Journal of Pediatric Surgery 51 (2016) 81–86

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Altered fecal short chain fatty acid composition in children with a history of Hirschsprung-associated enterocolitis☆,☆☆

Farokh R. Demehri [a], Philip K. Frykman [b], Zhi Cheng [b], Chunhai Ruan [c], Tomas Wester [d,e], Agneta Nordenskjöld [d,e], Akemi Kawaguchi [f], Thomas T. Hui [g], Anna L. Granström [d,e], Vince Funari [h], Daniel H. Teitelbaum [a,*], for the HAEC Collaborative Research Group (HCRG)

[a] Division of Pediatric Surgery, C. S. Mott Children's Hospital, University of Michigan, Ann Arbor, MI, USA
[b] Division of Pediatric Surgery and Departments of Surgery and Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[c] Michigan Comprehensive Metabolomics Research Core, University of Michigan, Ann Arbor, MI, USA
[d] Department of Pediatric Surgery, Astrid Lindgren's Children's Hospital, Karolinska University Hospital, Stockholm, Sweden
[e] Department of Women's and Children's Health and Center of Molecular Medicine-CMM, Karolinska Institute, Stockholm, Sweden
[f] Division of Pediatric Surgery, Children's Hospital Los Angeles, Los Angeles, CA, USA
[g] Division of Pediatric Surgery, Children's Hospital Oakland, Oakland, CA, USA
[h] Genomics Core Laboratory, Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 20 September 2015
Accepted 7 October 2015

Key words:
Hirschsprung disease
Hirschsprung-associated enterocolitis
Aganglionosis
Pediatric
Short chain fatty acids
Microbiome

ABSTRACT

Purpose: Children with Hirschsprung disease (HD) who have a history of enterocolitis (HAEC) have a shift in colonic microbiota, many of which are necessary for short chain fatty acid (SCFA) production. As SCFAs play a critical role in colonic mucosal preservation, we hypothesized that fecal SCFA composition is altered in children with HAEC.

Methods: A multicenter study enrolled 18 HD children, abstracting for history of feeding, antibiotic/probiotic use, and enterocolitis symptoms. HAEC status was determined per Pastor et al. criteria (12). Fresh feces were collected for microbial community analysis via 16S sequencing as well as SCFA analysis by gas chromatography–mass spectrometry.

Results: Nine patients had a history of HAEC, and nine had never had HAEC. Fecal samples from HAEC children showed a 4-fold decline in total SCFA concentration vs. non-HAEC HD patients. We then compared the relative composition of individual SCFAs and found reduced acetate and increased butyrate in HAEC children. Finally, we measured relative abundance of SCFA-producing fecal microbiota. Interestingly, 10 of 12 butyrate-producing genera as well as 3 of 4 acetate-producing genera demonstrated multi-fold expansion.

Conclusion: Children with HAEC history have reduced fecal SCFAs and altered SCFA profile. These findings suggest a complex interplay between the colonic metabolome and changes in microbiota, which may influence the pathogenesis of HAEC.

1. Background

Hirschsprung-associated enterocolitis (HAEC) is a life-threatening complication of congenital aganglionic megacolon, or Hirschsprung disease (HD). While surgical pull-through achieves satisfactory restoration of functional intestinal continuity in most patients, up to 40% of children experience at least one episode of HAEC after surgery [1]. Despite being the primary cause of morbidity for children with HD, the pathophysiology of HAEC is poorly understood. Several contributing factors have been identified, including dysfunctional host immunity, diminished epithelial barrier function, and altered gut microbiota, although no unifying causative agent has been identified [2].

A microbial etiology of HAEC has been postulated since initial reports of elevated *Clostridium difficile* toxin titers in children with HAEC [3], although carriage rates have since proven highly variable [4]. Analysis of changes in the gut microbiome using molecular microbiological techniques has shown decreased colonization of *Bifidobacterium* and *Lactobacillus* in children with HD who developed HAEC [5], while genomic approaches have found increased bacterial population diversity during HAEC episodes in children with HD [6], yet modest differences in children with a history of HAEC [7]. Similar findings have been noted in animal models of HD using neural crest cell specific endothelin receptor B (Ednrb) knockout mice [8]. At the genus level, Ednrb

☆ Conflicts of Interest: The authors declare no conflicts of interest.
☆☆ Level of Evidence: III.

* Corresponding author at: Section of Pediatric Surgery, University of Michigan, Mott Children's Hospital, 1540 E. Hospital Dr., SPC 4211, Ann Arbor, MI, 48109-4211. Tel.: +1 734 936 8464.
E-mail address: dttlbm@umich.edu (D.H. Teitelbaum).

http://dx.doi.org/10.1016/j.jpedsurg.2015.10.012
0022-3468/

knockout mice demonstrated decreased *Lactobacillus* and increased *Bacteroides* and *Clostridium* prior to enterocolitis. These findings suggest that disequilibrium in the gut microbiome – or dysbiosis – may result in an altered microbial ecosystem that leads to HAEC development.

The mechanisms by which altered colonic microbiota relate to the development of HAEC is not known. One important physiologic role of gut bacteria is the production of short chain fatty acids (SCFAs). Complex oligosaccharides and other organic, indigestible fiber matter not absorbed in the upper intestinal tract are fermented by the anaerobic microbial community of the colon, producing SCFAs (primarily acetate, propionate, and butyrate) and gas [9]. These SCFAs, of which butyrate is the best-studied, play a key role in maintaining gut homeostasis and epithelial integrity [10]. Butyrate serves as a principal energy source for colonocytes, regulates host gene expression via histone deacetylase inhibition [11]. This latter role has led to both an increase in IL-10 production and an anti-inflammatory role by inhibiting NF-κB signaling [12]. We hypothesized that children with a history of HAEC would have altered fecal SCFA composition, as well as disequilibrium of SCFA-producing microbiota.

This study was designed to evaluate the fecal SCFA makeup of patients who had completed definitive surgical treatment for HD. We compared 9 children with a history of at least one episode of HAEC to 9 children with no episodes of HAEC. We first performed gas chromatography–mass spectrometry (GC-MS) based quantification of fecal SCFA contents, and then compared this to 16S-based microbiota analysis of the same samples to elucidate the relationship between an altered microbiome and SCFA production.

2. Methods

2.1. Patient selection

This was a multi-institution study of children younger than 18 years of age who had completed definitive pull-through surgical treatment for a histopathological diagnosis of HD. Twenty children were enrolled by four member institutions of the HAEC Collaborative Research Group (HCRG): Cedars-Sinai Medical Center (CSMC), Los Angeles, CA; Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden; Children's Hospital Los Angeles, Los Angeles, CA; Children's Hospital of Oakland, Oakland, CA. Of these, 10 children had a history of HD without documented enterocolitis, and 10 had at least one episode of HAEC as defined by Pastor et al. criteria [13]. Two of these 20 children were excluded from analysis owing to the presence of a diverting ileostomy and active HAEC at the time of stool collection, respectively. Thus, a total of 9 HD and 9 HAEC samples were analyzed. Medical records were reviewed and parent interview was performed using standardized questionnaires. Data collected included demographics, medical/surgical history, diet in the first year of life, medications including antibiotics, probiotic use, and enterocolitis symptoms. This study was approved by the CSMC institutional review board (IRB no. CR00008054) as a multicenter study, as well as individual approval by all participating sites, and the University of Michigan IRB (HUM00079878). Stool was collected within one week of enrollment and immediately snap-frozen at −80 °C in air-tight containers to prevent the loss of volatile SCFAs. Frozen sample aliquots were shipped to the University of Michigan for SCFA analysis and to CSMC for bacterial DNA isolation.

2.2. Fecal SCFA analysis

Sample extraction was performed using aqueous extraction solvent containing 3% 1 M HCl (v/v) and isotope-labeled internal standards (d7-buytric acid and d11-hexanoic acid). Samples were then homogenized and centrifuged. Supernatants were transferred to new Eppendorf tubes for extraction by diethyl ether. After layer separation, the upper layer was transferred to an autosampler vial for GC-MS analysis. GC (Agilent 6890, Wilmington, DE) separation was performed using a ZB-Wax plus column, 0.25 μm × 0.25 mm × 30 m (Phenomenex, Torrance, CA). A single quadrupole mass spectrometer (Agilent, 5973 inert MSD) was used to identify and quantify SCFAs using Agilent Masshunter software, version B.06 [14]. Absolute quantities of SCFAs were normalized to sample mass.

2.3. Bacterial DNA isolation and amplicon preparation

Fecal samples were suspended in 50 mM Tris buffer (pH 7.5) containing 1 mM EDTA, 0.2% β-mercaptoethanol (Sigma) and 1000 U/ml of lyticase (Sigma). The mix was incubated at 37 °C for 30 min, and DNA was isolated using QIAamp DNA Stool Mini Kit (Qiagen). Bacterial 16S rRNA gene amplicons spanning variable regions 1–4 were generated in 20 μL PCR reactions using 20 ng of fecal DNA with 25 cycles using high-fidelity Phusion Polymerase (New England Biolabs, Beverly, MA) at 52.7 °C annealing using with degenerate 8F (AGAGTTTGATCM TGGCTCAG) and R357 (CTGCTGCCTYCCGTA) primers. All PCR reactions were purified using Agencourt AmPure Magnetic Beads (Beckman), resuspended in 20 μL of nuclease-free water and quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA).

2.4. 16S sequencing

Paired-end adapters with unique indexes were ligated to 100 ng of 16S amplicons and used to generate Ion Torrent sequencing libraries using the Ion Xpress Library Kit (Life Technologies, Carlsbad, CA). Library enrichment was performed with 10 cycles of PCR and purified using Agencourt Ampure Magnetic Beads (Beckman). All libraries were subjected to quality control using qPCR, DNA 1000 Bioanalyzer (Agilent), and Qubit (Life Technologies, Carlsbad, CA). Pooled libraries were assayed on Agilent Bioanalyzer (Santa Clara, CA) to check final sizing and KAPA Biosciences qPCR for quantitation. 16S samples were multiplexed and sequenced on the Ion Torrent PGM on a 318 chip with 400 bp chemistry. 250 single-end sequencing-by-synthesis was performed using the MiSeq Illumina sequencer (Illumina, San Diego, CA). Torrent reads shorter than 200 bp, or not containing the designed 16S primers (>2 nt mismatches) were discarded. 300 bp sequences of remaining high-quality reads were aligned to the Greengenes reference database (February 2011 release) using BLAST v2.2.22 in QIIME v1.5 wrapper [15] with an identity percentage ≥97% to select the operational taxonomic units (OTUs). Taxonomy for each sequence was assigned using the Ribosomal Database Project (RDP) classifier v2.2.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Comparison between two groups used the two-tailed, unpaired Student's T-test. All results are expressed as mean ± standard deviation unless otherwise specified. A p value of <0.05 was considered significant.

3. Results

3.1. Patient characteristics

The two groups (HD and HAEC) each included 9 patients, with an equal distribution of 8 males and 1 female (Table 1). Median age was 2.7 years (3 months to 8 years) for all children, 2.3 years for the HAEC group, and 3.5 years for the HD group ($p = 0.40$). There were no significant differences in length of aganglionosis, non-HAEC complications, early feeding type, or probiotics received. While no children in the HD group received antibiotics within 2 months of stool collection, three patients in the HAEC group had received antibiotics (two for prior HAEC treatment, and one for sickle cell prophylaxis). Trisomy 21 was present in one patient in the HD group and two patients in the HAEC group.