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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Metagenomic Alterations in Gut Microbiota Precede and Predict Onset of Colitis in the IL10 Gene-Deficient Murine Model

Jun Miyoshi,[1,2,a] Sonny T. M. Lee,[1,3,a] Megan Kennedy,[1,4] Mora Puertolas,[7] Mary Frith,[1,4] Jason C. Koval,[1] Sawako Miyoshi,[1,5] Dionysios A. Antonopoulos,[1,6] Vanessa Leone,[1,b] and Eugene B. Chang[1,b]

[1]*Department of Medicine, Knapp Center for Biomedical Discovery, University of Chicago, Chicago, Illinois;* [2]*Department of Gastroenterology and Hepatology, Kyorin University School of Medicine, Tokyo, Japan;* [3]*Division of Biology, Kansas State University, Manhattan, Kansas;* [4]*Medical Scientist Training Program, Pritzker School of Medicine, University of Chicago, Chicago, Illinois;* [5]*Department of General Medicine, Kyorin University School of Medicine, Tokyo, Japan;* [6]*Biosciences Division, Argonne National Laboratory, Lemont, Illinois; and* [7]*Department of Pediatrics, University of Chicago Knapp Center for Biomedical Discovery, Chicago, Illinois*

SUMMARY

Currently, predictive markers for the development and course of inflammatory bowel diseases (IBD) are not available. This study supports the notion that gut microbiome metagenomic profiles could be developed into a useful tool to assess risk and manage human IBD.

BACKGROUND & AIMS: Inflammatory bowel diseases (IBD) are chronic inflammatory disorders where predictive biomarkers for the disease development and clinical course are sorely needed for development of prevention and early intervention strategies that can be implemented to improve clinical outcomes. Since gut microbiome alterations can reflect and/or contribute to impending host health changes, we examined whether gut microbiota metagenomic profiles would provide more robust measures for predicting disease outcomes in colitis-prone hosts.

METHODS: Using the interleukin (IL) 10 gene-deficient (IL10 KO) murine model where early life dysbiosis from antibiotic (cefoperozone [CPZ]) treated dams vertically transferred to pups increases risk for colitis later in life, we investigated temporal metagenomic profiles in the gut microbiota of post-weaning offspring and determined their relationship to eventual clinical outcomes.

RESULTS: Compared to controls, offspring acquiring maternal CPZ-induced dysbiosis exhibited a restructuring of intestinal microbial membership in both bacteriome and mycobiome that was associated with alterations in specific functional subsystems. Furthermore, among IL10 KO offspring from CPZ-treated dams, several functional subsystems, particularly nitrogen metabolism, diverged between mice that developed spontaneous colitis (CPZ-colitis) versus those that did not (CPZ-no-colitis) at a time point prior to eventual clinical outcome.

CONCLUSIONS: Our findings provide support that functional metagenomic profiling of gut microbes has potential and promise meriting further study for development of tools to assess risk and manage human IBD. *(Cell Mol Gastroenterol Hepatol 2021;11:491–502; https://doi.org/10.1016/j.jcmgh.2020.08.008)*

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Inflammatory bowel diseases (IBD) are complex immune disorders that arise from convergence of environmental, microbial, and host genetic factors. More than 200 single nucleotide polymorphisms identified through genome-wide association studies are associated with increased risk for IBD development,[1–3] but few, if any, have been clinically useful to predict disease onset or outcomes. Environmental risk factors including diet, hygiene, and lifestyle are also important contributors in individuals with a background of IBD genetic susceptibility,[4] but even then, no single or combination of factors has emerged as strong disease predictors. On the other hand, the gut microbiome is highly sensitive to changes in environment and to states of host immune activation, which, in theory, could be highly informative in assessing health or impending disease. In this regard, numerous studies have shown both membership and functional changes in the gut microbiome of patients with active IBD, which are characterized by overall decrease in species diversity, reduced Firmicutes, and increased Proteobacteria compared with non-IBD controls.[5] However, almost without exception, these data have been acquired from samples obtained after disease onset, where immune activation can independently cause dysbiosis. Regardless of whether these changes are

[a]Authors share co-first authorship; [b]Authors share co-senior authorship.

Abbreviations used in this paper: CD, Crohn's disease; CPZ, cefoperazone; IBD, inflammatory bowel diseases; IL, interleukin; NI, no-treatment; PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate analysis of variance.

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cause or consequence of active disease, addressing the unmet need to find high performance predictors of disease onset or relapse in IBD remains a challenge. If achieved, subjects stratified as "high risk" would benefit from early interventions.

Most human IBD microbiome studies have used marker genes, such as the 16S ribosomal RNA gene, where bacterial membership can be easily profiled. However, these data provide no functional information, which is likely more important in understanding states of host-microbiome interactions. Here, we examine the utility of metagenomic analysis to provide more robust information about the gut microbiome functional state and membership. To do these studies, we used a murine model of vertically transmitted antibiotic (cefaperozone [CPZ])-induced dysbiosis from dams to offspring using interleukin (IL) 10 gene-deficient (IL10 KO) mice where the pups are prone to develop spontaneous colitis.[6] Like human IBD, disease penetrance in IL10 KO mice is low but can be increased when mice are subjected to certain factors such as high fat, low fiber Western-type diet,[7] pathobionts (*Helicobacter hepaticus*), and other environment shifts that cause gut microbiota perturbations. Thus, this model is sufficiently reflective of human IBD but also allows for temporal tracking of pre-disease dynamics in both host and microbiome, allowing us to identify potential predictive biomarkers for disease outcomes. We show that vertically transmitted maternal peripartum CPZ-induced dysbiosis into genetically susceptible offspring induces shifts in ontology pathways mapped by KEGG that precede and predict which mice go on to develop colitis, ie, CPZ-colitis vs CPZ-no-colitis. Prominent among them were alterations in pathways associated with microbial nitrogen metabolism, corroborating findings of other groups that had also identified these pathways as being associated with active IBD.[8–11] Also, offspring with vertically transmitted CPZ-induced dysbiosis showed persistent differences in functional profiles as well as microbial composition compared with control offspring of non–CPZ-treated dams. Together, our findings provide proof-of-concept that more robust measures of the gut microbiome, in this case metagenomic profiling, can be the harbingers of host health vs disease states. We believe the principles identified in our murine colitis model can be extrapolated to the management of IBD in humans. Further studies in humans are needed to delineate whether nitrogen metabolism can be used in a similar manner as a marker of dysbiosis. However, our study provides a framework from which to explore this concept and sets the stage for using gut microbiome metagenomics to identify disease markers in IBD patients that could predict relapse of disease that precedes clinical symptoms, unlike the markers of disease currently available, which indicate established relapse and are associated with active symptoms.

Results

Effects of Maternal Peripartum Antibiotic Exposure on Gut Microbiome Composition of Offspring

DNA was extracted and shotgun sequencing was performed on fecal samples harvested from our previously published study. The peripartum CPZ treatment protocol used is shown in Figure 1. We observed that the incidence of spontaneous colitis among pups from the no-treatment (NT) group was 0% in females (0/18) and 4.3% in males (1/23), whereas the incidence in pups from dams exposed to peripartum CPZ (CPZ group) increased to 12.5% in females (2/16) and 30.8% in males (8/26), respectively. Among the CPZ group mice that developed colitis late in life (CPZ-colitis mice), the mean age of onset was 16.5 weeks of age. MG-RAST[12] was used to assign microbial identity and functions to reads from 51 metagenome samples, representing 3 time points (3, 7, and 11 weeks of age) from representative animals, including 6 NT mice, 5 CPZ-colitis mice, and 6 CPZ-no-colitis mice (mice in CPZ group that did not develop overt colitis). Taxonomic compositional profiles including all bacterial and fungal community members were used to calculate Bray-Curtis dissimilarity, and principal coordinate analysis (PCoA) using permutational multivariate analysis of variance (PERMANOVA) revealed significantly distinct clustering of NT and CPZ groups at 3, 7, and 11 weeks of age (Figure 2*A*). When broken down into separate bacterial and fungal community analyses, this clustering was recapitulated by both communities across all time points (Figure 3).

Family-level relative abundances between CPZ and NT samples were significantly different for 86 bacterial taxa at week 3, and 101 taxa were different at week 7 (Figure 2*B*, Supplementary Table 1). Among these taxa, Bacteroidaceae exhibited the largest difference in relative abundance across treatment groups (week 3: corrected $P = .019$; week 7: corrected $P = .007$), with enrichment in relative abundance in the NT group (week 3: 33.06% ± 11.94%; week 7: 29.11% ± 9.22%) compared with the CPZ group (week 3: 6.97% ± 4.08%; week 7: 3.09% ± 1.05%). Enterococcaceae were also highly differentially abundant (week 3: corrected $P = .019$; week 7: corrected $P < .001$) but exhibited enrichment in CPZ mice (week 3: 12.56% ± 8.35%; week 7: 14.54% ± 3.57%) relative to NT mice (week 3: 2.50% ± 0.89%; week 7: 2.58% ± 0.67%). At week 11, CPZ and NT mice showed 54 significantly differentially abundant bacterial taxa, with Verrucomicrobiaceae exhibiting the largest differences (corrected $P < .001$) between CPZ (11.25% ± 2.76%) and NT mice (0.66% ± 0.72%) (Figure 2*B*, Supplementary Table 1).

No significant differences in gut bacterial community membership were observed between the CPZ-colitis and CPZ-no-colitis groups at weeks 3 or 7 (Figure 4*A*). However,

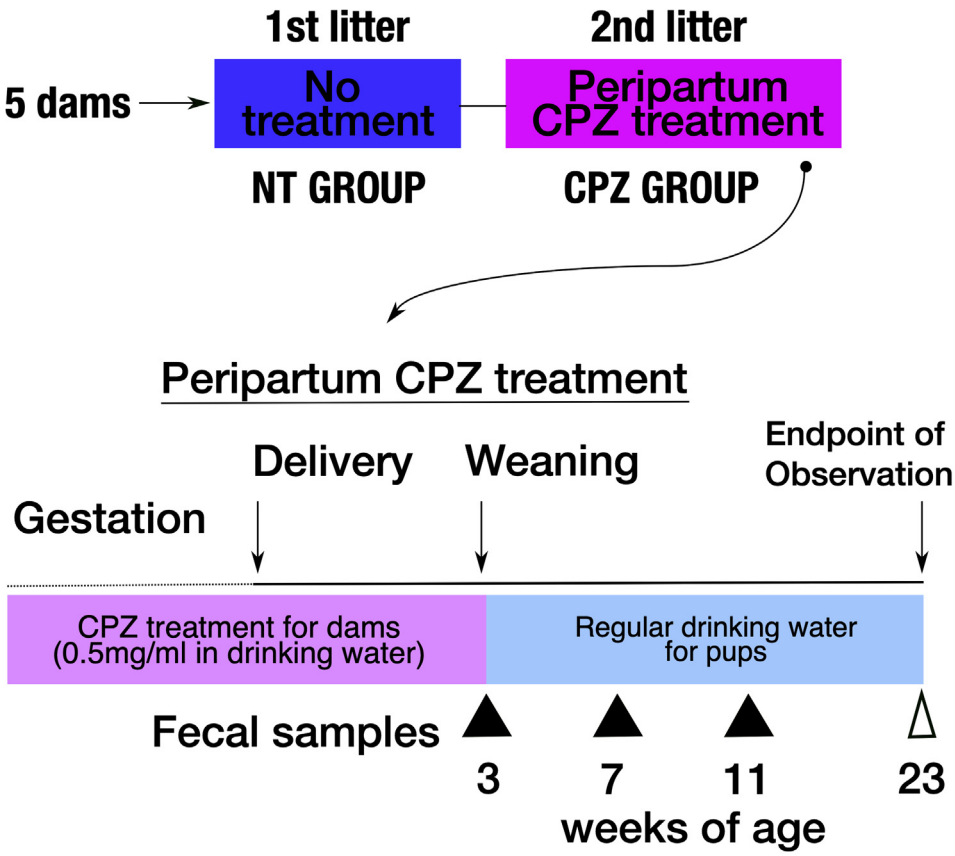

Figure 1. Maternal peripartum CPZ treatment. First and second litters from 5 dams were tracked as NT controls (no-treatment) and CPZ (antibiotics treatment) group, respectively. CPZ was administered in dam's drinking water (0.5 mg/mL) beginning at third week of second gestation until weaning of pups (3 weeks of age of pups). After weaning, all pups were provided regular drinking water.

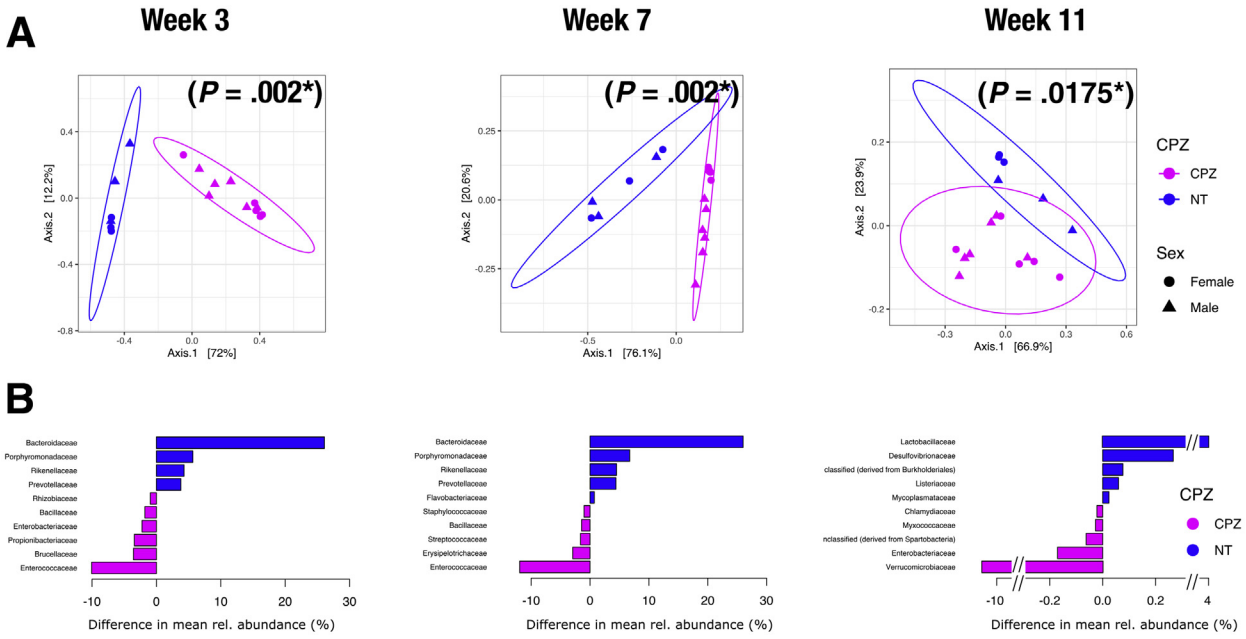

Figure 2. Microbial composition between litters with/without maternal peripartum CPZ exposure. Metagenomic shotgun sequencing data were assigned taxonomy with MG-RAST and analyzed by Bray-Curtis dissimilarity across groups. (*A*) PCoA plots show that offspring from CPZ-treated dams (*purple*) and NT controls (*blue*) formed significantly distinct clusters at all time points. Male mice are represented by *triangles* and female mice by *circles*. (*B*) Family-level relative abundances between CPZ and NT samples were compared, and top 10 differential mean abundances between the 2 treatment groups are presented.

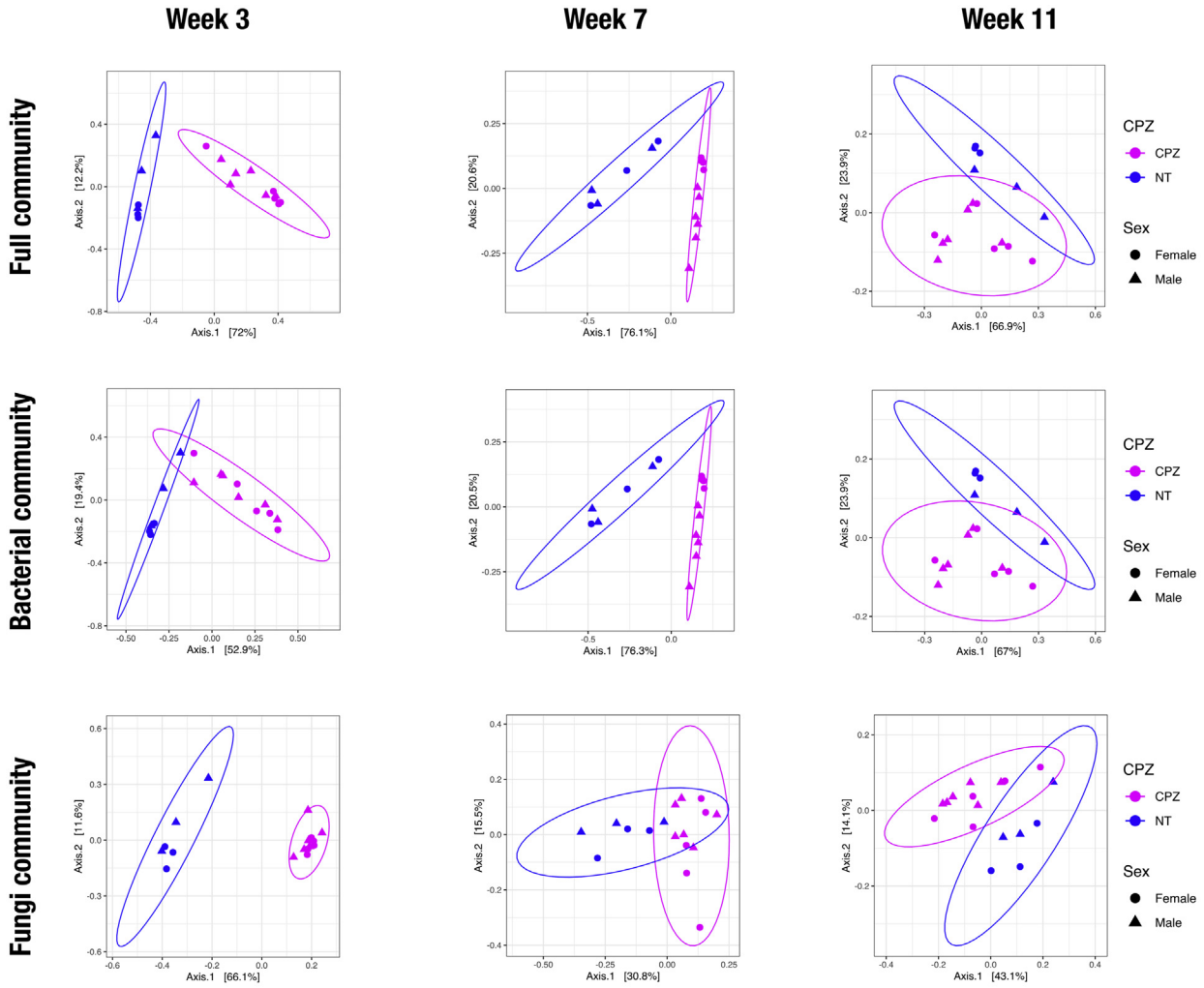

Figure 3. Microbial membership between litters with/without maternal peripartum CPZ exposure. Metagenomic shotgun sequencing data were assigned taxonomy with MG-RAST and analyzed by Bray-Curtis dissimilarity across groups. Similar to the overall microbial community membership (Figure 2*A*), composition of both microbiome and mycobiome varied between CPZ and NT groups at all time points and is different between pups from CPZ-treated dams (*purple*) and NT controls (*blue*), indicated by significantly distinct clusters at all time points.

at week 11, distinct and significant clustering became apparent ($P = .028$). These differences were mainly attributed to the presence and abundance of the families Lactobacillaceae (colitis: 0.76% ± 0.52%, no-colitis: 3.39% ± 2.64%) and Enterococcaceae (colitis: 1.09% ± 0.39%, no-colitis: 2.27% ± 0.76%), which exhibited the largest differences in relative abundances (Figure 4*B*, Supplementary Table 1).

Maternal Peripartum Antibiotic Exposure Impacts Offspring Gut Microbiota Functional Gene Dynamics That Correlate With Disease Course

In addition to significant changes in microbial community membership, we observed persistent functional dysbiosis in pups from CPZ-treated dams relative to those from NT dams. Community-wide functional representation was assessed by mapping metagenomic reads to KEGG level 3 pathways. PCoA plots that were based on Bray-Curtis dissimilarity and PERMANOVA clustering analysis revealed significant, distinct clustering of the CPZ and NT groups at all time points (Figure 5*A*). At weeks 3, 7, and 11, genes related to aminoacyl-tRNA biosynthesis (KEGG pathway ko00970) were overrepresented in NT mice compared with CPZ mice, whereas at weeks 3 and 7, genes related to ABC transporters (KEGG pathway ko02010) were enriched in CPZ-treated mice compared with NT mice (Figure 5*B*, Supplementary Table 2).

We next assessed whether functional pathway representation changed over time for either treatment group (Figure 6*A*). PCoA and clustering analysis revealed that for NT mice, the functional profile did not change significantly across time points (Figure 6*B*), whereas for

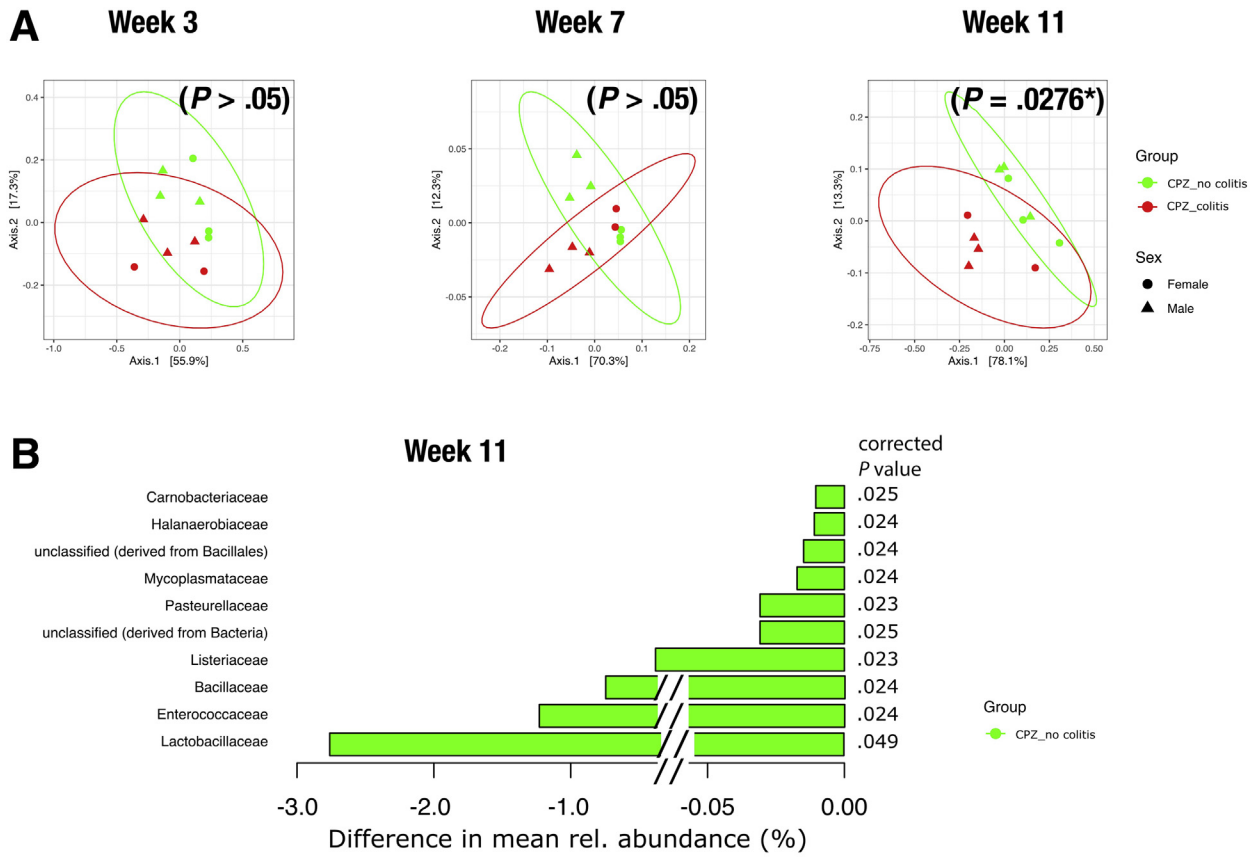

Figure 4. Microbial composition between pups that developed spontaneous colitis later in life and those that did not among maternal peripartum CPZ-treated offspring. Metagenomic shotgun sequencing data were assigned taxonomy with MG-RAST and analyzed by Bray-Curtis dissimilarity across groups. (*A*) Among CPZ group with maternal peripartum CPZ treatment, pups that developed colitis later in life (*red*; CPZ-colitis group) or did not develop colitis (*green*; CPZ-no-colitis group) clustered separately only at week 11. Male mice are represented by *triangles* and female mice by *circles*. (*B*) Family-level relative abundances between CPZ-colitis and CPZ-no-colitis samples at week 11 were compared. Top 10 differential mean abundances between the 2 resultant colitis phenotypes are presented.

CPZ mice, functional profiles formed significantly distinct clusters at each time point (Figure 6*C*; $P < .01$). Interestingly, at week 11, the CPZ-no-colitis group appeared to cluster between the CPZ-colitis group and the NT group (Figure 6*A*).

As we did with gut microbial taxonomic composition, we observed significant differences in functional pathway representation between CPZ-colitis and CPZ-no-colitis mice at week 11 but not weeks 3 or 7 (KEGG pathway level 3) (Figure 7*A*). At week 11, although none of the mice had yet developed frank symptoms of spontaneous colitis, genes related to nitrogen metabolism (KEGG pathway ko00910) and protein digestion and absorption (KEGG pathway ko04974) were significantly differentially abundant between the mice that would eventually develop colitis and those that would not (Figure 7*B*, Supplementary Table 2). Genes from these pathways were both enriched in the CPZ-colitis group (nitrogen metabolism: 0.009% ± 0.001%; protein digestion and absorption: 0.032% ± 0.002%) as compared with the CPZ-no-colitis group (nitrogen metabolism: 0.001% ± 0.001%; protein digestion: $5.41 \times 10^{-5}\%$ ± $5.11 \times 10^{-5}\%$).

Dynamics of Nitrogen Metabolic Capacity of Offspring Gut Microbiota Before Developing Colitis

Several previous studies suggest that microbial nitrogen metabolism is associated with the development of IBD.[8–11] We further assessed the nitrogen metabolic capacity of the gut microbiota community at week 11 to examine whether the genome-level alterations related to the metabolic pathway ko00910 is observed as physiological functional differences. Ninety-six substrates including a negative control on a Biolog PM3B MicroPlate Nitrogen Sources (Hayward, CA) plate were analyzed. The metabolic capacity of gut microbiota for each substrate was assessed in 2 ways, ie, the utilization rate (mu; metabolizing speed) and the consumption (area under the curve; amount of metabolized substrate) (Figures 8 and 9, Supplementary Table 3). In the assessment of the

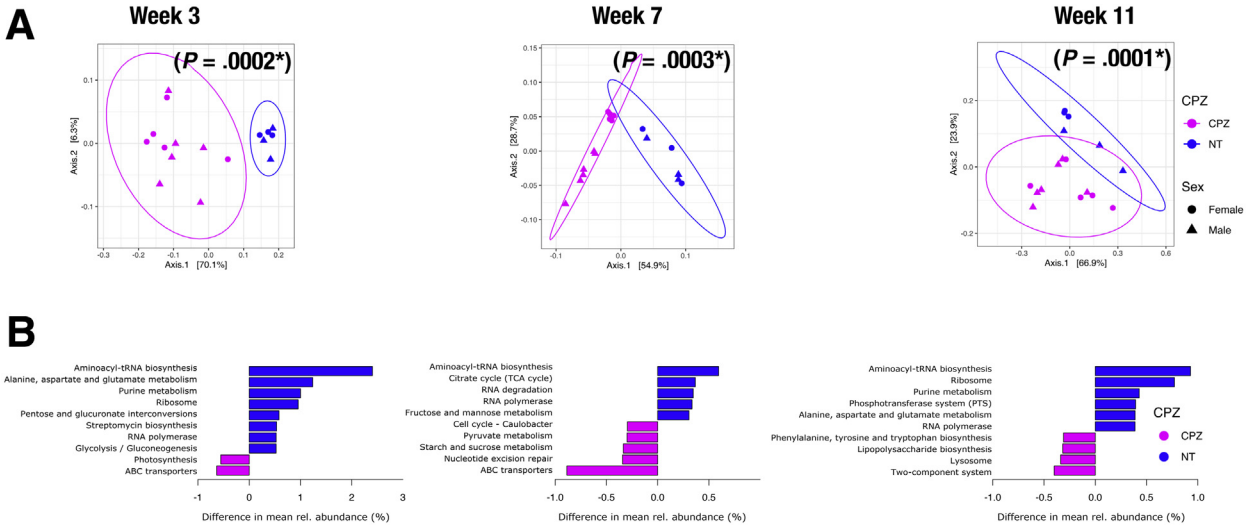

Figure 5. Functional pathways between litters with/without maternal peripartum CPZ exposure. Metagenomic shotgun sequencing data were mapped to KEGG level 3 functional pathways with MG-RAST and analyzed by Bray-Curtis dissimilarity across treatment groups. (*A*) PCoA plots of community-wide functional profile show that offspring from CPZ-treated dams (*purple*) and NT controls (*blue*) formed significantly distinct clusters at all time points. Male mice are represented by *triangles* and female mice by *circles*. (*B*) Top 10 differential mean abundance gene functional pathways across the CPZ-treated group (*purple*) and NT group (*blue*) at each time point are presented.

utilization rate, N-acetyl-D-galactosamine and e-amino-N-caproic acid tended to be different between CPZ-colitis and CPZ-no-colitis mice ($P < .01$, corrected $P = .122$, and $P = .024$, corrected $P > .99$, respectively). Meanwhile, in the consumption assessment, there was a tendency that Ala-Asp, ammonia, D-mannosamine, ethanolamine, formamide, L-methionine, Met-Ala, methylamine, and N-amylamine were different between these groups ($P < .01$, corrected $P = .247$; $P = .045$, corrected $P = .489$; $P = .044$, corrected $P = .607$; $P = .013$, corrected $P = .257$; $P = .025$,

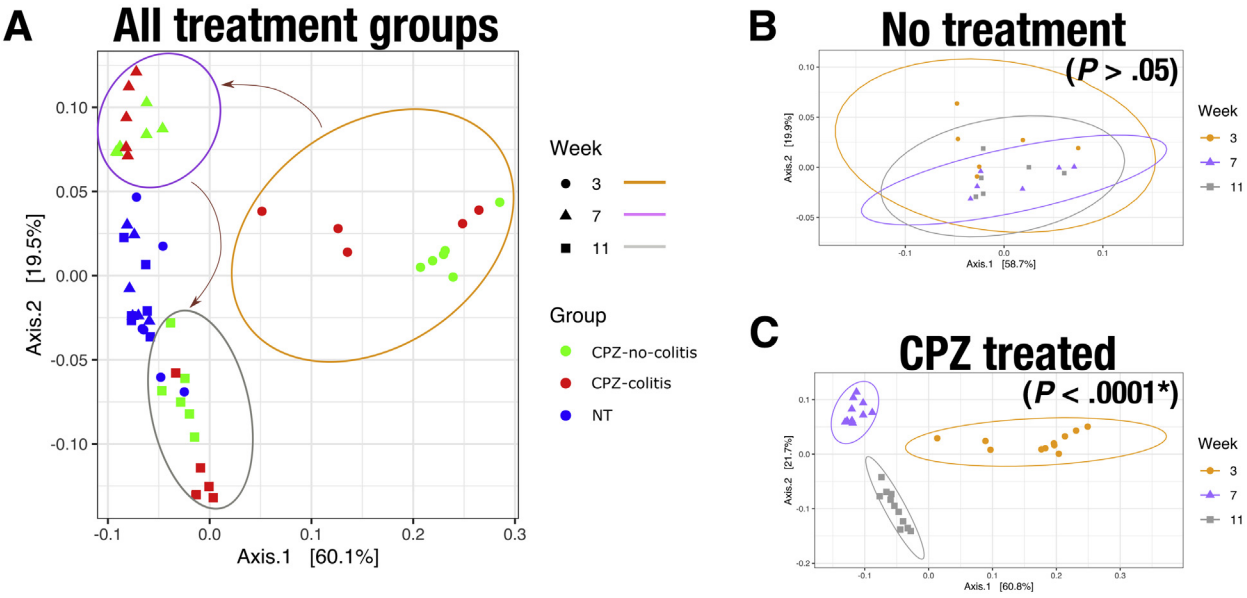

Figure 6. Functional pathway profiles of offspring with maternal peripartum CPZ treatment shifts over time. Metagenomic shotgun sequencing data were mapped to KEGG level 3 functional pathways, analyzed by Bray-Curtis dissimilarity across time points, and ordinated by PCoA.(*A*) NT control mice (*blue*), pups in CPZ-treated group that developed colitis later in life (*red*; CPZ-colitis group), and pups in CPZ-treated group that did not develop colitis (*green*; CPZ-no-colitis group) are plotted together. Weeks are denoted by shape; among the CPZ-treated group, weeks are further denoted by *colored ellipses*. *Arrows* indicate transitions of CPZ-treated mice over time. (*B*) NT control mice showed no significant differences in clustering across time points. (*C*) CPZ-treated mice clustered distinctly at each time point.

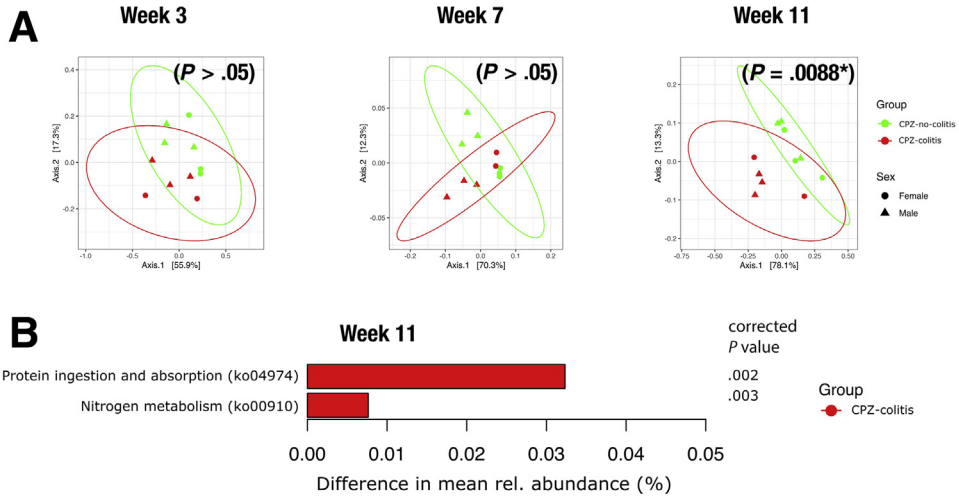

Figure 7. Functional pathways between pups that developed spontaneous colitis later in life and those that did not among offspring with maternal peripartum CPZ treatment. Metagenomic shotgun sequencing data were mapped to KEGG level 3 functional pathways with MG-RAST and analyzed by Bray-Curtis dissimilarity across treatment groups. (*A*) Among offspring from CPZ-treated dams, pups that developed colitis later in life (*red*; CPZ-colitis group) and those that did not (*green*; CPZ-no-colitis group) formed significantly distinct clusters only at week 11. Male mice are represented by *triangles* and female mice by *circles*. (*B*) *Bar plots* display significantly different functional pathways at week 11 between CPZ-colitis group (*red*) vs CPZ-no-colitis group (*green*).

corrected $P = .412$; $P < .01$, corrected $P = .211$; $P < .01$, corrected $P = .249$; $P = .044$, corrected $P = .533$; and $P < .01$, corrected $P = .237$; respectively). However, the differences in microbial community function between CPZ-colitis and CPZ-no-colitis at week 11 before developing overt colitis were not apparent.

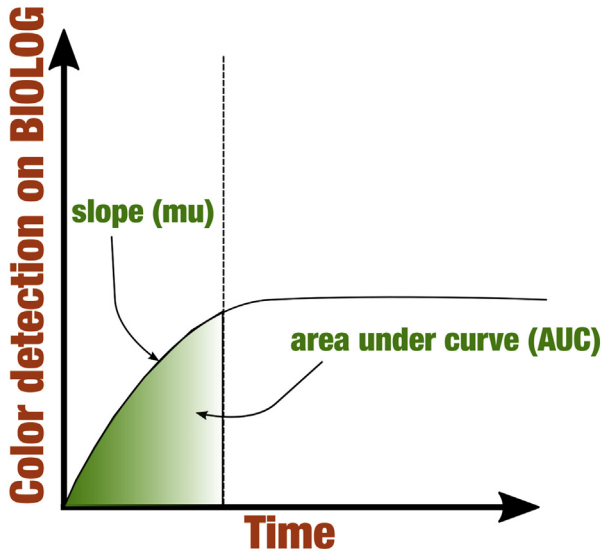

Figure 8. Assessment of microbial nitrogen metabolism capacity. Each well of the Biolog PM3B MicroPlate Nitrogen Sources plate contains tetrazolium dye that reacts irreversibly with reduced nicotinamide adenine dinucleotide, forming a distinct *purple color* in presence of actively respiring cells. The OmniLog system was used to incubate plates for 72 hours, and absorbance at 590 nm was measured every 15 minutes to detect colorimetric change and obtain the over-time change curve. We performed assessment of microbial nitrogen metabolism capacity before the color change reached the plateau. The slope of the curve (mu) represents microbial substrate utilization rate, and the area under the curve (AUC) represents the amount of consumed substrate.

Discussion

Currently no reliable clinical indicators of impending colitis development or relapse exist, and clinical outcomes in IBD remain unpredictable among genetically susceptible individuals. Advances in this area are hindered by the lack of longitudinal data preceding colitis onset mainly because the spontaneous nature of human IBD largely precludes such studies. The lack of indicators limits the ability to monitor treatment response and to implement preventative measures in the clinic. Because the gut microbiota is strongly implicated in IBD pathogenesis and clinical stool sampling is routine and noninvasive, identifying microbial signatures or metabolites that reliably predict disease onset or relapse would be a major advance in the clinical management of IBD. Here, to explore microbiota-based indicators of future IBD onset or relapse, we analyzed longitudinal changes in gut microbial metagenomes of colitis-prone mice raised in the same environment, some of which developed colitis, and others did not. Importantly, we found altered microbial metagenomic signatures (particularly those involved in nitrogen metabolism), beginning between 7 and 11 weeks of age, that defined the gut microbiota of mice that later developed colitis, although none of the mice showed clinical symptoms at these time points. Our findings highlight the potential for using alterations in microbial community function to warn of colitis onset and/or relapse in high risk individuals, facilitating timely precautionary measures or prompt intervention.

The present study was prompted by our previous observations that among genetically identical colitis-prone

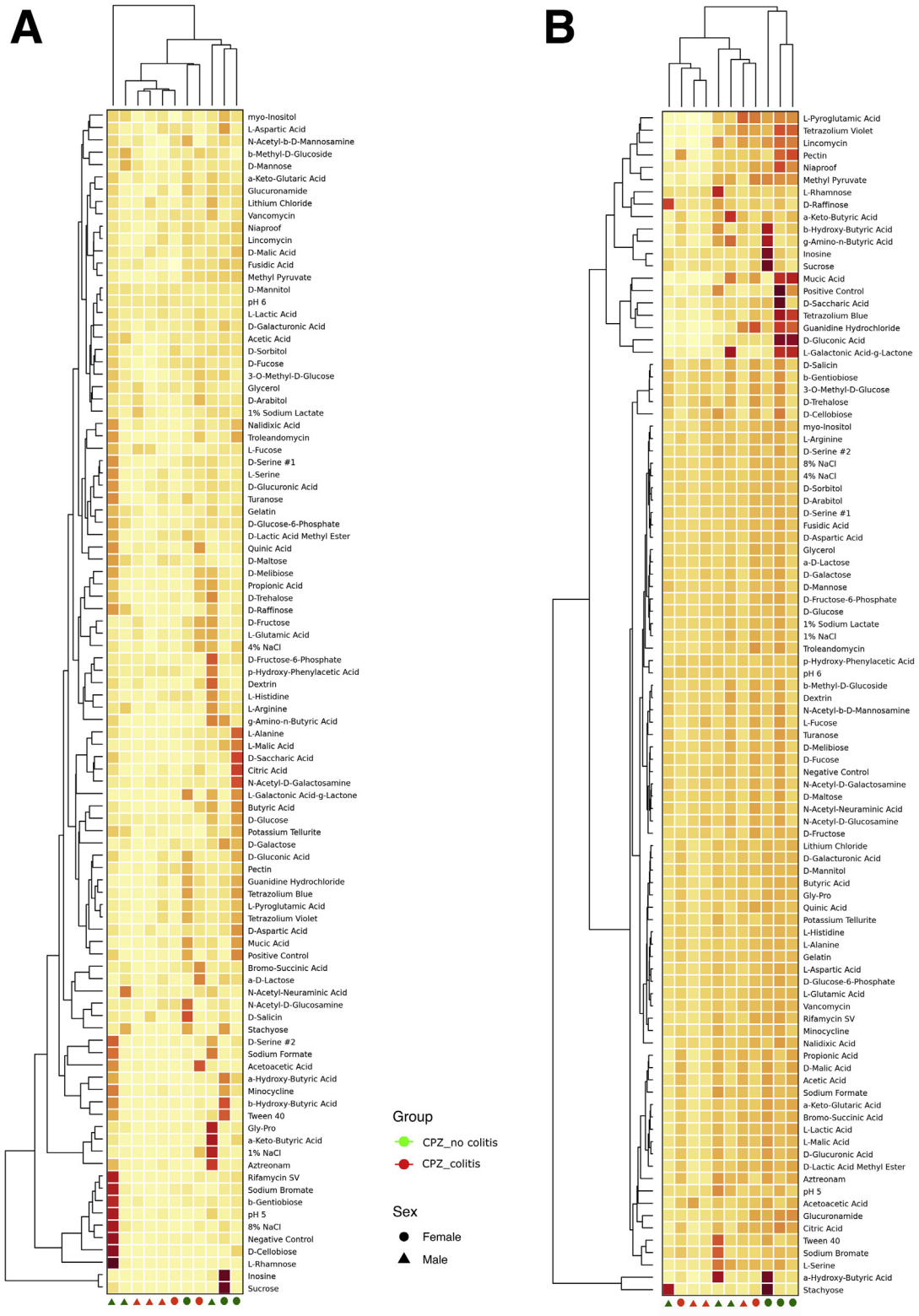

Figure 9. Microbial nitrogen metabolism capacity between offspring that developed spontaneous colitis later in life and those that did not with maternal peripartum CPZ treatment. Heatmap images for all substrates on the Biolog PM3B MicroPlate Nitrogen Sources plate were generated for (*A*) the substrate utilization rate (mu) and (*B*) the substrate consumption (area under the curve) among pups with maternal CPZ treatment that developed colitis later in life (*red*; CPZ-colitis group) or did not develop colitis (*green*; CPZ-no-colitis group). Male mice are represented by *triangles* and female mice by *circles*.

IL10 KO pups that acquire maternal antibiotic-induced dysbiosis through vertical transmission (CPZ group), about one third developed spontaneous colitis (CPZ-colitis mice), whereas the others did not (CPZ-no-colitis mice) in contrast to almost no spontaneous colitis development in the NT group.[6] This allowed us to investigate microbial predictors of colitis onset among susceptible pups in this model. We hypothesized that among susceptible individuals raised in the same environment, a divergence in the functional profile of the gut microbiota would precede colitis onset. Using fecal samples collected from these mice at 3, 7, and 11 weeks of age, our metagenomic shotgun sequencing phylogenetic and functional analyses revealed that although we observed no differences in gut microbial composition and function between CPZ-colitis and CPZ-no-colitis mice at 3 or 7 weeks of age, the microbiota of the 2 groups diverged at 11 weeks of age, with the CPZ-no-colitis group becoming more similar to the NT control group and the CPZ-colitis group diverging in a different direction. This divergence reflected alterations in several gene ontology KEGG pathways, but nitrogen metabolism, as indicated by the differing phylogenetic compositions and functional pathways enriched in the fecal microbiota between groups, was particularly prominent and has been observed by others in active IBD.[10] In spite of this shift, none of the animals showed clinical symptoms of colitis at 11 weeks of age, such as body weight loss, loose stool, or ruffled fur.[6] Thus, this compositional and functional divergence of the gut microbiota at the genome level preceded and predicted the colitis development.

Several previous studies support the notion that microbial nitrogen metabolism might be associated with the development of overt colitis. Metagenomic shotgun sequencing of fecal DNA samples from pediatric Crohn's disease (CD) patients revealed pathways involved in nitrogen metabolism were significantly more abundant in CD versus control samples.[9] In addition, bacterial nitrogen flux via urease activity in mice was associated with colitis severity,[10] and microbial ammonia biosynthesis pathways were more highly expressed in a mouse model of IBD compared with controls.[11] Winter et al[8] demonstrated that nitrate respiration, through the reduction of host-derived nitrate, provides a growth benefit to facultative anaerobes such as Enterobacteriaceae in the inflamed intestine. Because there are differences in the baseline gut microbiota between facilities and microbial taxon can drift over time, there is rationale in focusing on metabolic profiles of microbial communities in general that reflect adaptation and/or responses to shifts in host states and milieu. Because only five 11-week-old mice in CPZ-colitis group and 6 in CPZ-no-colitis group were analyzed, the present study could have a limitation in the statistical power. Nevertheless, the results from ex vivo system using live microbial community provided insights into the physiological functional changes in nitrogen metabolism of the gut microbiota and suggest the future targets in exploring metabolites as predictive surrogate biomarkers for the clinical onset or relapse of IBD.

Although different microbial functional profiles determined by metagenomic profiles between CPZ-colitis and CPZ-no-colitis mice are thought to be a reflection of multiple aspects of altered nitrogen metabolism, fundamentally they comprise a distinct signature in these mice that predicted impending colitis onset. We concede that a single universal indicator of impending colitis may not exist, but rather a functional divergence of the gut microbiota from a previous steady state might be the most reliable indicator of impending colitis onset or relapse. In a clinical context, longitudinal stool sampling of patients in remission until flare could help to identify patient-specific divergent factors that indicate risk for relapse. In particular, because of the high rates of colitis recurrence in ulcerative colitis patients after ileal pouch-anal anastomosis[13] and in CD patients after bowel resection,[14] these are clinical models in which prospective sampling could reveal microbiome-based signatures that predict colitis onset.

Another notable point is that 16S rRNA gene amplicon sequencing, which is standard in microbiome research and was used in our previous study,[6] could not resolve important differences between groups that were evident from metagenomic shotgun sequencing. Relying solely on 16S rRNA amplicon sequencing data would have been insufficient. Metagenomes provide much more robust information about microbial communities and enable phenotypic predictions with greater accuracy and precision[15] and should be considered in future studies, particularly in the context of IBD. In our model, we find that metagenomic functional profiles, ie, shifts in gene ontology implicating nitrogen metabolism, are predictive of high risk for developing experimental colitis. However, shifts in gut microbial metabolism of various nitrogen substrates were less apparent in the functional profiling using the ex vivo system with live gut microbial community. On a practical level, we believe metagenomic profiles will be more useful for detecting and predicting subjects that develop overt colitis later in life. Processing fecal samples for ex vivo functional profiling with live microbes is more likely to be less informative and encumbered by significant logistical challenges. Although routine metagenomic sequencing of patient samples is currently not practical or cost-effective, costs and turnaround time for metagenomic sequencing are likely to come down, making these types of measurement clinically useful for assessing patient risk for onset and relapse of IBD.

We recognized that there are several limitations of our study. First, it does not test microbially mediated mechanisms involved in colitis development, and further studies are needed to flesh out our finding that altered nitrogen metabolism preceded the onset of colitis and identify whether this is cause or effect. Nonetheless, our findings provide "proof of concept" that metagenome-derived information can be predictive of risk for disease, in this case the development of colitis associated with antibiotic-induced early life dysbiosis and loss of immune tolerance. These factors themselves were not sufficient to cause disease but set the stage for other factors that can tip the balance in a genetically susceptible host. Second, the murine and human microbiome, environment, diet, and genetics are not equivalent, and thus the microbial signatures that predicted colitis in this model may not represent predictive signatures

in humans. However, using a murine model enabled the collection of longitudinal data from birth to colitis onset in adulthood, which is unfeasible if not impossible to collect in humans. This model is therefore helpful in developing working paradigms that are relevant to human IBD. The specific metagenomic markers, mapping, and ontology pathways that will be useful to human IBD cannot rely on those found in mouse models and will have to be determined through clinical investigations done prospectively. Third, because the development of colitis was determined by criteria in body weight, fecal appearance, general appearance, activity, and the presence of rectal relapse, the possibility that asymptomatic animals might have subtle, subclinical colitis cannot be ruled out. Because the therapeutic goal for IBD is now mucosal healing, the inclusion of other markers such as fecal occult blood, lipocalin-2, calprotectin, and lactoferrin would provide information that collectively forms a more accurate and sensitive assessment of patients.

Nevertheless, this study has significant implications for the care of IBD patients, particularly those in remission, who are at high risk for relapse because of the nature of IBD. The state and changes of the gut microbiome are very sensitive to what is happening in the host; in turn, these changes can feed forward in contributing to the underlying problem or perturbation. We propose that the microbiome is, at minimum, the "canary in the coal mine" to alert physicians about states of normalcy or impending changes. These changes are quantifiable by clustering patterns or functional subsystems that can provide metrics for clinical state and course and can potentially provide insight into cause and/or effect relationships. Irrespective of cause and effect, however, the crucial point from this study is that metagenomic states and changes may serve as indicators of what is happening to the host. Such indicators would be clinically useful for introducing early interventions in the management of IBD. For example, if microbial community function of an individual in remission begins to diverge, this may be a sign of impending colitis; longitudinal sampling is needed to make this judgment call. In addition, serial sampling could provide an opportunity to steer a patient's diverging microbiome back to steady state by supplementation with the patient's own remission microbiota samples.

In conclusion, the findings of the present study strongly suggest that functional divergence of the gut microbiota, potentially in the form of microbial nitrogen metabolism, warrants further study as a potential indicator for IBD risk or relapse. The most practical application of such research in the clinical setting might be for monitoring patients in remission. One goal should be to develop a scalable and cost-effective assay to guide the implementation of preventative measures and to improve clinical management and monitoring of patient treatment response. The current lack of biomarkers and other metrics to guide clinical management of IBD highlights the critical need for further investigation into promising microbiome-based metrics.

Methods

Fecal DNA Samples for Metagenomic Shotgun Sequencing

DNA was extracted from feces of *H hepaticus*–free SPF IL10 KO mice with a C57BL/6J genetic background that were harvested in our previous study.[6] Briefly, 5 breeding pairs were prepared after the normalization of microbiota by bedding transfer[16] to obtain offspring of NT group and CPZ treatment group. The first and second litters from the 5 breeding pairs were tracked as NT controls and CPZ group, respectively. The peripartum CPZ treatment consisted of CPZ (0.5 mg/mL) administration in the dam's drinking water beginning at the third week of the second gestation until the weaning of pups (3 weeks of age of pups) as shown in Figure 1. The pups were tracked until 23 weeks of age. Collected feces were rapidly frozen at –80°C, and DNA was extracted as previously described.[17] NT litters did not develop overt spontaneous colitis throughout the observation period until 23 weeks of age, with the exception of 1 male, whereas the CPZ group showed a higher incidence of overt spontaneous colitis, ie, 2 of 16 females and 8 of 26 males developed colitis (the mean age of onset was 16.5 weeks of age). Because of this observation, fecal DNA samples described below were selected for metagenomic shotgun sequencing analysis. As representative samples of NT offspring, fecal DNA samples at 3, 7, and 11 weeks of age obtained from 3 females and 3 males in the NT group that did not develop frank, spontaneous colitis during the observation period were analyzed. For the CPZ group, fecal DNA samples at 3, 7, and 11 weeks of age from 3 females and 3 males that did not develop overt spontaneous colitis during the observation period were examined as the CPZ-no-colitis group. Meanwhile, the time-series samples from 2 females and 3 males that developed overt colitis later in life, ie, these mice did not show frank colitis symptoms at 11 weeks of age, were investigated as CPZ-colitis group.

Metagenomic Analyses

Metagenome libraries were generated by using the Illumina (San Diego, CA) Hiseq platform. An average of 16,438,078 sequences per sample was generated in this study (Supplementary Table 4). We used the quality control filter, an internal tool in the MG-RAST v4.0.3 pipeline,[12] and excluded an average of 764,372,216 reads from further analyses. Of these, dereplication identified an average of 5% as clusters of artificially replicated sequences. The filter parameters included a cutoff value of 0.9, with no length difference requirement and an initial base pair match of 3 base pairs. After quality filtering, an average of 1,405,454,114 sequences for the metagenomes was used in the metagenomic analyses. Approximately an average of 50% of our reads was annotated (e-value cutoff of $1e^{-05}$) to an assigned function or specific gene by MG-RAST v4.0.3 pipeline (Supplementary Table 5), against the SEED database.

Microbial Nitrogen Metabolism Capacity

On the basis of MG-RAST functional results, we selected 11 fecal samples from 11-week-old mice (5 samples from CPZ-colitis group and 6 from CPZ-no-colitis group) to investigate the nitrogen metabolism capacity of the gut microbiota. Briefly, under anaerobic conditions, fecal pellets were weighed and placed into 1.5 mL Eppendorf tubes. Five hundred μL of 1$\times$ phosphate-buffered saline was added, and fecal pellets were homogenized. One hundred to 300 μL of homogenized supernatant was added to 10 mL of inoculating tube-A, normalizing for initial fecal pellet weight, followed by addition of 100 μL to each well of a Biolog PM3B MicroPlate Nitrogen Sources plate. Both the inoculating tube-A and PM3B contain proprietary minimal media from Biolog. Each well of the plate contains tetrazolium dye that reacts irreversibly with reduced nicotinamide adenine dinucleotide, forming a distinct purple color in the presence of actively respiring cells. The OmniLog system (Biolog) was used to incubate plates at 30°C for 72 hours, and absorbance at 590 nm was measured every 15 minutes to detect colorimetric change and obtain the over-time change curve. The opm package for R[18] was used to analyze substrate utilization rate (slope of curve) and substrate consumption (area under the curve) for each substrate in each sample. The values of the substrate utilization rate and consumption were normalized before analyses. Heatmap images for all substrates and fecal samples were generated by using heatmap package for R.[19]

Statistics

PCoA plots were generated on the basis of Bray-Curtis dissimilarity using the R (https://www.R-project.org/) package phyloseq,[20] and clustering analysis was performed by PERMANOVA using the R package vegan.[21] Statistical differences between metagenome profiles were calculated with Storey's FDR multiple test correction approach using the software package STAMP v.2.1.3.[22] The criterion for statistical significance was set at $P < .05$ or FDR-corrected $P < .05$.

Data Availability

The raw metagenomic sequencing data in this study are publicly available at MG-RAST under the accession number mgp82768 and at the NCBI Sequence Read Archive under the accession number SRP252264.

Access to Data

All authors had access to the entire dataset and have reviewed and approved the final manuscript.

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Correspondence
Address correspondence to: Eugene B. Chang, MD, Knapp Center for Biomedical Discovery, Room 9130, University of Chicago, 900 East 57th Street, Chicago, Illinois 60637. e-mail: echang@medicine.bsd.uchicago.edu; fax: (773) 702-2281.

CRediT Authorship Contributions

Jun Miyoshi (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Writing – original draft: Lead)

Sonny T. M. Lee (Conceptualization: Lead; Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Visualization: Equal; Writing – original draft: Lead)

Megan Kennedy (Formal analysis: Lead; Methodology: Lead; Visualization: Lead; Writing – original draft: Equal),

Mora Puertolas (Conceptualization: Supporting; Writing – original draft: Equal)

Mary Frith (Conceptualization: Supporting; Writing – original draft: Equal)

Jason C. Koval (Investigation: Equal; Methodology: Equal),

Sawako Miyoshi (Conceptualization: Supporting; Data curation: Supporting; Investigation: Equal; Methodology: Supporting),

Dionysios A. Antonopoulos (Data curation: Supporting; Methodology: Supporting; Writing – review & editing: Supporting),

Vanessa Leone (Conceptualization: Lead; Funding acquisition: Supporting; Investigation: Equal; Supervision: Equal; Writing – original draft: Lead; Writing – review & editing: Lead),

Eugene B. Chang, MD (Conceptualization: Lead; Funding acquisition: Lead; Supervision: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

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