

Original Article

Early Postoperative Endoscopic Recurrence in Crohn's Disease Is Characterised by Distinct Microbiota Recolonisation

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Abstract

Background and Aims: Intestinal microbiota dysbiosis is implicated in Crohn's disease [CD] and may play an important role in triggering postoperative disease recurrence [POR]. We prospectively studied faecal and mucosal microbial recolonisation following ileocaecal resection to identify the predictive value of recurrence-related microbiota.

Methods: Mucosal and/or faecal samples from 121 CD patients undergoing ileocaecal resection were collected at predefined time points before and after surgery. Ileal biopsies were collected from 39 healthy controls. POR was defined by a Rutgeerts score ≥ 2 . The microbiota was evaluated by 16S rRNA sequencing. Prediction analysis was performed using C5.0 and Random Forest algorithms.

Results: The mucosa-associated microbiota in CD patients was characterised by a depletion of butyrate-producing species (false discovery rate [FDR] < 0.01) and enrichment of Proteobacteria [FDR = 0.009] and *Akkermansia* spp. [FDR = 0.02]. Following resection, a mucosal enrichment of Lachnospiraceae [FDR < 0.001] was seen in all patients but in POR patients, also Fusobacteriaceae [FDR < 0.001] increased compared with baseline. Patients without POR showed a decrease of Streptococcaceae [FDR = 0.003] and Actinomycineae [FDR = 0.06]. The mucosa-associated microbiota profile had good discriminative power to predict POR, and was superior to clinical risk factors. At Month 6, patients experiencing POR had a higher abundance of taxa belonging to Negativicutes [FDR = 0.04] and Fusobacteria [FDR = 0.04] compared with patients without POR.

Conclusions: Microbiota recolonisation after ileocaecal resection is different between recurrence and non-recurrence patients, with Fusobacteria as the most prominent player driving early POR.



These bacteria involved in the early recolonisation and POR represent a promising therapeutic strategy in the prevention of disease recurrence.

Key Words: Crohn's disease; postoperative recurrence; microbiota

1. Introduction

Crohn's disease [CD] is a chronic relapsing inflammatory bowel disease that mostly affects young adults, and that is characterised by a decreased quality of life due to symptoms of bloody diarrhoea, urgency to attend the bathroom, abdominal cramps, perianal pain, incontinence, and systemic symptoms of weight loss. IBD is a global health care problem with a substantial financial burden for patients but also for the health care system, and if treated suboptimally is characterised by a poor prognosis. Up to 70–80% of CD patients need a surgical intervention due to therapy failure and/or development of penetrating and/or stricturing complications of the disease.¹ Surgery is not curative and new lesions recur in the neoterminal ileum within months in up to 75% of patients.² As a consequence, many patients require again medical treatment and/or even re-resection with the risk for short bowel syndrome.^{2,3} The most significant risk factors for postoperative recurrence [POR] include active smoking, perforating disease, and previous resection, but also younger age of disease onset and short disease duration have been reported.¹ These clinical factors are far from perfect in predicting disease recurrence, and better markers to identify patients at risk are necessary in order to stratify postoperative management.

In this respect, several lines of evidence point to the involvement of the intestinal microbiota in disease recurrence. Nucleotide-binding oligomerisation domain 2 [NOD2] polymorphisms have been linked to ileocaecal resection and were shown to increase risk to develop POR and need for reoperation.⁴ In human studies, our group showed that the intestinal mucosa remains intact after diversion of the faecal stream, whereas exposure of the gut to luminal contents leads to rapid recurrence of ulcerations.⁵ These insights led to randomised trials with antibiotics [metronidazole and ornidazole] and have proven—although modest—effect in the prevention of POR.^{6,7}

The human POR model reflects the natural history of disease and is an excellent clinical situation to study the sequence of events that lead to onset or early recurrence of disease.

Better profiling of patients before or at the time of surgery could improve success rates. The microbiota have been extensively studied in patients with CD and findings showed a disequilibrium between beneficial and harmful bacteria, also called dysbiosis, in CD patients compared with healthy individuals. CD patients harbour fewer bacteria belonging to the Firmicutes phylum and *Bacteroides* genus and more bacteria belonging to the Actinobacteria and Proteobacteria phyla.^{8,9} Our group previously revealed signature species characterising faecal dysbiosis in CD.^{10,11}

In contrast, microbial studies in patients with POR are very limited. French researchers identified a higher abundance of the adherent invasive *Escherichia coli*, within early lesions in the neoterminal ileum compared with normal mucosa of CD patients and controls.¹² Neut *et al.* reported an increased colonisation of the normal and inflamed neoterminal ileum after resection by colonic bacteria.¹³ The authors further described a higher prevalence of enterococci, *Bacteroides*, and Fusobacteria in patients with early POR.¹³ A study by Sokol *et al.* revealed a reduction of *Clostridium coccoides* and *Faecalibacterium prausnitzii* in CD

patients with POR at Month 6.¹⁴ They furthermore associated a low abundance of *F. prausnitzii* at the time of surgery with an increased risk for POR of ileal CD. What is needed is prospective studies including both faecal and mucosal sampling in a longitudinal set-up. In the current study, we prospectively evaluated changes in the faecal and mucosal microbial communities in CD patients before and after ileocaecal resection. We hypothesised that microbial profiling at the time of surgery might aid to predict POR. Further, as it remains unknown whether dysbiosis is a cause or consequence of inflammation, we also investigated temporal changes of the mucosal and faecal microbiota in CD patients before and after surgery.

2. Materials and Methods

2.1. Participants

Study approval was obtained from the Ethics Committee of the University of Leuven [Ethics Committee approval, S52544 and S53684]. Written consent was given by all participants before sample collection.

All patients were prospectively recruited via University Hospitals Leuven [Belgium]. We included 121 patients with CD who underwent an ileocaecal resection between 2011 and 2016. One patient did not receive the postoperative endoscopy. The remaining 120 patients received a postoperative endoscopy at Month 6. POR was defined by a Rutgeerts score $\geq 2b$.

Faecal samples were provided before surgery and at predefined clinical visits at Month 1, 3, and 6 postoperatively. Mucosal biopsies were collected from different locations of the resected tissue at the time of surgery and from the neoterminal ileum during postoperative endoscopy at Month 6. The biopsies from the resected tissue were collected from macroscopically inflamed and non-inflamed segments using endoscopic forceps. Sections from adjacent tissue corresponding to the origin of the biopsies for microbial analyses were afterwards histologically defined as inflamed and non-inflamed by a clinical pathologist.

A cohort of 39 healthy individuals undergoing [surveillance] endoscopy, from whom we collected mucosal ileal biopsies, served as controls. Only subjects with normal endoscopy were included.

2.2. Faecal calprotectin

Calprotectin was measured in all faecal samples before and after surgery. Fresh cooled faecal samples were extracted within 24 h upon arrival using the Smart Prep extraction device [Roche Diagnostics, Mannheim, Germany]. Faecal extracts were stored at -20°C until measurement. Calprotectin was determined using an enzyme-linked immunoassay test [Bühlmann fCAL ELISA, Bühlmann Laboratories AG, Schönenbuch, Switzerland] according to the manufacturer's instructions.

2.3. Samples and DNA extraction

Cooled faecal samples [$n = 189$] were provided within 24 h after collection and aliquots were immediately stored at -80°C . Mucosal

Table 1. Characteristics of the CD cohort

Characteristics [<i>n</i> = 120]	Remission patients		Recurrence patients		<i>p</i> -value
	[i0 + i1 + i2a]		[i2b + i3 + i4]		
	<i>n</i> = 68		<i>n</i> = 52		
Number of mucosal samples*					
Inflamed resected ileum [m 0]	65		50		NA
Non-inflamed resected ileum [m 0]	61		47		NA
Neoterminal ileum [m 6]	55		41		NA
Number of faecal samples*					
m 0	31		23		NA
m 1	24		19		NA
m 3	24		19		NA
m 6	28		21		NA
Male/female [%]	31/37 [45.6/54.4]		26/26 [50/50]		0.632 ^b
Median [IQR] age [years]	53.9 [44.0–61.2]		48.1 [34.5–54.2]		0.893 ^a
Median [IQR] body mass index	20.5 [19.1–25.4]		23.1 [22.0–25.4]		0.343 ^a
Median [IQR] duration of disease at resection [years]	35.7 [22.2–38.1]		21.9 [16.8–29.0]		0.624 ^a
Maximum disease location [Montreal classification]					
L1 ileal [%]	29 [42.6]		21 [40.4]		
L2 colonic [%]	0 [0]		0 [0]		0.803 ^b
L3 ileocolonic [%]	39 [57.4]		31 [59.6]		
Disease behaviour at surgery [Montreal classification]					
B1 non-stricturing, non-penetrating [%]	0 [0]		0 [0]		
B2 stricturing [%]	36 [52.9]		30 [57.7]		0.604 ^b
B3 penetrating [%]	32 [47.1]		22 [42.3]		
p perianal disease modifier [%]	9 [13.2]		4 [7.7]		
Active smoking at resection [%]	15 [22.1]		17 [32.7]		0.192 ^b
Medication at resection					
Corticosteroids [%]	10 [14.7]		15 [28.8]		0.059 ^b
Immunosuppressants [%]	14 [20.6]		10 [19.2]		0.854 ^b
Anti-TNF [%]	8 [11.8]		14 [26.9]		0.033 ^b
Antibiotics [%]	15 [22.1]		6 [11.5]		0.133 ^b
Previous resection	20 [29.4.0]		21 [40.4]		0.280 ^b
Median [IQR] C-reactive protein at resection [mg/L]	2.6 [0.9–12.9]		12.4 [2.5–33.2]		0.185 ^a

IQR, interquartile range; m, Month; NA, not applicable; NS, not significant; CD, Crohn's disease; TNF, tumour necrosis factor.

Groups were compared by:

^aNon-parametric MannWhitney U test.

^bChi square test.

*Number of samples before rarefaction.

biopsies [during endoscopy or after resection] were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Bacterial DNA from faecal samples was extracted according to the previously described 'Godon' method.¹⁵ In short, after chemical and mechanical lysis with beads, nucleic acids were purified using ethanol precipitation and the pellet was eluted in 200 µl of Tris-EDTA buffer.

Microbial DNA from mucosal biopsies was extracted using the same 'Godon' method with slight modifications. Biopsies were first treated with 180 µl Buffer ATL [Qiagen] and 25 µl Proteinase K [Qiagen], and incubated at 53°C for a minimum of 2 h. The solution with suspended tissue was stored at -80°C and afterwards subjected to the same procedure as the faecal samples. However, the DNA pellet was eluted in 30 µl of Tris-EDTA buffer.

DNA was quantified using a NanoDrop ND-1000 Spectrophotometer [Nucliber] and the integrity was assessed using an Agilent 2100 Bioanalyzer with the DNA 12 000 kit.

2.4. 16S rRNA sequencing and analysis

The 16S rRNA sequencing was performed as previously described.¹¹ Briefly, the V4 region of the 16S rRNA gene was amplified using the 515F-806R primer pair with multiplex identifiers. Amplicons were first purified using the QIAquick PCR Purification Kit [Qiagen, Barcelona, Spain], quantified using a NanoDrop ND-1000 Spectrophotometer [Nucliber] and using an Agilent 2100 Bioanalyzer with the DNA 1000 kit, and then pooled in equal concentration. The pooled amplicons [2 nM] were then purified using HighPrep™ PCR magnetic beads [Magbio, Genomics Inc., Rockville, MD, USA] and subjected to sequencing using a MiSeq system [Illumina, San Diego, CA, USA] following standard Illumina platform protocols.

Sequences obtained from faeces and mucosal biopsies, together with negative controls from the extraction and PCR methods, were analysed with QIIME 1.9.1 using an in-house script.¹¹ Sequences were quality filtered [minimum Phred score of 20] and demultiplexed. A total of 92 158 976 high-quality sequences were

finally recovered, 71 546 690 for faeces and 20 912 286 for mucosal biopsies. Operational taxonomic unit [OTU] clustering at 97% sequence similarity was performed using USEARCH.¹⁶ Representative sequences were aligned using PyNAST against Greengenes template alignment [gg_13_8] and chimeric sequences were filtered out using ICHIME. Taxonomic OTU assignment was performed using the basic local alignment search tool [BLAST] and a combined database [Greengenes and PATRIC]. Reads corresponding to human sequences were removed from mucosal samples. An average sequence depth of 22 241 reads/faecal sample and 38 445 reads/mucosal sample was obtained and samples with fewer than 1000 filtered sequences were excluded from downstream analyses. Rarefaction is used to overcome cases in which read counts are not similar in numbers between samples. For intestinal mucosal samples, considered low biomass samples, we applied the decontamination procedure as described in Davis *et al.*¹⁷ Sequence data have been deposited in the NCBI database with the following access number: PRJNA514452.

2.5. Statistical analyses

General statistical tests were performed in SPSS [SPSS V.25.0 for Windows, SPSS, Chicago, IL, USA]. Groups were compared by non-parametric MannWhitney U test for numeric variables or chi square test for nominal variables.

Statistical microbiota analyses were performed in QIIME and R [<http://www.R-project.org>].¹⁸ The non-parametric KruskalWallis one-way test of variance was used to compare sequences between groups. Comparisons were performed at different taxonomic levels. The Wilcoxon signed rank test was used to compare paired samples between groups. The relative abundance of the microbial taxa was expressed in mean. Alpha diversity measurement was calculated using Chao1¹⁹ and Shannon diversity indexes.²⁰ Principal coordinate analyses [PCoA] analyses were performed based on unweighted and weighted UniFrac distance matrices. Significance of the clustering was defined using the PERMANOVA test; *p*-values significant after multiple testing using false discovery rate [FDR] were stated as 'FDR' < 0.05, and significant non-FDR-adjusted *p*-values were stated as '*p*' < 0.05.

Two different machine learning classification techniques were used for prediction analyses. First, the C5.0 algorithm was applied.^{21,22} The dataset was split randomly into a training sample [65–85%] and a testing sample [15–35%] using an iterative procedure maximising prediction accuracy. The classification trees were iteratively pruned in order to reach an optimal minimum number of cases per bin that maximised accuracy. Second, the Random Forest algorithm was used as an independent approach to confirm the relevance of the selected variables obtained by the C5.0 classification tree. The ranking of genera was calculated using mean decrease in node impurity [Gini index] in the 'randomForest' R package.²³ The predictive accuracy and area under the curve [AUC] were calculated. Receiver operating characteristic [ROC] analyses were performed using the 'pROC' package in R to compare the performance of the microbial profile at the time of surgery [genera], the clinical risk factors for POR [smoking, perforating disease, and previous resection] or a combination of both to predict POR.

3. Results

3.1. Study cohort

We prospectively included 121 patients with CD undergoing ileocaecal resection with ileocolonic anastomosis. One patient did not receive the postoperative endoscopy at Month 6 and was

excluded from the analyses. A total of 319 mucosal and 189 faecal samples were collected. Number of samples per location and time point, demographics, and clinical features of the study cohort are presented in Table 1.

At the time of postoperative endoscopy [Month 6], 68 patients [57%] remained in remission and 52 patients [43%] developed POR. A numerical higher percentage of the patients with POR were active smokers [*n* = 17; 33%] compared with patients without POR [*n* = 15; 22%] [*p* = 0.192]. Other clinical factors known to be associated with POR, such as penetrating disease and previous resection, did not differ significantly between both groups [Table 1]. At the time of surgery, a significant higher proportion of patients who later developed POR [*n* = 14; 27%] were on anti-tumour necrosis factor [TNF] therapy compared with the patients without POR [*n* = 8; 12%] [*p* = 0.033] [Table 1]. Faecal and mucosal microbial analyses [PCoA, alpha diversity, and taxonomic analyses] revealed no confounding effect due to anti-TNF use [Supplementary Figure 1, available as Supplementary data at ECCO-JCC online]. A small number of the patients recently received antibiotics before surgery [Table 1]. The number of patients on antibiotics was equally distributed between patients remaining in remission and patients developing recurrence. Consequently, this variable was not seen as a confounding factor for further microbial analyses. Fifteen patients received immediate postoperative prophylactic therapy [six thiopurines, 10 anti-TNF, one vedolizumab], eight [53%] remained in remission, and seven developed POR [47%] [*p* = 0.781].

Temporal analyses showed a significant drop of faecal calprotectin at the first month after surgery in both the future remission [*p* < 0.001] and POR patients [*p* < 0.001]. The calprotectin levels remained significantly lower at Month 6 compared with baseline in both groups (no POR: median 521 µg/g [Month 0], 100 µg/g [Month 6], *p* < 0.001; POR: median 603 µg/g [Month 0], 186 µg/g [Month 6], *p* = 0.003). The difference in calprotectin levels between patients with and without POR reached statistical significance at Months 3 and 6 [Month 3: *p* = 0.045; Month 6: *p* = 0.023] [Supplementary Figure 2, available as Supplementary data at ECCO-JCC online].

3.2. Mucosa-associated microbiota in CD and healthy subjects

To characterise the mucosa-associated microbiota in CD patients and healthy subjects, we compared ileal biopsies from healthy subjects with inflamed ileal biopsies from CD patients at the time of resection. Alpha diversity in the mucosal microbiome was significantly reduced in CD patients compared with healthy subjects, using Chao1 and Shannon indexes [both *p* = 0.001] [Figure 1A, B].

Weighted and unweighted PCoA analyses revealed that the mucosa-associated microbiota in CD patients deviated significantly from those in healthy subjects [*p* = 0.001] [Figure 1C; and Supplementary Figure 3, available as Supplementary data at ECCO-JCC online].

Taxonomic analyses at phylum level showed significant higher relative abundances of Proteobacteria [FDR = 0.009] and Verrucomicrobiota [FDR = 0.008] in CD patients, whereas Firmicutes [FDR < 0.001], Cyanobacteria [FDR = 0.009], Euyarchaeota [FDR = 0.016], and Tenericutes [FDR = 0.016] phyla were significantly reduced [Figure 1D].

Patients with CD also showed marked mucosal microbial dysbiosis at deeper taxonomic levels. In total, 25 genera were differently abundant between both groups, from which 22 genera were more abundant in the healthy subjects [*Coprococcus*, *Blautia*,

Ruminococcus, *Prevotella*, unidentified Lachnospiraceae, unidentified Christensenellaceae, *Lachnospira*, *Faecalibacterium*, unidentified Ruminococcaceae, Burkholderia, unidentified Clostridiales, unidentified Erysipelotrichaceae, unidentified Rikenellaceae, *Phascolarctobacterium*, *Paraprevotella*, [*Prevotella*], unidentified YS2, *rc4-4*, *Succinielasticum*, *Methanobrevibacter*, and *Serratia*; FDR <0.05] whereas only three genera [*Akkermansia*, *Megamonas*, and *Roseburia*; FDR <0.05] were more highly abundant in patients with CD. Many of the genera which were enriched in healthy subjects belonged to the Lachnospiraceae and Ruminococcaceae families [Figure 2; and Supplementary Table 1, available as Supplementary data at ECCO-JCC online]. The majority of these taxonomic differences were also observed when comparing the biopsies from healthy subjects with the biopsies taken from the non-inflamed resected tissue of CD patients [Supplementary Table 2, available as Supplementary data at ECCO-JCC online].

3.3. Dysbiotic variation between inflamed and non-inflamed resected tissues within one patient

To investigate whether localised dysbiosis in the tissue is the consequence of the inflammatory status, we compared the microbiome from the inflamed and non-inflamed ileum in paired biopsies from the resected tissue [$n = 89$]. Alpha diversity showed no differences between both regions and PCoA analyses indicated overlapping clusters of the mucosal communities from inflamed and non-inflamed areas [Supplementary Figure 4, available as Supplementary data at ECCO-JCC online]. Also unweighted pair group method

with arithmetic mean [UPGMA] clustering showed high similarities between both groups [Figure 3]. Taxonomically, Bacillales [FDR = 0.04] were more frequently detected in the non-inflamed tissue and Lachnospiraceae [FDR = 0.005] in the inflamed tissue.

All the following results were generated using the inflamed resected tissue as baseline sample for mucosa-associated microbiota analyses unless otherwise stated.

3.4. Recurrence-related microbiota at the time of resection and postoperative follow-up

Comparison of the microbial communities in faecal samples before surgery [Month 0], between patients developing POR and those without, only revealed modest taxonomic differences. POR patients harboured higher levels of four genera (*Atopium* [$p = 0.02$], *Gemella* [$p = 0.02$], *Corneybacterium* [$p = 0.04$], and *Rothia* [$p = 0.04$]) at baseline, whereas patients without POR were enriched in *Coprobacillus* [$p = 0.04$] and unknown Peptostreptococcaceae [$p = 0.01$], although comparisons were not significant after FDR correction [Supplementary Table 3, available as Supplementary data at ECCO-JCC online]. Also mucosal samples from the resected ileum [inflamed] showed no marked microbial differences between both patient groups. Taxonomically, five genera were differently distributed between patients with and without POR. In the resected tissue of POR patients, *Cloacibacterium* [$p = 0.02$] was increased, whereas *Actinomyces* [$p = 0.03$], *Peptostreptococcus* [$p = 0.03$], *Streptococcus* [$p = 0.045$], and an unknown genus belonging to Ruminococcaceae [$p = 0.03$] were significantly decreased before FDR correction,

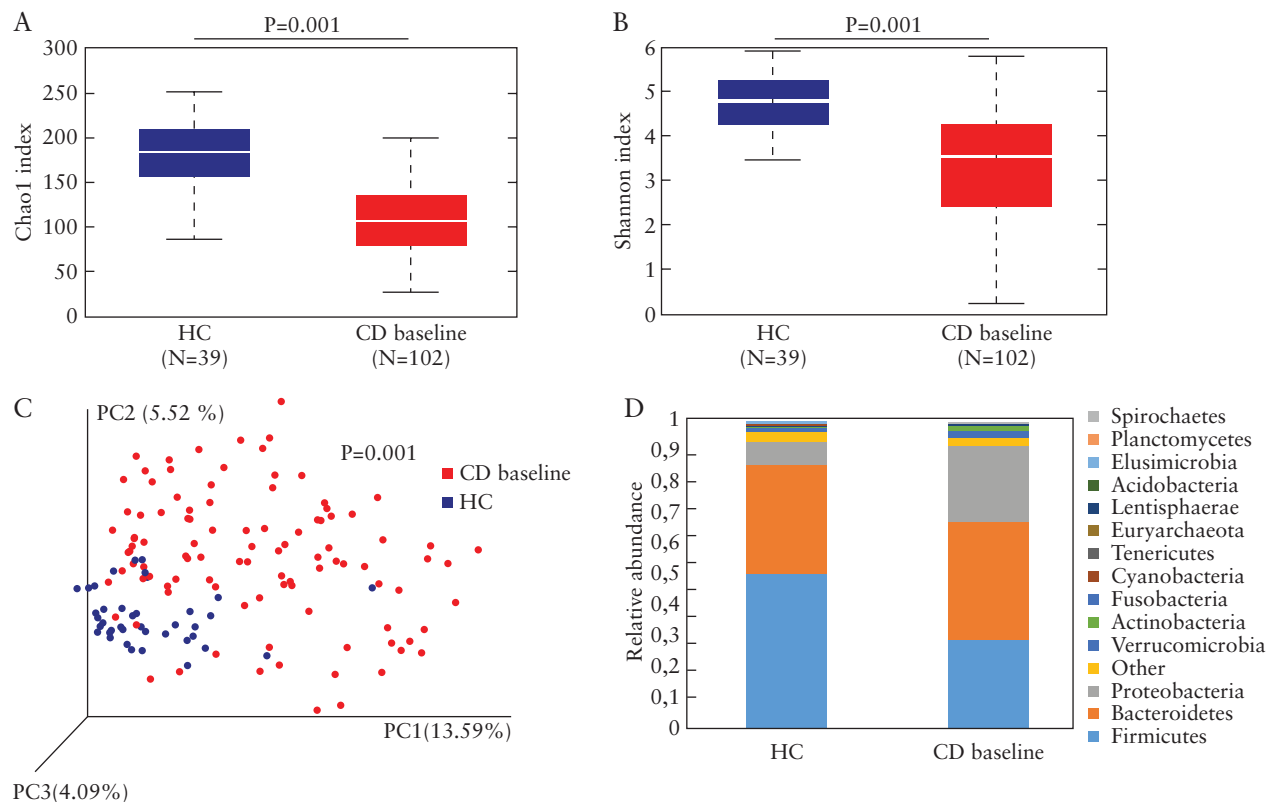


Figure 1. Characterisation of mucosa-associated microbiota in CD and healthy subjects. Alpha diversity based on [A] Chao1 and [B] Shannon indexes was significantly decreased in the inflamed tissue at the time of resection from patients with CD compared with non-inflamed tissue from healthy controls [HC]. [C] Principle Coordinate Analyses [PCoA] clustering based on the unweighted UniFrac distances revealed that the mucosa-associated microbiota in CD patients deviated significantly from the HC. [D] Taxonomic profiling revealed significant differences at phylum level. CD, Crohn's disease.

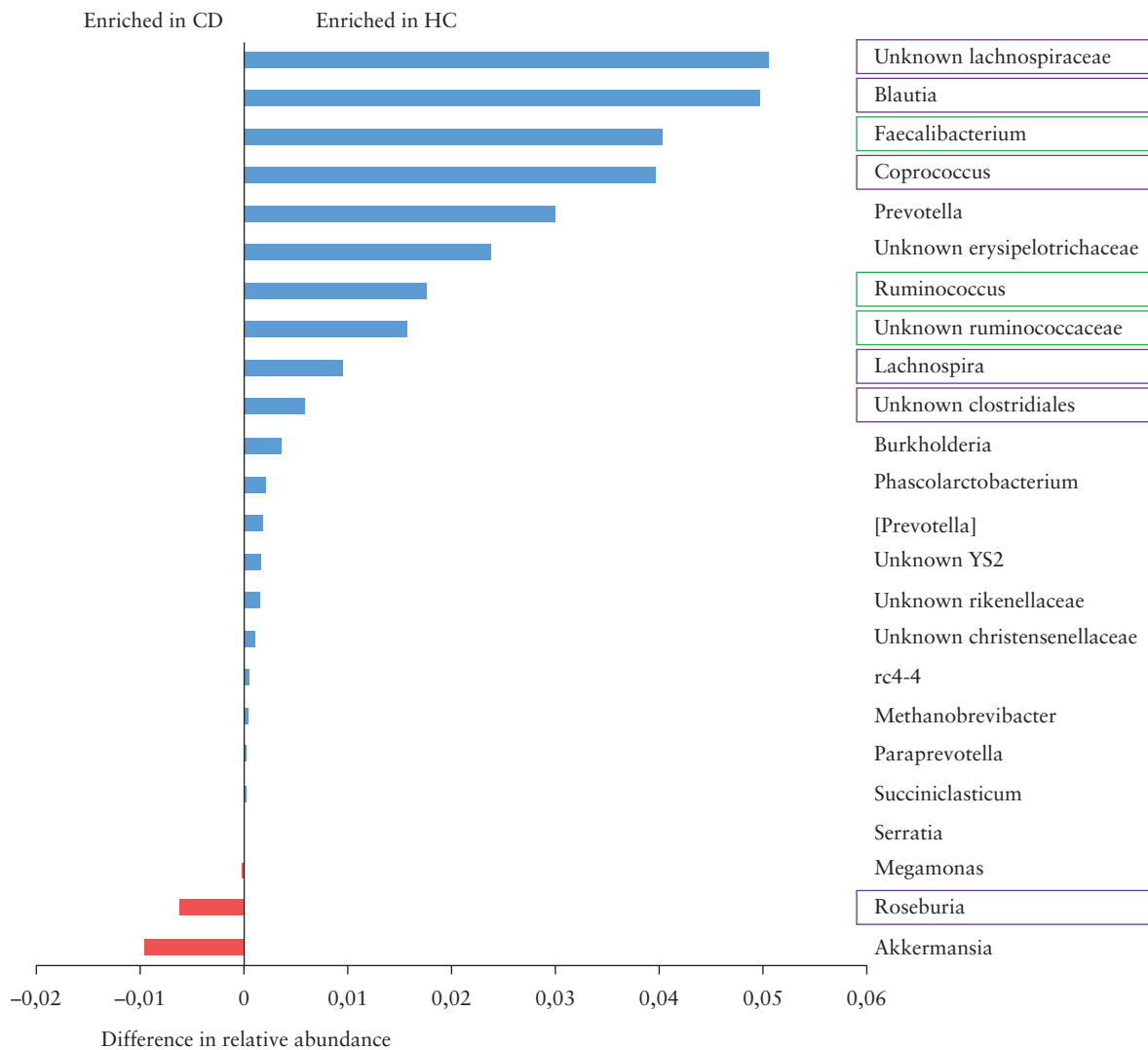


Figure 2. Relative abundance of genera that were differently distributed in the ileal mucosa between patients with CD and healthy controls [HC] [KruskalWallis; FDR <0.05]. Members of the Lachnospiraceae and Ruminococcaceae families are marked in purple and green respectively. CD, Crohn's disease; FDR, false discovery rate.

compared with patients remaining in remission [Supplementary Table 4, available as Supplementary data at ECCO-JCC online].

The faecal microbiota at Month 1 after surgery showed a higher relative abundance of Selenomonadales [$p = 0.04$] and an unknown genus of Lactobacillales [$p = 0.04$] in the POR group versus the remission group, but lacked significance after multiple testing correction [Supplementary Table 5, available as Supplementary data at ECCO-JCC online]. At Month 3, no taxonomically microbial differences at genus or higher taxonomic levels were detected in the faecal samples. At Month 6, patients with endoscopic recurrence possessed a higher relative abundance of *Fusobacterium* [$p = 0.01$] in their faecal samples and reduced relative abundance of *Bifidobacterium* [$p = 0.02$] compared with the patients in remission [Supplementary Table 6, available as Supplementary data at ECCO-JCC online]. These findings were confirmed in the mucosal samples. Patients with POR had a significant higher abundance of Fusobacteria [FDR = 0.04] and Negativicutes [FDR = 0.04] in the neoterminal ileum. At genus level, *Megasphaera* [$p = 0.003$], *Fusobacterium* [$p = 0.004$], *Odoribacter* [$p = 0.008$], *Paraprevotella* [$p = 0.017$], *Oscillospira* [$p = 0.018$],

unknown Peptostreptococcaceae [$p = 0.028$], *Coprococcus* [$p = 0.028$], and unknown Rikenellaceae [$p = 0.045$] were increased in mucosal samples of POR patients [Supplementary Table 7, available as Supplementary data at ECCO-JCC online].

3.5. Dynamics of the microbiome during the early disease course

Clustering analyses, using the PCoA method based on an [un] weighted UniFrac matrix, showed no identifiable clusters based on progress in time after resection. Faecal samples collected at the different time points clustered more in a subject-wise manner and not according to sampling time, revealing a higher intervariability than intravariability [Supplementary Figure 5, available as Supplementary data at ECCO-JCC online]. In general, both the remission and recurrence patients' microbiome showed a high overall stability over time.

Nevertheless, ileocaecal resection did modify the faecal gut microbiota at taxonomic level. Recolonisation after resection was marked by an increase in Negativicutes [FDR = 0.02] (*Veillonella* [$p = 0.002$], and *Dialister* [$p = 0.02$]) and decrease of Actinobacteria

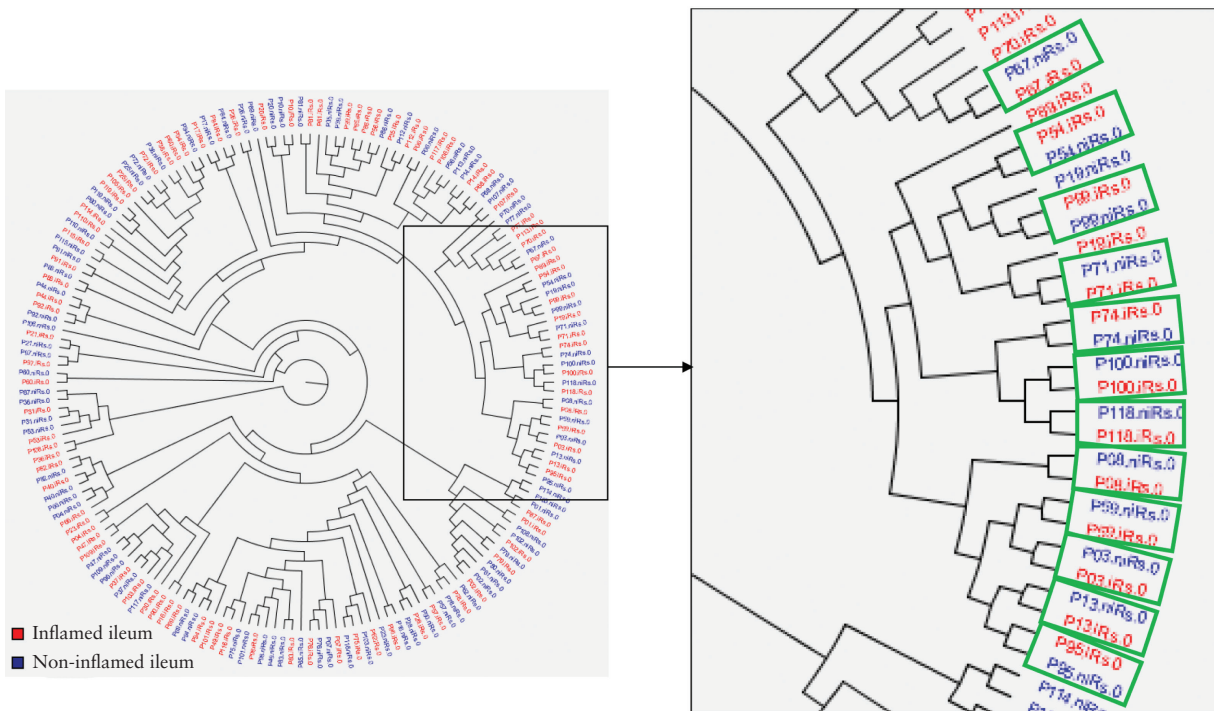


Figure 3. UPGMA clustering based on weighted UniFrac metric of 89 paired biopsies from inflamed and non-inflamed resected tissues. Green rectangles indicate paired samples from the same patient. UPGMA, unweighted pair group method with arithmetic mean.

[FDR = 0.02] [*Bifidobacterium* [$p = 0.006$]] in the total cohort of CD patients [Supplementary Figure 6 A, B, available as Supplementary data at ECCO-JCC online and Supplementary Table 8, available as Supplementary data at ECCO-JCC online]. When we studied the microbial changes after subdivision of the total patient cohort into the remission and recurrence subgroups, we confirmed the increase of Veillonellaceae in both subgroups [no-POR subgroup: $p = 0.02$; POR subgroup: $p = 0.03$][Supplementary Tables 9 and 10, available as Supplementary data at ECCO-JCC online]. Patients developing POR had a significant enrichment of Fusobacteria [FDR = 0.03] (*Fusobacterium* [FDR = 0.33, $p = 0.004$]) after resection [Supplementary Table 10]. Interestingly, this finding could not be observed in patients remaining in remission [Supplementary Figure 6C, available as Supplementary data at ECCO-JCC online].

Also the mucosa-associated microbiota showed significant changes after resection. Beta diversity in remission [$p = 0.001$] and POR [$p = 0.004$] patients differed significantly before and after resection, although unweighted UniFrac PCoA analyses did not reveal a clear separation for remission patients [Supplementary Figure 7 A, B, available as Supplementary data at ECCO-JCC online]. The mucosal microbiome differences in remission patients after surgery were mainly driven by a significant enrichment of Lachnospiraceae [FDR = 0.004], and by a decrease of Streptococcaceae [FDR = 0.003] and Actinomycetaceae [FDR = 0.011, $p = 0.005$] [Supplementary Figure 8 A C, available as Supplementary data at ECCO-JCC online, and Supplementary Table 11, available as Supplementary data at ECCO-JCC online]. Patients with POR also had increased Lachnospiraceae [FDR = 0.03] and uniquely experienced an increase of Fusobacteriaceae [FDR < 0.001] [Supplementary Figure 8D], Moraxellaceae [FDR = 0.07], Promicromonosporaceae [FDR = 0.07], and Erysipelotrichaceae [FDR = 0.07] [Figure 4; and Supplementary Table 12], available as Supplementary data at ECCO-JCC online.

3.6. Predictive potential of microbial and clinical factors

We next evaluated if clinical risk factors, microbial factors [faecal versus mucosal], and the combination of both could predict POR. Clinical risk factors alone had a poor discriminative power to predict POR [AUC = 0.612] [Figure 5B]. In contrast, mucosa-associated microbial factors revealed a much better predictive power [AUC = 0.738], which improved only slightly when combined with the clinical factors [AUC = 0.779]. C5.0 classification tree analyses based on the abundances of the mucosa-associated microbiota at the time of resection revealed four genera to predict POR [Figure 5A]. The model showed a good discrimination in the test set [AUC = 0.739] as well as in the entire dataset [AUC = 0.735] with correct classification of 82.7%. Validation using the Random Forest approach confirmed that clinical risk factors were insufficient to predict POR [AUC = 0.651], whereas microbial factors alone showed an excellent discriminative power, which could not be improved in combination with clinical factors [AUC = 1] [Figure 5C]. The confirmative power of both models was further strengthened by the retrieval of the four predictive genera from the decision tree in the Random Forests top 40 most important predictive variables [Supplementary Table 13, available as Supplementary data at ECCO-JCC online].

Similar analyses were performed using the faecal samples. The C5.0 model showed a better predictive power for POR based on the microbial factors alone [AUC = 0.79] compared with the clinical factors [AUC = 0.5], and the predictive power did not improve after combination of clinical and microbial factors [AUC = 0.79] [Figure 6B]. The C5.0 decision tree revealed three explanatory genera for development of POR based on their relative abundances [Figure 6A]. The performance of the decision tree was very good as demonstrated by an AUC of 0.875 in the test set and AUC of 0.79 in the entire dataset. Unfortunately, the predictive power using the C5.0 model could not be confirmed after validation using the Random Forest model [Figure 6C].

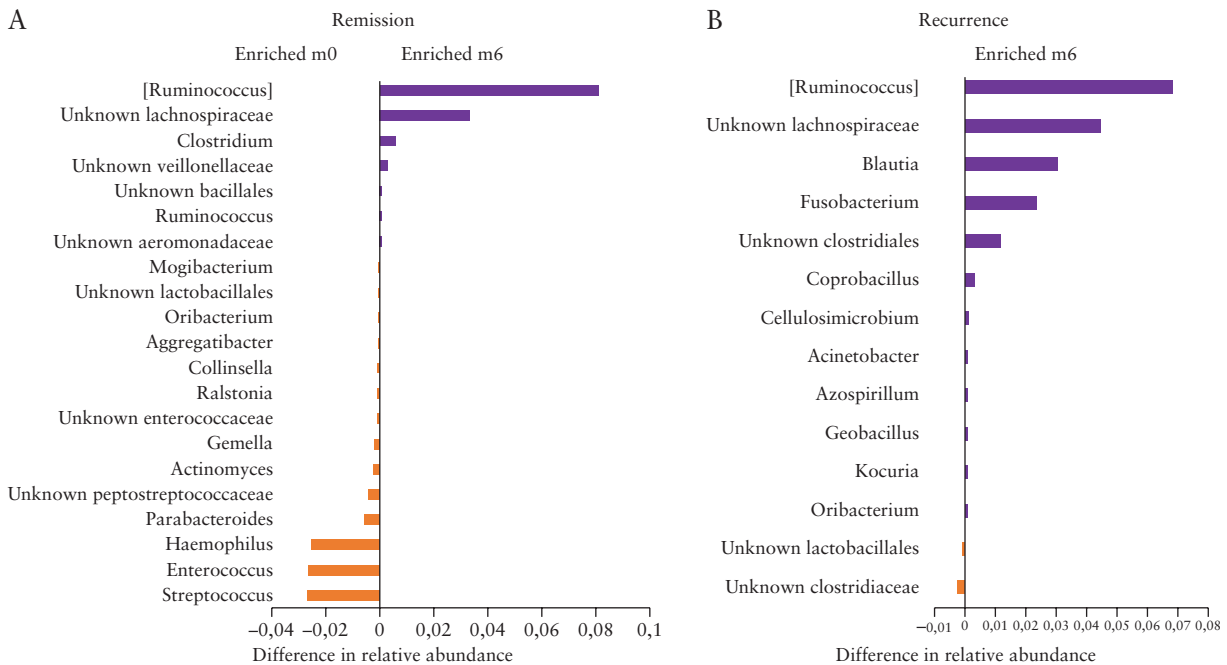


Figure 4. Relative abundance of genera that were differently distributed in the ileal mucosa before and after resection in [A] patients remaining in remission and [B] patients developing recurrence [KruskalWallis; $p < 0.05$]. Genera in orange and purple represent, respectively, an enrichment at the time of surgery and at postoperative endoscopy.

4. Discussion

In this study, we focused on microbiota changes in patients with CD in whom inflammation was cleared by surgery. These patients are at risk for disease recurrence, and previous preclinical and human studies have shown that the microbiota plays a pivotal role in this.^{2,5,24} To our knowledge, this study represents one of the largest prospective longitudinal cohorts investigating the faecal and mucosal microbiome in postoperative CD patients, using a sequencing-based approach. We identified distinct bacteria associated with POR which could predict POR with an AUC of 0.78. More specifically, we found in the postoperative recolonisation process differences in the abundances of bacteria involved in biofilm formation between patients with or without early POR.

We first showed that the ileal mucosa-associated microbiota in patients with CD had a reduced microbial diversity and a higher interindividual microbiota variation, which is in agreement with previous studies.^{25–27} This dysbiosis was characterised by a depletion of essential types of butyrate- and other short-chain fatty acid-producing members of the Firmicutes phylum, such as *Faecalibacterium*, *Coprococcus*, *Blautia*, *Lachnospira*, and *Ruminococcus* species. This is in alignment with literature, as genera belonging to the Lachnospiraceae and Ruminococcaceae families have repeatedly been identified as robust markers of IBD.^{28–30} Additionally, our study shows that Proteobacteria and Verrucomicrobia phyla [*Akkermansia* spp.] were more pronounced in the mucosa of patients with CD. Proteobacteria have been shown to be increased in patients with CD.⁸ The increase of the *Akkermansia* genus, with as type species *Akkermansia muciniphila*, a mucolytic mucosa-associated bacteria, is in discrepancy with previous studies in IBD. However, these studies were performed in patients with ulcerative colitis.^{31,32} One study reported a quantitative decrease of the mucolytic species in inflamed tissue of CD patients.³³ *Akkermansia* is generally known to exert several beneficial properties, such as the provision of acetate

for butyrate-producers by degrading mucin and the production of propionate through the use of pseudovitamin B12.³⁴ The CD patients in our cohort also showed a severe depletion of essential types of butyrate-producing bacteria, and previous studies have reported that CD patients [certainly patients with ileal involvement or previous ileal resections] are at increased risk for vitamin B12 deficiency.³⁵ This, in combination with its capacity to stimulate low-level pro-inflammatory interleukin 8 secretion by enterocytes,³⁶ might rather have a detrimental effect. Furthermore, an enrichment of *Akkermansia* spp. might lead to excessive mucin degradation which may facilitate the access of luminal antigens to the intestinal immune system, thereby contributing to IBD.³⁷ Accumulating evidence from animal colitis models points to a potentially negative role of *A. muciniphila*, which exacerbated Salmonella-induced intestinal inflammation,³⁷ has colitogenic capacities, and can act as a pathobiont in a genetically susceptible host.³⁸ Additionally, *Akkermansia* may bloom after use of antibiotics,³⁹ supporting the hypothesis of *Akkermansia* as an opportunistic bacterium that may flourish after disruption of an ecosystem.

Furthermore, we investigated whether early POR could be predicted using the microbiota information at the time of surgery. We applied two complementary machine learning models on the generated data and studied the microbiota as a system, taking interactions into account. We were able to predict early POR based on the mucosa-associated genera, and this performed much better than clinical factors alone. The predictive power based on the baseline faecal samples was inferior to that of the mucosal samples, which is in alignment with a previous study,²⁸ but also might be due to the lower sample size of the faecal cohort. The identification of microbial predictors of disease recurrence after surgery in CD patients may help clinicians to better predict patients at risk. Our findings suggest that microbial screening at the time of surgery can stratify patients in high and low risk for early POR, and help the decision to initiate prophylactic treatment.

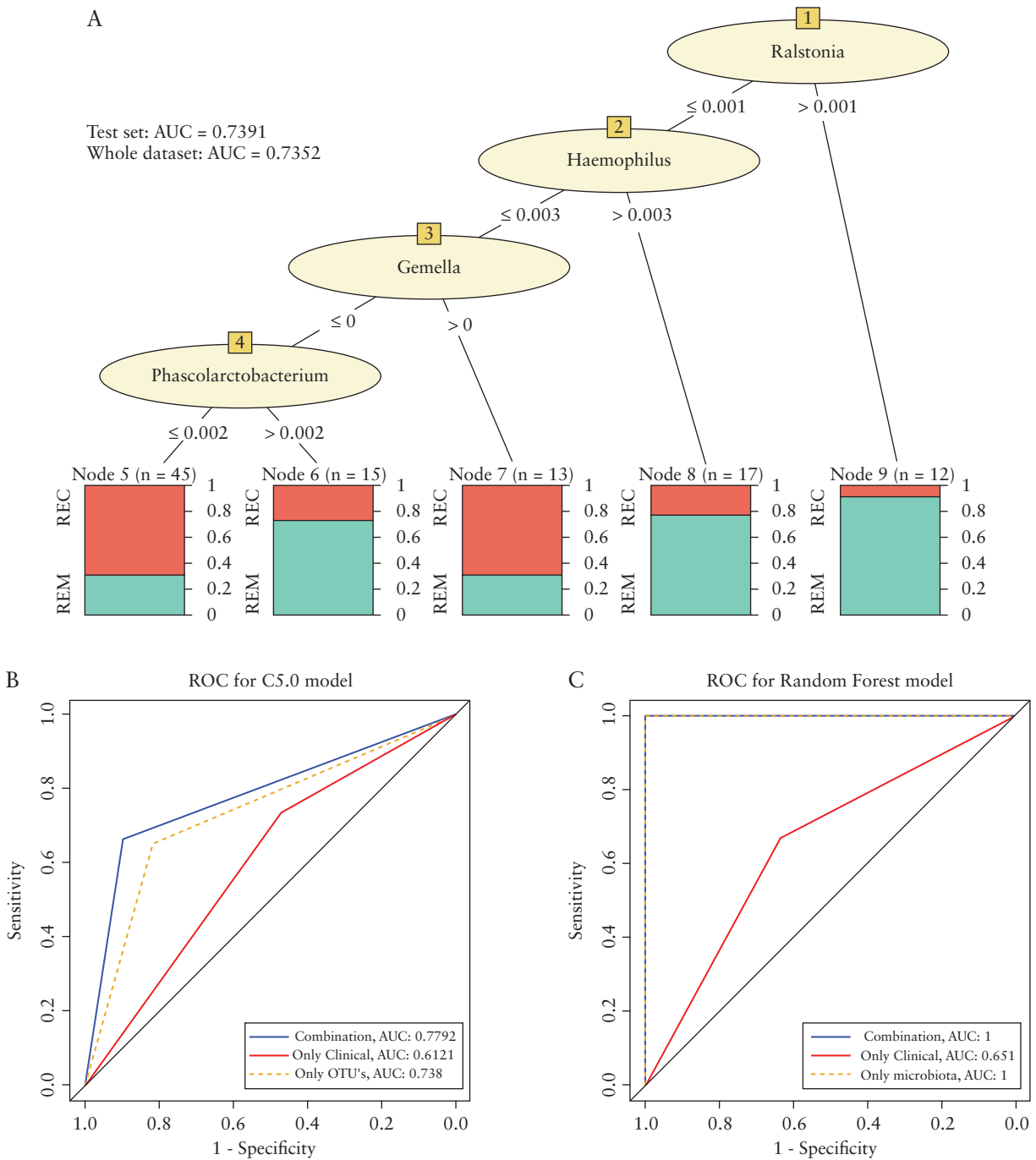


Figure 5. Endoscopic postoperative recurrence prediction analyses based on the baseline mucosal cohort. [A] C5.0 derived decision tree to predict postoperative endoscopic recurrence based on the mucosal abundances of *Ralstonia*, *Haemophilus*, *Gemella*, and *Phascolarctobacterium* at the time of resection. The AUC of the decision tree is reported for the test set and the entire dataset. Minimum cases per bin on the tree = 12. Training data proportion = 0.77. Prediction performance of the [B] C5.0 and Random Forest [C] models using receiver operating characteristic [ROC] analyses based on clinical risk factors alone, mucosa-associated genera alone and combination of clinical and microbial factors. AUC, area under the curve.

Few studies have investigated associations of the microbial community and POR in both faecal and mucosal samples.^{27,40} We observed an increased abundance of Fusobacteria in the mucosal community of POR patients at the time of postoperative endoscopy, which was also reflected in the faecal community. Neut *et al.* were the first to report a frequent isolation of Fusobacteria in tissue from POR patients, using a culture-dependent technique.¹³ Recently, another study

using pyrosequencing, also found a higher abundance of Fusobacteria in biopsies of POR patients.⁴¹ Both studies included a small number of patients without longitudinal follow-up, and only investigated mucosal samples. We here describe an increase in both the luminal and adherent microbial communities. Fusobacteria are proteolytic bacteria with invasive capacities which can exacerbate inflammation and can behave as a pathogen.⁴² In this study, we furthermore observed

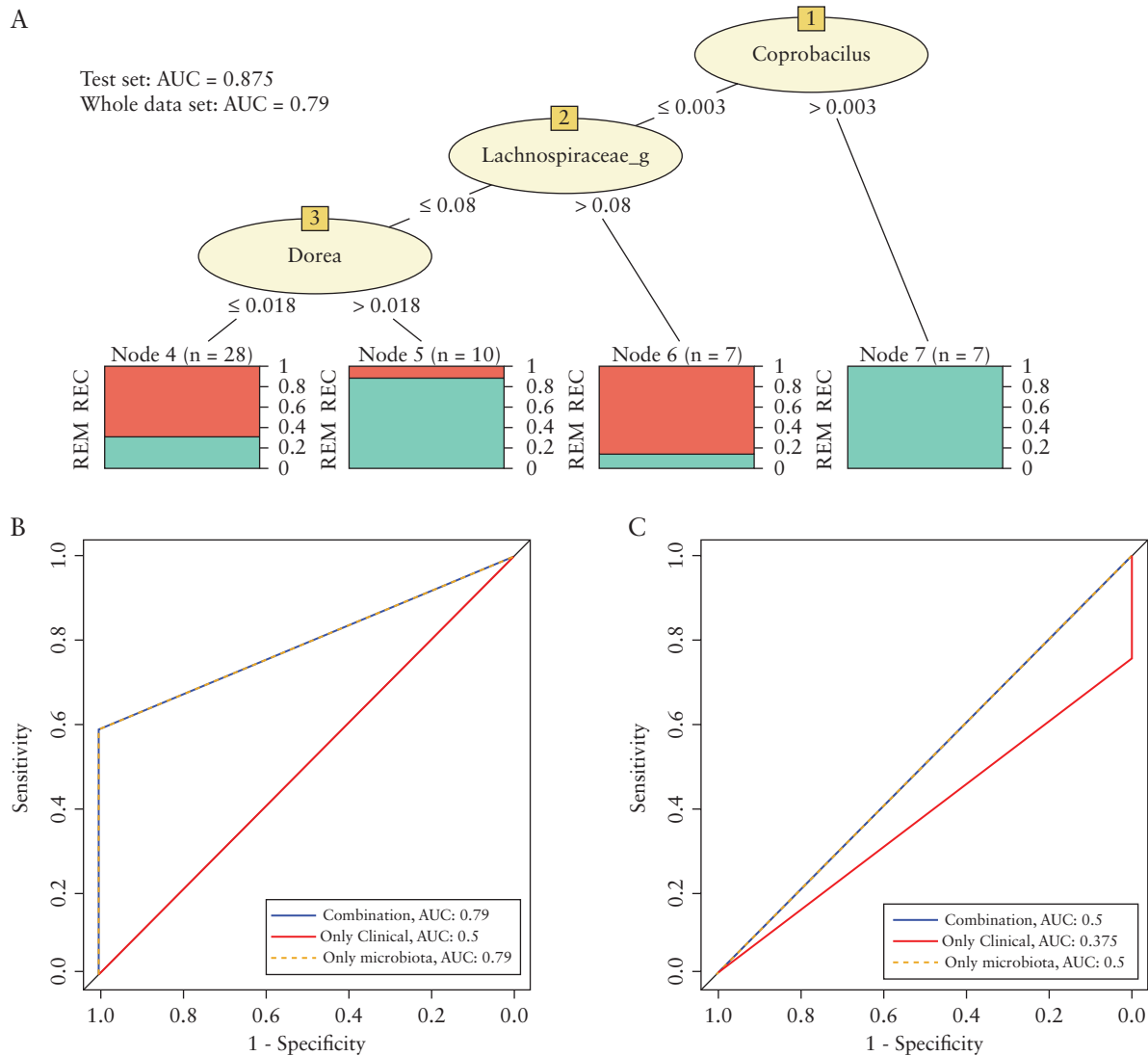


Figure 6. Endoscopic postoperative recurrence prediction analyses based on the baseline faecal cohort. [A] C5.0 derived decision tree to predict postoperative endoscopic recurrence based on the faecal abundances of *Coprobacillus*, unidentified *Lachnospiraceae* genus, and *Dorea* at the time of resection. The AUC of the decision tree is reported for the test set and the entire dataset. Minimum cases per bin on the tree = 6. Training data proportion = 0.83. Prediction performance of the [B] C5.0 and Random Forest [C] models using receiver operating characteristic [ROC] analyses based on clinical risk factors alone, faecal-associated genera alone, and combination of clinical and microbial factors. AUC, area under the curve.

that the mucosa-associated microbiota from POR patients harbour a higher relative abundance of Negativicutes, more specifically, members of the Veillonellaceae family. Gevers *et al.* also reported an increase of mucosal Veillonellaceae and Fusobacteriaceae in paediatric patients with new onset of CD.²⁸ These findings point to an important and possibly common role of these species in the onset of disease, in both paediatric and adult patients.

We have also identified differences in the recolonisation process after surgery. Lachnospiraceae increased following surgery in both patients with and those without POR, which is in agreement with other studies.^{26,40,43,44} Lachnospiraceae have been shown to be reduced in CD patients and have been associated with disease activity.⁴⁵ A novel finding and striking difference in the recolonisation process after resection, was the increase of Fusobacteria in patients developing early POR but not in patients remaining in remission. This finding was also reflected in the faecal community, even though Fusobacteria usually are hard to retrieve in faeces. Fusobacteria have been linked to colorectal cancer,⁴⁶ which is known to be a high-risk

condition for patients with IBD.⁴⁷ They are believed to outcompete the initial microbial drivers and exert tumour-promoting properties in a later stage of colorectal cancer development.⁴⁸ Also in our study, Fusobacteria might seem to have a role at a later stage in POR development, seen its low abundance in the earliest months after surgery.

Fusobacteria also have been associated with chronic inflammatory diseases of the oral cavity. In the oral cavity, this pathogen plays a central role in the formation of polymicrobial communities.⁴⁹ This intermediate coloniser establishes a bridge between early colonisers like *Actinomyces*, streptococci, and Veillonellae, and the late pathogenic colonisers.⁴⁹ The initial colonisers do not possess the ability to coaggregate with commensals or with late pathogenic colonisers, whereas Fusobacteria possess the ability to coaggregate with all other bacteria, and consequently reach out to late pathogenic colonisers.⁴⁹

In the current study, we observed different dynamics of those bacteria with ability for biofilm formation in patients with and without POR. The mucosal community in patients with remission status, but not in patients developing POR, showed a decrease of

Streptococcaceae and Actinomycinae after surgery, which is in alignment with Sokol *et al.*⁴⁴ In addition, Veillonellaceae in the faecal community increased after surgery in all the subjects. Although purely speculative, these findings might point to similar mechanisms and formation of polymicrobial communities in the early development of POR, with Fusobacteria as the crucial player. Fusobacteria are capable of producing several toxins, and *F. nucleatum* is a well-known pro-inflammatory species and has previously been detected in patients with IBD.⁵⁰ Its presence was furthermore associated with non-responders to faecal microbiota transplantation.⁵¹ Another potential pathogenic strategy of Fusobacteria, in altering the innate host defences and triggering host inflammatory responses, is the induced secretion of mucin.⁵² Altered mucin secretion might affect the integrity of the mucosal barrier, and has been described to play a role in the onset and maintenance of IBD.⁵³ Fusobacteria are susceptible to certain types of antibiotics, including metronidazole.⁵⁴ The prophylactic use of metronidazole and ornidazole was effective in decreasing POR in at least three randomised controlled trials [RCTs].^{6,7,55}

Despite the association between Fusobacteria and POR in both the mucosal and the faecal communities, it remains unclear if this is the cause or consequence of the disease. The POR model is a good model, reflecting the early onset and natural history of CD. In our study, this was confirmed by the decrease of faecal calprotectin levels after surgery in all the patients. Although calprotectin levels increased again in patients developing POR, they remained lower at all postoperative time points when compared with calprotectin levels before surgery. This suggests that the increase of Fusobacteria after resection is not a marker of inflammation, and that these species might be involved in the early pathogenesis of the disease. Sequential faecal sampling revealed that the relative abundance of Fusobacteria in POR patients remained low at Month 1 and Month 3 after surgery, but reached high levels at Month 6. These results suggest that the colonisation starts between Month 3 and Month 6.

In conclusion, we demonstrated that the mucosa-microbiota in patients with CD undergoing ileocaecal resection is characterised by a depletion of butyrate-producing species [eg *Faecalibacterium* spp] and enrichment of Proteobacteria and *Akkermansia* spp. The recolonisation after ileocaecal resection differs between patients with early POR and patients without POR, by an increase of microbiota members belonging to Fusobacteria, in both faecal and mucosal communities. Patients without POR are characterised by a decrease of potential harmful bacteria. Further independent external validation is required to confirm these findings. Future randomised trials should evaluate whether prevention of the colonisation by and consequent overgrowth of Fusobacteria during the postoperative period might reduce the development of early CD recurrence.

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Conflict of Interest

GDH's institution [KU Leuven] received fees for his work as central pathology reviewer in clinical trials for Centocor J&J and Takeda. MF received financial support for research from Pfizer, Takeda, and Janssen; lecture fees from Ferring, Boehringer-Ingelheim, Chiesi, Merck Sharpe & Dohme, Tillotts,

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Author Contributions

KM: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis, and technical support; MP: analysis of data; AM: analysis of data; ZX: analysis of data; VPA: analysis of data; JS: technical support and interpretation of data; AS: technical support; DC: technical support; AW: critical revision of the manuscript for important intellectual content; AD: critical revision of the manuscript for important intellectual content; GDH: acquisition of data, interpretation of data, critical revision of the manuscript for important intellectual content; MF: critical revision of the manuscript for important intellectual content; CM: acquisition of data, interpretation of data, drafting of the manuscript, obtained funding, material support, and study supervision; SV: study concept and design, acquisition of data, interpretation of data, drafting of the manuscript, obtained funding, material support, and study supervision. All the authors revised and approved the manuscript.

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Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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