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Altered Gut Microbiota Is Present in Newly Diagnosed Pediatric Patients With Inflammatory Bowel Disease

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ABSTRACT

Background and aims: Clinical and experimental data suggest that gut microbiota plays an important role in the pathogenesis of inflammatory bowel disease (IBD). The aim of this study was to determine intestinal microbiota in newly diagnosed patients with IBD and to compare it with patients' healthy siblings who share same genetic and environmental background and to healthy unrelated controls.

Methods: Molecular approach targeting 16S ribosomal RNA was employed for analyzing the gut microbiota of participants' stool samples. Terminal restriction fragment length polymorphphism analysis was performed.

Results: Newly diagnosed pediatric patients with IBD (n = 19, 68.4% Crohn disease [CD], mean age 14.8 ± 0.65 years), their unaffected healthy siblings (n = 20, mean age 12.8 ± 0.85 years), and unrelated healthy controls (n = 19, mean age 10.7 ± 0.8 years) were included. Microbial diversity differed significantly between IBD patients, healthy siblings, and healthy controls ($P = 0.018$ for *MspI* digestion, $P = 0.013$ for *HhaI* digestion). No significant difference in microbial diversity was found between healthy siblings and healthy controls. In patients reduced presence of genus *Eubacterium, Lactobacillus, Enterobacter* and *Clostridium*, and increased presence of genus *Streptococcus*, *Prevotella* and *Escherichia*, compared with healthy siblings and healthy controls, was found.

Conclusion: Newly diagnosed pediatric patients with IBD show significantly less diverse microbiota and microbial composition compared with healthy siblings and healthy controls.

Key Words: children, Crohn disease, microbiology, microbiome, pediatrics, ulcerative colitis

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What Is Known

- Clinical and experimental data suggests that microbiome plays an important role in the development of inflammatory bowel disease.
- Both increased and decreased abundance of specific bacterial taxa, as well as difference in microbial diversity has been described in patients with inflammatory bowel disease.

What Is New

- This study found significant difference in microbiota composition in newly diagnosed, treatment-naive pediatric patients with inflammatory bowel disease compared with healthy siblings and healthy controls.
- Lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in patients compared with healthy siblings and healthy controls.

The etiopathogenesis of inflammatory bowel disease (IBD) is unclear, but clinical and experimental data suggests the crucial role of a microbiome, intestinal mucosal barrier, and of the immune system (1,2). Therefore, it is proposed that in IBD, there is an unrestrained abnormal immune response to gut microbiota/content occurring in genetically predisposed individuals (2). The largest pediatric study to date by Gevers et al (3) has confirmed the result of other smaller studies (4–6) that composition of microbiota in treatment-naïve pediatric IBD patients is altered compared with healthy subjects. Both increased and decreased abundance of specific bacterial taxa, as well as difference in microbial diversity has been described (3). A reduced diversity of microbiota, the lower abundance of ''beneficial'' bacteria, mainly Firmicutes and increases in abundance of ''detrimental'' bacteria, such as Proteobacteria (mainly *Escherichia coli*), was observed in patients with IBD (7). The division of certain bacterial species into either beneficial or detrimental has, however, recently been challenged. Paradoxically, in patients treated with exclusive enteral nutrition (EEN) (8–11) or Crohn disease (CD) treatment-with-eating diet (CD-TREAT) (12), a decrease in proportion of potentially beneficial bacteria has been described. Contrary, Levine et al (13) have recently demonstrated that exclusion of dietary components by EEN or Crohn Disease Exclusion Diet (CDED) reduced potentially harmful Proteobacteria while increasing potentially beneficial Firmicutes. Not only that the results of different studies differ but also it is not clear whether dysbiosis in IBD patients is merely a consequence of the disease, or it has a role in the disease development.

The gut microbiota has multiple functions, including supplying energy and nutrients to the host, such as vitamin K and water-soluble vitamins synthesized by human commensal bacteria (14). Furthermore, short-chain fatty acids (SCFA) produced by the phyla Firmicutes and Bacteroidetes serve as a primary energy source for colonic epithelial cells (15). It was observed that IBD patients have diminished ability to produce SCFA, which further alters microbiota composition and consequently influences intestinal and immune homeostasis (16). Finally, microbial products released into the bowel lumen can epigenetically influence the long-term function of both, intestinal immunity and the mucosal barrier (17,18).

In approximately a quarter of patients, disease is diagnosed before the age of 18 years with the significant increase in incidence being observed in this specific age group (19,20). Thus, it is of utmost importance to understand the contributing factors for disease development, which may provide the possibility for disease prevention and/or treatment that is more efficient. With the exception of rare monogenic diseases, most commonly occurring in the first years of life (21), only about 7.5% of incidence of CD and 13.6% of ulcerative colitis (UC) can be explained by genetics (22). Therefore, the current emphasize is on different environmental factors, which could play a crucial role in the etiopathogenesis of IBD (23). Studying currently healthy siblings of patients with IBD, who share both, the genetic background and environmental exposures, may provide further insights into IBD pathogenesis.

To our knowledge, only few studies have compared fecal microbiota of IBD patients to their unaffected siblings/relatives (24–29). The results of these studies have not been conclusive. Some studies have shown that dysbiosis was present in both IBD patients and healthy siblings, suggesting that dysbiosis in IBD patients is not merely a consequence of intestinal inflammation (24,28). However, not all studies have confirmed these results (26,29).

The aim of this study is, therefore, to determine the composition of intestinal microbiota in newly diagnosed IBD patients and to compare it with patients' healthy siblings and healthy unrelated controls. To our knowledge, this is the first study that compared microbiota of newly diagnosed, treatment-naive pediatric IBD patients with that of healthy pediatric siblings and healthy unrelated pediatric controls.

MATERIALS AND METHODS

Patients and Study Design

Newly diagnosed pediatric IBD patients and their unaffected healthy siblings were recruited at the Referral Centre for Pediatric Gastroenterology and Nutrition at the Children's Hospital Zagreb from June 2016 to April 2019. Unrelated healthy controls were recruited by circular e-mail sent to hospital staff who accepted their children's participation. All participants older than 9 years of age and their parents gave written informed consent. The diagnosis of IBD was established according to the revised Porto criteria (30), whereas disease location was defined using the Paris classification (31). Severity of the disease was estimated by Pediatric Crohn disease activity index (PCDAI) and Pediatric ulcerative colitis activity index (PUCAI) (32,33). Exclusion criteria in healthy controls included chronic illness or family history positive for chronic intestinal diseases (celiac disease, IBD, gastrointestinal carcinoma). In healthy siblings, exclusion criteria included unintentional weight loss in the last 6 months, changes in stool frequency or consistency or other symptoms suggestive of undiagnosed IBD. For each participant, retrospective data on type of delivery (vaginal birth or C-section), months of total breast-feeding, months of exclusive breast-feeding, time of weaning, number of siblings, and owning of pets were collected. Stool samples of all participants were collected in sample containers, before therapy introduction. They were stored in the hospital or at home at −20°C for a maximum of 24 hours, after which they were transferred in the cold packs to the Department of Clinical Microbiology at the University Hospital for Infectious Diseases and stored at −80°C. Stool samples of newly diagnosed patients were collected before therapy introduction.

Total fecal bacterial DNA extraction, from ∼150 mg of stool samples, was performed using Quick-DNATM Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions.

PCR Amplification and Terminal Restriction Fragment Length Polymorphphism Analysis

PCR amplification and terminal restriction fragment length polymorphphism (T-RFLP) analysis were performed according to Andoh et al (34) with slight modifications. 6'-Carboxyfluorescein (6-FAM)-labeled 27-F primer (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Thermo Fisher Scientific) were used for the amplification of the *16S rRNA* gene from the human fecal DNA (34). The PCR amplification (20 ng of DNA) was performed in 50 μL reactions, in triplicates, according to previously described protocol (35). Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Germany) and diluted in 50 μL of elution buffer.

HhaI and *MspI* enzymes were used for the restriction of amplified *16S rRNA* genes (34). One hundred and twenty nanograms of purified PCR product was digested separately in 30 μL reaction volumes, using 1 μL of FastDigest *HhaI* and FastDigest *MspI* (Thermo Fisher Scientific) at 37°C for 1 hour. Restriction products were purified by ethanol/sodium acetate/EDTA precipitation and resuspended in 12 μL deionized formamide (Thermo Fisher Scientific) to a final concentration of 10 ng/μL (36). 3 μL of restriction digest product (∼36 ng) was mixed with 11 μL of deionized formamide and 0.5 μL of 4-fold diluted GS2500ROX (Thermo Fisher Scientific). The length of the terminal restriction fragments (T-RFs) was determined with an ABI PRISM 310 genetic analyzer in GeneScan mode (20 s injection time; 15 kV, and 60°C for 48 minutes for each sample) (Thermo Fisher Scientific) (37).

Fragment sizes were estimated by using the Local Southern Method GeneMapper 3.7 software (Thermo Fisher Scientific). T-RFs in the range of 50 to 810 bp with a peak height greater than 25 fluorescence units were included in the analysis. Alignment of T-RFs was performed by T-REX software (*http://trex.biohpc.org/*) (38). Binning treshold of 2 bp was used for assignment of T-RFs to operational taxonomic units (OTUs) (36). The OTUs were quantified as the percentage values of an individual OTU per total OTU area, and this was expressed as the percentage area of the underpeak curve (% AUC) (39).

Assignment of OTUs to bacterial taxa was performed in silico using the web-based analysis tool (PAT+) provided by MiCA3 (*http://mica.ibest.uidaho.edu/pat.php*), based on the RDP (Ribosomal Database Project) release 10 *16s rRNA* gene database (40).

Statistics

The differences between categorical variables were assessed by chi-square test. The differences for noncategorical variables were assessed based on distribution and number of groups by ANOVA or *t* test and Kruskal-Wallis or Mann-Whitney *U* test. The relative abundance of OTUs was used to calculate Shannon-Wiener diversity index in order to compare diversity between different sample groups. Cluster analyses were performed using

TABLE 1. Baseline characteristics of study population

	IBD (n = 19)	Healthy siblings (n = 20)	Healthy controls (n = 19)	*P* value
Male, n (%)	12 (63.2%)	7 (35%)	9 (47.4%)	0.212
CD, n (%)	13 (68.4%)			
UC, n (%)	6 (31.6%)			
Age, years, mean (SD)	14.77 (0.65)	12.84 (0.85)	10.72 (0.84)	0.005
Breast-feeding, months, mean (SD)	13.29 (2.59)	13.7 (3.39)	10.0 (1.57)	0.606
Weaning, months, mean (SD)	5.4 (0.3)	5.35 (0.27)	5.26 (0.21)	0.994
Number of siblings, mean (SD)	1.4 (0.11)	1.4 (0.11)	1.05 (0.21)	0.055
Vaginal delivery, n (%)	15 (79.0%)	16 (80%)	14 (73.7%)	0.881
Owning of pets, n (%)	12 (63.2)	12 (60%)	12 (63.2%)	0.973
PCDAI, mean (SD)	21.4 (7.5)			
PUCAI, mean (SD)	36.7 (21.4)			
Localization of the disease (CD patients)*	L1 (ileal): 4 (30.8%)			
	L2 (colonic): 1 (7.7%)			
	L3 (ileocolonic): 8 (61.5%)			
Localization of the disease (UC patients)*	E4 (pancolitis): 6 (100.0%)			

CD = Crohn's disease; IBD = inflammatory bowel disease; PCDAI = Pediatric Crohn disease activity index; PUCAI = Pediatric ulcerative colitis activity index; UC = ulcerative colitis.
*Paris classification of the inflammatory bowel disease (31).

BioNumerics software (Applied Maths, Belgium) based on the *HhaI* or *MspI* T-RFLP patterns. A dendrogram representing calculated similarity distances was generated using Pearson similarity coefficient analysis and the unweighted pair-group methods with arithmetic means (UPGMA). P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (Chicago, IL) statistical software.

Study was approved by Ethics Committee of the Children's Hospital Zagreb (IRB number: 21102014).

RESULTS

Baseline characteristics of enrolled participants (IBD patients, healthy siblings, and healthy controls) are summarized in Table 1. Overall, with the exception of age (healthy controls being significantly younger compared with IBD patients and healthy siblings), there were no differences in study populations in respect to sex, duration of breast-feeding, time of weaning, number of siblings, mode of delivery, and owning of pets. At the time of assessment, 12 CD patients (92.3%) had mild disease according to PCDAI scoring (PCDAI score between 10 and 30), whereas 1 patient (7.7%) had moderate-to-severe disease (PCDAI score higher than 30). In UC patients, 3 patients (50%) had mild disease (PUCAI score between 10 and 34) and 3 patients (50%) had moderate disease (PCDAI score between 35 and 64) according to PUCAI score.

The fecal microbiota profiles of all 3 groups are illustrated by a dendogram (Fig. 1). A setting of similarity generated 2 major clusters. Most of healthy controls (17/19 with *HhaI* and 18/19 with *MspI*) and healthy siblings (20/20 by *HhaI* and 20/20 by *MspI*) were classified in cluster I. In IBD patients, 57.9% (*HhaI* digestion) and 52.6% (*MspI* digestion) were classified into cluster II (Table 2). In CD patients, there was a significant difference between cluster and disease localization ($P = 0.002$); all patients with CD classified in cluster I (n = 4) had L1 localization of the disease whereas none of the patients in cluster II had L1 localization (8 patients had L3 and 1 patient had L2 localization).

There was no association between cluster distribution and disease severity based on PUCAI ($P = 0.10$) or PCDAI score ($P = 0.825$), age ($P = 0.503$ for CD and $P = 1.0$ for UC), symptoms duration ($P = 0.414$ for CD and $P = 1.0$ for UC), presence of perianal disease in patients with CD ($P = 0.098$) and disease localization in UC ($P = 1.0$).

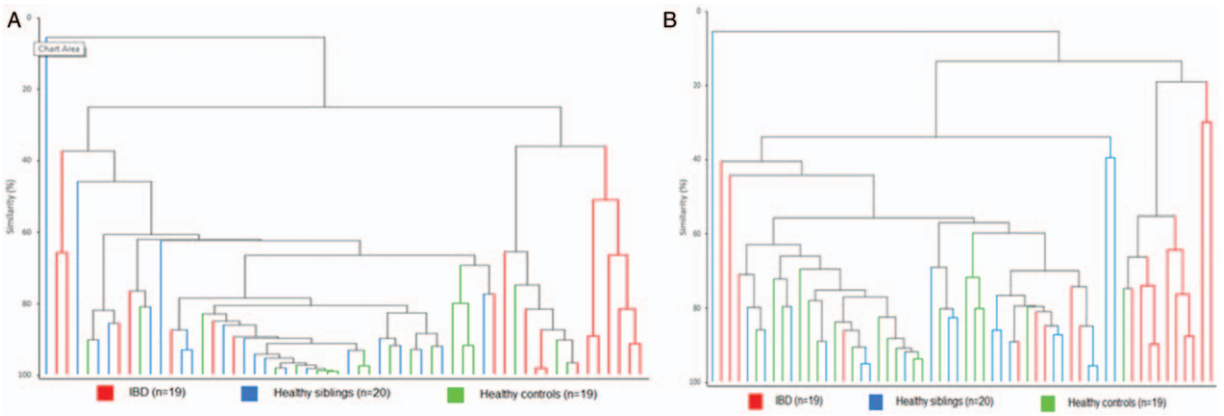

FIGURE 1. Dendrogram of the fecal microbiota profiles of inflammatory bowel disease patients, healthy unrelated controls and healthy siblings: (A) *HhaI* digestion and (B) *MspI* digestion.

TABLE 2. Distribution of fecal microbiota profiles in inflammatory bowel disease patients, healthy siblings, and healthy controls

	Cluster I (*HhaI* digestion/*MspI* digestion)	Cluster II (*HhaI* digestion/*MspI* digestion)
IBD patients, n = 19	8 (42.1%)/9 (47.4%)	11 (57.9%)/10 (52.6%)
CD patients, n = 13	5 (38.5%)/5 (38.5%)	8 (61.5%)/8 (61.5%)
UC patients, n = 6	3 (50%)/3 (50%)	3 (50%)/3 (50%)
Healthy siblings, n = 20	20 (100%)/20 (100%)	0 (0%)/0 (0%)
Healthy controls, n = 19	17 (89.5%)/18 (94.7%)	2 (10.5%)/1 (5.2%)

CD = Crohn's disease; IBD = inflammatory bowel disease; UC = ulcerative colitis.

On the basis of the result of *HhaI*/*MspI*-digested T-RF patterns, microbial diversity of IBD patients was reduced compared with that of healthy siblings and healthy unrelated controls (Table 3). Post hoc analysis revealed the difference was significant only for IBD patients versus healthy siblings when estimated by *HhaI* digestion ($P = 0.011$), and in IBD patients versus healthy siblings and healthy controls (IBD vs healthy siblings, $P = 0.035$; IBD vs healthy controls, $P = 0.05$) when estimated by *MspI* digestion. No significant difference in microbial diversity has been observed between healthy siblings and healthy controls.

Supplemental Tables 1 and 2 (Supplemental Digital Content, *http://links.lww.com/MPG/B764*) show OTUs with significant changes after *HhaI* and *MspI* digestion. The relative abundance of 37 of 149 (24.8%, *MspI* digestion) and 27 of 169 (16%, *HhaI* digestion) OTUs differed significantly between the 3 groups. All *HhaI*- and *MspI*-associated OTUs predicting the genus *Clostridium,* among others, were significantly decreased in IBD patients at the time of diagnosis compared with healthy siblings and healthy controls. There was no significant difference between OTUs predicting the genus *Clostridium* between healthy siblings and healthy controls. However, all mentioned OTUs were lower in healthy siblings compared with healthy controls and were approaching OTU values of IBD patients.

Some other *HhaI*- and *MspI*-associated OTUs representing phylum Firmicutes, which include bacteria from the genus *Paenibacillus*, *Bacillus*, *Lactobacillus*, *Blautia*, *Eubacterium*, *Roseburia*, and *Ruminoccocus* were significantly reduced in IBD patients compared with healthy siblings and healthy controls (Supplemental Tables 1 and 2, Supplemental Digital Content, *http://links.lww.com/MPG/B764*). Although the difference was not significant, the same bacteria were lower in healthy siblings compared with healthy controls and were approaching values of IBD patients. Only 1 *MspI*-associated OTU (128-bp *MspI* OTU) representing the genus *Citrobacter*, *Collinsella*, and *Paenibacillus* differed significantly between healthy siblings and healthy unrelated controls, with healthy siblings having lower values, similarly to that in IBD patients.

On the contrary, the genus *Streptococcus*, *Lactococcus*, and *Enterococcus* predicted by the *MspI*-associated 555-bp and 563-bp were significantly increased in IBD patients. The same has been noticed for the phylum Proteobacteria, represented by genus *Enterobacter*, *Citrobacter*, *Escherichia*, and *Klebsiella* (495-bp *MspI* OTUs). For 1 *HhaI*-associated OTU (374-bp OTU) representing phylum Proteobacteria, abundance was significantly lower in patients with IBD.

DISCUSSION

In this study, we have replicated previous findings that newly diagnosed pediatric patients with IBD have not only significantly less diverse microbial composition compared with healthy controls but also to healthy siblings. Furthermore, lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in IBD patients compared with healthy siblings and healthy controls, whereas no significant difference in microbiota composition was observed in healthy siblings and healthy controls. To our knowledge, this was the first study that compared microbiota of newly diagnosed, treatment-naive pediatric IBD patients with healthy pediatric siblings and healthy unrelated controls.

We identified 6 studies that have compared microbiota composition of IBD patients with that of healthy siblings/relatives (24–29). Of those, 4 have included healthy unrelated controls (24,26,29) and only 3 have included pediatric patients with IBD (25–27). Methodologies of these studies have differed by the type of IBD patients (both CD and UC patients, only CD patients), age of control group (healthy minor or adult siblings/relatives), disease activity (patients with active or inactive disease), and methods used for microbiota analysis (DNA extraction, sequencing methodology, and data analysis).

Previous studies have consistently shown that de novo pediatric IBD is strongly associated with microbiota alterations (7). Microbial communities of new pediatric IBD patients could be differentiated with high accuracy from those of healthy unrelated controls (3,41). In our study, dendrograms comparing the gut microbiota separated patients and controls into 2 major clusters. Almost all healthy siblings and healthy controls were included in 1 cluster, whereas about 55% or IBD patients were included in a second cluster. Remaining IBD patients were included in the same cluster with healthy siblings and healthy unrelated controls. In CD group of patients, all patients that were in cluster 1 (together with healthy siblings and controls) had L1 localization of the disease (ileal/ileocecal disease), whereas all CD patients with colonic involvement were in cluster 2. In a study by Andoh et al (34) almost all healthy individuals were included in 1 cluster, whereas

TABLE 3. Comparison of fecal bacterial diversity between inflammatory bowel disease patients, healthy siblings, and healthy controls

Shannon index	IBD, n = 19	Healthy siblings, n = 20	Healthy controls, n = 19	*P* value
MspI digestion, mean (SD)	2.11 (0.12)	2.45 (0.85)	2.44 (0.74)	0.018**
HhaI digestion, mean (SD)	1.75 (0.12)	2.14 (0.81)	1.99 (0.66)	0.013*

The Shannon diversity index was calculated from the *HhaI*- and *MspI*-digested T-RF patterns. Post hoc analysis. IBD = inflammatory bowel disease.
*$P < 0.05$ for IBD versus healthy siblings.
**$P < 0.05$ for IBD versus healthy siblings and IBD versus healthy controls. IBD = inflammatory bowel disease.

74.6% of adult CD patients (active disease, remission-achieved, remission-maintained patients) were forming 2 separate clusters. Similarly, in a study by Ijaz et al (26), the gut microbiota community structure (β-diversity) of pediatric CD patients was different to the microbiota of the unaffected adult relatives of CD children and adult healthy unrelated controls. Additionally, similar to our results, no difference in the gut microbiota community structure between the healthy relatives and healthy unrelated controls was seen (26).

Dysbiosis, in addition to reduced microbial diversity, involves also changes in abundances of potentially pathogenic and/or beneficial taxa (7,42). Consistent with the results of other studies (24–29), in our study, microbial diversity of IBD patients was significantly lower compared with healthy siblings and healthy controls. Furthermore, abundance of specific taxa has been increased/reduced in IBD patients. In their review, Ni et al (43) have reported that the phylum Firmicutes is often reduced in adult IBD patients (44–48). Our study confirmed these results and has shown lower abundance of the phylum Firmicutes in IBD patients, more specifically OTUs representing bacteria from the genus *Paenibacillus*, *Bacillus*, *Lactobacillus*, *Blautia*, *Eubacterium*, *Roseburia,* and *Ruminoccocus.* OTUs representing phylum Proteobacteria have been increased in patients with IBD, observation, which has also been reported previously (44,49). Previously mentioned changes in the composition of the gut microbiota could lead to metabolite alterations, primarily reduction in amino acid biosynthesis and carbohydrate metabolism pathways and increase in expression of genes related to oxidative stress, that are likely to have a role in the IBD pathogenesis (43). However, there are studies in pediatric patients showing different results, implicating that the abovementioned pathogenesis may not be valid—at least not in pediatric patients (10,13).

Comparing microbiota profiles of healthy siblings with that of healthy unrelated controls, we have not found significant differences neither in microbiota diversity nor in specific bacteria genus, which is not in accordance with previous research findings (24,25,27). In our study, only 1 *MspI*-associated OTU (representing genus *Citrobacter*, *Collinsella*, and *Paenibacillus*) differed significantly between healthy siblings and healthy unrelated controls. However, some differences observed between healthy siblings and healthy controls were similar to the differences observed when comparing IBD patients to healthy controls. More specifically, most OTUs representing bacteria from phylum Firmicutes, but not from phylum Proteobacteria observed in healthy siblings, differed from that of healthy controls and were approaching values observed in IBD patients. Those differences were not statistically significant but we might have been underpowered to find a significant difference in our cohort. The former may indicate that, to some extent, dysbiosis does exists in the microbiota of healthy siblings of IBD patients, but only with regard to reduced 'beneficial' bacteria, which has been observed in other study as well (24). However, as speculated by Ijaz et al (26), dysbiosis reported in paeditric IBD patients occurs to a much lower extent in their healthy genetically linked counterparts. Similarly, in a study by Joossens et al (29), dysbiosis signature found in adult patients with CD was markedly characteristic for the disease as it was not observed in unaffected relatives. The described pattern of decrease in ''beneficial'' bacteria in microbiota of siblings could be attributed to shared genetics and environmental exposures in siblings. Even more so, shared exposures to different risk factors in early perinatal and postnatal life when the microbiota is developing and becoming stabilized could lead to permanent changes in microbiota, that could influence the risk of developing IBD later in life (18).

The main limitation of this study is small number of subjects, which is nevertheless, comparable with other studies. Furthermore, by using other sequencing methodology, such as strain-level shotgun metagenomics with deep sequencing, we would have been able to provide strain-level taxonomic classification. There are several strengths to our study. First, all controls, healthy unrelated and siblings, were younger than 18 years of age, ensuring that age was not a confounder in this cohort. Second, all the patients were treatment-naïve and recruited at the time of diagnosis, which excludes the effect of treatment on the microbiota profiles.

In conclusion, significantly different microbiota composition is present already at diagnosis, in treatment-naive patients with IBD. Although the conundrum on the causes and consequences cannot be resolved by our findings, we have also identified the differences in microbiota composition between healthy siblings and healthy unrelated controls but the extent of them is small.

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