

The Association Between Fecal Biomarkers of Environmental Enteropathy and Rotavirus Vaccine Response in Nicaraguan Infants

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Background: Environmental enteropathy (EE) is a common intestinal condition among children living in low- and middle-income countries and is associated with diminished enteric immunity to gastrointestinal pathogens, and possibly to oral vaccine antigens. The goal of this study was to examine associations between biomarkers of EE and immunogenicity to the pentavalent rotavirus vaccine (RV5).

Methods: Infants were recruited 1 day before their first RV5 immunization in León, Nicaragua, from public health rosters. Infants provided a preimmunization blood and stool sample, and a second blood sample 1 month after receipt of RV5. We measured immunoglobulin A (IgA) seroconversion to the first dose of RV5 and concentrations of 4 previously identified fecal biomarkers of EE (alpha-1 antitrypsin, neopterin, myeloperoxidase and calprotectin). We then assessed associations between concentrations of these biomarkers, both individually and as combined scores, and seroconversion to the first dose of RV5.

Results: Of the 43 enrolled infants, 24 (56%) seroconverted after the first dose of RV5. As compared with infants who seroconverted, those who did not seroconvert had higher median concentrations of both myeloperoxidase (3.1 vs. 1.1 $\mu\text{g/mL}$, $P = 0.002$) and calprotectin (199.1 vs. 156.2 $\mu\text{g/mL}$, $P = 0.03$). Further, those who did not seroconvert had a higher median combined score of the 4 biomarkers as compared with those who seroconverted (6.5 vs. 4.5, $P = 0.017$).

Conclusions: We found an association between biomarkers of EE and seroconversion to the first dose of RV5. It is possible that interventions that prevent or ameliorate EE may also improve oral rotavirus vaccine response.

Key Words: rotavirus vaccines, myeloperoxidase, calprotectin, biomarkers, Nicaragua

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evoke a less robust immune response in LMIC infants as compared with infants living in high-income settings, which corresponds to lower efficacy and effectiveness.¹ For example, in Nicaragua, the pentavalent RV (RV5; RotaTeq, Merck & Co., Kenilworth, NJ) provides about 46% effectiveness against hospitalization for rotavirus diarrhea, as compared with 82%–95% in the United States.^{2,3} Several host factors that may contribute to poor RV response are currently under investigation. If a causal relationship can be established between a certain host factor and poor RV response, then an intervention can be designed that alters the host factor to improve the RV response.

To mount a robust immune response to an oral vaccine, it is essential to have a well-functioning enteric immune system. Impaired enteric immunity against enteric pathogens is often seen in individuals with environmental enteropathy (EE).⁴ EE has been recognized since the 1960s as a series of histopathologic alterations of the gut mucosa including decreased villus height, increased crypt depth and lymphocytic infiltration of the lamina propria, in settings with repeated exposure to enteric pathogens.^{5–7} These changes can be found early in infancy^{8,9} and are associated with increased intestinal permeability^{10–12} and a chronic inflammatory state.⁴ This chronic inflammatory state may result in hyporesponsiveness to individual antigens,⁴ such as RV antigens.

Because the definitive diagnosis of EE requires intestinal biopsy, investigators are currently identifying noninvasive biomarkers that are consistent with features of EE. Kosek et al¹³ identified 3 fecal biomarkers associated with increased permeability or intestinal inflammation that are also associated with linear growth deficits and are not correlated to one another: alpha-1 antitrypsin (AAT), neopterin (NEO) and myeloperoxidase (MPO). AAT is a serum protein resistant to proteolysis¹⁴ that has been shown to be excreted into the gut lumen in greater quantities when intestinal permeability is increased.¹⁵ NEO is a metabolic product of macrophages and dendritic cells and is elevated in celiac disease, a disease with features similar to EE.^{16,17} MPO is found in the granules of neutrophils, and fecal MPO levels are elevated in inflammatory bowel disease.¹⁸ A subsequent study included fecal calprotectin (CAL) as a biomarker of EE.¹⁹ CAL, a protein present in the cytosol of neutrophils, is resistant to intestinal degradation, is elevated in inflammatory bowel disease^{20,21} and was not well correlated with the 3 other fecal biomarkers noted above.¹⁹

The goal of this study was to examine the association between previously identified fecal biomarkers of EE and immunogenicity to the first dose of RV5. We hypothesized that infants with high concentrations of these biomarkers consistent with EE would mount a weak immune response to the vaccine.

MATERIALS AND METHODS

Setting

The study was conducted in León, Nicaragua. Nicaragua is a LMIC in Central America with an annual per capita Gross National Income (GNI) of \$1870.²² At the time the study was conducted, the Nicaraguan National Immunization Schedule included 3 doses of RV5 at 2, 4 and 6 months of life.

Low- and middle-income countries (LMICs) carry the greatest burden of rotavirus disease; however, oral rotavirus vaccines (RVs)

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Study Population

Study participants were recruited between September and November 2014 from public health rosters of recent deliveries and pregnancies in the Perla Maria and Subtiava Health Sectors of León, Nicaragua. Eligibility criteria included gestational age at birth ≥ 35 weeks, birthweight of 2500–4500 g, no contraindication to receive RV5 according to the package insert, no known chronic health conditions and no known blood transfusion. Recruitment occurred at the household, 1 day before the infant's first immunization visit, at 2 months of age. The study was approved by institutional review boards (IRBs) of the Universidad Nacional Autónoma de Nicaragua, León (IRB study 110) and the University of North Carolina at Chapel Hill (IRB study 14–1136).

Data and Sample Collection

At study enrollment, a survey was administered to each mother to collect information on personal, family and household characteristics. The survey collected information on any prior or current diarrhea episodes, defined as an increase in stool frequency to at least 3 loose or watery stools per 24-hour period or as a substantial change in stool consistency (bloody, looser than normal). Next, a preimmunization blood sample was obtained from each infant and stored at 4°C until transport to the Universidad Nacional Autónoma de Nicaragua, León Microbiology Laboratory. At the same visit, a fresh stool sample was obtained from the infant's diaper. This stool sample was diluted 20-fold and homogenized in sterile prerduced anaerobic saline-0.1 M potassium phosphate buffer (pH 7.2) containing 15% glycerol (vol/vol). The stool samples were then snap frozen by putting them in liquid nitrogen, transported to the microbiology laboratory on dry ice and stored at -80°C until used. The day after enrollment, infants received RV5 according to the national immunization schedule in a public health facility. A study nurse visited the public health facilities to confirm RV5 receipt by direct observation or from the medical record for each of the participating infants. Four weeks after the first dose of RV5, a second blood sample was obtained from each infant.

Laboratory Methods

Rotavirus-specific IgA Assays

Serum rotavirus-specific IgA titers were determined by enzyme-linked immunosorbent assay (ELISA) using a method previously described.²³ In brief, microtiter plates of 96 wells (Greiner Bio-One, Kremsmünster, Austria) were coated with RV5 vaccine diluted 1:100 in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After 2 hours of blocking with phosphate-buffered saline-bovine serum albumin 3%, a total of 100 μL of diluted serum (1:25 and 1:50) was added and incubated at 37°C for 1 hour, followed by addition of peroxidase-conjugated anti-human IgA (P0216; DakoCytomation, Glostrup, Denmark) diluted 1:2000, and incubated at 37°C for 1 hour. The reaction was then developed for 10 minutes with tetramethylbenzidine Q3 (Thermo Fisher Scientific, Waltham, MA) and stopped with 2M H_2SO_4 . Optical density was determined at 450 nm in each well. Titer was assigned by comparing the absorbance reading of both dilutions with a database of absorbance readings from serum titers (50, 100, 200, 400, 800 and 1:600) of RV5-vaccinated children. Phosphate-buffered saline was used in all experiments for background monitoring.

Fecal Biomarker ELISA

ELISA for AAT, NEO, MPO and CAL were performed on all stool samples. ELISA kits were purchased from commercial vendors (α -1-antitrypsin in stool human ELISA: BioVendor, Asheville, NC; NEO ELISA: Genway Biotech Inc, San Diego, CA; MPO ELISA: ALPCO, Salem, NH; CAL ELISA: Eagle Biosciences, Nashua,

NH) and run per the manufacturer's directions unless otherwise stated. Samples were diluted 1:100 with assay buffer before use in the NEO ELISA kit and 1:500 for use in the MPO ELISA kit. Final concentrations were obtained using the standard curve method.

Statistical Analysis

Fold change of IgA was calculated as the ratio of postimmunization divided by preimmunization rotavirus-specific IgA titers. Seroconversion to the first dose of RV5 dose was defined as ≥ 4 -fold increase in rotavirus-specific IgA titers.

Combined scores of biomarkers were generated. First, we calculated the "Kosek EE Score" with values ranging between 0 and 10, including 3 biomarkers (AAT, NEO and MPO) as previously described by Kosek et al.¹³ Briefly, for each biomarker, 0 points were assigned for a concentration of each biomarker ≤ 25 th percentile, 1 point was assigned for a concentration between 25th and 75th percentile, and 2 points were assigned for a concentration ≥ 75 th percentile. The Kosek EE Score was then calculated as follows: 2 (AAT score) + (NEO score) + 2 (MPO score). Following Kosek et al.,¹³ we also performed a principal component analysis using data from all 4 biomarkers (AAT, NEO, MPO and CAL) tested to define a new "4 biomarker EE score." As for the Kosek EE Score, 0 points were assigned for a concentration of each biomarker ≤ 25 th percentile, 1 point was assigned for a concentration between 25th and 75th and 2 points were assigned for a concentration ≥ 75 th percentile. Based on our findings from principal component analysis, the 4 biomarker EE score was weighted as follows: (AAT score) + (NEO score) + 2 (MPO score) + 1.5 (CAL score), and ranged between 0 and 11.

Associations between concentrations of biomarkers were assessed using Spearman's rank correlation coefficient (ρ). Wilcoxon rank sum tests were used to compare concentrations of biomarkers and EE scores between infants who did versus did not seroconvert to the first dose of RV5. Fisher exact tests were used to compare the proportions with EE scores in the highest percentile between infants who did versus did not seroconvert. Spearman's rank correlation coefficients were also estimated to examine 1) associations between concentrations of each biomarker and fold change in rotavirus-specific IgA titers and 2) associations between EE scores and fold change in rotavirus-specific IgA titers.

RESULTS

Participants

Of the 50 infants enrolled in the study, 45 completed study requirements (3 dropped out because mother declined second blood draw, 1 did not receive RV5 and 1 moved). An additional 2 children were dropped from the analysis because of insufficient sample to perform all of the required laboratory analyses. The remaining 43 infants had a mean age of 8.8 weeks upon study entry, 44% were female, 88% were receiving breastmilk, none had a weight-for-age Z score below -2 , and the majority lived in homes with municipal piped water, indoor toilets and a nondirt floor (Table 1). While none of the infants had diarrhea at the time of RV5 immunization, 7% had experienced a prior diarrhea episode.

Laboratory Findings

Of the 43 infants, 24 (56%) seroconverted after the first dose of RV5. Among infants who seroconverted, 3 (23%) were IgA seropositive before immunization, while among the 19 infants who did not seroconvert, 10 (53%) were IgA seropositive before immunization. The median concentrations of AAT, NEO, MPO and CAL in infants' stools were, respectively, 138.2 $\mu\text{g}/\text{mL}$ [interquartile range (IQR): 96.2–180.3], 412.4 nmol/L (IQR: 248.0–901.1), 1.5 $\mu\text{g}/\text{mL}$ (IQR: 0.7–3.3) and 162.3 $\mu\text{g}/\text{mL}$ (IQR: 153.9–246.3). The median

TABLE 1. Characteristics of Participants (N = 43)

Characteristic	Frequency or Mean			
	All	Seroconversion	Nonseroconversion	P*
Gender (% female)	44.1% (19/43)	45.8% (11/24)	42.1% (8/19)	1
Age at study entry (wk, SD)	8.8 (0.5)	8.7 (0.3)	9.0 (0.7)	0.2
Birthweight (kg, SD)	3.1 (0.3)	3.1 (0.3)	3.1 (0.3)	0.4
Breastfed yesterday (% yes)	88.3% (38/43)	91.7% (22/24)	84.2% (16/19)	0.6
Exclusively breastfed (% yes)	27.9% (12/43)	33.0% (8/24)	21.0% (4/19)	0.5
Maternal education (% with any secondary education)	69.7% (30/43)	70.8% (17/24)	68.4% (13/19)	1
Household water source (% municipal piped water)	97.6% (42/43)	100.0% (24/24)	94.7% (18/19)	0.4
Household sanitation (% indoor toilet)	83.7% (36/43)	91.7% (22/24)	73.6% (14/19)	0.2
Floor type (% nondirt floor)	72.1% (31/43)	79.1% (19/24)	63.1% (12/19)	0.3
Animal(s) present in the home (% yes)	69.7% (30/43)	70.8% (17/24)	68.4% (13/19)	1
Diarrhea episode before receipt of first rotavirus vaccine dose	7.0% (3/43)	4.1% (1/24)	10.5% (2/19)	0.6

*Binary variables compared using Fisher exact tests; continuous variables compared using two-sample *t* tests. SD indicates standard deviation.

Kosek EE Score was 4.0 (IQR: 2.5–6.0), and the mean of 4 biomarker EE score was 5.5 (IQR: 3.5–8.5).

Correlations Between Biomarkers

Concentrations of the biomarkers were moderately to strongly correlated to one another (Table 2). AAT was strongly correlated to NEO ($\rho = 0.81$, $P < 0.001$), and moderately correlated to MPO ($\rho = 0.50$, $P < 0.001$) and CAL ($\rho = 0.36$, $P = 0.016$). NEO was moderately correlated to both MPO ($\rho = 0.50$, $P < 0.001$) and CAL ($\rho = 0.32$, $P = 0.033$). MPO and CAL were strongly correlated ($\rho = 0.70$, $P < 0.001$).

Associations Between Immunogenicity to RV5 and Biomarker Concentrations

Concentrations of the 4 biomarkers by seroconversion status are shown in Table 3 and Figure 1. As compared with infants who did seroconvert, infants who did not seroconvert had higher median concentrations of both MPO (respectively, 1.1 vs. 3.1 $\mu\text{g/mL}$, $P = 0.002$) and CAL (156.2 vs. 199.1 $\mu\text{g/mL}$, $P = 0.03$). There were no statistically significant differences in concentrations of AAT and NEO between the groups of infants who seroconverted versus who did not seroconvert. Also, as compared with infants who did seroconvert, those who did not seroconvert had a higher median Kosek

TABLE 2. Correlation Between Biomarkers for Environmental Enteropathy (N = 43)

Biomarker	AAT ($\mu\text{g/mL}$)	NEO (nmol/L)	MPO ($\mu\text{g/mL}$)	CAL ($\mu\text{g/mL}$)
AAT ($\mu\text{g/mL}$)	1	0.81* (<0.001)	0.50 (<0.001)	0.36 (0.016)
NEO (nmol/L)	0.81 (<0.001)	1	0.50 (<0.001)	0.32 (0.033)
MPO ($\mu\text{g/mL}$)	0.50 (<0.001)	0.50 (<0.001)	1	0.70 (<0.001)
CAL ($\mu\text{g/mL}$)	0.36 (0.016)	0.32 (0.033)	0.70 (<0.001)	1

*Spearman's correlation coefficient ρ (*P* value).

TABLE 3. Environmental Enteropathy Biomarker Concentrations* and Scores in Infants Who Did Versus Did Not Seroconvert† to the First Dose of the Pentavalent Rotavirus Vaccine

Biomarker	Seroconversion (n = 24)	Nonseroconversion (n = 19)	P
Alpha-1 antitrypsin ($\mu\text{g/mL}$)	129.5 (87.3–164.8)	152.3 (104.1–224.3)	0.4‡
Neopterin (nmol/L)	408.7 (235.9–946)	412.4 (334.0–747.5)	0.7
Myeloperoxidase ($\mu\text{g/mL}$)	1.1 (0.5–1.9)	3.1 (1.8–4.0)	0.002
Calprotectin ($\mu\text{g/mL}$)	156.2 (152.0–174.6)	199.1 (158.5–321.8)	0.03
Kosek EE Score % in the first quartile	2/24 (8.3%)	6/19 (31.6%)	0.1
Kosek EE Score	3.5 (2–4.3)	5.0 (3.5–7)	0.03
4 Biomarker EE Score % in the first quartile	2/24 (8.3%)	9/19 (47.4%)	0.005
4 Biomarker EE Score	4.5 (2.8–5.8)	6.5 (4.5–9.5)	0.017

*Concentrations and scores shown as medians with interquartile ranges.

†Seroconversion defined as a 4-fold or greater increase in rotavirus-specific IgA titers in the infant's postimmunization serum sample as compared with the preimmunization serum sample.

‡Continuous and ordinal variables compared between seroconverters versus nonseroconverters using Wilcoxon rank sum tests. Binary variables compared using Fisher exact tests.

§Kosek EE Score is defined in reference 13; 4 biomarker EE score is described in Materials and Methods.

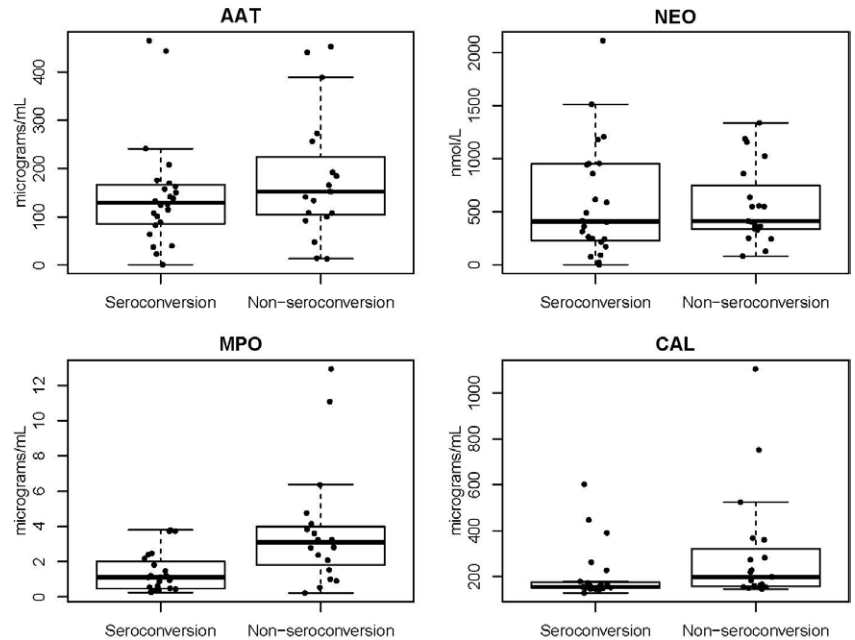


FIGURE 1. Biomarker concentrations by seroconversion status. Boxes show the interquartile ranges and bold lines represent medians. AAT indicates alpha-1 antitrypsin; CAL, calprotectin; MPO, myeloperoxidase; NEO, neopterin.

TABLE 4. Correlations Between Biomarker Concentrations and Fold Change in Rotavirus-specific IgA Titers After the First Dose of the Pentavalent Rotavirus Vaccine

Correlation	AAT	NEO	MPO	CAL	Kosek EE Score*	4 Biomarker EE Score*
IgA fold change	-0.13 [†] (<i>P</i> = 0.4)	-0.12 (<i>P</i> = 0.4)	-0.43 (<i>P</i> = 0.003)	-0.21 (<i>P</i> = 0.178)	-0.30 (<i>P</i> = 0.050)	-0.29 (<i>P</i> = 0.056)

*Kosek EE Score described in reference 13; 4 biomarker EE score described in Materials and Methods section.

[†]Spearman's correlation coefficient ρ (*P* value).

EE Score and 4 biomarker EE score (respectively, 3.5 vs. 5.0, *P* = 0.03 and 4.5 vs. 6.5, *P* = 0.017).

When IgA response to RV5 was treated as a continuous variable (fold change in rotavirus-specific IgA titer), it was found to be moderately negatively correlated with MPO concentration ($\rho = -0.43$, *P* = 0.003). Correlations between fold change in IgA titer and concentrations of the other biomarkers are shown in Table 4.

DISCUSSION

In this preliminary study, we found that 2 of the 4 fecal EE biomarkers examined, MPO and CAL, were associated with failure to seroconvert to the first dose of RV5. For example, only 30% of infants in the highest quartile of MPO concentration seroconverted to the first dose of RV5, as compared with 82% of infants in the lowest quartile of MPO concentration. Further, high combined scores of biomarkers were associated with poor RV5 immunogenicity. The PROVIDE study conducted in Bangladesh²⁴ also found an association between fecal EE biomarkers and oral vaccine response; AAT was associated with poor response to the monovalent RV1, while CAL and other fecal EE biomarkers were associated with poor response to oral polio vaccines. Approximately 80% of infants in the PROVIDE study had EE biomarker concentrations that exceeded nontropical normal values. In comparison, 47%, 33% and 14% of infants in our study exceeded nontropical normal values for MPO, CAL and AAT, respectively. These differences in biomarker

concentrations between infants in both studies may be because of the younger age of our study participants (2 months of age as compared with 3 months of age of infants in the PROVIDE study), as EE progresses over time. Further, high access to municipal piped water sources in our participants' households may result in less intense enteropathogen exposure. It is possible that the smaller numbers of infants in our study with impaired intestinal permeability (as measured by AAT concentration) may explain why we did not observe a similar association between AAT concentration and RV immunogenicity as was observed among the PROVIDE infants. Finally, in our study, we found that the EE biomarkers examined were highly correlated to one another, in contrast to previous studies by Kosek et al¹³ and George et al.¹⁹

As MPO and CAL are both found within neutrophils, this study raises the question of the role of neutrophils in the development of EE and poor RV responses. MPO is found in the granules of neutrophils and is used in the intracellular killing of pathogens. During neutrophil activation and neutrophil death, MPO is released. MPO is also present within "neutrophil extracellular traps," used in the extracellular killing of pathogens. CAL is a protein present within the cytosol of neutrophils, and is also released upon neutrophil activation and neutrophil death. Upon release, it has bacteriostatic and cytokine-like effects locally, and is also capable of sequestering zinc.²⁵ While this observational study cannot be used to understand the interactions between neutrophils, the development of EE and poor RV responses, it suggests that

future studies may be warranted to better understand the role of neutrophils in vaccine-elicited enteric immunity. If these findings are confirmed within a larger sample size and in different populations, future studies should be conducted to determine if interventions that decrease EE, or specifically, neutrophil activation, may improve RV response.

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