Microbiota Separation and C-reactive Protein Elevation in Treatment-naïve Pediatric Granulomatous Crohn Disease

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ABSTRACT

Objectives: In patients with inflammatory bowel diseases (IBDs), the presence of noncaseating mucosal granuloma is sufficient for diagnosing Crohn disease (CD) and may represent a specific immune response or microbial-host interaction. The cause of granulomas in CD is unknown and their association with the intestinal microbiota has not been addressed with high-throughput methodologies.

Methods: The mucosal microbiota from 3 different pediatric centers was studied with 454 pyrosequencing of the bacterial *16S rRNA* gene and the fungal small subunit (SSU) ribosomal region in transverse colonic biopsy specimens from 26 controls and 15 treatment-naïve pediatric CD cases. *Mycobacterium avium subspecies paratuberculosis* (MAP) was tested with real-time polymerase chain reaction. The correlation of granulomatous inflammation with C-reactive protein was expanded to 86 treatment-naïve CD cases.

Results: The CD microbiota separated from controls by distance-based redundancy analysis ($P = 0.035$). Mucosal granulomata found in any portion of the intestinal tract associated with an augmented colonic bacterial microbiota divergence ($P = 0.013$). The granuloma-based microbiota separation persisted even when research center bias was eliminated ($P = 0.04$). Decreased *Roseburia* and *Ruminococcus* in granulomatous CD were important in this separation; however, principal coordinates analysis did not reveal partitioning of the groups. CRP levels >1 mg/dL predicted the presence of mucosal granulomata (odds ratio 28 [6–134.32]; 73% sensitivity, 91% specificity).

Conclusions: Granulomatous CD associates with microbiota separation and C-reactive protein elevation in treatment-naïve children; however, overall dysbiosis in pediatric CD appears rather limited. Geographical/center bias should be accounted for in future multicenter microbiota studies.

Key Words: Crohn disease, fungi, granuloma, inflammatory bowel disease, microbiota

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Inflammatory bowel diseases (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are disorders that affect >4 million people worldwide (1). The incidence of IBD has significantly increased globally in the 20th century and recent observations indicate their ongoing emergence (2,3). The diagnosis of IBD is based on clinical history, physical examination, serology, radiological studies, endoscopy, and histology (4). Although no ideal noninvasive biomarker exists for IBD, the assessment of C-reactive protein (CRP) is commonly employed during the diagnostic process. CRP serum levels have shown value in indicating prognosis, response to therapy, and disease activity in CD (5–7).

About 25% of patients with IBD present during childhood or adolescence (8) with somewhat different disease characteristics than adults (9). The distinction between CD and UC in as many as 15% of adult and 20% of pediatric IBD cases can be challenging (10). The occurrence of a granuloma is pathognomic of CD and is helpful in distinguishing Crohn colitis from UC. The cause of noncaseating granulomas in CD is unknown and their presence has not been conclusively linked to specific microorganisms, including *Mycobacterium avium subspecies paratuberculosis* (MAP) (11,12). In the meantime, the intestinal microbiota plays a significant role in the pathogenesis of CD in which disease phenotype as well as host genotype is associated with specific shifts in composition (13) depending on the location (ileum, colon) and sample (feces, mucosa) examined (14,15). The fecal microbiota differs from mucosal (16) with the latter likely being more relevant for intestinal immunomodulation (17).

The advancement of culture-independent methodologies has shed light on the complexity and dynamic structure of the human microbiota; however, variation between interrogation methods, sampling, and the IBD populations studied has confounded the

clear characterization of disease-associated microbiota and its potential pathogenic role (18). Moreover, the possible confounding effects of long-standing inflammation and ongoing anti-inflammatory treatment have rarely been accounted for in microbiota studies of IBD. In the meantime, findings demonstrated the significant microbiota-modifying effects of frequently used pharmacotherapy in the disease group (19).

Pediatric IBD cases present a unique opportunity to examine the biological components of IBD pathogenesis following a suspected shorter duration of the disease than in adults. Furthermore, if samples are obtained at the time of the diagnostic procedure, subjects will most likely be treatment naïve. The largest microbiota investigation on pediatric colonic mucosal samples from patients with IBD with limited medication exposure was conducted on 12 CD cases and 17 controls with bacterial cultures and real-time polymerase chain reaction (PCR) of the bacterial *16S rRNA* gene for 14 separate species (20). Although yeasts are acknowledged as potential pathogens in CD (21), high-throughput fungal metagenomic analyses have not been applied to pediatric cases of IBD.

In the present study, we examined transverse colonic mucosal biopsy samples by massively parallel pyrosequencing of the bacterial *16S rRNA* gene and the fungal small subunit (SSU) ribosomal region in 15 treatment-naïve pediatric CD cases and 26 controls. The microbiota associations of CD were correlated with histological, clinical, and laboratory characteristics of the patients.

METHODS

Patients and Samples

Control patients (abdominal pain: 8, irritable bowel syndrome: 4, hematochezia: 3, solitary juvenile polyp: 3, diarrhea: 2, perianal fissure: 2, gastroesophageal reflux disease: 2, and 1 of each: gastritis, healthy [polyposis in sister]) were recruited before endoscopy following informed consent through the institutional review board approved tissue banks of the Charles University, (EK-1796/08); the Pediatric Inflammatory Bowel Disease Consortium Registry at the Baylor College of Medicine (H-17654); and the Massachusetts General Hospital (MGH; 2009p001287) (Table 1). Patients only with grossly and histologically normal mucosa at colonoscopy were designated as controls.

Treatment-naïve CD cases were recruited before their first diagnostic colonoscopy in Baylor and MGH whose disease was determined based upon clinical, biochemical, and histological characteristics. Neither patients with CD nor controls reported the use of antibiotics within 6 months of sampling. Transverse colonic mucosal samples were snap-frozen on dry ice or in liquid N_2 immediately after biopsy and stored at $-80°C$ until further analysis. Table 1 shows demographic characteristics and Montreal classification of the patients studied. Age, sex, ethnicity, and disease location were similar between granuloma-positive and -negative patients. Age and sex did not differ significantly between CD and controls. Studies indicate that the proximal and distal colonic microbiota may differ (22). Therefore, we aimed to interrogate the middle/average of the mucosal bacterial community by studying transverse colonic mucosa samples.

CRP correlation was extended to 86 treatment-naïve pediatric CD cases. Granulomatous CD was defined as the presence of granuloma or a distinct giant cell in at least 1 biopsy specimen. Histological severity of inflammation was graded between 0 and 3 (none to severe) based on the pathology reports incorporating epithelial damage, architectural distortion, and white blood cell infiltration of the lamina propria and epithelium by a physician observer (S.M.). Only those patients who had both esophago-

gastroduodensocopy and colonoscopy evaluation were included. Only CRP values obtained 1 week before or at endoscopy were considered. Histological assessment of terminal ileum was made at similar frequency between granulomatous and nongranulomatous CD groups.

DNA Extraction

After thawing, the colonic mucosal biopsies were centrifuged at 14,000 rpm for 30 seconds and resuspended in 500 μL of RLT buffer (Qiagen, Valencia, CA) (with β-mercaptoethanol). Sterile 5 mm steel beads (Qiagen) and 500 μL of sterile 0.1-mm glass beads (Scientific Industries, New York) were added for complete bacterial lyses in a Qiagen TissueLyser (Qiagen), run at 30 Hz for 5 minutes. Samples were centrifuged briefly, and 350 μL of RTL and 200 μL of 100% ethanol were added to a 100-μL aliquot of the sample supernatant. This mixture was added to a DNA spin column, and DNA recovery protocols were followed as instructed in the QIAamp DNA Mini Kit (Qiagen) starting at step 5 of the tissue protocol. DNA was eluted from the column with 30 μL of water and samples were diluted accordingly to a final concentration of 20 ng/μL. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

Massively Parallel bTEFAP

Bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) was performed as described previously (24). (For details, see supplementary Methods, *http://links.lww.com/MPG/A115*.)

Bacterial Diversity Data Analysis

Before analysis, sequences shorter than 300 bp were removed and sequences were depleted of any nonbacterial ribosome sequences and chimeras using the Black Box Chimera Check software B2C2 (described and freely available at *http://www.researchandtesting.com/B2C2.html*). To determine the identity of bacteria in the remaining sequences, they were first queried using a distributed BLASTn.NET algorithm (25) against a database of high-quality *16S rRNA* bacterial sequences derived from NCBI. (For details, see supplementary Methods, *http://links.lww.com/MPG/A115*.)

Fungal Diversity Analysis

Multivariate analysis of bacteria and fungi were based on measures of multivariate distance (ie, dbRDA); however, the specific distance measure most used for bacteria (UniFrac) was not available for fungi. Therefore, the Bray-Curtis distance measure was used in this case, which is a robust measure of distance for community datasets (26). The primer set to detect fungal SSU ribosomal region was as follows: SSU-F: 5′-TGG AGG GCA AGT CTG GTG-3′; SSU-R: 5′-TCG GCA TAG TTT ATG GTT AAG-3′. Barcoding, quality filtration, and taxonomic assignment were performed as in the bacterial analyses.

MAP Testing

The DNA extraction steps for MAP samples were the same as for the bacterial diversity testing. The only difference was that we used Qiagen TissueLyser to run at 30 Hz for 20 minutes to break MAP cells. The designed MAP primers and probe were as follows:

TABLE 1. Demographics and characteristics of treatment-naïve Crohn disease patients and controls

| | Age, y | Sex | Ethnicity | Montreal classification | | | Terminal ileum intubation | No. biopsy | Site of granuloma |
				A	Location	B			
Crohn disease									
Granulomas	15.6	F	AA	A1	L3 L4	B1	Yes	32	>4 sites
	13.5	M	W	A1	L2 L4	B1	Yes	29	Duo, sigmoid
	16.3	F	W	A2	L3 L4	B1	Yes	71	Stom, TI, Trans, RS
	13	F	W	A1	L3 L4	B1	Yes	43	Stom, TI, Des, RS
	17.3	M	AA	A2	L3 L4	B1	Yes	43	Des
	15	F	W	A1	L2 L4	B2	No	16	Sigmoid
	8.11	F	W	A1	L3 L4	B1	Yes	25	Rectum
	16.9[*]	M	W	A2	L3 L4	B1	Yes	30	>4 sites
No granulomas	7.1	F	W	A1	L2 L4	B1	No	29	
	16.9	M	W	A2	L3	B1	Yes	25	
	8.1	F	W	A1	L2 L4	B1	Yes	30	
	15	M	W	A1	L3 L4	B1	Yes	28	
	16.1	M	W	A2	L3	B1	Yes	32	
	9.6[*]	M	W	A1	L3	B1	Yes	15	
	13[*]	F	W	A1	L2	B2	No	6	

Center				Diagnosis
Control				
Baylor	5	M	W	Juvenile polyp
	15	F	W	Abdominal pain
	8	F	W	Abdominal pain
	13	M	AA	Abdominal pain
	5	F	W	Abdominal pain
	8	F	W	Hematochezia
	12	M	W	Diarrhea
	13	M	W	Abdominal pain
	10	M	W	Abdominal pain
MGH	14	F	W	Diarrhea
	17	F	W	Hematochezia
	11	F	W	Gastritis
	11	F	W	Perianal fissure
	12	M	U	GERD
	16	M	W	IBS
	18	M	W	GERD
	11	M	W	IBS
	18	M	W	Abdominal pain
CZ	3.5	M	W	Juvenile polyp
	13.5	F	W	IBS
	14	M	W	Juvenile polyp
	15	F	W	Hematochezia
	16.5	M	W	Perianal fissure
	17	F	W	Abdominal pain
	17	F	W	IBS
	17.5	F	W	Healthy

A = age; AA = African American; B = behavior; Baylor = Baylor College of Medicine; CZ = Czech Republic; des = descending colon; duo = duodenum; GERD = gastroesophageal reflux disease; IBS = irritable bowel syndrome; MGH = Massachusetts General Hospital; RS = rectosigmoid colon; Stom = stomach; trans = transverse colon; U = unknown; W = white. Montreal classification (23) was based on microscopic findings. The granuloma-positive group had gastric involvement more commonly than granuloma-negative patients ($P = 0.026$). Terminal ileal (TI) sampling and the number of biopsies analyzed by traditional histology did not differ significantly between the granuloma-positive and -negative groups.
[*] Crohn disease patients from MGH; others were from Baylor.

MAPIS-F: 5′-TGG GTT GAT CTG GAC AAT GAC GGT-3′; MAPIS-R: 5′-TAA CCA TGC AGT AAT GGT CGG CCT-3′; MAPIS-probe: 5′-/56-FAM/TAC GGA GGT GGT TGT GGC ACA A/3IABkFQ/-3′. MAP ATCC strains (19689, 19851, 43544, 15769, 19074, 25219) were used as positive controls, and *Mycobacterium intracellulare* ATCC strains (13950, 16985, 25122) were used to test primer cross-reaction. The quantitative real-time PCRs were performed by a Roche Light Cycler 480. The PCR reactions were as follows: deactivation, 95°C for 10 seconds; amplification, 35 cycles of 95°C for 15 seconds, 60°C for 1 minute (27).

Further Statistical and Bioinformatic Analysis

Unpaired, 2-tailed *t*-tests, odds ratio (OR) and 2-sided Fisher exact test calculations were also used in the group comparisons. Statistical significance was declared at $P < 0.05$. Error bars represent standard error of the mean (SEM).

RESULTS

Crohn Disease Associates With Significant Bacterial Microbiota Separation

Distance-based redundancy analysis (dbRDA) showed significant separation between the CD-associated colonic mucosal microbiota and the microbiota of controls ($P = 0.035$; Fig. 1A). The most abundant genera differences were in *Roseburia* (decrease), *Sutterella* (increase), *Eubacterium* (decrease), and *Subdoligranulum* (decrease) in CD compared to control (supplementary Table 1, *http://links.lww.com/MPG/A116*). Interestingly, 9 of the 15 CD samples separated more distantly from controls (Fig. 1A).

Mucosal Granuloma Indicates Distinct Microbiota Separation in CD

When the clinical and histological data of the 9 patients with CD with more distinct microbiota separation were reviewed, the only significant finding was the presence of granuloma or a distinct giant cell in at least 1 biopsy sample from either the upper or the lower gastrointestinal tract in 8 of the 9 patients (Fig. 1A). None of the other 6 patients in the study whose microbiota separated less distinctly had granuloma or giant cell observed. The presence of granuloma significantly predicted a discrete colonic mucosal microbiota separation in CD ($P = 0.0014$) with 88.8% sensitivity and 100% specificity. dbRDA incorporating the presence or absence of granuloma supported this observation because the microbiota separation was more prominent when this histological parameter was taken into account ($P = 0.013$, Fig. 1B). In the meantime, principal coordinates analysis (PCoA) did not show significant microbiota divergence between the groups (supplementary Fig 1, *http://links.lww.com/MPG/A117*) revealing the utility of dbRDA when limited dysbiosis is present in association with disease, or disease subtypes.

Heatmap of the indicator species analysis at the genera (Fig. 2) and the species (supplementary Fig 2, *http://links.lww.com/MPG/A117*) level showed that patients with CD with granuloma had a higher number of genera and species significantly differentiating the colonic mucosal microbiota from controls than patients without granuloma. Analysis of variance (ANOVA) results for the genera and species comparisons between granuloma-positive, granuloma-negative CD, and controls are presented in supplementary Tables 2 and 3 (*http://links.lww.com/MPG/A118*).

Although there were 32 genera differing (with Student *t* test $P < 0.05$) between granuloma-positive CD patients and controls (supplementary Table 4, *http://links.lww.com/MPG/A119*), only 4 could be detected in granuloma-negative cases (supplementary Table 5, *http://links.lww.com/MPG/A119*). None of the 4 genera separating granuloma-negative CD from controls represented >0.4% of the bacterial populations in any of the samples studied.

The most prominent genera distinguishing granulomatous CD from nongranulomatous were *Ruminococcus*, *Roseburia*, *Eggerthella* (decrease), and *Porphyromonas* (increase) (Fig. 2 and supplementary Table 6 [*http://links.lww.com/MPG/A119*]). There was a trend for the genera *Faecalibacterium* to be decreased in the transverse colonic mucosa of granulomatous patients with CD compared with granuloma-negative ones (supplementary Table 6
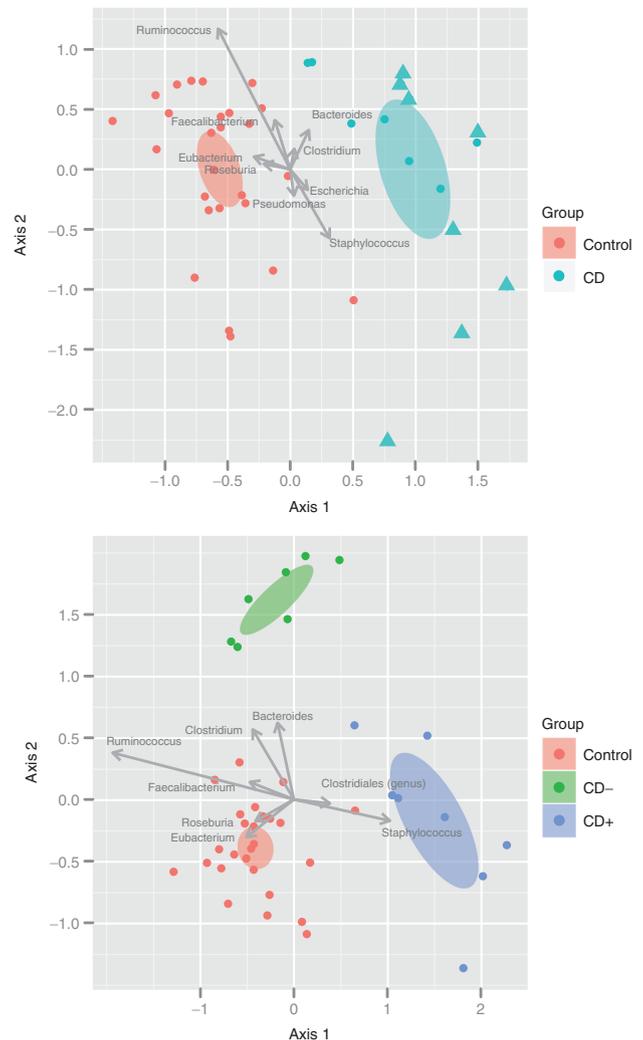

FIGURE 1. Distance-based redundancy analysis (dbRDA) biplots of bacterial genera. Upper panel, Biplot of the dbRDA results for bacteria (genera) comparing the control (C, red) and Crohn disease (CD, blue) groups. Ellipses represent the 95% confidence interval around group centroids. Arrows indicate the contribution of individual taxa to the dbRDA axes, and only those taxa with the largest contributions are shown. Samples of patient with Crohn disease (CD) designated with triangles had granuloma detected in their intestinal mucosal biopsies. Patients with granulomatous CD were observed to separate more distinctly from controls than nongranulomatous CD. There was only 1 nongranulomatous CD sample, which separated with the granulomatous samples (ie, outside the 95% CD confidence interval away from controls: dot among triangles: total of 9 CD samples [1 nongranulomatous, 8 granulomatous] parting more than the other 6 nongranulomatous samples). C to CD separation was significant ($P = 0.035$). Lower panel, Biplot of the dbRDA results for bacteria (genera) comparing the control (C, red), CD with granuloma (CD+, blue), and CD without granuloma (CD−, green) groups. Partitioning between these groups was more significant than ($P = 0.013$) between control and CD alone.

[*http://links.lww.com/MPG/A119*]; $P = 0.073$). Additionally, *Dialister* (Fig. 2) was absent in 7 of 8 granuloma-positive cases, but detectable in 6 of 7 granuloma-negative CD cases (OR 0.0238 [0.0012–0.4679]; $P = 0.0087$). On the contrary, *Porphyromonas*
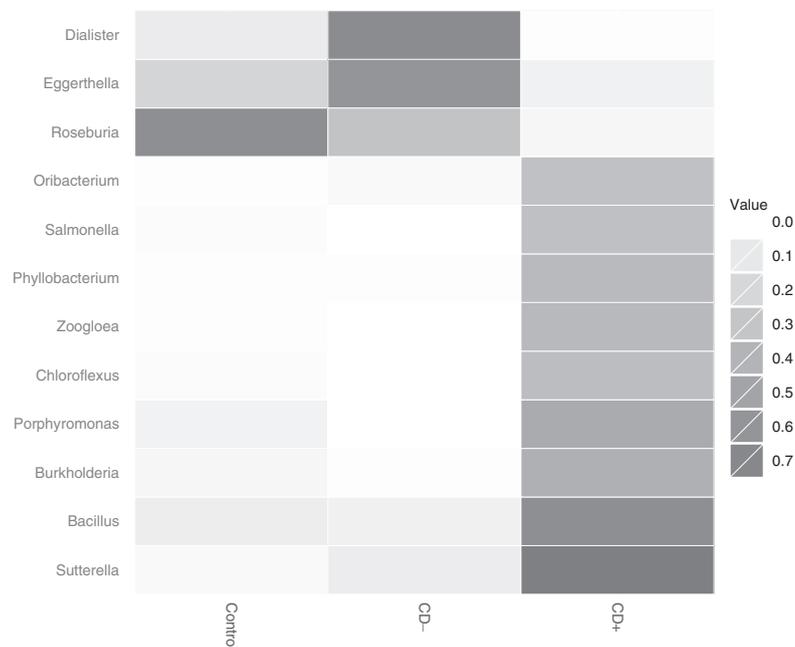
FIGURE 2. Heatmap summary of the indicator species analysis performed on the bacterial microbiota at the genera level. Only those genera that contained indicator values with $P < 0.05$ are shown. The heatmap values are indicator scores, calculated based upon the relative frequency and the relative average abundance in the groups. Only abundance-based numeric comparisons for this figure are provided in supplementary Tables 2 to 4. C = control group; CD− = granuloma-negative Crohn disease; CD+ = granuloma-positive Crohn disease.

was present in 5 of 8 granulomatous patients with CD, but was undetectable in the granuloma-negative cases ($P = 0.026$). Histological severity of inflammation in the transverse colon (in adjacent biopsies to ones analyzed for microbiota) was not significantly different between granuloma-positive and -negative cases ($P = 0.212$). Only the abundance of *Ruminococcus* showed significant correlation (inverse correlation: supplementary Fig 3, *http://links.lww.com/MPG/A117*) with microscopic inflammation among the above 6 genera (the others had no significant correlation with inflammation, not shown).

There were no species differentiating granuloma-positive from -negative CD; however, there were 16 species differing between CD and controls (supplementary Table 7, *http://links.lww.com/MPG/A119*). When the presence of granuloma was taken into account, there were 61 species significantly differing in average abundance between granulomatous CD and controls (supplementary Table 8, *http://links.lww.com/MPG/A119*) whereas only 18 such could be detected between granuloma-negative CD and controls (supplementary Table 9, *http://links.lww.com/MPG/A119*). Notably, *Ruminococcus gnavus* was significantly increased in granuloma-negative CD only (supplementary Table 7, *http://links.lww.com/MPG/A119*). *Faecalibacterium prausnitzii*, while detected and distinguished by our microbiota analysis, was not among the significantly partitioning species in any of the comparisons.

Bacterial richness and diversity analyses did not reveal significant differences between control, granuloma-positive, and granuloma-negative groups (supplementary Figs 4–6, *http://links.lww.com/MPG/A117*).

Fungal *Malassezia* Genus Associates With Granuloma-positive CD

Fungal dbRDA showed no significant separation between controls and granuloma-positive or -negative cases of CD

(supplementary Fig 7, *http://links.lww.com/MPG/A117*); however, the genus *Malassezia* was significantly associated with granulomatous CD. ANOVA for fungal genera showed only *Malassezia* to differentiate significantly between the observed groups (false discovery rate 0.02). A significantly higher proportion of granulomatous patients with CD had *Malassezia* exceeding a cutoff value of 1% (6 of 7 [1 sample could not be amplified]; OR 25.2 [2.45–259.24], $P = 0.0025$) compared with control (5 of 26). Samples from granuloma-positive patients with CD more commonly had *Malassezia* >1% compared to granuloma-negative cases (2 of 7) in the transverse colonic mucosa as well (OR 15 [1.03–218.31], $P = 0.102$).

MAP Not Found in the Biopsy Specimens

We tested for the presence of *MAP* by real-time PCR. None of the samples studied was positive.

Significant Geographical Bias

Center and geographical bias was not addressed during the initial evaluations with the intention to identify common microbiota associations (independently from geographical location) of CD in industrialized countries; however, control and disease samples were skewed by sites (Table 1). Therefore, geographical effects on microbiota composition (28) may have significantly influenced our results. Indeed, although PCoA did not separate the sites, dbRDA did (supplementary Fig 8, *http://links.lww.com/MPG/A117*), indicating potential center bias. Consequently, we separately analyzed the results from the center from which most samples were obtained (Baylor). Geographical/center bias on microbiota composition could have resulted from multiple factors including genetic and dietary differences between the populations studied, variation

in bowel-cleansing regimens (29), and differing methods of sample freezing (dry ice vs liquid nitrogen).

Center bias modified the results of bacterial genus and species comparisons between the control, CD, and granuloma-based comparisons. Nevertheless, granuloma-based microbiota separation persisted (*P* = 0.04; supplementary Fig 9, *http://links.lww.com/MPG/A117*) and several bacterial genera and species remained different between the groups of the Baylor cohort (supplementary Tables 10–16, *http://links.lww.com/MPG/A119*). *Roseburia* and *Eubacterium* were consistently decreased in CD mucosa (supplementary Table 10, *http://links.lww.com/MPG/A119*), whereas *Roseburia* and *Ruminococcus* (including *R bromii, callidus, gnavus, obeum,* and as-yet unclassified *Ruminococcus* species) were less abundant in granulomatous CD than in non-granulomatous, with *Porphyromonas* being more abundant in the colonic mucosa of granuloma-positive patients (supplementary Table 12, *http://links.lww.com/MPG/A119*). At the species level, *Eubacterium ramulus* and *Roseburia* sp (as-yet unclassified *Roseburia* sp) were consistently decreased in CD (supplementary Table 13, *http://links.lww.com/MPG/A119*). Granulomatous CD cases were mostly the reason for this separation with respect to *Roseburia* sp (supplementary Tables 14 and 16, *http://links.lww.com/MPG/A119*). Importantly, *Dialister* was <0.045% abundant in all granulomatous CD samples, whereas >0.07% in all nongranulomatous CD cases in the Baylor cohort (*P* = 0.0013). *Dialister invisus* specifically was less abundant (<0.044%) in all granulomatous CD samples, whereas it was present at 0.044% or more in nongranulomatous CD (*P* = 0.0152).

When the Baylor cohort was analyzed by fungal metagenomics, the association of *Malassezia* with granulomatous CD was less prominent (5 of 6 CD+ cases >1%, whereas 3 of 9 in control; *P* = 0.12); however, *Saccharomyces* (2 of 11 in CD, 8 of 9 in control; *P* = 0.0055) and *Candida* (3 of 11 CD, 7 of 9 control; *P* = 0.07) were surprisingly less frequently detectable in all the Baylor CD samples (same between granulomatous and nongranulomatous) than controls. These findings pertained to *Saccharomyces cerevisiae* (detectable in 2 of 11 in CD, 8 of 9 in control; *P* = 0.0055) and *Candida albicans* (detectable in 1 of 11 in CD, 5 of 9 in control; *P* < 0.05) as well. These findings warrant further investigation because opposing results (ie, increased abundance of *C albicans* specifically) were obtained from mouth swabs and stool specimens from patients and family members with CD (30).

Mucosal Granuloma Predicted by CRP Levels in Treatment-naïve Pediatric CD

When the clinical and laboratory parameters of the patients with CD were compared, the patients with granulomatous CD had a trend for higher levels of serum CRP than patients without granuloma. We decided to use an arbitrary cutoff value of CRP (>1 mg/dL). By this means, a CRP >1 mg/dL predicted granulomatous CD by 80% sensitivity and 100% specificity in the microbiota study cohort (*P* = 0.007). This level of CRP would have forecasted the observed more significant microbiota separation as well (OR 16 [1.09–243.26]; *P* = 0.089; 80% sensitivity, 80% specificity). The analysis was extended to a larger population of treatment-naïve pediatric CD cases (86 patients, 56% of whom had granuloma/giant cell detected in at least 1 biopsy sample from the upper or the lower intestine). Within this larger population CRP >1 mg/dL significantly predicted granulomatous CD (OR 28 [6–134.32]; *P* < 0.00001; 73% sensitivity, 91% specificity). The average level of serum CRP was also significantly higher in the granulomatous CD group than in patients without granuloma (*P* = 0.0059; Fig. 3). CRP testing for the 86 patients was performed
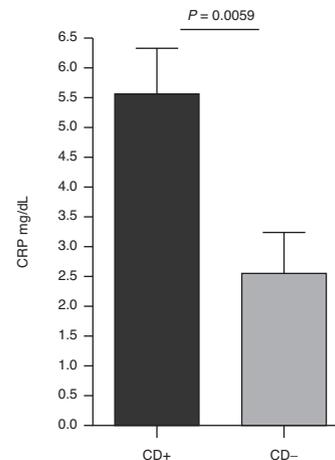

FIGURE 3. C-reactive protein (CRP) results. CRP levels were higher (average 5.58 mg/dL) in granulomatous CD (CD+) than in patients without granuloma (CD−; average 2.54 mg/dL). Error bars represent standard error of the mean.

in >4 different laboratories; however, CRP for 53 patients was tested in the same laboratory. If we examined only these cases, sensitivity (76%) and specificity (100%) for CRP >1 mg/dL predicting granulomatous CD increased (*P* < 0.00001) compared to the collective cohort tested in multiple laboratories.

DISCUSSION

The commensal microbiota is recognized to play an important role in a number of common human disorders including IBD (31–33). In the meantime, it is extremely difficult to overcome or incorporate the tremendous number of confounding variables, which characterize IBD-focused clinical microbiota research (31). Therefore, the pathogenic role of IBD-related dysbiosis and its potential therapeutic implications remain questionable (32).

In the present study we analyzed pediatric transverse colonic mucosal biopsy samples from treatment-naïve CD cases and controls, which enabled us to overcome several of the potential confounding issues of microbiota analysis in IBD, such as examining a clinical subpopulation of patients (9) (early onset, with limited time for chronic inflammation); elimination of treatment bias (19); studying the mucosa-associated microbiota, relevant for intestinal immunomodulation (17); and avoiding colonic location–dependent microbiota variation (16). Several limitations of our analysis are, however, still present: small sample size arising from the strict selection of patients; the nature of the control population, for whom a colonoscopy was indicated and a number of whom may have dysbiosis compared to healthy children regardless of having no obvious intestinal inflammation (34); lack of data regarding specific host genetic factors, such as polymorphisms in IBD susceptibility genes, which can associate with microbiota variation (13,15); and significant center bias according to dbRDA. Nevertheless, our data suggest a microbiota separation within pediatric CD colonic mucosa (Fig. 1) that is modulated by the presence of granulomatous inflammation in any part of the intestinal tract independently from geographic bias (Fig. 1 and supplementary Figure 8, *http://links.lww.com/MPG/A117*). In a similar study of colonic mucosal samples from adult patients, only about 30% of CD cases separated from controls (35); however, the latter study tested mucosal samples from surgical cases (likely manifesting in patients with long-lasting and/or more aggressive inflammation) without accounting for the

effects of treatment, and using a control population of largely (62%) colon cancer patients who themselves may have significant dysbiosis (36). In addition, although we directly tested for differences in the microbiota (using dbRDA), they did not (using PCoA to summarize overall variation). In fact, direct comparison between the PCoAs of the study by Frank et al (35) and ours (supplementary Figure 1, *http://links.lww.com/MPG/A117*) indicates an even less prominent dysbiosis in treatment-naïve pediatric CD than in the chronically treated, adult, surgical cases. The limited dysbiosis is further emphasized by the fact that none of the direct taxa comparisons in the present study were significant after correcting for multiple tests (supplementary Tables 1–16, *http://links.lww.com/MPG/A116*, *http://links.lww.com/MPG/A118*, *http://links.lww.com/MPG/A119*); however, this latter result may be a consequence of the small sample sizes originating from the challenges in obtaining treatment-naïve samples. Therefore, the direct taxa comparisons represent only trends, which need to be verified in further studies with larger sample sizes.

We also detected a significant correlation between granulomatous CD and colonic mucosal microbiota variation by dbRDAs. Granuloma is a pathognomic feature of CD in the clinical setting of IBD and can be detected in 21% to 60% of patients, with a higher frequency in children (10,37,38). The pathogenesis of granuloma formation in CD is unknown, although some studies hypothesize that specific bacterial components may play a role (39). Granulomas in pediatric CD have been associated with an increased incidence of perianal disease and gastritis (10). MAP has been detected in a higher number of pediatric patients with granulomatous CD than in controls (40), but these observations were not confirmed with similar methodology from adult patients with CD in biopsies containing mucosal granuloma (12). Therefore, the importance of MAP in regards to IBD pathogenesis remains questionable.

Roseburia was decreased in the colonic mucosa of our CD proband, which was largely attributable to the subjects with granuloma irrespective of geographic bias. This was not observed by Willing et al (14), who only found significant decreases of this genus in the ileum and feces of patients with ileal CD, but not in the mucosa of the large bowel of patients with colonic CD, perhaps indicating that patients with granulomatous CD were more common in the ileal CD group of their cohort than in the group with colonic disease. Interestingly, a decrease in *Roseburia* was recently found in the colonic mucosa of young adult mice sensitive to experimental colitis secondary to maternal dietary modification (41), and in patients with UC as well (42).

A decrease in *Ruminococcus* (supplementary Tables 4, 6, 12, *http://links.lww.com/MPG/A119*) was detected in our patients with granulomatous CD, which was also correlated with the severity of mucosal inflammation (supplementary Fig 6, *http://links.lww.com/MPG/A117*). Similar results were obtained from the colonic mucosa of adults (43), but not controlled for therapy, granulomas, or inflammation. Perhaps this may be the reason for the discordance between our results and this later manuscript in respect to *Eubacterium*, where we found a CD-associated decrease (supplementary Table 3, *http://links.lww.com/MPG/A118*) as opposed to Verma et al (43).

Although we could associate granulomatous CD with the decreased presence of *Dialister*, and increased detection of *Porphyromonas* and *Malassezia*, none of these bacterial and fungal taxa were exclusive for differentiating this CD phenotype when examined in all the samples. *Malassezia* can induce granulomatous inflammation (44), and at least 1 case has been reported of *Malassezia furfur* sepsis in a patient with CD (45); however, when geographical bias was considered, the association of *Malassezia* with granulomatous CD was less prominent. In the meantime, *Dialister* separated all granulomatous samples with being less

abundant than in nongranulomatous CD from the Baylor cohort, which pertained to *D invisus* specifically as well. A decrease in *D invisus* has been observed in stool from patients with CD and their relatives, supporting our findings (46).

Our results emphasize that giant cells and granulomas in CD are important associates of microbiota composition and should be incorporated into future work in the metagenomics of this disorder.

The finding of CRP levels >1 mg/dL correlating with granulomatous CD and microbiota separation in treatment-naïve patients is also novel and important for indicating this molecule as a potential biomarker for host-microbial interactions in this disease group. Previous investigations did not reveal association between granulomatous CD and CRP (10), but it is unclear whether this was selectively examined in treatment-naïve patients, and a cutoff point of significance was not established.

The present study includes the first high-throughput microbiota analysis of treatment-naïve CD incorporating bacterial and fungal metagenomics on colonic mucosal specimens. Therefore, it can be considered as pilot study for future, larger-scale, high-throughput metagenomic investigations in treatment-naïve intestinal samples from patients with IBD. Our findings support that stringent, geographic/center, clinical, molecular, and histological selection of patients can further our understanding of dysbiosis in IBD and the relation between the microbiota and immune responses.

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