

ORIGINAL ARTICLE

Duodenal Microbiota in Stunted Undernourished Children with Enteropathy

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ABSTRACT

BACKGROUND

Environmental enteric dysfunction (EED) is an enigmatic disorder of the small intestine that is postulated to play a role in childhood undernutrition, a pressing global health problem. Defining the incidence of this disorder, its pathophysiological features, and its contribution to impaired linear and ponderal growth has been hampered by the difficulty in directly sampling the small intestinal mucosa and microbial community (microbiota).

METHODS

In this study, among 110 young children (mean age, 18 months) with linear growth stunting who were living in an urban slum in Dhaka, Bangladesh, and had not benefited from a nutritional intervention, we performed endoscopy in 80 children who had biopsy-confirmed EED and available plasma and duodenal samples. We quantified the levels of 4077 plasma proteins and 2619 proteins in duodenal biopsy samples obtained from these children. The levels of bacterial strains in microbiota recovered from duodenal aspirate from each child were determined with the use of culture-independent methods. In addition, we obtained 21 plasma samples and 27 fecal samples from age-matched healthy children living in the same area. Young germ-free mice that had been fed a Bangladeshi diet were colonized with bacterial strains cultured from the duodenal aspirates.

RESULTS

Of the bacterial strains that were obtained from the children, the absolute levels of a shared group of 14 taxa (which are not typically classified as enteropathogens) were negatively correlated with linear growth (length-for-age z score, $r = -0.49$; $P = 0.003$) and positively correlated with duodenal proteins involved in immunoinflammatory responses. The representation of these 14 duodenal taxa in fecal microbiota was significantly different from that in samples obtained from healthy children ($P < 0.001$ by permutational multivariate analysis of variance). Enteropathy of the small intestine developed in gnotobiotic mice that had been colonized with cultured duodenal strains obtained from children with EED.

CONCLUSIONS

These results provide support for a causal relationship between growth stunting and components of the small intestinal microbiota and enteropathy and offer a rationale for developing therapies that target these microbial contributions to EED. (Funded by the Bill and Melinda Gates Foundation and others; ClinicalTrials.gov number, NCT02812615.)

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ENVIRONMENTAL ENTERIC DYSFUNCTION (EED) is a disease of the small intestine for which the causative factors are poorly understood. The condition was first described in the 1960s by pathologists who were examining biopsy samples obtained from the proximal small bowel of adult Peace Corps volunteers who had lived in areas with high levels of fecal–oral contamination and in whom diarrhea and intestinal malabsorption developed.¹ Histopathological changes included diminution in the number and height of intestinal villi with associated loss of absorptive surface area, disruption of the epithelial barrier, and a chronic inflammatory infiltrate.

Several studies have documented an association between altered absorptive function of the small intestine (as defined by dual-sugar permeability tests) and both asymptomatic infection with one or more enteropathogens and growth stunting.^{2,3} Stunting is associated with poor developmental outcomes, including reduced intellectual capacity and an impaired response to oral vaccines.^{4,7} Despite considerable effort, neither nutritional interventions nor initiatives to improve water quality, sanitation, and hygiene practices have proved to be effective in reducing the incidence of stunting.^{8,9} These results support the hypothesis that subclinical enteric dysfunction contributes to stunting.¹⁰

Most studies of EED have relied on nonvalidated fecal or plasma biomarkers because esophagogastroduodenoscopy (EGD) is rarely performed in undernourished pediatric populations to obtain duodenal biopsy samples because of its inherent risks. Consequently, there are conflicting data about the incidence of EED and its relationship to stunting.¹¹ Evidence that perturbed development of the microbiota contributes to undernutrition is provided by studies involving the analysis of fecal samples,¹²⁻¹⁴ and recent data suggest a potential role for bacteria in the upper gastrointestinal tract.¹⁵

To examine the role of the duodenal microbiota in the pathogenesis of EED and its relationship to stunting, we initiated the Bangladesh Environmental Enteric Dysfunction (BEED) study involving young children who live in an urban slum (Mirpur) in Dhaka.¹⁶ This study has two components: an interventional phase, which was designed to test whether a nutritional interven-

tion administered for 3 months would improve linear growth in children between the ages of 12 and 18 months who were stunted or at risk for stunting,¹⁷ and a diagnostic phase involving children who did not have a response to the nutritional intervention and who subsequently underwent EGD with duodenal biopsy. The results of the diagnostic phase of the study are reported here.

METHODS

STUDY DESIGN AND OVERSIGHT

The BEED study was approved by the ethical review committee at the International Centre for Diarrhoeal Disease Research, Bangladesh. Written informed consent was obtained from each child's parent or guardian to participate in the study and to undergo EGD if the child met the criteria for being stunted or at risk for stunting. Children who had a length-for-age z score greater than 2 standard deviations below that of children in a healthy reference cohort of the World Health Organization (i.e., length-for-age z score, less than -2) were classified as stunted, and those with a score between -2 and -1 were classified as being at risk for stunting. For 3 months, children in the two groups received one large egg (weight, 60 to 65 g), 150 ml of cow's milk, and multiple micronutrients for 6 days per week, plus an anthelmintic medication.¹⁶ A lack of response to the nutritional intervention was defined as continuing to have a z score of less than -2 in the stunted group and a score of between -1 and -2 in the at-risk group. All the children who did not have a response to the nutritional intervention and who did not have a coexisting illness that causes undernutrition¹⁶ were deemed to be qualified to undergo EGD. At the time of the EGD procedure, we obtained plasma, fecal samples, and duodenal aspirates and biopsy samples (Tables S1 and S2 in Supplementary Appendix 2, available with the full text of this article at NEJM.org). In addition, we obtained 21 plasma samples and 27 fecal samples from age-matched healthy children (who were defined as having a z score of -1 or more for both weight-for-length and length-for-age measures) living in Mirpur. None of the children undergoing EGD received antibiotics for at least 2 weeks before the procedure.

PROTEOMIC ASSAYS

We performed proteomic assays (SOMAScan, SomaLogic) to quantify several thousand proteins spanning a broad range of biologic functions.¹⁸ In this study, we analyzed 4077 plasma proteins and 2619 duodenal proteins that passed quality-control filtering. Details about these analyses are provided in the Methods section in Supplementary Appendix 1.

DNA SEQUENCING AND ENTEROPATHOGEN DETECTION

We analyzed bacterial taxa present in duodenal aspirates and fecal samples by sequencing amplicons that were generated from variable region 4 of bacterial 16S ribosomal DNA (rDNA) genes using a polymerase-chain-reaction (PCR) assay. We grouped V4-16S rDNA reads into amplicon sequence variants (i.e., sequences in which artifacts generated by sequencing errors have been removed) and then normalized their levels using DESeq2.¹⁹ The levels of amplicon sequence variants were then scaled according to bacterial load and were \log_{10} -transformed before the performance of additional analyses. A PCR-based assay was used to quantify duodenal and fecal levels of 18 bacterial, viral, and protozoan enteropathogens.²⁰

ANALYSIS IN GNOTOBIOTIC MICE

In these experiments, which were approved by the Washington University animal studies committee, 5.5-week-old, male, germ-free C57BL/6J mice were fed a diet representative of that consumed by 18-month-old children living in Mirpur. (Details regarding the composition of the typical diet are provided in Table S9 in Supplementary Appendix 2.) A collection of 39 bacterial strains cultured from duodenal samples obtained from the study children who had histopathological evidence of EED was then administered to the mice by oral gavage. Age-matched mice with the same genetic background (C57BL/6J) that had also been raised under sterile conditions and fed the same diet received the cecal microbiota of a conventionally raised C57BL/6J mouse by gavage; these animals served as conventionalized controls. After the mice had been euthanized, we extracted DNA from luminal contents obtained from different regions of the intestines and determined the levels of bacterial

strains. Details are provided in the Methods section in Supplementary Appendix 1. In addition, we performed gene-expression analysis, immunoassays, and histologic analysis to characterize duodenal tissue.

STATISTICAL ANALYSIS

We determined the strength of the association between the presence of EED and duodenal protein modules, levels of plasma proteins, levels of duodenal amplicon sequence variants, the histopathological severity score, and length-for-age z score according to the Pearson correlation coefficient. Spearman's rank correlation was used to identify relationships between the length-for-age z score and the enteropathogen burden. Relationships between the duodenal proteome and absolute levels of duodenal amplicon sequence variants were assessed by means of singular value decomposition of a cross-covariance matrix. Reported P values have not been adjusted for multiple comparisons; a P value of less than 0.05 was considered to indicate statistical significance for analyses that did not require correction for multiple hypothesis testing. Q values were adjusted for the false discovery rate²¹; a value of less than 0.1 was considered to indicate statistical significance. Point estimates and dispersion are reported as means and 95% confidence intervals unless otherwise indicated.

RESULTS**STUDY PATIENTS**

From July 2016 through July 2018, we enrolled 525 children in our study. Of these enrollees, 110 children (mean [\pm SD] age, 18 \pm 2 months) who did not have a response to the nutritional intervention underwent EGD. During the nutritional intervention, these children had not shown significant improvement in linear growth (mean change from baseline in the length-for-age z score, 0.03; 95% confidence interval [CI], -0.03 to 0.08; $P=0.40$), although they had shown significant improvement in the weight-for-age z score (change from baseline, 0.15; 95% CI, 0.08 to 0.22; $P<0.001$) (Table S1 in Supplementary Appendix 2).

We determined the severity of EED according to histologic analysis of duodenal biopsy samples obtained from the children. Included in the

Table 1. Clinical Characteristics of the Study Children, According to Subgroup.*

Characteristic	No Benefit from Nutritional Intervention (N=110)	Biopsy-Confirmed EED with Available Plasma and Duodenal Samples (N=80)
Demographic		
Age — mo	18.4±2.1	18.3±2.1
Female sex — no. (%)	64 (58)	48 (60)
Anthropometric findings at the time of endoscopy		
Length-for-age z score	-2.19±0.82	-2.29±0.86
Weight-for-age z score	-1.76±0.87	-1.85±0.91
Weight-for-length z score	-0.96±0.91	-1.02±0.93
Histopathological score for EED on duodenal biopsy sample — no. (%)		
No evidence	6 (5)	0
Mild	41 (37)	34 (42)
Moderate	13 (12)	10 (12)
Severe	50 (45)	36 (45)
Median fecal biomarker levels (IQR)		
Alpha-1 antitrypsin — mg/g	0.33 (0.17–0.50)	0.33 (0.17–0.50)
Myeloperoxidase — ng/ml	2325.5 (799.6–4569.1)	2006.0 (809.9–4315.0)
Neopterin — nmol/liter	1116.5 (495.5–2552.5)	1146.0 (506.3–2575.3)
EED biomarker score†	2.60 (1.52–4.25)	2.77 (1.52–4.24)

* Plus–minus values are means ±SD. To convert the values for alpha-1 antitrypsin to grams per liter, multiply by 0.01.

† Percentages may not total 100 because of rounding. IQR denotes interquartile range, and NA not applicable.

‡ The biomarker score for environmental enteric dysfunction (EED) is a continuous measurement starting at 0, with higher scores indicating greater levels of a group of fecal biomarkers of inflammation (alpha-1 antitrypsin, myeloperoxidase, and neopterin).

composite scoring system was the presence of infiltrating immune cells, villus atrophy, and crypt hyperplasia (Table 1 and Fig. S1 in Supplementary Appendix 1, where all the supplementary figures in this article are provided). Biopsy samples were graded on a scale of 0 to 3, in which grade 0 indicated normal histologic features (in 6 children), grade 1 mild histopathological changes (in 41 children), grade 2 moderate abnormalities (in 13 children), and grade 3 severe abnormalities (in 50 children). According to this definition, there was no significant correlation between the length-for-age z score and either the histopathological severity score ($P=0.57$) or an EED biomarker score that is based on fecal levels of three markers of intestinal inflammation and barrier disruption (myeloperoxidase, alpha-1 antitrypsin, and neopterin) ($P=0.36$) (Table 2).

Matched sets of plasma samples and duodenal biopsy samples were collected from 84 of the 110 children. Of these biopsy samples, 4 had normal histologic features, which left 80 children with matched samples for analysis (Table 1 and Tables S1 and S2 in Supplementary Appendix 2). There was sufficient fluid in the duodenal lumen of 38 of these 84 children to obtain an aspirate for analysis of the microbial community content; of the 38 children with sufficient fluid, 2 had normal biopsy samples (Fig. S2).

PLASMA PROTEOMES IN AFFECTED CHILDREN

Because biopsy samples were not obtained before the nutritional intervention, we could not confirm whether EED was present at the time of enrollment. In addition, only four children who had normal biopsy samples also had available plasma samples, and a single normal finding on

Table 2. Correlations between Length-for-Age z Scores and Features of Duodenal Microbiota, Fecal Biomarkers, and Histopathological Severity among Children with EED.*

Variable	Children in Subgroup	Pearson Correlation Coefficient	Coefficient (95% CI)†	P Value	Q Value‡	Mean Relative Level (95% CI)
<i>n</i> .						
36						
Correlation between length-for-age z score and features of duodenal microbiota						<i>percent</i>
<i>Streptococcus</i> sp.		-0.40	-0.31 (-0.54 to -0.07)	0.02	0.10	45.2 (38.3 to 52.2)
<i>Rothia mucilaginosa</i>		-0.45	-0.31 (-0.53 to -0.10)	0.006	0.06	2.37 (1.30 to 3.43)
<i>Veillonella</i> sp.		-0.44	-0.32 (-0.54 to -0.10)	0.007	0.06	0.79 (0.57 to 1.00)
Total bacterial load						
All bacteria§		-0.41	-0.32 (-0.56 to -0.08)	0.01	0.10	33.0 (2.67 to 63.3)¶
Collection of 14 core taxa		-0.49	-0.38 (-0.61 to -0.15)	0.003	0.04	38.9 (34.1 to 43.8)¶
Correlation between length-for-age z score and fecal biomarkers and histopathological severity						
	110					
Myeloperoxidase		-0.19	-0.19 (-0.31 to 0.00)	0.05	—	—
Alpha-1 antitrypsin		-0.15	-0.13 (-0.29 to 0.08)	0.11	—	—
Neopterin		0.11	0.09 (-0.07 to 0.24)	0.27	—	—
EED biomarker score		-0.09	-0.07 (-0.23 to 0.08)	0.36	—	—
Histopathological severity score**		-0.06	-0.06 (-0.27 to 0.15)	0.57	—	—

* Shown are data for two subgroups of study children with EED: the 36 children who had an available aspirate sample that was analyzed by means of a sequence-based, culture-independent method, for whom duodenal microbiota data are presented; and the 110 children who had no response to the nutritional intervention and were eligible for endoscopy, for whom data regarding fecal biomarkers and histopathological severity are presented. The dashed lines indicate that the determination of Q values and mean relative levels is not applicable because multiple comparisons were not performed and because these measurements were calculated on an absolute scale.

† Values for the total bacterial load and levels of duodenal bacterial taxa were log-transformed and z-scored before determining their relationships with the length-for-age z score. Thus, in this category, coefficients represent the effect of a unit change in the standard deviation of the bacterial load or the absolute deviation of bacterial taxa on the length-for-age z score.

‡ Q values are P values that have been adjusted for the false discovery rate.

§ The total bacterial load represents the inverse of the fractional level of the *Alicyclobacillus acidiphilus* spike-in.

¶ Absolute values rather than percentages are reported for all bacterial loads. Absolute levels were defined as the inverse of the relative levels of a spiked-in bacterial strain not typically found in the human gut microbiota (*A. acidiphilus*).

|| The total bacterial load of core group taxa represents the total of the absolute levels of the 14 bacterial amplicon sequence variants found in more than 80% of duodenal aspirates at a relative level of more than 0.01%.

** The histopathological severity score ranges from 0 to 3, with higher scores indicating more severe histopathological abnormalities.

biopsy does not rule out the possibility of disease at another duodenal site. All these considerations led us to focus our analysis on post-intervention plasma samples obtained from children with a histopathological diagnosis of EED. Among the five plasma proteins with levels that most strongly correlated with the length-for-age z score, four proteins — insulin-like growth factor 1 (IGF-1), IGF acid-labile subunit (IGFALS), IGF binding protein 3 (IGFBP-3), and procollagen C endopeptidase enhancer 2 (PCOLCE2) — were positively correlated, whereas IGFBP-2 was negatively correlated (Table S3A in Supplementary Appendix 2). Two of these proteins, IGFALS and IGFBP-3, stabilize IGF-1, which increases its half-life and bioavailability. Consistent with this finding is our observation that IGFBP-2, a protein that sequesters and inhibits the growth-potentiating functions of IGF-1, was negatively correlated with the length-for-age z score.²² PCOLCE2 cleaves the C-terminal propeptide of type I procollagen and is thought to facilitate bone formation, which is consistent with our observation of the positive correlation between this protein and the length-for-age z score.²³

We created a linear model to examine the relationship between IGF-1 and the length-for-age z score and the effect (interaction) of other members of the plasma proteome on this relationship (see the Methods section in Supplementary Appendix 1). Of the 4076 proteins tested for association with IGF-1 levels as related to the z score, osteoprotegerin (OPG or TNFRSF11B), a decoy receptor for receptor activator of nuclear factor- κ B (RANK) ligand, which promotes bone growth by inhibiting osteoclastic activity,²⁴ ranked fourth ($\beta=0.22\pm 0.08$; unadjusted $P=0.005$), and phosphate-regulating neutral endopeptidase (PHEX), the product of the gene responsible for X-linked hypophosphatemia and hereditary rickets,²⁵ ranked sixth ($\beta=0.25\pm 0.08$; unadjusted $P=0.004$) (Table S4 in Supplementary Appendix 2). Comparisons of the plasma proteomes of the 80 children with EED and 21 age-matched healthy children before and after the nutritional intervention are provided in Figure S3 and Table S5A through 5F in Supplementary Appendix 2.

DUODENAL MICROBIOTA AND PROTEOME ANALYSES

We characterized the composition of the duodenal microbiota in the 36 children with EED who

had an available aspirate sample. (For ethical reasons, duodenal specimens were not obtained from healthy children living in Mirpur.) Shotgun sequencing of aspirate DNA revealed that bacteria dominated the microbial community (Table 1 in Supplementary Appendix 3). Sequencing analysis of V4-16S rDNA revealed 165 distinct amplicon sequence variants (taxa) with relative levels of at least 0.01% in 1 or more of the 36 samples. A core group of 14 bacterial taxa were present in at least 80% of the aspirates obtained from children with EED (Table S6A in Supplementary Appendix 2). Permutational multivariate analysis of variance revealed no significant effects of breast-feeding status on duodenal bacterial composition before, during, or after the nutritional intervention.

We used an additional duodenal biopsy sample that had been obtained from each of the 80 children and had not been used for histopathological analysis to quantify expressed proteins. Our goal was to obtain a mechanistic definition of enteropathy that was more comprehensive than that provided by a tissue section stained with hematoxylin and eosin. We reasoned that such a definition could serve as a starting point for identifying features in the duodenal habitat that might correlate with the composition of the duodenal microbiota and the length-for-age z score. We performed independent components analysis (ICA), a method that is used to identify modules of coexpressed genes that belong to distinct biologic pathways,^{26,27} to search for groups of duodenal proteins that are involved in inflammation, injury, and metabolic dysfunction and thus could underlie the enteropathy observed in these children. Our analysis of 2619 proteins in the 80 duodenal proteomic data sets resulted in the grouping of 901 proteins into 14 modules containing between 63 and 183 proteins. The “biologic process” terms, as defined in the Gene Ontology project, that were most represented in each module are provided in Table 2A through 2N in Supplementary Appendix 3. Module 1 contained proteins enriched in Gene Ontology terms related to neutrophil activation, acute inflammation, and host immune responses against pathogens (Table S7A and S7B in Supplementary Appendix 2).

We performed singular value decomposition of the cross-correlation matrix between levels of

duodenal proteins and absolute levels of core group bacterial taxa to determine whether features of the duodenal proteome and duodenal microbiota were significantly correlated.²⁸ Figure S4 shows the 50 proteins with the most significant positive correlations and the 50 having the most significant negative correlations with the absolute levels of these core taxa. Of these 100 proteins, 22 were members of ICA Module 1 (hypergeometric test of enrichment, $P < 0.001$). Figure 1A and 1B summarize the top 10 positive correlations, including two antimicrobial peptides (cathelicidin antimicrobial peptide [CAMP] and chitinase-3-like protein 1 [CHI3L1]), plus an established fecal biomarker of intestinal inflammation, lipocalin-2 (LCN2). In addition, there was a negative correlation between the levels of these core taxa and the levels of 10 proteins produced by absorptive enterocytes (e.g., cadherin-related family member 5 [CDHR5], which is required for brush border formation)²⁹ (Fig. S4A and S4B).

There was a significantly negative correlation between the length-for-age z score and both the total bacterial load in the duodenum (the sum of the absolute levels of all organisms) and the absolute levels of 13 of the 14 core group taxa, including the 3 organisms that were most strongly correlated with duodenal inflammatory proteins in Module 1: a veillonella species, a streptococcus species, and *Rothia mucilaginosa* ($Q < 0.10$ after adjustment for false discovery rate) (Fig. 1C through 1F, Table 2, and Table S6B in Supplementary Appendix 2). (Details regarding duodenal proteins that were correlated with the length-for-age z score are provided in Table S3B in Supplementary Appendix 2.) Neither the total bacterial load nor the levels of any of these 13 taxa were significantly correlated with the severity of the histopathological features. PCR assays of 18 enteropathogens that are commonly observed in the study population²⁰ showed no significant correlation between their levels in duodenal aspirates and the length-for-age z score ($P > 0.10$ by Spearman's correlation) (Table S8A in Supplementary Appendix 2).

We expanded our analysis to include plasma samples and duodenal biopsy samples obtained from the 44 children from whom we could not obtain duodenal aspirates (i.e., for a total of 80 children) (Fig. S1). We correlated the levels of plasma proteins with the levels of 100 duodenal

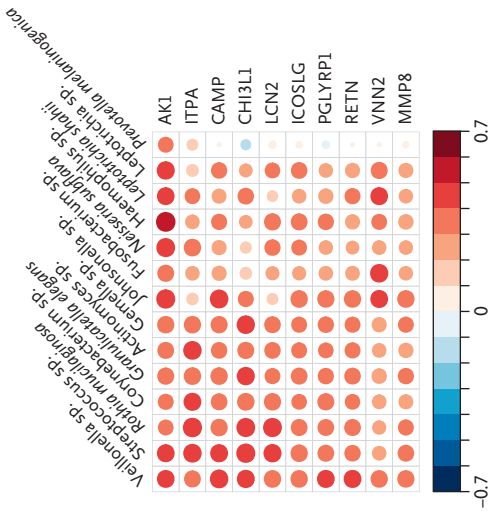
proteins that were most positively or negatively associated with the 14 core taxa (50 duodenal proteins in each direction). Plasma levels of regenerating family member 3 alpha (REG3A) had the strongest correlation ($P = 6.9 \times 10^{-6}$; $Q = 0.03$), followed by LCN2 ($Q = 0.09$) and 6-pyruvoyl tetrahydropterin synthase (PTS) ($Q = 0.10$), an enzyme involved in the production of tetrahydrobiopterin (BH_4 , also called sapropterin), a cofactor for several enzymes including those involved in the production of nitric oxide and several neurotransmitters. A summary of positive correlations between plasma levels of REG3A and the core taxa-associated duodenal proteins is provided in Figure 1G; the strongest correlation was with LCN2 expressed in the duodenum. (LCN2 is also expressed in other tissues, including the esophageal mucosa and minor salivary glands.) (Details regarding duodenal proteins that were correlated with plasma levels of PTS and LCN2 are provided in Figure S5).

In a comparison of levels of each of the 14 duodenal core taxa in fecal samples obtained from 27 healthy children and 48 children with EED, there was a significantly elevated level of veillonella species, the bacterium that was most positively correlated with proteins involved in duodenal inflammation in samples obtained from the children with EED ($P = 0.001$; $Q = 0.01$). This organism has also been associated with stunting in a study involving children living in Madagascar and the Central African Republic.¹⁵ An even greater difference could be appreciated when all 14 taxa were considered together ($P < 0.001$ by permutational multivariate analysis of variance) (Fig. S6). In children with EED and in healthy children, the most frequently detected pathogens in the fecal microbiota were *Escherichia coli* strains (EPEC, ETEC, and EAEC), along with giardia and campylobacter species. Although there were no significant between-group differences in the levels of any one enteropathogen ($P > 0.05$ by Mann-Whitney U test), a significantly greater diversity in pathogens was detected in children with EED than in healthy controls (mean number of unique pathogens, 4.6 ± 2.0 vs. 2.7 ± 1.4 ; $P = 0.002$ by Mann-Whitney U test) (Table S8B in Supplementary Appendix 2). Among the children with EED, there was no significant correlation between the length-for-age z score and either the number of different pathogens de-

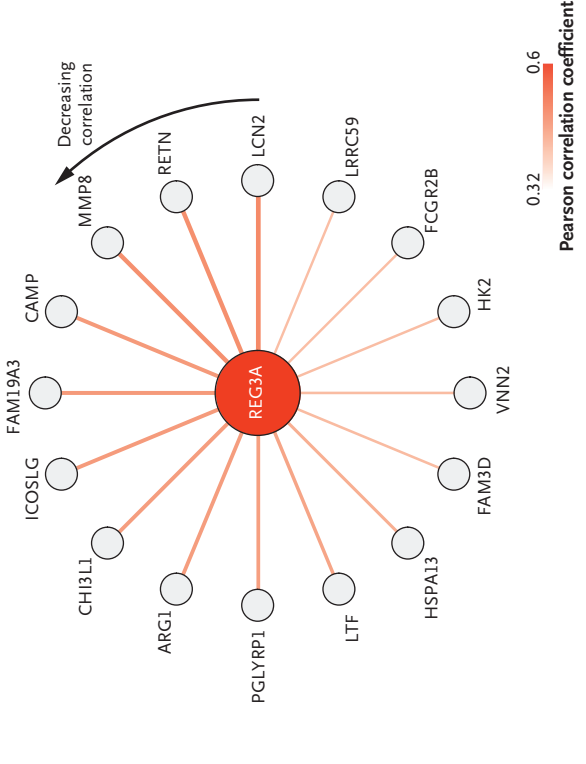
B Details of Proteins Listed in Panels A and G

Symbol	Name	Function
AK1	Adenylate kinase isoform 1	Regulation of adenine nucleotide balance; energy homeostasis
ARG1	Arginase 1	Marker for activated macrophages
CAMP	Cathelicidin antimicrobial peptide	Innate immune defense against bacteria
CHI3L1	Chitinase-3-like protein 1	Biomarker of inflammation in multiple disease contexts
FAM19A3	TFAA chemokine like family member 3	Chemokine-related protein
FAM3D	Cytokine-like protein EF-7	Neutrophil chemokine
FCGR2B	Fc gamma receptor II-B	Regulation of immunoglobulin-dependent phagocytosis
HK2	Hexokinase 2	Glucose metabolism; regulation of monocyte differentiation
HSPA13	Heat shock protein family A, member 13	Stress response and protein export
ICOSLG	Inducible costimulator ligand	Immune ligand involved in wound healing
ITPA	Inosine triphosphatase	Hydrolysis of noncanonical purine nucleotides
LCN2	Lipocalin-2	Proinflammatory cytokine up-regulated during intestinal inflammation
LRRCS9	Leucine-rich repeat containing 59	Regulation of toll-like receptor and interferon-gamma signaling
LTF	Lactotransferrin	Antimicrobial peptide; mucosal defense
MMP8	Matrix metalloproteinase 8	Neutrophil collagenase up-regulated in intestinal inflammation
PGLYRP1	Peptidoglycan recognition protein 1	Bactericidal proinflammatory cytokine
RETN	Resistin	Proinflammatory cytokine; induces insulin resistance
VNN2	Vascular noninflammatory molecule 2	Biotinidase involved in hematopoietic-cell trafficking

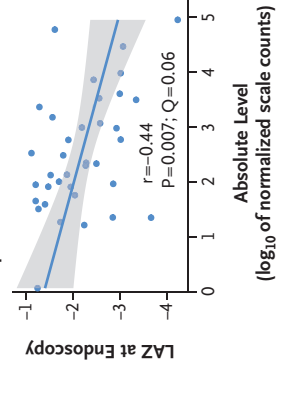
A Top 10 Correlations between 14 Core Bacterial Taxa and Duodenal Proteins



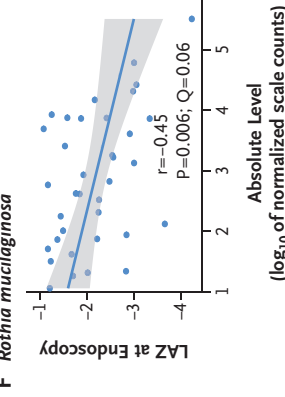
C Star Network of Correlations between Plasma Levels of REG3A and Core Taxa-Associated Duodenal Proteins



D Veillonella sp.



E Streptococcus sp.



F Rothia mucilaginosa

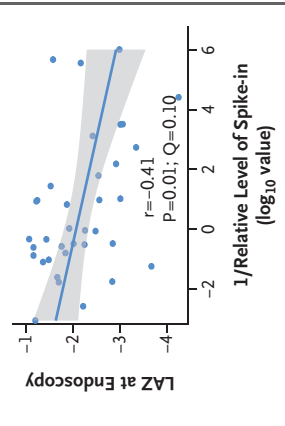


Figure 1 (facing page). Correlations between Components of the Duodenal Proteome, the Absolute Levels of Duodenal Bacterial Taxa, and Stunting.

Panel A shows the top 10 positive correlations between members of the 14 core taxa among the study children with environmental enteric dysfunction (EED) and duodenal proteins. A larger size and darker color of the circle represent a greater magnitude of the correlation. Negative correlations were included to match the color bar in Figure S4 in Supplementary Appendix 1. Panel B shows annotations of proteins that are indicated in Panels A and G. Panels C through F show the total duodenal bacterial load and levels of the three organisms that were most positively correlated with duodenal inflammatory proteins and that had a significantly negative correlation with the length-for-age z score (LAZ). A spike-in is a known amount of a bacterium that is not typically found in the human gut microbiota (*Alicyclobacillus acidiphilus*). The spike-in is added to each duodenal aspirate sample to determine the absolute levels of each bacterium as well as the total density of bacteria per volume of aspirate (i.e., the bacterial load). Absolute levels are calculated by normalizing the levels of each bacterium to the levels of the spike-in organism. The least-squares regression line is depicted in blue, and shaded regions denote 95% confidence intervals. The letter r indicates the Pearson correlation coefficient. Panel G shows a star network of correlations between plasma levels of REG3A (regenerating family member 3 alpha), a secretory protein that has been associated with cell proliferation or differentiation and that had the strongest correlation with the 14 core taxa, and each of the core taxa-associated duodenal proteins. As indicated by the color key, darker-colored lines connecting REG3A to the various proteins indicate a stronger correlation. The duodenal protein with the strongest correlation, lipocalin-2 (LCN2), is indicated by the tail of the arrow line at the 3 o'clock position; duodenal proteins with progressively weaker correlations with REG3A are distributed in a counterclockwise manner from this position.

tected in feces or the level of any single pathogen ($Q > 0.05$ by Spearman's correlation).

TRANSMISSION OF ENTEROPATHY TO MICE

After identifying an association between a shared group of bacterial taxa that were present in the duodenal microbiota of children with EED and a proteomic signature of duodenal inflammation and growth stunting, we evaluated whether these taxa were causally related to the pathogenesis of this enteropathy when modeled in a mouse. We first cultured 39 bacterial strains from duodenal aspirates, including 11 of the 14 core group taxa. (Functional annotations of the genomes of these taxa are provided in Figure S7 and also in Table 3 in Supplementary Appendix 3.)

This collection of microbes (EED collection) was administered by oral gavage to one group of mice; the control mice received cecal microbiota from a conventionally raised mouse (Fig. 2A). Both groups of mice were fed an animal-protein-deficient diet representing the typical diet of 18-month-old children living in Mirpur (Table S9). We detected 23 of the 39 bacterial strains at a mean relative level of more than 0.01% at one or more locations along the gastrointestinal tract in mice that received the EED collection (Fig. 2B, Fig. S8, and Table S10 in Supplementary Appendix 2). Of these 23 strains, 9 corresponded to core duodenal bacterial amplicon sequence variants (including the veillonella species) that had a negative correlation with length-for-age z scores.

Unlike the control mice, the mice that received the EED collection had an inflammatory infiltrate in the lamina propria of the small intestine that was dominated by mononuclear cells (including lymphocytes), with associated disruption of the overlying epithelium and sloughing of epithelial cells from the upper portion of villi (Fig. 2C, 2D, and 2E). Crypt elongation, a regenerative response to epithelial damage, was the most consistent feature of architectural distortion (Fig. 2F). These histopathological changes occurred in a patchy distribution along the length of the small intestine, whereas the colon was spared. Consistent with the innate immune response that has been documented in children with EED, levels of messenger RNA (mRNA) transcripts encoding the bactericidal C-type lectins Reg3 β and Reg3 γ were higher in the duodenum of mice that received the EED collection than in control mice that received a normal mouse microbiota (Fig. 2G and Table S11 in Supplementary Appendix 2; Table S12 shows the characterization of small-intestine immune responses based on flow cytometry). Levels of matrix metalloproteinase 8 (MMP8), a protein with levels that are correlated with the absolute levels of duodenal bacterial taxa in children with EED, were significantly higher in serum samples and along the length of the small intestine of mice that received the EED collection than in control mice (Fig. 2H). The observed disruption of the small intestinal epithelial barrier is consistent with the relatively decreased levels of mRNA transcripts encoding seven intercellular tight junction components (e.g., zonula occludens, claudin 4, and adherens junction formation fac-

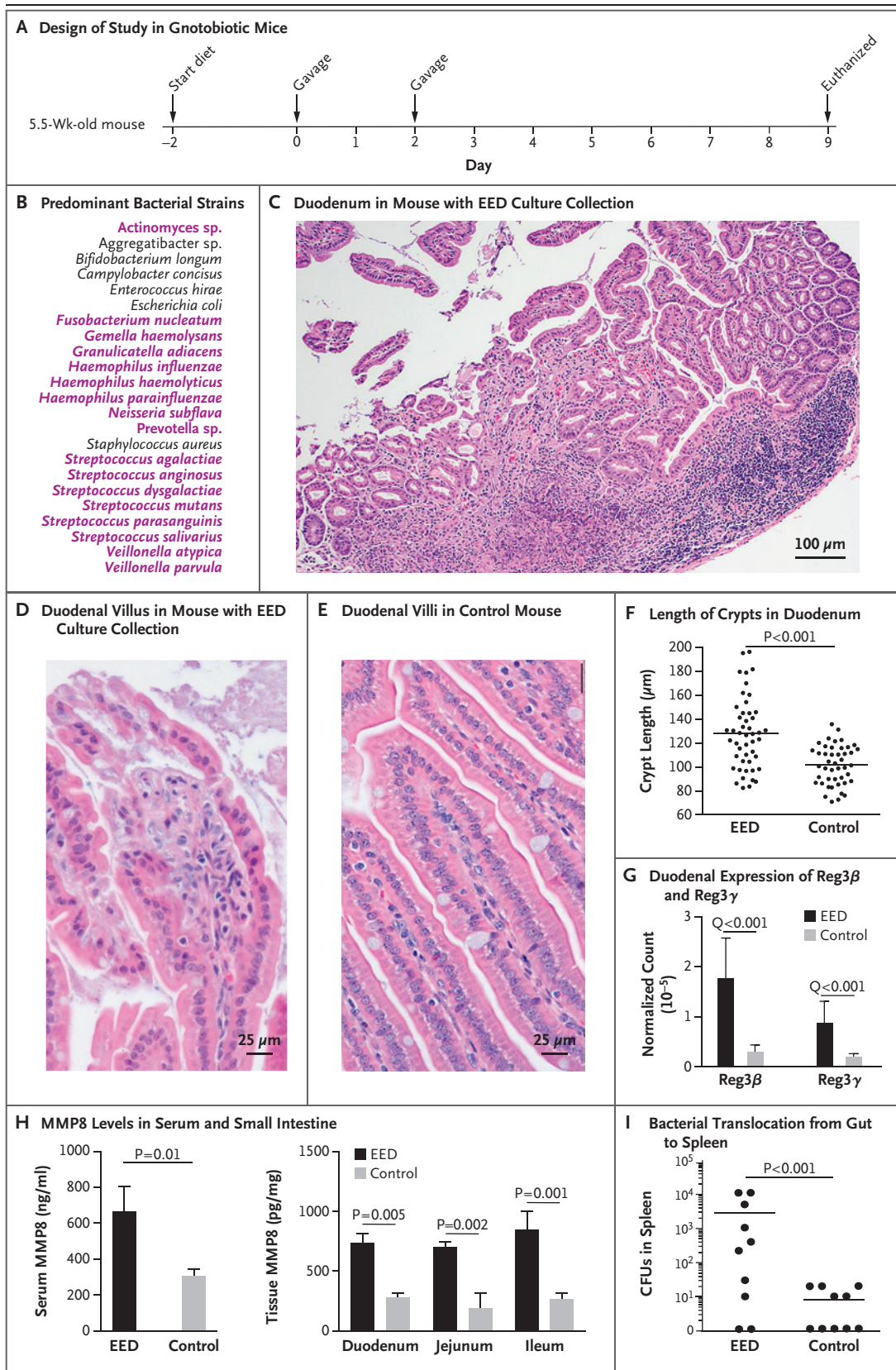


Figure 2 (facing page). Transmission of Enteropathy to Gnotobiotic Mice by Duodenal Bacteria Obtained from Study Children with Environmental Enteric Dysfunction (EED).

Panel A shows the design of the experiment in which gnotobiotic mice received either a collection of 39 bacterial strains cultured from duodenal samples obtained from the study children with histopathological evidence of EED (EED collection) or cecal microbiota of a conventionally raised mouse (control collection), all administered by oral gavage. Four of the mice that received the EED collection became moribund 5 days after the second gavage and were euthanized. At this time point, these mice had lost 20% of their starting body weight, as compared with a loss of 4.7% for all other mice that received the EED collection and a loss of 4.9% in the control group. Panel B shows 23 bacterial strains that colonized mice in the EED group at a relative level of more than 0.01% at one or more locations along the intestine. (The biogeographic features of colonization are provided in Figure S8 in Supplementary Appendix 1.) The 17 strains belonging to the 14 core taxa that were detected at the genus level in the duodenums of children with EED are highlighted in purple. Panels C, D, and E show representative histopathological changes in the duodenal epithelium and lamina propria of mice colonized with the EED collection (Panels C and D, respectively) as compared with control mice (Panel E) (hematoxylin and eosin staining). (Details regarding flow cytometric analysis of immune-cell populations are provided in Table S12 in Supplementary Appendix 2.) Panel F shows the quantification of crypt length in the proximal 3 cm of the small intestine in the two groups. The 10 best-oriented crypts were measured in five mice in each study group. Each dot represents one measurement; the horizontal lines denote the mean value. Panel G shows the differential expression of duodenal *Reg3β* and *Reg3γ* messenger RNAs, as determined by means of DESeq2 ($Q < 0.001$ for both comparisons after adjustment for false discovery rate). The numbers in the y axis are shown at 0.00001 of the actual counts. Panel H shows levels of matrix metalloproteinase 8 (MMP8) in the serum and small intestine of the study mice. Panel I shows the number of colony-forming units (CFUs) of bacteria translocated from the gut to the spleen in the two study groups. Each dot represents a splenic homogenate from an individual mouse in two independent experiments involving five mice per experiment; the horizontal line indicates the mean value. All the translocated bacteria that were recovered from mice that received the EED collection were identified as *Escherichia coli* (lacking the virulence-associated markers of diarrheagenic strains) and *Enterococcus hirae*. Rare *Enterococcus faecalis*, *Acinetobacter lwoffii*, and *A. radioresistens* were recovered from the spleens of the control mice.

tor) in the duodenums of mice that received the EED collection (Table S11 in Supplementary Appendix 2). Moreover, there was bacterial translocation into the systemic circulation, as evidenced

by recovery of viable *E. coli* and *Enterococcus hirae* in the spleens of mice that received the EED collection but not in the spleens of the controls (Fig 2I). Together, these results support a causal relationship between bacteria cultured from the duodenums of children with EED and the pathogenesis of enteropathy.

DISCUSSION

Our previous work to characterize undernourished Bangladeshi children focused on those with acute malnutrition and revealed a causal relationship between the impaired development of their fecal microbial community and the occurrence of wasting.^{12,13} The BEED study provided an opportunity to assess the contribution of the proximal small intestinal microbiota to stunting in an undernourished Bangladeshi pediatric population who had no benefit from a nutritional intervention. Endoscopy allowed us to establish a diagnosis of EED on the basis of characteristic histopathological changes in biopsy specimens of duodenal mucosa. The lack of correlation between the histopathological scoring system used here and the length-for-age z score is consistent with the findings of other studies that used more elaborate scoring methods.³⁰ Follow-up analyses of biospecimens uncovered a strong correlation between the absolute levels of a group of 14 duodenal bacterial taxa and the degree of stunting in these children and identified duodenal proteins with levels that corresponded to the absolute levels of these bacterial taxa. Plasma proteins (e.g., REG3A and LCN2) with levels that correlate with features of the duodenal proteome (e.g., MMP8) together with the levels of these 14 duodenal taxa in feces represent candidate biomarkers of EED. A gnotobiotic mouse model provided support for a causal relationship between the duodenal microbiota and enteropathy.

Further studies are needed to determine whether our findings generalize to other populations of children with stunting. The microbial community of the small intestine remains a “terra incognita,” and its relationship to the pathogenesis of gut barrier dysfunction, enteropathy, and stunting has been largely unexplored. Our results emphasize the need for techniques for performing imaging and sampling of the small intestine that are less invasive than those

currently available in routine practice.³¹ Ultimately, such methods could allow one limitation of the current study — the absence of information about the duodenal microbiota of healthy children — to be surmounted. Comparing the oral microbiota of children with EED with those of healthy children may also be informative. The possibility that a healthy child's duodenal or even fecal microbiota may contain factors that ameliorate or prevent the development of EED has therapeutic implications.

The findings from our gnotobiotic mouse model, which provided support for a causal relationship between the microbiota in the proximal small intestine and the pathological features of EED, suggest new opportunities for investigation. These avenues include further dissection of interactions between members of the collection of bacterial strains associated with enteropathy and defining factors that negatively affect their fitness, a goal that may be facilitated by *in silico* metabolic reconstructions of their nutrient requirements (Fig. S7 and Table 3 in Supplementary Appendix 3). Exploration of the mechanisms by which members of the small intestinal microbiota affect the mediators of linear growth of young children may result in the development of new therapeutic approaches for stunting.

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APPENDIX

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