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Mara Zambruni
The University of Texas

Theresa J Ochoa
The University of Texas

Anoma Somasunderam
The University of Texas

Miguel M Cabada
University of Texas Medical Branch

Maria L Morales
Universidad Peruana Cayetano Heredia

See next page for additional authors

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Recommended Citation

Zambruni, Mara; Ochoa, Theresa J; Somasunderam, Anoma; Cabada, Miguel M; Morales, Maria L; Mitreva, Makedonka; Rosa, Bruce A; Acosta, Gonzalo J; Vigo, Natalia I; Riveros, Maribel; Arango, Sara; Durand, David; Berends, Maitreyee N; Melby, Peter; and Utay, Netanya S, "Stunting is preceded by intestinal mucosal damage and microbiome changes and Is associated with systemic inflammation in a cohort of Peruvian infants." *The American of Tropical Medicine and Hygiene*. 101, 5. 1009 - 1017. (2019).
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Authors

Mara Zambruni, Theresa J Ochoa, Anoma Somasunderam, Miguel M Cabada, Maria L Morales, Makedonka Mitreva, Bruce A Rosa, Gonzalo J Acosta, Natalia I Vigo, Maribel Riveros, Sara Arango, David Durand, Maitreyee N Berends, Peter Melby, and Netanya S Utay

Stunting Is Preceded by Intestinal Mucosal Damage and Microbiome Changes and Is Associated with Systemic Inflammation in a Cohort of Peruvian Infants

Mara Zambruni,¹ Theresa J. Ochoa,^{1,2} Anoma Somasunderam,³ Miguel M. Cabada,^{4,5} Maria L. Morales,⁴ Makedonka Mitreva,^{6,7} Bruce A. Rosa,⁶ Gonzalo J. Acosta,² Natalia I. Vigo,² Maribel Riveros,² Sara Arango,² David Durand,² Maitreyee N. Berends,⁵ Peter Melby,⁵ and Netanya S. Utay^{3*}

¹Department of Pediatrics, The University of Texas Health Science Center at Houston Medical School, Houston, Texas; ²Instituto de Medicina Tropical “Alexander von Humboldt,” Universidad Peruana Cayetano Heredia, Lima, Peru; ³Division of Infectious Diseases, The University of Texas Health Science Center at Houston Medical School, Houston, Texas; ⁴Universidad Peruana Cayetano Heredia–University of Texas Medical Branch Collaborative Research Center Cusco, Universidad Peruana Cayetano Heredia, Cusco, Peru; ⁵Infectious Diseases Division, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas; ⁶The McDonnell Genome Institute, Washington University in St. Louis, St. Louis, Missouri; ⁷Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri

Abstract. Stunting, defined as height-for-age Z score equal to or lower than -2 , is associated with increased childhood mortality, cognitive impairment, and chronic diseases. The aim of the study was to investigate the relationship between linear growth, intestinal damage, and systemic inflammation in infants at risk of stunting. We followed up 78 infants aged 5–12 months living in rural areas of Peru for 6 months. Blood samples for biomarkers of intestinal damage (intestinal fatty-acid-binding protein [I-FABP] and zonulin) and systemic inflammation (interleukin- 1β , interleukin-6, tumor necrosis factor α [TNF- α], soluble CD14, and lipopolysaccharide-binding protein [LBP]) and fecal samples for microbiome analysis were collected at baseline and closure of the study. The children’s growth and health status were monitored through biweekly home visits by trained staff. Twenty-one percent of the children became stunted: compared with non-stunted children, they had worse nutritional parameters and higher levels of serum I-FABP at baseline. The likelihood of becoming stunted was strongly associated with an increase in sCD14 over time; LBP and TNF- α showed a trend toward increase in stunted children but not in controls. The fecal microbiota composition of stunted children had an increased beta diversity compared with that of healthy controls throughout the study. The relative abundance of *Ruminococcus 1* and 2, *Clostridium sensu stricto*, and *Collinsella* increased in children becoming stunted but not in controls, whereas *Providencia* abundance decreased. In conclusion, stunting in our population was preceded by an increase in markers of enterocyte turnover and differences in the fecal microbiota and was associated with increasing levels of systemic inflammation markers.

INTRODUCTION

Worldwide, 155 million children are stunted, meaning that their height or length is less than two SDs from the median value expected for age and gender.¹ Childhood stunting is associated with increased childhood mortality, cognitive impairment, and a higher risk of chronic diseases later in life² and, therefore, represents a serious public health concern. The suboptimal linear growth may be in part explained by a chronically inadequate diet. In fact, most stunted individuals come from food-insecure households.³ However, interventions solely focused on improving nutrition fail to restore their normal trajectory of growth.⁴ An alternative or additional cause may be chronic systemic inflammation as suggested by multiple studies showing that stunted children have an increased level of blood inflammation markers.^{5–8} The origin of this inflammatory status could be a sustained, aberrant activation of the mucosal immune system. Indeed, there is an established association between stunting and the environmental enteric disorder (EED), a small-intestine enteropathy highly prevalent in low-resource countries and characterized by mucosal inflammation, small intestine villi flattening, and increased intestinal permeability.^{9–14} The triggers for this disorder are probably inadequate diet, continuous exposure to environmental contaminants, and/or an alteration of the normal commensal flora.

Here, we present the results of a study investigating the relationship between linear growth, markers of intestinal damage, and systemic inflammation among infants in rural Peru.

METHODS

Study design and description of the setting. The study was approved by the Institutional Review Boards of the University of Texas (UT) Health Science Center at Houston, UT Medical Branch in Galveston, Texas, and the Universidad Peruana Cayetano Heredia in Lima, Peru. The Ministry of Health Authorities of Moyobamba and Urubamba endorsed the study.

This was a pilot prospective study. Infants from two communities of rural Peru were enrolled and followed up for 6 months between December 2014 and May 2015, during the summer/raining season. The research sites were situated in the Urubamba district of the Cusco region, in the Peruvian Andes highlands, and in the Moyobamba district of San Martin region, in the Northeastern part of the Peruvian Amazon. According to a 2014 survey (Instituto Nacional de Estadística e Informática, 2015), the prevalence of stunting among children aged less than 5 years in the two regions was 18% and 16%, respectively.

Subjects. Infants aged 5–12 months were identified using local health centers’ databases. Their families were home-visited by the research team, and written consent was obtained from both parents. Children were eligible if they had no history of prematurity, severe chronic illness, or previously diagnosed failure to thrive; they had a length-for-age Z (LAZ) score greater than -2 at enrollment; and their families agreed

* Address correspondence to Netanya S. Utay, Division of Infectious Diseases, The University of Texas Health Science Center at Houston Medical School, 6431 Fannin St., MSB 1.122, Houston, TX 77030. E-mail: netanya.s.utay@uth.tmc.edu

on close follow-up. Patients could withdraw from the study at any time.

At the end of the study, we classified children as “stunted” if their last LAZ score at the end of the follow-up was equal to or lower than -2 , whereas we defined “controls” as children who had a last LAZ score above -2 .

Household characteristics and nutritional surveys.

Caregivers were interviewed on enrollment using a structured questionnaire to gather information regarding family composition and household characteristics. Nutritional surveys were conducted at the beginning and closure of the study and aimed at collecting information regarding duration and pattern of breastfeeding and diet characteristics in the first 2 years of life. A diet diversity score (DDS) was calculated for each child as per international guidelines.¹⁵

Growth monitoring. Participants’ weight and length were measured at enrollment and monthly thereafter by trained staff using a calibrated hanging scale and a length board. Weight-for-age Z (WAZ), LAZ, and weight-for-height Z scores were calculated using WHO Anthro software, version 3.2.2 (World Health Organization, Geneva, Switzerland).

Health status monitoring. Children were home-visited every 2 weeks by a trained nurse. At each encounter, information regarding intercurrent episodes of acute illness and use of antibiotics, as recalled by caregivers, was gathered.

Specimen collection. The study design included collection of blood samples for the measurement of markers of mucosal damage and systemic inflammation and a fecal sample for microbiota composition at baseline and the end of follow-up. Blood samples were collected by venipuncture at the research center, left to coagulate, and centrifuged to remove the serum within 5 hours of blood collection. Stool samples for microbiome analysis were collected on the same day of blood samples and conserved on ice for a maximum of 4 hours. Serum and stool samples were then frozen in dry shippers and sent to the reference laboratory where they were stored at -70°C until analysis.

Measurement of serum biomarkers. Serum-soluble CD14 (sCD14) was assessed using the human magnetic Luminex screening assay and interleukin- 1β (IL- 1β), IL-6, and tumor necrosis factor alpha (TNF- α) using the human magnetic Luminex performance assay (both R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. Lipopolysaccharide-binding protein (LBP), intestinal fatty-acid-binding protein (I-FABP), and zonulin were measured using commercially available ELISA kits (Abnova, Taipei City, Taiwan; R&D Systems; and IDK[®] Zonulin ELISA kit, Bensheim, Germany, respectively) according to the manufacturer’s protocol.

Microbiome analysis. *Microbial DNA extraction, 16S rRNA gene amplification, and deep-sequencing.* Bacterial genomic DNA was extracted from human feces using the MoBio PowerMag Microbiome DNA/RNA isolation kit (Carlsbad, CA) following the manufacturer’s instructions, followed by amplification of the V4 regions of the 16S rRNA gene using individually barcoded universal primers containing linker adapters for Illumina sequencing. Sequencing was performed using a MiSeq system (Illumina Inc., San Diego, CA).

16S data analysis. Sequence processing and analysis were performed using specific software for comparison and analysis of microbial communities (QIIME and mothur). Pair-end reads of length 2×250 bp were demultiplexed and quality-filtered according to the following parameters: a required

minimum average quality score of 35 over a 50-bp sliding window, no homopolymer longer than 6 bp, no ambiguous bases allowed, two primer mismatches allowed, and one barcode mismatch allowed. Sequences were clustered and binned into operational taxonomic units (OTUs) (based on 97% identity), and singleton reads were removed from the dataset. The resulting OTU table was subsampled to the smallest number of reads associated with any one sample.

Both the DNA extraction and 16S data analysis were carried out at the Alkek Center for Metagenomics and Microbiome Research, Baylor College of Medicine, Houston, Texas.

Statistical analysis. Continuous data distribution was expressed in terms of medians and interquartile range. A Chi-square test was used to compare categorical data. For each biomarker, samples with levels below the lower limit of detection were given an arbitrary value equal to one-half of the value of the lowest measurable level. Similarly, samples with levels above the upper limit of detection were set as twice the value of the highest measured level. A Mann-Whitney U test was used to compare the relative distribution of continuous variables, unless otherwise specified. Spearman rank correlation coefficient was used to measure the correlation between continuous variables. Cytokine concentration values were log $_{10}$ -transformed for regression analysis.

The proportional change in serum biomarkers, measured as the difference between the baseline and end-of-follow-up concentration divided by the baseline concentration, was used as a measure of variation of the biomarkers over time.

Multiple logistic regression models were used to measure the association between stunting and the change of each biomarker during the follow-up period, after controlling for age and LAZ score at enrollment.

For the microbiome analysis, we compared children who became stunted at the end of the study ($N = 16$) with a restricted group of children with an LAZ score above -1.5 at both enrollment and end of follow-up ($N = 36$), as this classification allowed for the best discrimination of groups (Supplemental Figure 2).

Pairwise Bray–Curtis diversity values were calculated between each sample pair based on their overall microbiome profiles, and groups of samples were compared using an unpaired two-tailed t -test with unequal variance (data were normally distributed; Shapiro–Wilk value of 0.96). The linear discriminant analysis effect size (LEfSe) method¹⁶ was used for differential taxa abundance testing, using default recommended settings according to the authors’ instructions, and an adjusted $P \leq 0.05$ and an linear discriminant analysis (LDA) effect size > 2 for significance. The LEfSe algorithm performs class comparison tests, validates for biological consistency, and is able to consider all taxonomic levels for comparison simultaneously. Taxa were classified according to the adjusted P -values for significance.

STATA version 12 (StataCorp., College Station, TX), GraphPad Prism version 6 (La Jolla, CA), MS Excel 2016 (Microsoft Corp., Redmond, WA), and a Galaxy server (LEfSe) were used for the analysis.

RESULTS

Participants. Seventy-eight children were enrolled in the study. Three children were withdrawn from the study when the parents moved away. The baseline characteristics of the children are reported in Table 1. Individuals from the two sites were similar in terms of age, gender distribution,

TABLE 1
Characteristics of the children at baseline, by research site

	Urubamba (n = 38)	Moyobamba (n = 40)	P-value
Median age at enrollment (months) (25th, 75th)	9 (8, 10)	8.5 (7–12)	0.9†
Female, n (%)	15 (47)	17 (53)	0.8‡
Birth weight* (g) (25th, 75th)	3,346 (3160, 3532)	3,310 (3,000, 3,770)	0.9†
Length at birth* (cm) (25th, 75th)	49.5 (48, 51)	49.5 (48–50)	0.9†
Breastfeeding, any duration (months), n (%)	38 (100)	36 (90)	0.04‡
Length of exclusive breastfeeding (months) (25th, 75th)	6 (3, 6)	6 (3, 6)	0.9†
Age at introduction of solid food (months) (25th, 75th)	6 (6, 6)	6 (6, 6)	0.1†
Diet diversity score (25th, 75th)	4.5 (4, 5)	4 (3, 5)	0.9†
Consumption of animal foods at least 1/day, n (%)	30 (80)	26 (65)	0.2‡
Household characteristics			
Maternal age (years) (25th, 75th)	26.5 (20–32)	26.5 (22–34)	0.6†
Level of maternal education, n (%)			
None	0 (0)	1 (2.6)	
Grammar school	1 (2.6)	15 (39.5)	
High school	24 (63.1)	17 (44.7)	
Technical school or university	13 (34.2)	5 (13.2)	< 0.001‡
Median paternal age (years) (25th, 75th)	28.5 (25–38)	32.5 (30–40)	0.07
No. of people living in the house (25th, 75th)	4 (3–5)	5 (4–6)	0.05†
Households with indoor piped water provision, n (%)	38 (100)	19 (47.5)	< 0.001‡
Households with a refrigerator, n (%)	19 (50)	26 (65)	0.1‡
Households connected to public sewage, n (%)	32 (84.2)	17 (42.5)	0.001‡
Weekly average expense for food provision (nuevo sol) (25th, 75th)	140 (100, 150)	50 (40, 50)	< 0.001†

* Information derived from child's growth cards.

† Mann-Whitney U-test.

‡ Chi-square test.

anthropometric data, and diet habits, although children from Moyobamba came from lower socioeconomic status.

Health status and growth trajectories. Every child enrolled in the study received a median of two visits per month. Overall, the prevalence of acute infections during the 6-month follow-up was low: we observed a median (IQR) of one (0–2) episode of diarrhea (> 3 loose or watery stools in 24 hours) per child in Urubamba and 0 (0–1) episode in Moyobamba ($P = 0.05$) and two^{1,2} episodes of upper respiratory tract infections (nasal congestion, sore throat, or cough with or without fever > 1 day) in Urubamba and 1 (0–2) in Moyobamba ($P = 0.03$); no other episodes of acute illness or hospitalizations were reported. No child in either site received more than one course of antibiotics during the 6 months of follow-up. We did not observe differences in the incidence of acute infections between children who became stunted and controls.

Sixty-one of 78 children (79%) presented a decline of their LAZ scores during the study (Figure 1). By the end of the

follow-up, 16/75 (21%) children met the criteria for definition of stunting (LAZ < -2). These children were slightly older at enrollment (median age 10.5 months versus 8 months, $P = 0.01$), had lower baseline WAZ scores (-0.82 versus 0.1, $P = 0.0002$), had lower baseline LAZ scores (-1.54 versus -0.42, $P < 0.0001$), and showed a larger decrease in LAZ score over time than controls at the end of the follow-up (-1.11 versus -0.29, $P = 0.01$) (Figures 1 and 2). Based on their birth records, the birth weight and length of children who became stunted were not significantly lower than those of children who did not become stunted (Supplemental Table 1).

No differences were observed between stunted children and controls in terms of household characteristics or dietary patterns, including breastfeeding duration, average (DDS¹⁵), daily consumption of animal food, or weekly household expenditure for food supply (Supplemental Table 1). However, given the small sample size, nonstatistically significant differences should be interpreted with caution.

FIGURE 1. Changes in anthropometric measures during follow-up. (A) Median changes in weight-for-age, length-for-age, and weight-for-length Z scores during follow-up (75 children) and (B) median change in length-for-age Z score: stunted children (16 children) vs. non-stunted (59 children). ** $P < 0.001$, Mann-Whitney U-test.

FIGURE 2. Monthly changes in length-for-age Z (LAZ) score, stunted children ($N = 16$) vs. non-stunted ($N = 59$) children. The children who developed stunting at the end of the follow-up had a lower LAZ score at enrollment and at each time point during the follow-up (each point indicates the median value per group; error bars indicate 95% CI). At the end of the follow-up, the median decrease in LAZ score was -1.1 for children meeting the criteria for stunting vs. -0.29 for non-stunted children ($P = 0.01$).

Three children were lost to follow-up between the 4th and 5th months of follow-up. Based on their last available measurement, one had become stunted by the time he left the study and the other two had shown a decrease in their LAZ score.

Markers of enteropathy. Sixty-six of 78 children enrolled had blood samples collected at baseline and the end of the follow-up; the following results refer to this subset of participants. The remaining eight children had only one sample of blood collected, either because the parents could not come on the days set for the blood draw or because they refused a second venipuncture after the first attempt failed.

Intestinal fatty-acid-binding protein is a protein found in the cytosol of intestinal epithelial cells and released into the bloodstream on cell death.¹⁷ The I-FABP concentrations were 112 pg/mL to 14,959 pg/mL, values much higher than those reported for healthy children in industrialized countries, 20 to 200 pg/mL.¹⁸ In our cohort, only 2/142 serum samples had a concentration below 200 pg/mL.

Intestinal fatty-acid-binding protein baseline levels were significantly higher in the group of children who later became stunted (2252 pg/mL versus 1,448 pg/mL, $P = 0.005$), whereas there was no difference between the stunted and control subjects at the end of the study, as I-FABP concentration increased in the latter group (Supplemental Table 2). The I-FABP concentration change over time, however, was not statistically significant between stunted and control groups (Figure 3A, Supplemental Figure 1).

Zonulin is a protein secreted by viable gut epithelial cells and is an important modulator of tight junctions.¹⁹ The concentration of this marker in our cohort and its change over time were not significantly different between stunted and control children (Figure 3B, Supplemental Table 2).

Proinflammatory cytokines, sCD14, and LBP. Interleukin-6, IL-1 β , and TNF- α are mediators of the acute phase response and have been implicated in growth delay of children with chronic inflammatory diseases.²⁰ Stunted and control children had comparable values of serum IL-1 β and IL-6 at both time points (Figure 3C–E, Supplemental Table 2, Supplemental Figure 1). Serum TNF- α showed a tendency to increase more over time in children who became stunted

(proportional change 0.05 versus -0.15 , $P = 0.10$), although the result was statistically significant only when we conducted the analysis using stricter criteria for controls (only children who had an LAZ score > 1.5 both at enrollment and closure of the study; Supplemental Figure 1).

Soluble CD14, which is shed by monocytes after stimulation by bacterial lipopolysaccharide (LPS), augments macrophage and neutrophil response to endotoxin. The serum concentration of this marker is elevated in patients with enteropathy and endotoxemia.^{21–23} Stunted children and controls had similar levels of sCD14 at baseline, but by the end of the study stunted children had significantly higher concentrations (median 2.01×10^6 pg/mL versus 1.78×10^6 pg/mL, $P < 0.01$; Figure 3F, Supplemental Table 2).

Lipopolysaccharide-binding protein is a type-1 acute phase protein involved in the systemic response to LPS.²⁴ Lipopolysaccharide-binding protein levels were similar between stunted children and controls at baseline and showed a tendency to increase among stunted children, although the change was not statistically significant (0.46 versus -0.14 , $P = 0.07$; Figure 3G, Supplemental Table 2, Supplemental Figure 1). The change in concentrations of sCD14 and LBP was strongly correlated ($r = 0.76$, $P < 0.0001$; Figure 3H).

Associations between linear growth and biomarkers. Among stunted children, the baseline log-transformed concentration of I-FABP was strongly and inversely correlated with change in LAZ scores over the following months ($r = -0.78$, $P < 0.01$; Figure 4A). Conversely, among controls, the baseline log-transformed concentration of I-FABP was positively correlated with the change in LAZ scores ($r = 0.67$, $P = 0.04$; Figure 4B). Among stunted children, the log-transformed concentration of I-FABP at the end of the follow-up showed a modest inverse correlation with change in LAZ scores, but this was only a statistical trend (Figure 4C). There was no correlation between the log-transformed concentration of I-FABP at the end of the follow-up and change in LAZ scores in the control children (Figure 4D).

A binary logistic analysis showed that the odd of becoming stunted was significantly associated with older age at enrollment (OR 1.4, $P = 0.03$), a lower length-for-age Z score (LZA) at enrollment (OR 0.08, $P = 0.002$), and an increase in sCD14 concentration over time (OR 9.7, $P = 0.01$). No other statistically significant associations were noted between stunting at the end of the follow-up and change in markers, although there was a trend toward increased odd of becoming stunted with increasing levels of LBP (OR 5.4, $P = 0.08$). In a multiple logistic analysis, the odds of becoming stunted were still significantly associated with the increase in sCD14 level over time even after controlling for age and HAZ score at enrollment (OR 8.7, $P = 0.03$).

Fecal microbiome. Seventy-five children had two sets of stool samples available for microbiome studies, but, as described in the Methods section, we only compared the fecal microbiome of the children stunted at the end of the follow-up ($N = 16$) with the microbiome of children whose LAZ score was above -1.5 throughout the study ($N = 36$). For this analysis, we compared children who met the criteria for stunting at the end of the follow-up (LAZ < -2) with children with an LAZ score greater than -1.5 at both enrollment and end of the follow-up, as this classification allowed for the best discrimination of data (Supplemental Figure 2).

The alpha diversity (within-sample diversity) of the fecal microbiota, measured as the Shannon index, increased with

FIGURE 3. Proportional changes in blood levels of seven serum biomarkers during the 6-month follow-up, stunted children ($N = 16$) vs. non-stunted ($N = 59$) children. Graphs A–F report medians with the 25th–75th percentile (Mann–Whitney U-test). Graph H shows the correlation between changes in sCD14 and LBP over time (Spearman rank correlation).

age in both cases and control. We did not observe differences based on gender, site of residence (highlands versus jungle), or nutritional status (data not shown), but the study was not powered to show differences between these subgroups.

With regard to beta diversity (between-sample diversity), Bray–Curtis analysis (Figure 5) showed that 1) the average beta diversity of intestinal microbiota was significantly different between children developing stunting and controls at both baseline and end of follow-up ($P = 0.047$ and $P = 0.0015$, respectively); 2) control children had a more heterogeneous microbiota composition at enrollment, but heterogeneity decreased over time ($P = 0.021$); 3) children who developed stunting had a less heterogeneous composition at baseline, but there was a change toward more diversity ($P = 0.0043$) at the end of follow-up (Figure 5).

The relative abundance of bacterial taxa in the gut microbial communities was assessed using the previously described LEfSe method. As expected, during the follow-up, we observed a reduction of taxa belonging to the phylum Actinobacteria (Bifidobacteriaceae) and Proteobacteria

(Enterobacteriaceae) and an increase in taxa belonging to Firmicutes (members of Lachnospiraceae family, such as *Roseburia*, *Blautia*, and *Pseudobutyrvibrio*, and Ruminococcaceae family) and Bacteroidetes in both groups. However, when we compared the fecal microbiome of stunted children versus controls, we observed some differences. The relative abundance of some specific genera belonging to the families of Ruminococcaceae (*Ruminococcus 1* and 2) and Coriobacteriaceae (*Collinsella*) increased over time in the children who became stunted but not in children with normal growth. Conversely, in these children, there was a decrease in the relative abundance of one genus of Enterobacteriaceae (*Providencia*) which was not observed in the microbiome of controls (Table 2 and Supplemental Figure 3).

DISCUSSION

In this pilot study conducted among Peruvian infants living in rural regions, we found that 1) during a 6-month observation period, 79% of the children showed a decrease in their LAZ

FIGURE 4. Correlation between intestinal fatty-acid-binding protein (I-FABP) levels and change in length-for-age Z scores (LAZ) in stunted children (16) vs. non-stunted children (59). The concentration of serum I-FABP was inversely correlated with changes in LAZ scores among stunted children, whereas it seemed to have a weak positive correlation with LAZ score change in children who did not become stunted. r = Spearman correlation coefficient.

score and 21% developed clinical stunting; 2) almost all participants had higher than expected levels of I-FABP; 3) an increase in sCD14 was associated with becoming stunted; and 4) the fecal microbiome of stunting children followed a different trajectory compared with controls.

This observational study confirms that stunted growth remains a widespread problem, even in countries like Peru where acute malnutrition is becoming rare. If we consider that stunting may be the most evident feature of a complex syndrome associated with long-lasting metabolic, immunological, and cognitive changes, the public health relevance of this phenomenon appears evident.

In our cohort, we did not observe differences in household wealth, diet, or frequency of acute infections between stunted and control children, but we did note interesting differences in the serum concentrations of markers of intestinal cell turnover and innate immune system activation.

In two repeated occasions, the young infants living in Uru-bamba and Moyobamba showed levels of serum I-FABP well

above the reference values.^{22,25,26} Another study conducted in Zimbabwe on a birth cohort of children living in impoverished conditions reported comparable data.⁶ Intestinal fatty-acid-binding protein is almost solely expressed in the enterocytes of the small intestine and it is more concentrated in the cells at the top of the villi.¹⁷ Studies conducted in patients with celiac disease (a disorder sharing many of the histological and functional features of EED) showed that this marker correlates with disease activity and the extent of villi flattening.^{18,26} In the few infants included in celiac disease studies, serum I-FABP levels were extremely high at the time of diagnosis but rapidly normalized after gluten removal, probably reflecting not only the exquisite vulnerability of a fast-growing mucosa to external insults but also the potential for rapid healing.

The relationship between I-FABP levels and linear growth in cases and controls was contradictory but may be explained by considering this as a marker of intestinal functional reserve rather than just enterocyte death.²⁷ Children living in low-resource, unhygienic settings are continuously exposed to a vast array of intestinal pathogens,^{28–31} and it is conceivable that the increased levels of I-FABP represent the accelerated turnover of a rapidly growing mucosa subjected to ongoing damage and forced to continuously regenerate itself. In the presence of a reduced availability of nutrients however, the rate of mucosal damage may exceed the healing potential of the host, leading to a vicious cycle of reduction of the enterocyte mass, further compromise of nutrient harvesting, damage of the intestinal barrier integrity, innate immune activation, and growth delay. It is tempting to hypothesize that children who developed stunting in our study were already at

FIGURE 5. Bray-Curtis diversity comparisons among fecal microbiome samples collected at baseline and end of follow-up, stunted children vs. non-stunted children. P -values are calculated using t -test.

TABLE 2
Gut microbiome analysis: changes in the relative abundance of selected taxa

Phylum	Class	Order	Family	Genus	Not stunted		Stunted	
					LDA	P-value	LDA	P-value
Taxa with relative abundance increasing over time in stunted children but not in non-stunted								
Firmicutes	—	—	—	—	4.61	0.110	4.98	0.007
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcus 1</i>	2.22	0.088	2.85	0.008
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcus 2</i>	2.59	0.615	3.05	0.025
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcaceae UCG 014</i>	2.94	0.185	3.42	0.043
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	—	4.46	0.097	4.74	0.037
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Uncultured	2.12	0.068	2.64	0.012
Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	—	3.73	0.364	3.85	0.031
Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	<i>Clostridium sensu stricto 1</i>	3.20	0.526	3.60	0.043
Actinobacteria	Coriobacteria	Coriobacteriales	Coriobacteriaceae	<i>Collinsella</i>	2.39	0.693	2.59	0.035
Taxa with relative abundance increasing over time in both stunted and non-stunted children								
Firmicutes	Clostridia	—	—	—	4.78	0.018	4.96	0.013
Firmicutes	Clostridia	Clostridiales	—	—	4.78	0.018	4.96	0.013
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Lachnospiraceae UCG 004</i>	2.14	0.008	2.78	0.002
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Roseburia</i>	3.50	0.010	3.83	0.007
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Blautia</i>	3.25	0.018	3.60	0.010
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Lachnospiraceae NK4A136</i> group	3.44	0.004	3.91	0.017
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Fusicatenibacter</i>	3.53	0.013	3.66	0.023
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Pseudobutyribrio</i>	4.17	0.024	4.04	0.032
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	<i>Lachnospiraceae UCG 010</i>	2.55	0.005	2.61	0.048
Firmicutes	Clostridia	Clostridiales	Christensenellaceae	—	3.25	0.001	2.62	0.025
Firmicutes	Clostridia	Clostridiales	Christensenellaceae	<i>Christensenellaceae R-7</i> group	3.24	0.007	2.61	0.025
Firmicutes	Clostridia	Clostridiales	Family XIII	—	2.01	0.031	1.99	0.028
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	<i>Ruminococcaceae UCG 002</i>	3.61	0.008	3.64	0.047
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	<i>Butyrivimonas</i>	2.40	0.021	2.32	0.036
Taxa with relative abundance decreasing in stunted children but not in non-stunted children								
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Providencia</i>	3.07	0.191	2.88	0.036
Taxa with relative abundance decreasing over time in both stunted and non-stunted children								
Actinobacteria	—	—	—	—	4.57	0.004	4.56	0.003
Actinobacteria	Actinobacteria	—	—	—	4.57	0.004	4.53	0.004
Actinobacteria	Actinobacteria	Bifidobacteriales	—	—	4.56	0.005	4.53	0.004
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	—	4.56	0.005	4.53	0.004
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	<i>Bifidobacterium</i>	4.56	0.005	4.53	0.004
Proteobacteria	Gammaproteobacteria	—	—	—	4.70	0.013	4.71	0.048
Proteobacteria	Gammaproteobacteria	Enterobacteriales	—	—	4.64	0.027	4.79	0.013
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	—	4.64	0.027	4.79	0.013
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Escherichia-Shigella</i>	4.59	0.028	4.78	0.014

For the purpose of this analysis, only non-stunted children having an LAZ ≥ -1.5 at the end of the follow-up ($N = 36$) were used as comparison vs. stunted children (defined as children with an LAZ ≤ -2 at the end of the follow-up) ($N = 16$) as this classification allowed for the best discrimination of data.

the limit of their functional reserve at enrollment and, over the following, months suffered from a progressive loss of mucosal surface, which eventually resulted in clinical signs of stunting.

Consistent with other studies,^{5,6,10} we found stigmata of innate immune activation in children who became clinically stunted. Both membrane-bound and sCD14 accelerate LPS-LBP complex recognition by Toll-like receptor-4 on monocytes, macrophages, and dendritic cells.²⁴ Our sCD14 and LBP findings suggest increased microbial translocation across the intestinal mucosa and increased LPS-induced immune activation.^{21,22} Our findings may indeed signify that the origin of immune activation in stunted children is a sustained low-grade microbial translocation through an exhausted intestinal mucosa. They can also partially explain the increased risk of death due to sepsis, tuberculosis, meningitis, or nonspecific febrile episodes in children with severe stunting³² because chronic, low-grade exposure to bacterial products may induce a status

of tolerance, whereby immune cells become relatively anergic to endotoxin stimulation,³³ or priming, whereby immune cells exhibit an exaggerated inflammatory response.³⁴ In either case, the abnormal inflammatory response will make the host less likely to survive to a severe infection.

It is increasingly recognized that commensal flora plays a critical role in host metabolic and immune homeostasis. The intestinal microbiota participates in nutrient harvesting and interacts with the host epithelial and immune cells to maintain the equilibrium between tolerance toward exogenous molecules and preparedness against pathogen invasion. This symbiosis is particularly fascinating in the early stages of life, as the predictable, ordered assembly of the gut microbiome and its functional capacity appear to be essential for the healthy development of the child.^{35–37} Many factors, including genetics, mode of delivery, age, diet, use of antibiotics, and the mother's microbiome, can affect the infant gut microbiota composition.

However, the interindividual diversities (β -diversity) decrease with age, and after the first 3 years of life, there is a remarkable consistency among healthy individuals.^{35,37} Aberrant microbiota composition has been associated with numerous diseases in childhood and later in life.^{38,39}

The analysis of the fecal microbiota evolution of these children over the 6-month follow-up showed some expected changes in both cases and controls. First, the within-sample diversity of the samples increased with age, reflecting the acquisition of new bacterial strains from the environment. Second, some of the changes in the bacterial communities followed the predictable age-dependent pattern: Enterobacteriaceae and Bifidobacteriaceae, typically predominant in the intestine of very young infants, decreased over time, whereas members of the Lachnospiraceae expanded.^{35,36,39} Bacteria belonging to this family often produce short-chain fatty acids that regulate host immunity through the induction of regulatory T cells and help maintain enterocyte health and have been considered markers of a healthy microbiota.^{40,41}

Interestingly, despite the limited sample size, we were also able to appreciate differences between the gut microbiota of stunted and non-stunted children. First, differences in fecal microbiota composition between cases and controls were already present at baseline, before any child had developed signs of clinical stunting, and persisted throughout the study (Figure 5), possibly signifying that the intestinal microbiome is a driver, or shares an upstream mediator, with stunting. If changes in the gut microbiota result in decreased energy harvesting from food, interventions aimed at restoring a healthy commensal flora may be just as important as providing an age-appropriate diet to cure stunting. An arrest of the maturation of intestinal microbiome has indeed already been described in children with severe acute malnutrition.^{42,43} Previous studies comparing the gut microbiota of healthy versus malnourished children have often found dissimilarities between the groups but the bacteria identified as discriminant are not consistent, probably due to differences in age, geography, type of malnutrition, and sample-processing techniques.^{42–45} Inferring the potential significance of such differences is arduous: the human microbiome possesses an extraordinary functional redundancy, and the true significance of changes in the relative abundance of some species needs to be investigated with sophisticated metagenomic analyses that were beyond the scope of this pilot study.

Our study has other limitations¹: The sample size is small and probably explains some of the nonstatistically significant findings.² Our children were of slightly different ages; although the differences were in terms of months, the tremendous pace of changes in the physiology and microbiota characteristics of this period of life makes them remarkable and potentially confounds some of the results.³ The microbiome analysis was performed on fecal samples which are not necessarily representative of the bacterial communities populating different segments of the intestine.

In summary, we found that the clinical onset of stunting is preceded by the appearance of markers of accelerated mucosal turnover, systemic inflammation, and changes in the gut microbiota. Future studies aimed at characterizing the progressive functional maturation of the intestinal mucosa and microbiome and their cross-talk with the developing immune system in the early stages of life may offer new approaches for the prevention and cure of the stunting syndrome.

Received December 8, 2018. Accepted for publication July 23, 2019.

Published online September 3, 2019.

Note: Supplemental tables and figures appear at www.ajtmh.org.

Acknowledgments: We would like to thank Mark Manary and Isabel Ordiz for their valuable technical advice and encouragement; the Peruvian field team: Darwin Silva and nurse Veronica Vela Perez from Moyobamba and Renzo Gambetta and nurse Mitsy Larico from Uribamba, who made this study possible; and Ochoa and his wife for the hospitality, encouragement, and support provided to the field team.

Financial support: This study was supported by the Thrasher Research fund.

Authors' addresses: Mara Zambruni, Department of Pediatrics, The University of Texas Health Science Center at Houston Medical School, Houston, TX, E-mail: mara.zambruni@uth.tmc.edu. Theresa J. Ochoa, Gonzalo J. Acosta, Natalia I. Vigo, Maribel Riveros, Sara Arango, and David Durand, Instituto de Medicina Tropical "Alexander von Humboldt," Universidad Peruana Cayetano Heredia, Lima, Peru, E-mails: theresa.j.ochoa@uth.tmc.edu, [gjacobstagarica@houstonmethodist.org](mailto:gjacostagarcia@houstonmethodist.org), natalia.vigo@outlook.com, maribel.riveros@upch.pe, sara.arango29@gmail.com, and david.durand@upch.pe. Anoma Somasunderam and Netanya S. Utay, Division of Infectious Diseases, The University of Texas Health Science Center at Houston Medical School, Houston, TX, E-mails: anoma.somasunderam@uth.tmc.edu and netanya.s.utay@uth.tmc.edu. Miguel M. Cabada, Maitreyee N. Berends, and Peter Melby, Infectious Diseases Division, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, E-mails: micabada@utmb.edu, maitreyee.n.berends@ttuhsc.edu, and pcmelby@utmb.edu. Maria L. Morales, Universidad Peruana Cayetano Heredia—University of Texas Medical Branch Collaborative Research Center Cusco, Universidad Peruana Cayetano Heredia, Cusco, Peru, E-mail: maria.morales.f@upch.pe. Makedonka Mitreva, Division of Infectious Diseases, Department of Medicine, Washington University School of Medicine, St. Louis, MO, E-mail: mmitreva@wustl.edu. Bruce A. Rosa, The McDonnell Genome Institute, Washington University in St. Louis, MO, E-mail: barosa@wustl.edu.

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