Title: Fecal Microbiota Transplant in Pediatric Patients

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MATERIALS AND METHODS

Study design

This single center, open-label prospective trial was conducted in children with the primary objective of observing safety and secondary objective was to examine and correlate clinical response with microbiome changes before and after FMT. This study enrolled subjects with clinically active IBD despite standard medical therapy and was conducted at Children's Hospital of Pittsburgh of UPMC (University of Pittsburgh) from October 2014 to October 2016. Subjects were mostly recruited from the institution's outpatient gastroenterology clinic or enrolled from across the country by self-referral or at the recommendation of their gastroenterologist. This institutional review board-approved study was conducted under IND 015758 and was registered at clinical trials.gov (NCT02108821).

Eligibility Criteria and Study

Subjects eligible for screening were between the ages of 2-22 years with IBD (CD, UC, or indeterminate colitis (IC)) diagnosed based on the Porto criteria by their treating gastroenterologists[29]. Due to their small numbers, subjects with IC were enrolled and assessed similarly to UC patients. Subjects were defined as being medically refractory if they had clinically active disease despite an adequate trial of standard therapy dictated by their treating gastroenterologist.

Inclusion criteria: (i) Those undergoing a medically indicated colonoscopy for clinically active mild-moderate disease (ii) Subjects on infliximab were eligible only if they failed to respond after a full induction dose (iii) No changes in medications or their dosage for at least 4 weeks prior to transplantation (iv) Mild to moderate disease defined by a Pediatric Crohn's Disease Activity Index (PCDAI) or the Pediatric Ulcerative Colitis Activity Index (PUCAI) in the range of 10 to 40 or 10-64 respectively. (v) The biomarkers of disease activity (calprotectin or lactoferrin) were at

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least more than 2 times the upper limit of normal value (when employed as sole enrollment criteria)[30].

Exclusion criteria: (i) Active infections including *Clostridium difficile*, (ii) they had severe disease defined by PUCAI ≥ 65 or PCDAI > 40, (iii) they were receiving immunosuppression with high-dose steroids (1 mg/kg or 30 mg/day or equivalent) in combination with a biological agent, (iv) they had a central venous catheter in place, (v) they were critically ill with life support such as vasopressors, assisted ventilation etc., or (vi) they had CD with disease limited to small bowel (at diagnosis), or presence of a stricture or bowel obstruction, phlegmon, an abscess, perforation, or active fistulizing disease (at screening), (vii) medical therapy was changed within last 4 weeks.

Donors were healthy family members, first-degree relatives, or trusted friends. Donor inclusion criteria included not being on prescription drugs, no antibiotic exposure in the previous three months, body mass index < 30, being free of any current or past history of malignancy, chemotherapy, chronic systemic or gastrointestinal disease, or functional disorders including chronic fatigue, irritable bowel syndrome, and fibromyalgia. Informed consent and assent (where applicable) was obtained prior to performing screening procedures.

Subjects and donors were tested for infections within 35 days prior to FMT and were re-evaluated if there was any change in symptoms. Blood work included screening for hepatitis A, B, C, syphilis, and human immunodeficiency virus (HIV). Stool tests included routine culture (*Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Escherichia coli* O157), *Clostridium difficile* DNA testing, and microscopic examination for ova, parasites, Giardia, Cryptosporidium, Cyclospora, and Isospora. The donor stool was additionally tested for *Helicobacter pylori* antigen. Female patients and donors were screened for pregnancy at the time of enrollment and before the procedure. All donors were additionally screened for risk of communicable

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diseases by the American Association of Blood Banks donor questionnaire. (http://www.aabb.org/tm/questionnaires/Documents/dhq/v2/DHQ%20v2.0.pdf)

Fecal microbiota transplantation

Pre-transplant preparation: Stool was collected from all donors and subjects for microbiome analysis before starting pre-transplant medications. All subjects were given antibiotics for 5 days starting 7 days prior to procedure with either Metronidazole or Vancomycin (10 mg/kg/dose with maximum dose of 500 mg/dose three times daily for either drug). Subjects also took omeprazole (1 mg/kg/dose up to 20 mg twice daily) or equivalent starting 5 days before the procedure for 7 days. The donors were allowed to use over-the-counter laxatives prior to FMT if needed. All subjects received 2 to 4 mg of loperamide two hours prior to the procedure.

FMT procedure: Fresh donor stool was collected on the day of the procedure up to 3 - 4 hours prior to FMT. Approximately 150 g of stool was blended using 250 - 300 ml of non-bacteriostatic normal saline in a new blender at high speed for 2 - 3 minutes. Stool slurry was then sieved through 2 layers of gauze to remove large particles. Stool suspension was drawn into multiple 60 ml syringes and labeled for use.

All patients received general anesthesia and an endotracheal intubation for airway protection. A pediatric colonoscope (PCF-140) was used for the upper endoscopy to facilitate jejunal intubation. Biopsies were first obtained from the esophagus, stomach, and duodenum followed by infusion of 20-30 ml of the fecal suspension into the distal duodenum or proximal jejunum followed by a 15 ml flush of normal saline. The subjects then underwent a colonoscopy and biopsies were obtained from all segments of the colon as the scope was being advanced into the terminal ileum. Approximately 200-250 ml of the fecal suspension was then delivered into the terminal ileum and right colon. The subjects were transferred to the post-operative recovery

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unit and discharged after 1 - 2 hours of observation. Subsequently they were advised to continue omeprazole for 2 additional days and use loperamide at a dose of 1 - 2 mg every 6 hours for 24 hours after FMT.

Post-FMT follow-up

Adverse events and clinical response were assessed at 1 week, 1 month, and 6 months following FMT. Adverse events were recorded using the NIH common terminology criteria as mild, moderate, severe and life threatening. Each event was then evaluated for being related, possibly related or unrelated to the study procedure. All subjects documented adverse events in a diary for 1 week following transplantation and all adverse events were recorded until 6 months after fecal transplantation. Disease activity was assessed by PCDAI or PUCAI depending on the underlying diagnosis. All subjects underwent routine blood counts, erythrocyte sedimentation rate and C-reactive protein testing at 1 and 6 months. Fecal biomarkers were determined locally at the discretion of treating gastroenterologists at 1 and 6 months after FMT. Stool was evaluated for infection only in subjects having diarrhea or bloody stools at follow-up. Subjects from out-of-region were allowed to follow up with their local gastroenterologists.

Response criteria: The clinical response was assessed at 1 month and 6 months after FMT. Response was defined as a decrease of 15 points in PUCAI or 12.5 points in PCDAI at 1 month as used in previous studies[30, 31,37]. Remission was defined as normalization of previously elevated fecal biomarkers and a PCDAI/PUCAI of 0 points. If a subject required escalation of medical therapy prior to 1-month evaluation they were considered to be non-responders. Subsequently, any escalation of medical therapy was considered as a loss of response.

Clinical Data and Statistical Analysis:

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Descriptive statistics are reported as means and standard deviation or median with interquartile range for continuous variables and frequency with percentage for categorical variables. Differences in outcome between responders and non-responders at 30 days were assessed using independent t-tests or Wilcoxon rank-sum test for continuous variables and Chi-square or Fisher's exact test for categorical variables, as appropriate based on cell size. Subgroup statistical analysis within Disease types of CD and IC/UC between responders and non-responders was not feasible because of small sample sizes (n = 7 and n=14 respectively).

All statistical tests were two-sided and conducted at the alpha = 0.05 level. Statistical analysis was done using The SAS software v 9.4 (Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA).

Microbiome Analysis

Stool samples were collected for microbiome analysis from subjects and donors prior to the transplant procedure and from subjects at 1 week, 1 month and 6 months after FMT.

<u>Bacterial DNA extraction and sequencing</u>

Microbial DNA was extracted from stool samples using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA). Bacterial 16S rRNA gene sequences were amplified and sequenced on the Illumina MiSeq platform. 16S amplicons were produced using fusion primers adapted for the Illumina MiSeq that target the V4 region (515F and 806R primers). Samples were sequenced with blank extraction and no-template added PCR controls at the University of Illinois Roy J. Carver Biotechnology Center, Urbana, Illinois. USA.

<u>Sequence processing and analysis</u>

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Sequence data were analyzed using Quantitative Insights into Microbial Ecology (QIIME) with default parameters and normalized numbers of sequencing reads. Samples were rarefied to 1,500 sequences. Rarefaction curves of all samples show that at 1,500 reads the curves are in the linear segment of the curve (Supplemental Figure I). Rarefaction to 15,000 sequences showed no significant differences compared to 1,500 sequences (Supplemental Figure II, III, IV). Alpha diversity (observed OTUs metric) and beta diversity were calculated using QIIME[31]. Significant differences were assessed using ANOVA and post-hoc Tukey HSD test where appropriate to account for multiple hypothesis testing. Variations in beta diversity were assessed with the PERMANOVA and PERMDISP algorithms in QIIME. Linear discriminant analysis effect size (LEfSe) was used to determine differentially abundant taxa across groups of samples[32]. Only taxa with an average relative abundance >1% in at least one group of samples was considered for this analysis. A p-value of 0.05 was used to determine significance in all statistical tests.

Accession numbers

All 16S rRNA gene sequences have been deposited to the National Center for Biotechnology Information under the BioProject ID PRJNA380944.

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