be due to
257 increased microbial utilisation of butyrate [14–16], although butyrate levels in CGD colitis have not
258 previously been measured.

259 In the third cluster, which demonstrated a strong positive correlation with FCP, one of the notable
260 pathways was the PWY-6700 (queuosine biosynthesis I) pathway. Queuosine plays an essential role
261 in tRNA modifications, and the gut microbiome is thought to be a major provider of this micronutrient
262 to the host [17,18]. However, it was shown to be upregulated under Zn limiting conditions, so again
263 increased FCP may potentially be responsible for its increased abundance in active colitis patients
264 [19,20].

265

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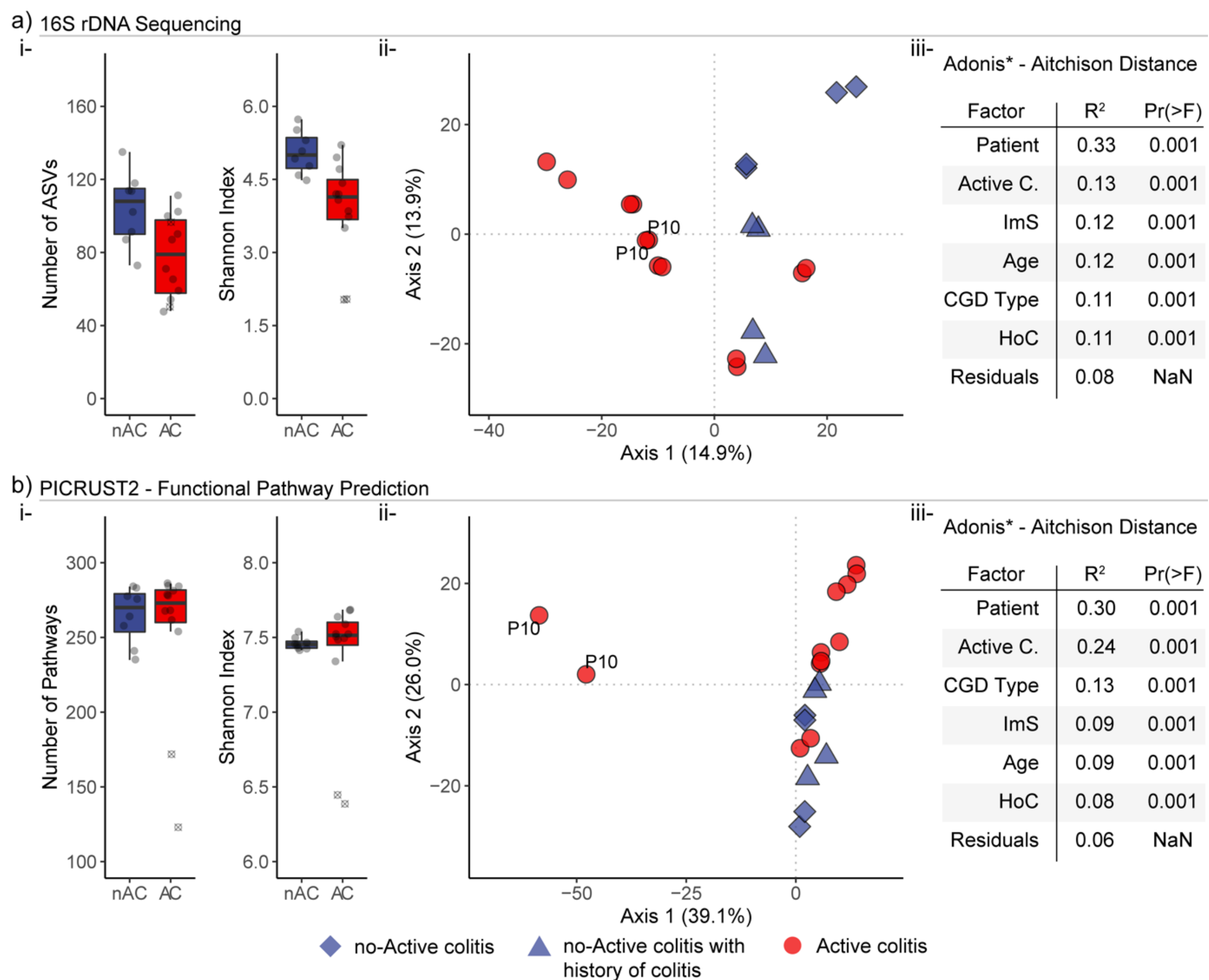
266 **Analysis of microbial functional pathways reveals differences between CGD patients with and**
267 **without colitis**

268 Multivariate analysis using Aitchison distances on functional metabolic pathway predictions also
269 showed significant effects of active colitis, history of colitis (HoC), age, CGD type and use of
270 immunosuppressants (ImS). Notably, the amount of variation explained by active colitis was almost
271 double (at 24%) that observed in the 16S sequencing data (13%). However, individuality remained the
272 largest explanatory factor at 30% (Figure 2B-iii). In terms of alpha-diversity, only active colitis and
273 having a history of colitis resulted in significant differences on Shannon Index (Supplementary Table
274 E2). Although the mean difference between groups was small (~0.1) for both factors, ANCOM test
275 revealed differentially abundant pathways. Three pathways were significantly enriched in patients
276 with colitis. These were PWY-6590, CENTFERM-PWY and FAO-PWY, all of which also showed
277 significant correlation with the FCP levels, as described in the next section (Supplementary Figure
278 E1B). On the other hand, GLUCARDEG-PWY (the D-glucarate degradation I pathway) had higher
279 abundance in patients with prior colitis.

280



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281 **Figure 3: Alpha and beta diversity of the patient cohort in relation to active colitis and health.** (a) based on
282 16S rDNA sequencing data, and (b) based on functional pathways predicted by the PICRUST2 software. (i)
283 Richness (number of ASVs) and diversity (Shannon Index) in patients with active colitis (AC) versus those with
284 no active colitis (nAC). Data are shown from the standardised data set with two bowel segments (rectum and
285 sigmoid) per patient. Patient P10, who had an enterococci dominated microbiota is shown with crossed circles (
286). (ii) Community level clustering of the no colitis and active colitis groups; patients with a history of colitis
287 are also indicated. (iii) Multivariate analysis by Adonis on the Aitchison distances. Adonis formula; *distance ~*
288 *Active Colitis + Immunosuppression + History of Colitis + CGD Type + Age + Gender + Patient.* Statistical
289 significance between groups is reported in supplementary Table E2. Active colitis, n = 6; no active colitis, n = 4.
290 Active C., active colitis; ImS, immunosuppression; HoC, history of colitis.

291

292 **Systemic inflammatory markers correlate with the abundance of certain bacterial genera**

293 We next sought to investigate the relation between blood inflammatory markers and mucosal
294 microbiome composition. Correlation analysis showed distinct patterns clustered under four groups
295 (Supplementary Figure E3). Group-1 mostly consisted of positively correlated genera and included
296 the strongest significant association between *Lachnoclostridium* and IL12 ($r^2 = 0.83$, $p = 0.01$). This
297 genus also had increased abundance in patients with active colitis in our cohort, and has been
298 previously shown to have increased abundance in colitis but not in Crohn's disease [12].

299 Group-3 and Group-4 were almost entirely represented by negative correlations while differing in the
300 associated inflammatory markers. The former included mucosal non-colitic *Blautia*, *Alistipes* and
301 *Faecalibacterium* genera showing significant negative correlations with IL12, sCD14 and IL1β. In the
302 latter, there were significant negative associations between the innate cytokines (IL6, IL1β and
303 TNFα) and *Agathobacter*, *Bifidobacterium*, *Anaerostipes* and *Lachnospiraceae* NK4A136 group. The
304 majority of these genera (*Alistipes*, *Faecalibacterium, Bifidobacterium* and *Lachnospiraceae*
305 NK4A136 group) are short-chain fatty acid (SCFAs; acetate, propionate and butyrate) producing
306 bacteria that can reduce inflammation [21]. In particular, an association between increased abundance
307 of *Faecalibacterium prausnitzii* coupled with increased butyrate and decreased levels of sCD14 has
308 previously been reported in HIV-infected individuals [22]. Interestingly, across the top 20 genera, we
309 were not able to identify as many significant positive as negative correlations with the levels of
310 inflammatory markers. In particular, despite *Bacteroides* genus showing significant correlations with
311 the FCP levels and the endoscopic UCEIS score, it did not significantly correlate with any of the
312 systemic inflammatory markers. This lack of positive associations also suggested that there might be
313 additional factors other than colitis which mediate the interplay between mucosal microbiome and
314 systemic inflammation.

315

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316 **Microbial composition and functional pathway alpha-diversity metrics differ between patients**
317 **with high and low levels of systemic inflammation**

318 To explore this possibility, we introduced high (n = 4) and low (n = 4) systemic inflammation groups
319 based on the total rank scores of the inflammatory markers (Supplementary Table E1). Notably, a
320 patient with no colitis and one with the mildest disease were classified into the 'High' inflammation
321 group while two patients with active colitis were found to have 'Low' ranks of inflammatory markers,
322 implying a lack of clear correlation between colonic and systemic inflammation.

323 Revisiting alpha-diversity metrics, both taxonomic and functional pathway richness showed
324 significant differences between the High and Low systemic inflammation groups (Supplementary
325 Table E2). More importantly, inflammation was the only factor that resulted in a significant
326 separation of the functional pathway richness. The Low systemic inflammation group had a richness
327 mean of 282 (±3) unique functional pathways compared to 261 (±17) in the High inflammation group.
328 Together, these results suggest either that increased systemic inflammation is reducing functional
329 pathway richness in the gut microbiome, or conversely (and more plausibly) that reduced functional
330 richness due to disruption of gut microbiome might be inducing inflammation.

331

332 **Multivariate analysis reveals a strong association between systemic inflammation and the gut**
333 **microbiome**

334 To further investigate the association between gut mucosal microbiome and systemic inflammation,
335 we performed robust Aitchison PCA limited to two dimensions. The initial multivariate analyses
336 showed active colitis as a significant factor explaining 22% and 21% of the variation in the 16S rDNA
337 and functional pathway data, respectively (Supplementary Table E3). However, the greatest
338 explanatory factor was systemic inflammation accounting for 37% of variation for 16S rDNA and
339 59% for functional pathway data, even greater than the inter-individual differences. Use of
340 immunosuppressants, CGD type and history of colitis had more modest effects. The relationship
341 between systemic inflammation and functional and compositional characteristics was further
342 confirmed by PERMANOVA showing a significant and large effect size for both data types (Figure
343 3A,B).

344 Subsequently, ANCOM test revealed one genus and five pathways which were differentially abundant
345 between the High and Low systemic inflammation groups (Supplementary Figure E4). The single
346 genus that differed between the systemic inflammation groups was the *Lachnospiraceae* NK4A136
347 group, which was completely absent in the patients with High systemic inflammation. This group has
348 been shown to be health associated in various studies [23,24], as well as considered to have anti-
349 inflammatory properties since it is a SCFA producer [25].

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350 We also sought to investigate whether there was any overlap between the genera associated with
351 colitis and systemic inflammation (Figure 4). *Blautia*, *Alistipes* and *Bifidobacterium* were exclusively
352 found to differentiate patients with both non-colitic colon and low systemic inflammation, whereas
353 *Lachnoclostridium* was exclusively observed as a significant feature in both active colitis and high
354 systemic inflammation. However, five further taxa differentiated colitis from non-colitis without
355 being implicated in systemic inflammation, while six taxa were differentially abundant between the
356 high and low inflammation groups without varying according to colitis status. Again, this implies that
357 the relationship between gut mucosal microbiome and systemic inflammation is more complex than
358 simply reflecting the severity of colitis.

359 Three of the functional pathways, P562-PWY (myo-inositol degradation I), PWY-5304
360 (superpathway of sulphur oxidation), P461-PWY (hexitol fermentation to lactate, formate, ethanol
361 and acetate), had increased abundance in the Low inflammation group. Myo-inositol is abundant in
362 the gut, and higher abundance of its degradation pathway in low systemic inflammation may reflect
363 the presence of a non-dysbiotic mucosal microbiome. PWY-5304 is related to sulphur metabolism in
364 which hydrogen sulphide, L-cysteine and inorganic sulphate can be produced by microorganisms such
365 as *Desulfovibrio* species that may have immune-regulatory properties [26–28]. Lastly, P461-PWY
366 was found to be more abundant in the Low Inflammation group. This pathway is responsible for
367 fermentation of sugar alcohols and has been associated with *Anaerostipes hadrus* [29].

368 By contrast, the remaining two pathways had increased abundance in the High inflammation group,
369 and both were involved in the biosynthesis of vitamin B6 (pyridoxal 5'-phosphate, PLP). These were
370 PWY0-845 (superpathway of PLP biosynthesis and salvage) and PYRIDOXSYN-PWY (PLP
371 biosynthesis I). Gut microbiota is one of the main sources of Vitamin B6, though the mechanism by
372 which it affects host-microbiome interplay is not well established as there are conflicting reports
373 regarding its relationship to inflammation [30–33].

374

375 **The abundance of certain microbial metabolic pathways correlates with systemic inflammatory**
376 **markers**

377 To further clarify the relation between the most implicated microbial pathways and inflammation, we
378 performed correlation analysis between the top 20 contributors to axis-1 in the Aitchison PCA and
379 individual cytokines (Figure 3C). Primarily, pathways that were associated with a lower level of
380 systemic inflammation clustered in Group-1 and Group-2, and the majority of the significant
381 interactions (14/18) occurred with IL1β and TNFα. The three pathways (PWY-562, PWY-5304,
382 P461-PWY) that were found to have increased abundance in patients with Low systemic
383 inflammation also showed significant negative correlations with one or more of the monocyte derived

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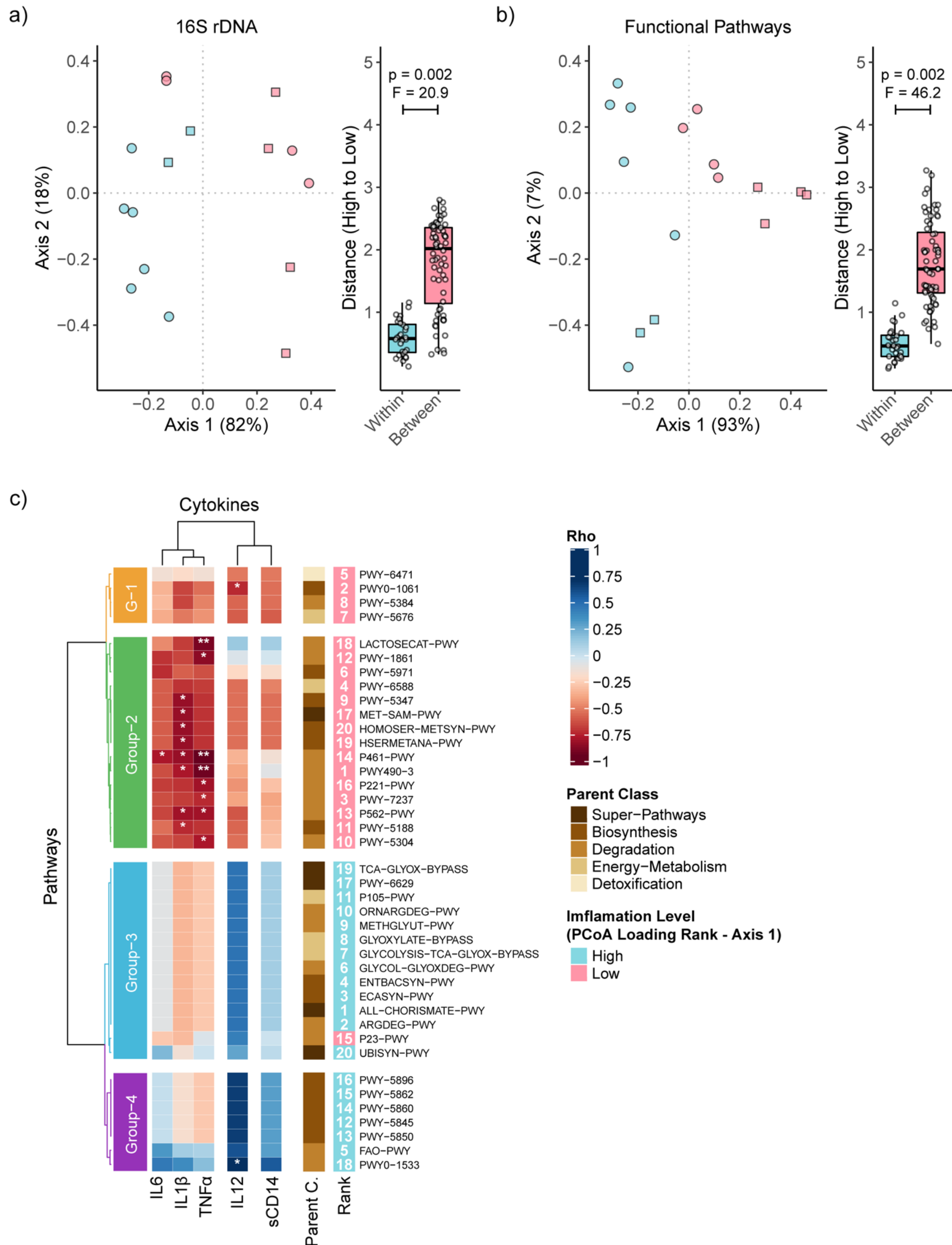

384

385 **Figure 3: Functional pathway and taxonomic associations between mucosal microbiome and systemic**
386 **inflammation.** PCoA of robust Aitchison distances on the basis of systemic inflammation using (a) ASVs from
387 16S rDNA sequencing, and (b) functional pathway predictions. Samples with active colitis are shown as circles
388 (O) and no active colitis as squares ( ), while fill colours represent High or Low systemic inflammation. (c)

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389 Correlation analysis between the top 20 loadings (for both High and Low systemic inflammation groups) on axis
390 1 for functional pathways and inflammatory markers. Significance was tested by PERMANOVA as detailed in
391 Supplementary Table E4.

392

393 cytokines. In particular, P461-PWY negatively correlated with IL6, IL1β and TNFα, which has not
394 been reported previously. In addition, four methionine biosynthesis pathways, PWY-5347
395 (superpathway of L-methionine biosynthesis), MET-SAM-PWY (superpathway of S-adenosyl-L-
396 methionine biosynthesis), HOMOSER-METSYN (L-methionine biosynthesis I), HSERMETANA (L-
397 methionine biosynthesis III) showed negative correlations with inflammatory markers. Similarly, a
398 P562-PWY related pathway, PWY-7237 (myo-, chiro- and scillo-inositol degradation), showed
399 negative correlation with TNFα.

400 In contrast, Group-3 and Group-4 mostly consisted of positive correlations, although there was only
401 one significantly correlating pathway (PWY0-1533, methylphosphonate degradation I). This pathway
402 had a strong correlation ($r^2 = 0.71$) with the IL12 level, and its abundance was increased in the High
403 inflammation group compared to the Low inflammation group. Notably, this pathway has been
404 reported to be enriched in the dysbiotic gut microbiome of severe acute malnutrition patients with
405 acute diarrhea [34]. It was also notable that the FAO-PWY pathway which was positively associated
406 with increased FCP level and identified to have increased abundance in colitis, showed positive
407 correlation ($r^2 = 0.59$) with levels of IL12.

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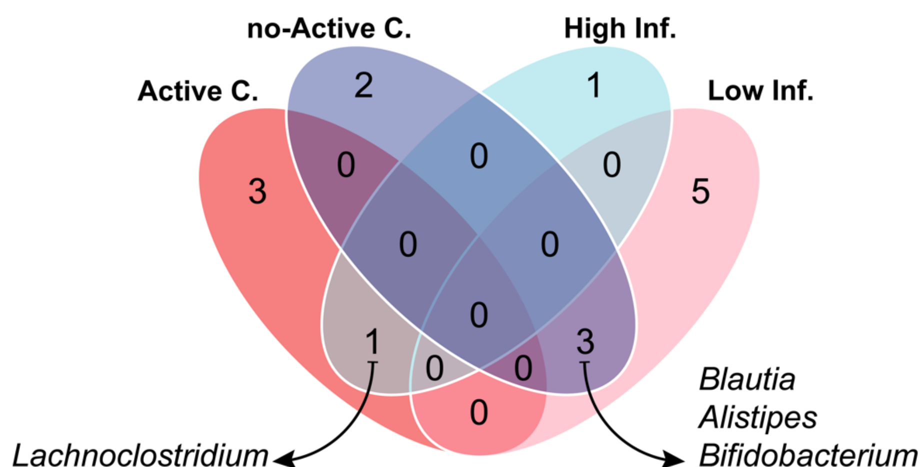

416 **Figure 4: Genus level taxonomic associations shared across active colitis and systemic inflammation**
417 **groupings.** The Venn diagram concatenates those genera that were identified as significantly different between
418 groups in previous analyses. *Lachnoclostridium* was shared between colitis and high levels of systemic
419 inflammation, while *Blautia, Alistipes* and *Bifidobacterium* was associated with non-colitic colon and low level
420 of systemic inflammation. C,, colitis; Inf., inflammation.

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421 **Discussion**

422 The current study is the first to report on the mucosal microbiome of patients with CGD in relation to
423 colitis and systemic inflammation. Given the rarity of this condition, conducting a large scale
424 investigation would be challenging, and to our knowledge, there is only one previous study describing
425 the faecal microbiome of 11 patients with this immunodeficiency condition [5]. Although this
426 previous study provided valuable first insights on the gut microbiome in CGD, other groups have
427 demonstrated that the localised mucosal microbiota along the gut can significantly differ from that of
428 the faecal in health as well as disease. Therefore, investigating mucosal microbiota in CGD patients
429 may further help us in understanding the regulatory role of the gut microbiota in relation to both
430 colitis and systemic inflammation.

431 As revealed by the genus level taxonomic profiles, the intra-individual differences along the gut
432 segments were marginal in most cases. In agreement with some [35-38] but not all [10] previously
433 reported studies, the differences between the colitic and non-colitic segments within individual
434 patients were also minimal in terms of taxonomic composition and alpha diversity. On the other hand,
435 the expected inter-individual difference between patients was the greatest explanatory factor in the
436 initial beta diversity analyses independent from colitis status [39,40]. However, a number of genera
437 were found to associate with either colitic or non-colitic gut. In particular, our results showed that
438 elevated abundance of *Subdoligranulum* was indicative of normal gut mucosa. Although this was
439 consistent with other studies suggesting its preventative role In IBD [11,12,41], one earlier study
440 linked its reduced abundance in IBD patients to administration of antibiotics [42]. All patients in our
441 study were on antibiotics, although we were unable to analyse according to the agents received as
442 nearly all were on co-trimoxazole. The one patient with an *Enterococcus* dominated microbiome was
443 receiving ciprofloxacin and metronidazole and we cannot exclude that these antibiotics were a
444 contributory factor. For example, although the same pattern was not seen in the other patient on
445 ciprofloxacin (plus doxycycline), metronidazole might theoretically have reduced *Bacteroides*
446 abundance.

447 Another interesting finding was the strong negative correlation between *Blautia* and endoscopic
448 assesment scores, in parallel with its increased abundance in non-colitis patients. This bacterium's
449 presumably protective role in CGD colitis contrasts with a recent study in which it was associated
450 with the IBD-related microbial network in the gut [43]. We also observed a significant positive
451 correlation between the *Bacteroides* genus and markers of colitis severity. Interestingly, the
452 characteristics of the members of this genus may differ substantially, even at species level. For
453 example, some *B. fragilis* can have anti-inflammatory and protective properties against colitis
454 [11,44,45], while the enterotoxigenic *B. fragilis* can induce inflammation and promote IBD [46,47].

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455 In a related study conducted by Fiedorova and colleagues [39], the authors emphasize the
456 inconsistencies among studies in identifying certain bacteria associating with gastrointestinal disease
457 severity or health in CVID patient cohorts. While there are several host-related (e.g. genetic makeup
458 and CVID characteristics) and environmental factors (e.g. geographical origin, cohort size and
459 methodology) that could impact the results of such studies, it could also imply that the taxonomic
460 changes may not have to be consistent because the underlying driver is the total functional metabolic
461 capability of the gut microbiota.

462 With this approach, i.e. analysing on the basis of microbial functional pathways, we identified an
463 increased number of significant associations by correlation analyses and abundance testing, which
464 improved the contribution of colitis as an explanatory factor in beta diversity analyses (albeit patient
465 individuality still remained the main factor).

466 However, the lack of a clear separation between active colitis and no active colitis groups led to the
467 introduction of systemic inflammation levels as a new variable. Surprisingly, the effect size of the
468 existing explanatory factors (e.g. colitis status) and in particular individuality, changed drastically
469 meaning that systemic inflammation was independent from colitis disease severity as reported
470 previously [35]. A dysbiotic gut microbiota was shown to induce systemic inflammation in mice [48],
471 and our findings suggest that high systemic inflammation in CGD can be associated with altered gut
472 microbial composition and, especially, functional capability. Collectively, these results strongly
473 implicate the colonic mucosal microbiome in the systemic inflammatory phenotype of CGD,
474 independently of the impact of colitis.

475 The gut microbiome is continuously shaped by a combination of factors, and it can reveal explicit
476 relationships in diseases such as colitis. Our findings also support the concept that there is not a single
477 universal healthy gut microbiota composition. Host lifestyle factors, genetic background, health or
478 specific diseases, the environment as well as aging drive microbiota composition and may result in
479 several shifts and alterations over time [49–51]. For example, we discovered a modest but significant
480 impact of age and genetic type of CGD (X-linked versus autosomal recessive) on beta diversity in our
481 cohort. The latter might relate to differences in residual neutrophil function, although we have no
482 clear evidence for this at present. Nevertheless, ultimately the microbiome should reach stable
483 homeostasis with the host in terms of metabolic functional capability. These properties of a ‘healthy’
484 gut microbiome are crucial for understanding the interaction with the host as well as personalised
485 treatment approaches, particularly in immunocompromised patients.

486 There are a number of limitations to our study that need to be acknowledged. Firstly, our findings are
487 limited to the changes within a CGD patient cohort and did not include healthy individuals for
488 comparison since recruitment of healthy patients was not possible due to sampling by colonoscopy.
489 Secondly, the functional pathway data was generated using a computational prediction tool and may

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490 not completely reflect the true functional profiles. Lastly, non-bacterial members of the gut microbiota
491 such as fungi may provide additional insights but were not analysed here.

492

493 **Conclusions**

494 In this work, we have first demonstrated that patients with CGD-associated colitis exhibit reduced
495 diversity in microbial populations at the level of the gut mucosa and identified bacterial taxa which
496 appear to differentiate between non-colitic and colitic colon; the abundance of *Bacteroides* appears to
497 correlate positively and *Blautia* negatively with disease activity. Very severe colitis may be associated
498 with dominance of a single pathogenic species (e.g. *Enterococcus*). We have also demonstrated
499 differences in microbial metabolism between patients with and without colitis and identified
500 metabolic pathways which associate with disease severity, possibly due to an interaction with faecal
501 calprotectin. Many of the changes in microbiota appear to persist even with mucosal healing and
502 similar patterns are observed in both affected and unaffected segments of patients' colons, implying a
503 microbial 'risk phenotype' for the development of colitis. It will be interesting to study whether this
504 resolves after successful haematopoietic stem cell transplant or gene therapy.

505 We have also demonstrated changes in microbial taxa and metabolism corresponding with systemic
506 inflammation, which is not fully explained by the presence of colitis. Indeed, inflammation appears to
507 show clearer and more significant associations with the gut microbiota than colitis itself. Our data
508 therefore imply that CGD patients' microbiome may influence the inflammatory phenotype of this
509 disease and this demands further investigation. If confirmed in other cohorts, strategies to modify the
510 gut microbiome (including faecal transplant) should be explored as therapies in CGD.

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521 **Acknowledgements**

522 Not applicable.

523

524

525 **Declarations**

526 **Availability of data and material**

527 The datasets generated and analysed during the current study are available in the NCBI Sequence
528 Read Archive (http://www.ncbi.nlm.nih.gov/sra) at NCBI BioProject ID: PRJNA613382

529 **Code availability**

530 Not applicable

531 **Ethics approval and consent to participate**

532 All patients provided written informed consent (NHS Research Ethics Committee (REC)
533 15/LO/1334).

534 **Consent for publication**

535 All participants consented for the results of the study to be published. No individual details, images or
536 videos are included in this manuscript.

537

538

539 **Authors' contributions**

540 MD performed laboratory and bioinformatic analyses. SH collected clinical data. PJS and CDM
541 helped to conceive the study, performed endoscopy with assessment of colitis activity and obtained
542 biopsies. DML conceived the study, recruited patients and supervised the analysis. All authors read
543 and approved the final manuscript.

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