Associations between Gut Microbial Colonization in Early Life and Respiratory Outcomes in Cystic Fibrosis

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Objective To examine patterns of microbial colonization of the respiratory and intestinal tracts in early life in infants with cystic fibrosis (CF) and their associations with breastfeeding and clinical outcomes.

Study design A comprehensive, prospective longitudinal analysis of the upper respiratory and intestinal microbiota in a cohort of infants and young children with CF followed from birth was performed. Genus-level microbial community composition was characterized using 16S-targeted pyrosequencing, and relationships with exposures and outcomes were assessed using linear mixed-effects models, time-to-event analysis, and principal components analysis.

Results Sequencing of 120 samples from 13 subjects collected from birth to 34 months revealed relationships between breastfeeding, microbial diversity in the respiratory and intestinal tracts, and the timing of onset of respiratory complications, including exacerbations and colonization with *Pseudomonas aeruginosa*. Fluctuations in the abundance of specific bacterial taxa preceded clinical outcomes, including a significant decrease in bacteria of the genus *Parabacteroides* within the intestinal tract prior to the onset of chronic *P aeruginosa* colonization. Specific assemblages of bacteria in intestinal samples, but not respiratory samples, were associated with CF exacerbation in early life, indicating that the intestinal microbiome may play a role in lung health.

Conclusions Our findings relating breastfeeding to respiratory outcomes, gut diversity to prolonged periods of health, and specific bacterial communities in the gut prior to respiratory complications in CF highlight a connection between the intestinal microbiome and health and point to potential opportunities for antibiotic or probiotic interventions. Further studies in larger cohorts validating these findings are needed. *(J Pediatr 2015;167:138-47)*.

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Cystic fibrosis (CF) is the most common life-limiting autosomal recessive genetic disorder among people of European descent. It is characterized by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, which result in viscous epithelial secretions beginning at birth as a consequence of abnormal sodium and chloride transport. Individuals with CF experience progressive lung disease, pancreatic insufficiency, and profound impacts on growth and nutrition.[1,2] Infants and young children with CF are at risk for chronic infection and inflammation, resulting in significant morbidity and early mortality.[3,4] In both animal models and in observational studies of humans, CF has been associated with atypical microbial colonization of the intestinal and the respiratory tracts, findings that are attributable to loss of *CFTR* function and the resulting altered microenvironments.[4-10] The natural history of microbial acquisition in the respiratory and intestinal tracts in patients with CF beginning at birth and the impact of these communities on clinical outcomes are largely unexplored.

Microbial colonization patterns in infancy are influenced by environmental exposures (including delivery mode, infant diet, hospitalizations, and medications), human genetics, and immune function.[11-13] Microbial colonization of the respi-

CF	Cystic fibrosis
rRNA	Ribosomal RNA
SDI	Simpson diversity index

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Supported by the Cystic Fibrosis Foundation and the Harry Shwachman Career Development Award, The Joshua Burnett Career Development Award through the Hitchcock Foundation, the Geisel School of Medicine at Dartmouth Department of Pediatrics (to J.M.), the Neukom Institute (to G.O. and J.M.), the Cystic Fibrosis Foundation Research Development Program (STANTO07R0 [to G.O.]) the National Institutes of Health (K01LM011985 [to A.H.], R01AI59694 [J.M.], R37AI83256-06 [G.O.], 4UH3DK083993 [M.S. and H.M.], K24AT003683 [to P.H.], P20GM104410 [to A.H., M.K., J.M.], P01ES022832 [to M.K.]), and Environmental Protection Agency (RD-83459901 [to M.K.]).

Portions of the study were presented as an abstract at the North American Cystic Fibrosis Conference, Salt Lake City, UT, October 17-19, 2013.

http://dx.doi.org/10.1016/j.jpeds.2015.02.049

ratory tract of infants with CF has been shown to be significantly influenced by dietary exposure to breast milk, and colonization of the gut by specific assemblages of microbes appears to precede colonization of the lungs, highlighting potential interactions between nutrition, intestinal colonization, and respiratory outcomes.[3] Seminal studies in germ free animals highlight the importance of gut microbial colonization for immune programming in the neonatal period, ultimately affecting lifelong systemic disease risk.[14,15] The aberrant respiratory and gastrointestinal microbial colonization patterns in young children with CF[1,4,16] and the success of probiotics trials that have demonstrated benefits of altering the gut microbiome in an effort to ameliorate risk of respiratory compromise in CF[17,18] both point to a knowledge gap in our understanding of the interactions between intestinal microbial colonization and CF disease progression in early life.

The purpose of this study was to investigate the hypothesis that microbial acquisition patterns relate to risk of CF-related complications and clinical markers of CF disease progression. Associations between intestinal microflora composition and clinical outcomes in young children with CF may ultimately help to inform nutritional and probiotic treatment strategies that ameliorate colonization with pathogens, maintain a more health-promoting microbiota, and decrease morbidity and mortality.

Methods

Institutional review board approval was obtained in April 2010 (Center for the Protection of Human Subjects at Dartmouth number 21761) with yearly renewal of approval in 2011 through 2013, and parents of subjects provided written informed consent.

Eligibility criteria included diagnosis with CF prenatally or postnatally based upon newborn screening results and subsequent confirmation with sweat chloride and genetic testing. Subjects were eligible if their care for CF would be at the Dartmouth-Hitchcock Medical Center- (Lebanon or Manchester, New Hampshire) affiliated clinics. Enrollment occurred prior to 1 month of life with follow-up prospectively at routine CF clinic visits, which facilitated collection of detailed clinical information as well as samples every 3 months until the maximum age of 34 months for this data analysis. Enrollment and prospective collection is ongoing. Subjects were excluded if they had non-CF chromosomal anomalies.

Detailed clinical information was collected prospectively and included demographics, birth history, medical history at each visit both with parental interviews and medical record review, growth measurements, outcome data including information regarding outcomes of interest: CF pulmonary exacerbations (as diagnosed by an attending physician using the Akron Children's CF exacerbation score if appropriate,[19] and defined by findings including wheezing, cough lasting for more than 3 days, shortness of breath, weight loss, decrease in oxygen saturations below 95%, and treatment with antibiotics), growth failure (defined as height, weight, and head circumference <10th percentile or a loss of >10% in growth measurements between visits), hospitalization for any CF-related complication, and onset of *P aeruginosa* colonization (as identified during routine clinic oropharyngeal culture, requiring therapy with antibiotics). Clinical culture results were collected prospectively, along with medical interventions and exposures including medications, supplements, and dietary and environmental histories. Detailed antibiotic exposure data were collected and used to adjust linear mixed effects models of changes in the microbiome development over time.

Oropharyngeal and stool samples were collected at regularly scheduled CF clinic visits which occurred every 3 months beginning in the first month of life for up to 3 years. Oropharyngeal swabs were collected by doubling specimen collection swabs for routine clinical surveillance oropharyngeal culture, which allowed for the collection of a research specimen simultaneously with the routine clinical specimen with minimal additional burden for participating children. Research swabs were placed in individual 2 mL microcentrifuge tubes, stored at −20°C, and transported to the lab on dry ice.

Respiratory swabs were submerged in 2 mL RNAlater (Qiagen, Venlo, The Netherlands) and vortexed for 1 minute. Swabs were removed with sterile forceps to a clean tube where they were placed cotton side up and centrifuged for 2 minutes at 14 000 × g in order to recover fluid from the absorbent material of the swab, and any recovered fluid was combined with the rest of the sample. Swabs were discarded, and samples were frozen at −80°C until further processing. Bacterial DNA was extracted using the MoBio Powersoil bacterial DNA isolation kit (MoBio, Carlsbad, California) according to the manufacturer's instructions. Stool samples were collected by parents at home using a sterile wooden spatula and placed in a sterile collection cup in the freezer; 50 mg aliquots of stool samples were stored in sterile tubes with 1 mL RNAlater and frozen at −80°C. Thawed stool samples were dissolved in phosphate buffered saline, followed by DNA extraction as above.

DNA extracts were used to construct polymerase chain reaction amplicon libraries for 454 pyrosequencing targeting the V4-V6 regions of the bacterial 16S ribosomal RNA (rRNA) gene, performed at the Josephine Bay Paul Center at the Marine Biological Laboratory.[3] Titanium amplicon sequencing initially targeted the 16S V6 hypervariable region and ultimately the V4-V6 regions. These 2 approaches have been assessed for their similarities and were not statistically significantly different,[3] therefore, the data were pooled for the analyses presented here. A mix of multiple fusion primers containing degeneracies in the 16S-specific domain designed to capture the range of diversity of rRNA sequences represented in molecular databases[20,21] were used. Primer sequences were: 967FPP: 5′-CNACGCGAAGAACCTTANC, 967FAQ: 5′ CTAACCGANGAACCTYACC, 967FU2: 5′-CAACGCGMARAACCTTACC, 967FU3: 5′-ATACGCGARGAACCTTACC, 518F: 5′-CCAGCAGCYGCGGTAAN, and 1064R: 5′-CGACRRCCATGCANCACCT.

Samples were amplified in triplicate and no-template controls were included for each primer set. Positive polymerase