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ORIGINAL RESEARCH

A Disease-Associated Microbial and Metabolomics State in Relatives of Pediatric Inflammatory Bowel Disease Patients

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SUMMARY

Healthy first-degree relatives of pediatric inflammatory bowel disease patients can have a fecal microbial and metabolomics profile seen in many patients with quiescent inflammatory bowel disease. This potential predisease state was associated with an increased likelihood of increased fecal calprotectin levels.

BACKGROUND & AIMS: Microbes may increase susceptibility to inflammatory bowel disease (IBD) by producing bioactive metabolites that affect immune activity and epithelial function. We undertook a family based study to identify microbial and metabolic features of IBD that may represent a predisease risk state when found in healthy first-degree relatives.

METHODS: Twenty-one families with pediatric IBD were recruited, comprising 26 Crohn's disease patients in clinical remission, 10 ulcerative colitis patients in clinical remission, and 54 healthy siblings/parents. Fecal samples were collected for 16S ribosomal RNA gene sequencing, untargeted liquid chromatography–mass spectrometry metabolomics, and calprotectin measurement. Individuals were grouped into microbial and metabolomics states using Dirichlet multinomial models. Multivariate models were used to identify microbes and metabolites associated with these states.

RESULTS: Individuals were classified into 2 microbial community types. One was associated with IBD but irrespective of disease status, had lower microbial diversity, and characteristic shifts in microbial composition including increased Enterobacteriaceae, consistent with dysbiosis. This microbial community type was associated similarly with IBD and reduced microbial diversity in an independent pediatric cohort. Individuals also clustered bioinformatically into 2 subsets with shared fecal metabolomics signatures. One metabotype was associated with IBD and was characterized by increased bile acids, taurine, and tryptophan. The IBD-associated microbial and metabolomics states were highly correlated, suggesting that they represented an integrated ecosystem. Healthy relatives with the IBD-associated microbial community type had an increased incidence of elevated fecal calprotectin.

CONCLUSIONS: Healthy first-degree relatives can have dysbiosis associated with an altered intestinal metabolome that may signify a predisease microbial susceptibility state or subclinical inflammation. Longitudinal prospective studies are required to determine whether these individuals have a clinically significant increased risk for developing IBD. *(Cell Mol Gastroenterol Hepatol 2016;2:750–766; http://dx.doi.org/10.1016/j.jcmgh.2016.06.004)*

Keywords: Microbiome; Metabolomics; Inflammatory Bowel Disease; Family Cohort.

The inflammatory bowel diseases (IBDs)—comprising Crohn's disease (CD) and ulcerative colitis (UC)—are chronic inflammatory diseases with a growing prevalence worldwide.[1] IBD is believed to arise from a combination of genetic susceptibility and environmental factors that trigger an inappropriate mucosal inflammatory response.[2] A pathogenic role for the intestinal microbiome at the junction of genetics and environment is supported by the resistance of germ-free mice to experimental colitis models and the transmissibility of colitis by microbiota derived from mice with genetic defects in mucosal immunity.[3–5] IBD patients have reduced microbial diversity and alterations in the composition and function of the intestinal microbiome compared with healthy controls.[6–10] Founder effects and diet are important environmental factors in microbial composition, and are associated

Abbreviations used in this paper: **AUC, area under the curve; CD, Crohn's disease; IBD, inflammatory bowel disease; LC/MS, liquid chromatography/mass spectrometry; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PCR, polymerase chain reaction; rRNA, ribosomal RNA; ToFMS, time-of-flight mass spectrometry; UC, ulcerative colitis; UPLC, ultra-performance liquid chromatography.**

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with IBD disease risk.[11] These findings support the hypothesis that IBD involves a dysbiosis arising from a combination of environmental and genetic perturbations of the mucosal habitat controlling microbial composition.[12,13]

It remains unclear which differentially abundant microbes in IBD patients are instigators of disease rather than bystanders responding to the altered environment of the inflamed intestine. An important prospect for resolution is the expanding recognition of bioactive microbial products that profoundly can affect mucosal immune responses and epithelial homeostasis.[14] For instance, IBD patients have reduced fecal concentrations of short-chain fatty acids such as butyrate that limit inflammatory responses in colitis models.[15–17] This may be attributable to shifts in the microbiome including reduced abundance of the prominent butyrate-producer *Faecalibacterium prausnitzii*.[8,9,18] Administration of *F prausnitzii* or its culture supernatant ameliorated experimental colitis; however, treatment of mice with butyrate at the concentration in culture supernatant did not affect colitis severity.[18] This underscores the need to more fully characterize the IBD-associated metabolome and the scope of microbial products relevant to disease pathogenesis.

If IBD develops as a consequence of a host response to a proinflammatory microbiome and its associated microbial products, as has been reported in animal models, IBD patients would be predicted to have harbored dysbiosis before the development of disease. We performed a family-based study of the microbiome and metabolome of pediatric IBD patients and their unaffected first-degree relatives, who are at heightened risk for developing IBD. We hypothesized that unaffected siblings and parents—previously reported to have increased fecal calprotectin levels compared with healthy unrelated controls—may carry a predisease microbial risk state owing to shared genetic and environmental factors with the IBD proband.[19,20] We found that individuals in the family cohort grouped into 1 of 2 states based on fecal microbial and metabolomics profiles, which could be defined bioinformatically as OTU types and metabotypes (multidimensional community clusters characterized by differences in the abundance of signature taxa or metabolites, respectively).[21] These OTU types and metabotypes were correlated highly with one another, and with disease status. We propose that in families at risk for IBD, a subset of healthy individuals harbor a stable intestinal microbial/metabolomic state that may confer increased susceptibility to IBD.

Materials and Methods

Cohort Recruitment and Sample Collection

Twenty-one CD and UC probands younger than the age of 18 were recruited from the Pediatric IBD Center at the Cedars-Sinai Medical Center. All patients were in clinical remission at the time of collection as evidenced by a Harvey–Bradshaw Index score of less than 5 for CD patients or a partial Mayo score of less than 2 for UC patients. Family members of these probands were recruited and assessed by detailed questionnaire for history of IBD. Six siblings and 9 parents were known to have IBD. Non-IBD family members were validated by lack of symptoms or medications suggestive of undiagnosed IBD. Participants were provided with a toilet hat, sample containers, and cold packs for home collection. Freshly defecated feces were frozen immediately at the clinic or at home and then brought to the clinic on cold packs for storage at -80°C. Frozen fecal samples were ground with a mortar and pestle in the presence of liquid nitrogen and then aliquoted for microbiome and metabolome analysis. Fecal calprotectin was measured from frozen aliquots of stool by an enzyme-linked immunosorbent assay performed by Quest Diagnostics (Los Angeles, CA). This research was approved by the Cedars-Sinai Medical Center Institutional Review Board under IRB 3766. All authors had access to the study data and reviewed and approved the final manuscript.

16S Ribosomal RNA Gene Sequencing

Genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) with a 30-second, beat-beating step using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK).[22] Polymerase chain reaction amplification of bacterial 16S ribosomal RNA (rRNA) genes was performed using extracted genomic DNA as the template. The 100-μL reactions contained 50 mmol/L Tris (pH 8.3), 500 μg/mL bovine serum albumin, 2.5 mmol/L $MgCl_2$, 250 μmol/L of each of the 4 deoxynucleotide triphosphates, 400 nmol/L of each primer, 4 μL of DNA template, and 2.5 U JumpStart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO). The polymerase chain reaction (PCR) primers (F515/R806) targeted the V4 hypervariable region of the 16S rRNA gene, with the reverse primers including a 12-bp Golay barcode.[23] Thermal cycling was performed in an MJ Research PTC-200 (Bio-Rad, Inc, Hercules, CA) with the following parameters: 94°C for 5 minutes; 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds; and 72°C for 5 minutes. PCR products were purified using the MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA). DNA sequencing was performed using an Illumina HiSeq 2000 (Illumina, Inc, San Diego, CA). Clusters were created using template concentrations of 1.9 pmol/L and PhiX at 65 K/mm^2. Sequencing primers targeted 101 base pair reads of the 5' end of the amplicons and 7 base pair barcode reads.[23] Reads were filtered using the following parameters: minimum Q-score, -20; maximum number of consecutive low-quality base calls allowed before truncating, - 3; and maximum number of N characters allowed, - 0. All filtered V4 reads had a length of 101 bp. The number of sequences per sample ranged from 17,946 (an outlier; the second lowest sequence depth was 478,168) to 848,638, with a mean of 620,720. Raw 16S rRNA sequence data were deposited under National Center for Biotechnology Information BioProject ID PRJNA324147. Operational taxonomic units (OTUs) were picked in QIIME 1.8.0 against the May 2013 version of the Greengenes database (http://greengenes.secondgenome.com), prefiltered at 97% identity.[24] The number of reads assigned to 97% OTUs ranged from 445,985 to 805,639, excluding the lowest depth sample (16,941).