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Haley Liakakos Date

The Long-Term Impact of Subclinical *Shigella* Infections on Environmental Enteropathy in Children Under 2

By

Haley Liakakos
Master of Science in Public Health

Epidemiology

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Dr. Elizabeth Rogawski McQuade
Committee Chair

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2021

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Abstract

The long-term impact of subclinical *Shigella* infections on environmental enteropathy in children under 2

By Haley Liakakos

Shigellosis, caused by the enteric pathogen *Shigella*, is one of the leading causes of diarrheal death from a bacterial pathogen among children living in low- and middle-income countries (LMICs). Clinical and subclinical *Shigella* infections have both been associated with long-term adverse effects such as impaired linear growth. The impact from subclinical infections has been theorized to occur through the mechanisms of environmental enteropathy (EE). A prospective birth cohort study of 1,715 children living in 8 different LMICs was conducted. Over the course of 24 months, monthly non-diarrheal stool samples were collected from each child and analyzed for subclinical *Shigella* infections through quantitative PCR methods. EE was reflected by elevated concentrations of 3 fecal biomarkers: myeloperoxidase (MPO), neopterin (NEO), and alpha-1-antitrypsin (AAT). MPO concentrations were found to be significantly higher by 0.30 log(nm/mL) (95% CI: 0.23, 0.37) in the initial month of *Shigella* detection among stools with subclinical *Shigella* infections compared to stools without *Shigella* infections. After the *Shigella* infection, MPO concentrations declined throughout the following 6 months, and concentrations were lower after 5- and 6-months post-infection among stools within an initial infection compared to stools without an initial infection [MPO 5-month difference: -0.18 log(nm/mL) (95% CI: -0.35, -0.00); MPO 6-month difference: -0.16 log(nm/mL) (95% CI: -0.26, -0.04)]. Subclinical *Shigella* infections had no effect on NEO concentration levels within the initial month of *Shigella* detection. Post-infection, NEO concentrations were lower among stools with an initial infection compared to stools without an initial infection. Subclinical *Shigella* infections had no effect on AAT concentration levels until 6-months post-infection when AAT levels among stools with an initial infection were lower than stools without an initial infection [AAT difference: -0.13 log(mg/g) (95% CI: -0.24, -0.03)]. These findings, both initially and longitudinally, did not differentiate by cephalosporin, macrolide, fluoroquinolone, or any antibiotic use around time of initial infection. Enteric pathogens contribute to the vicious, prolonged cycle of EE, and *Shigella* is among one of the highest burden pathogens among those most at risk. Our study suggests the persistent role of *Shigella* on EE is not captured by these fecal biomarkers.

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INTRODUCTION

Shigella is an infectious bacterial pathogen which causes shigellosis, a high burden diarrheal disease. Diarrheal diseases are the fifth leading cause of death globally, yet they disproportionately affect children under 5 years living in low- and middle-income countries (LMICs) with diarrheal diseases being the second leading cause of death.[1–3] Among these children, *Shigella* was attributed to 63,700 deaths in 2015, making it one of the leading causes of bacterial diarrheal death.[4] Recent application of molecular diagnostics to identify *Shigella* has demonstrated that the burden has been underestimated.[5–8] Some of the defining characteristics of a *Shigella* infection include diarrhea and dysentery, yet an analysis of the Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health (MAL-ED) study found that, among children under 2 years, only 15% of *Shigella*-attributed diarrheal stools was accompanied by dysentery and 64% of children with non-diarrheal samples that tested positive for *Shigella* never had a *Shigella*-attributed diarrheal episode.[8,9] Subclinical infections, despite the decrease in acute severity, have been associated with poor long-term outcomes comparable to clinical infections. These infections have been associated with sustained impaired child growth throughout the first 2-5 years of life.[10]

While the mechanism for how *Shigella* and other enteric infections could lead to these longer-term adverse effects is unknown, one proposed and under-studied theory is through environmental enteropathy (EE).[11–13] EE occurs when a person is infected with multiple pathogens, symptomatic or asymptomatic, over a prolonged period usually from living in areas of poor sanitation. It is a condition of the intestines characterized by inflammation, greater intestinal permeability, and shortening of villi which results in malabsorption of nutrients.[13,14] It is important to note that malnutrition, in turn, affects intestinal functionality which can lead to

greater susceptibility to enteric pathogens. This cycle contributes to the unclear etiology of EE and provides insight as to why there is a higher burden in LMICs.[13–15] While EE in the short-term is generally asymptomatic, the longer-term impacts have been theorized to include growth stunting, poor cognitive development, and poor vaccine effectiveness in children.[13] *Shigella* infections cause inflammation of the intestines which is theorized to lead to both the clinical symptoms of diarrhea and dysentery and the long-term impact on the intestines through EE.[16]

Diagnosing EE is difficult and expensive as it requires invasive intestinal biopsies and physical symptoms can be general and sometimes not apparent. This, along with the lack of a clear definition adopted by practitioners, hinders the accuracy among burden estimates. Many researchers are looking to measure other less invasive indicators, such as biomarkers, for more efficiency.[14,17] There are several fecal biomarkers that translate multiple aspects of gut health that are commonly used in EE analyses.[17] Myeloperoxidase (MPO) reflects inflammation from neutrophil activity. MPO is unique compared to other inflammatory biomarkers as secretion in the stool is not dependent on being breastfed and there is direct evidence for intestinal disease activity.[12,17–19] Neopterin (NEO) reflects inflammation from gut immunity. Elevated levels indicate TH1 immune activity backed up by intestinal disease studies, and NEO is resistant to being broken down in stool.[12,17,18,20] Alpha-1-antitrypsin (AAT) reflects intestinal permeability. AAT is a clear indicator of protein loss and is also resistant to being broken down in stool.[12,17,18,21,22] These biomarkers together provide a non-invasive examination of intestinal function and health.

Studies in LMICs have shown consistent findings of above average fecal biomarker levels that have been associated with poor linear growth.[11,12,23] Furthermore, both clinical and subclinical *Shigella* infections have been associated with elevated fecal biomarker

concentrations.[10–12,16,21,24] Specifically, *Shigella* has been associated with significant fecal MPO elevations and a dose response relation of MPO increasing by 0.21 logs for every log of *Shigella* quantity detected. [8,12] While the evidence is consistent, one common limitation in these studies stems from the cross-sectional nature of the association between *Shigella* and biomarkers. For *Shigella* to affect longer-term outcomes like growth, the effects of *Shigella* on inflammation and EE would be hypothesized to persist beyond the initial *Shigella* infection. This study evaluates the longitudinal effects of subclinical *Shigella* infections on the intestines through fecal biomarkers to interrogate the potential mechanism for *Shigella* to impact growth through EE. Furthermore, even though *Shigella* is one of the more commonly antibiotic-treated diarrheal illnesses, little is known on how antibiotics aid in the recovery of intestines from EE.[25] The second aim of this study is provide insight about whether the effects of *Shigella* on EE differ depending on recent antibiotic therapy.

METHODS

Aims of Primary Study

We performed a secondary analysis of the MAL-ED prospective birth cohort study. The aim of the study was to take a holistic approach in providing context for a broad spectrum of enteric pathogens infecting children living in LMICs. Unique to previous studies, MAL-ED documented a multitude of factors to observe their interactions and associations with each other. They collected information from birth to 2 years of age on illnesses, anthropometry, cognitive development, maternal and environmental factors, living conditions, socioeconomic status (SES), demographics, nutrition, and, important for the focus of this study, infections by over 20 enteric pathogens, EE biomarkers, and medication use.[26]

Study Population

From November 2009 through February 2012, infants were enrolled into the MAL-ED study and followed for 24 months. There was a focus on resource-limited settings within LMICs, and at least 200 children were enrolled in each of the following 8 sites: Dhaka, Bangladesh; Fortaleza, Brazil; Vellore, India; Bhaktapur, Nepal; Loreto, Peru; Naushahro Feroze, Pakistan; Venda, South Africa; and Haydom, Tanzania. Healthy infants were enrolled within the 17 days after birth if their caregiver reported they planned to live within the study area for the next 6 months and approved of twice weekly home visits for 24 months. Infants were excluded from study enrollment if they were hospitalized for anything besides a healthy delivery, were diagnosed by a medical doctor for a severe or chronic condition, diagnosed for enteropathies, or weighed less than 1,500 grams at birth. Infants were also excluded if their family anticipated living outside the study area for more than 30 consecutive days within the first 6 months, if the

infant was not a singleton, or if the mother was younger than 16 years of age or had another child enrolled in the study.[26]

Sample Collection

Field MAL-ED researchers visited households twice weekly to collect basic health and dietary information as well as to complete disease surveillance measures. Sociodemographic information was collected at study enrollment, and updated every 6 months.[26] After 9 months, information on diet and breastfeeding was collected monthly rather than during the twice weekly visits.[27] Enteric pathogen infections and EE biomarker concentrations were measured in stool samples. Non-diarrheal stool samples were collected monthly. To ensure clear separation from diarrhea, non-diarrheal samples were taken at least 3 days before or 3 days after a maternal reported diarrhea episode.[28] These samples went under quantitative polymerase chain reaction (qPCR) as previously described for identification of 29 pathogens, including the following which were identified as the most prevalent pathogens in MAL-ED: adenovirus 40/41, astrovirus, *Campylobacter*, *Cryptosporidium*, *Entercytozoon bieneusi*, enteroaggregative *Escherichia coli*, enterotoxigenic *Escherichia coli*, typical enteropathogenic *Escherichia coli*, atypical enteropathogenic *Escherichia coli*, *Giardia*, norovirus, sapovirus, and *Shigella*.[10] To measure the EE biomarkers, quantitative ELISAs were run for MPO, NEO, and AAT on the non-diarrheal stool samples collected monthly during the first 12 months and collected on months 15, 18, 21, and 24.[26] Data on recent antibiotic use of any antibiotics, cephalosporins, macrolides, and fluoroquinolones were collected during the twice weekly household questionnaires.[28]

Statistical Analysis

Shigella infections were identified in non-diarrheal stool samples by qPCR detection of the *ipaH* gene. A quantitative cycle threshold of 35 was considered negative. Cycle threshold values were considered a case of a subclinical *Shigella* infection. The EE biomarkers were measured in log10 concentrations of ng/mL for MPO, nmol/L for NEO, and mg/g for AAT.[17]

The effects of *Shigella* infections on EE biomarkers from the initial *Shigella* infection through 6-months post-infection were estimated through multiple linear regression models. Specifically, for each non-diarrheal stool, detection of *Shigella* compared to no *Shigella* detection was associated with MPO, NEO, and AAT concentrations respectively during the same month and for each following month up to 6-months post-infection. These 21 models were adjusted for country of residence, age at the time of the infection, sex, the dietary habits of whether the child had been exclusive breastfed up until time of sample, stool consistency, previous *Shigella* infection, and coinfection covariates. Recent previous *Shigella* infections and current coinfections were necessary covariates. A sample was documented as coming from a child with a previous *Shigella* infection if *Shigella* was detected in another stool sample from the prior 3 months. Coinfections at the time of the initial *Shigella* infection were included in the models if they were independently associated with the EE biomarker outcome in the concurrent stool sample. To determine which pathogens were associated with the EE biomarkers, multiple linear regression models were run for each pathogen (defined by detection at a cycle threshold <35) against stool concentrations of MPO, NEO, and AAT respectively while adjusting for country site and age. The pathogens that were statistically significantly ($p<0.05$) associated with the biomarker were included as covariates in the models for that biomarker.

In sensitivity analyses, we detrended biomarker concentrations by age, included additional adjustment for coinfections at the time of the biomarker outcome measurement, and additional adjustment for *Shigella* detection at the time of the biomarker outcome measurement. This was done by running the basic model of initial *Shigella* infection on MPO biomarker concentration adjusting for country of residence, sex, exclusive breastfeeding, stool consistency, previous *Shigella* infection, and MPO coinfection pathogens. The basic model was run adjusting for age to observe the difference in the model between including longitudinal coinfections and longitudinal secondary *Shigella* infections. Another sensitivity analysis investigated the changes in MPO biomarker concentrations among children who had a *Shigella* infection within the first 18 months of life. This model was adjusted for country of residence, age, sex, exclusive breastfeeding, stool consistency, previous *Shigella* infection, and MPO coinfection pathogens.

To determine if recent antibiotic treatment with different classes of antibiotics impacted the effect of *Shigella* on the concentrations of MPO, NEO, and AAT, the primary multiple linear regression models were run with the interaction factor between *Shigella* infection and antibiotic use. One set of models investigated antibiotic use 15 days before or after the initial *Shigella* infection, and the other set of models investigated antibiotic use 30 days before or after the initial *Shigella* infection. The different classes of antibiotics examined were cephalosporins, macrolides, and fluoroquinolones, and the last set of models looked at any antibiotic class used.

Ethics Statement

The MAL-ED study collected data on and from human participants under the age of 5 along with maternal consent. The study design was approved by the University of Virginia's institutional review board. Approval was given within each of the 8 sites. The Bangladesh site

had approval from the Ethical Review Committee, International Centre for Diarrhoeal Disease Research, Bangladesh. The Brazil site had approval from the Committee for Ethics in Research, Universidade Federal do Ceara, and the National Ethical Research Committee, Health Ministry, Council of National Health. The India site had approval from the Institutional Review Board, Christian Medical College, Vellore, and the Health Ministry Screening Committee, Indian Council of Medical Research. The Nepal site had approval from the Institutional Review Board, Institute of Medicine, Tribhuvan University, the Ethical Review Board, Nepal Health Research Council, and the Institutional Review Board, Walter Reed Army Institute of Research. The Peru site had approval from the Institutional Review Board, Johns Hopkins University, and the PRISMA Ethics Committee, Health Ministry, Loreto. The Pakistan site had approval from the Ethical Review Committee, Aga Khan University. The South African site had approval from the Health, Safety and Research Ethics Committee, University of Venda, and the Department of Health and Social Development, Limpopo Provincial Government. The Tanzania site had approval from the Medical Research Coordinating Committee, National Institute for Medical Research, and the Chief Medical Officer, Ministry of Health and Social Welfare. This study's secondary analysis did not require approval from Emory University's institutional review board, as it did not fall under human subjects research since the data from the primary MAL-ED study is public and deidentified.

RESULTS

EE Biomarker Concentrations Throughout 6 Months After Shigella Infection

Over the course of 2 years, 1,715 children in the MAL-ED study contributed 34,654 non-diarrheal stool samples. Of these, 10% (N = 3,505 samples) tested positive for *Shigella* infection, detected through qPCR methods. Details of the *Shigella* distribution by demographic characteristics can be viewed in Table 1. In stools with subclinical *Shigella* infections, the inflammatory EE biomarker, MPO, was elevated by 0.30 log(ng/mL) (95% CI: 0.23, 0.37) compared to stools without *Shigella* during the initial month of *Shigella* detection. 1-4 months after the identification of the subclinical infection, the concentration of MPO was no different compared to those following stools without *Shigella.* MPO concentrations then after were slightly lower relative to those following stools without *Shigella* (Table 2). Samples with *Shigella* had MPO concentration levels 0.18 log(ng/mL) (95% CI: 0.00, 0.35) and 0.16 log(ng/mL) (95% CI: 0.04, 0.26) lower than samples without *Shigella* detection after 5-6 months. There was no clear difference in NEO concentrations, the EE biomarker characterizing gut immunity, in the initial detection month between those with and without subclinical *Shigella* infections [NEO difference: 0.01 log(nmol/L) (95% CI: -0.05, 0.07)]. The subsequent 6 months showed that stools following subclinical *Shigella* infections had lower concentrations of NEO compared to those not following an infection (Table 2). In the initial month of detecting subclinical *Shigella* infections, there was no clear difference in concentrations of the EE biomarker, AAT, reflecting intestinal permeability [AAT difference: -0.02 log(mg/g) (95% CI: -0.08, 0.04)]. AAT concentrations were lower among stools following a subclinical *Shigella* infection, particularly after 6 months [AAT difference: -0.13 log(mg/g) (95% CI: -0.24, -0.03)],

compared to stools 6 months after no *Shigella* infection (Table 2). The longitudinal associations of *Shigella* infections and biomarker concentrations are depicted in Figure 1.

Sensitivity Analysis of Age and Coinfection Covariates

The associations between *Shigella* infection and MPO biomarker concentrations showed little difference when adjusting for age or alternatively when detrending the biomarker concentrations for age. When both adjusting for age and detrending for age, the estimates were nearly the same as those only adjusting for age. The second sensitivity analysis demonstrated that when additionally adjusting for longitudinal coinfections (i.e. coinfections at the time of the biomarker measurement) and then adjusting for both longitudinal coinfections and *Shigella* infections there is not much difference in stool-collected MPO concentration (Figure 2).

Sensitivity Analysis of Children 18 Months and Younger

The association between *Shigella* infection and MPO biomarker concentration at initial month of *Shigella* detection in stool had statistical significance between the difference among those younger than 24 months of age compared to those younger than 18 months of age. Both age groups had significant elevation of MPO levels at month of *Shigella* detection, but the MPO elevation was significantly greater when examining stools from children up to 24 months of age compared to stools from children up to 18 months of age. There was little difference of NEO and AAT levels between stools from children younger than 24 months and stools from children younger than 18 months at initial month of *Shigella* detection and 3-months post-detection. This trend was similar when measuring the difference in MPO levels 3-months post-detection. For months 1 and 2 and months 4 through 6, there was no difference in MPO, NEO, and AAT

biomarker levels between stools from children younger than 24 months and stools from children younger than 18 months (Figure 3).

Differential Impact of *Shigella* Infections on Longitudinal EE Biomarker Concentrations by Antibiotic Treatment

There was no clear difference in *Shigella's* impact on MPO, NEO, or AAT concentrations for children who were and were not taking cephalosporin, macrolide, fluoroquinolone, or any antibiotic in general between a 30-day range or a 60-day range around initial *Shigella* infection (Figure 4). Children who took macrolides within the 15 days before or after the *Shigella* infection had lower MPO concentrations long-term starting around 4 months after the infection compared to children who took macrolides but did not have a *Shigella* infection. Specifically, MPO was significantly lower among children with macrolide antibiotic use 15 days before or after infection at 5-months post-infection only. There was no significant difference in *Shigella's* impact on NEO concentrations by different antibiotic treatment though NEO concentrations were more elevated throughout time when antibiotics were used. *Shigella's* impact on AAT concentrations by different classes of antibiotics was similar to the results for NEO concentrations, though elevated AAT levels when antibiotics were used were mostly seen in the first 4-months post-infection.

Table 1. Characteristics of non-diarrheal stool samples from 1715 children in MAL-ED study

	Shigella Infection (N=3,505)	No Shigella Infection (N=31,149)	Total (N = 34,654)
Country			
Bangladesh	564 (16%)	3754 (12%)	4318 (12%)
Brazil	139 (4%)	2700 (9%)	2839 (8%)
India	592 (17%)	4176 (13%)	4768 (14%)
Nepal	290 (8%)	4755 (15%)	5045 (15%)
Peru	574 (16%)	3638 (12%)	4212 (12%)
Pakistan	268 (8%)	4373 (14%)	4641 (13%)
South Africa	321 (9%)	4257 (14%)	4578 (13%)
Tanzania	757 (22%)	3496 (11%)	4253 (12%)
Sex			
Female	1726 (49%)	15305 (49%)	17031 (49%)
Age (days)			
Median [Q1, Q3]	545 [394, 640]	366 [184, 548]	395 [212, 576]
Stool Consistency			
Watery	5 (0.1%)	132 (0.4%)	137 (0.4%)
Liquid	50 (1%)	1056 (3%)	1106 (3%)
Soft	1101 (31%)	13011 (42%)	14112 (41%)
Formed	398 (11%)	3283 (11%)	3681 (11%)
Exclusively Breastfed			
Exclusive	20 (1%)	1703 (5%)	1723 (5%)

Shigella Infection within Prior 3 Months			
Prior Infection	1544 (44%)	4451 (14%)	5995 (17%)
Coinfection Pathogens			
Adenovirus 40/41 Coinfection	564 (16%)	3716 (12%)	4280 (12%)
Campylobacter Coinfection	1418 (40%)	8290 (27%)	9708 (28%)
Giardia Coinfection	1677 (48%)	9575 (31%)	11252 (32%)
Norovirus Coinfection	628 (18%)	4757 (15%)	5385 (16%)
Sapovirus Coinfection	598 (17%)	3751 (12%)	4349 (13%)
EAEC Coinfection	2198 (63%)	3751 (12%)	16890 (49%)
ETEC Coinfection	1631 (47%)	8058 (26%)	9689 (28%)
tEPEC Coinfection	676 (19%)	3350 (11%)	4026 (12%)
aEPEC Coinfection	1100 (31%)	7632 (25%)	8732 (25%)
Antibiotic Use			
Any Antibiotic Use			
+/- 15 Days from Stool Collection	1085 (31%)	9617 (31%)	10702 (31%)
+/- 30 Days from Stool Collection	1675 (48%)	13972 (45%)	15647 (45%)
Any Cephalosporin Use			
+/- 15 Days from Stool Collection	225 (6%)	2401 (8%)	2626 (8%)
+/- 30 Days from Stool Collection	381 (11%)	3920 (13%)	4301 (12%)
Any Macrolide Use			
+/- 15 Days from Stool Collection	202 (6%)	1942 (6%)	2144 (6%)
+/- 30 Days from Stool Collection	384 (11%)	3274 (11%)	3658 (11%)
Any Fluoroquinolone Use			
+/- 15 Days from Stool Collection	60 (2%)	404 (1%)	464 (1%)
+/- 30 Days from Stool Collection	119 (3%)	761 (2%)	880 (3%)

Table 2. Monthly EE biomarker concentration associations from time of initial subclinical *Shigella* detection through 6-months post-detection

Month after initial *Shigella* detection	MPO log[ng/mL] (95% CI)	NEO log[nmol/L] (95% CI)	AAT log[mg/g] (95% CI)
0	0.30 (0.23, 0.37)	0.01 (-0.05, 0.07)	-0.02 (-0.08, 0.04)
1	-0.04 (-0.17, 0.08)	-0.18 (-0.28, -0.07)	-0.04 (-0.15, 0.07)
2	0.06 (-0.08, 0.20)	-0.13 (-0.25, -0.01)	-0.09 (-0.22, 0.03)
3	-0.04 (-0.13, 0.06)	-0.14 (-0.22, -0.05)	-0.08 (-0.16, 0.01)
4	-0.08 (-0.23, 0.07)	-0.07 (-0.20, 0.07)	-0.08 (-0.20, 0.05)
5	-0.18 (-0.35, -0.00)	-0.14 (-0.30, 0.01)	0.02 (-0.12, 0.17)
6	-0.16 (-0.26, -0.04)	-0.13 (-0.24, -0.02)	-0.13 (-0.24, -0.03)

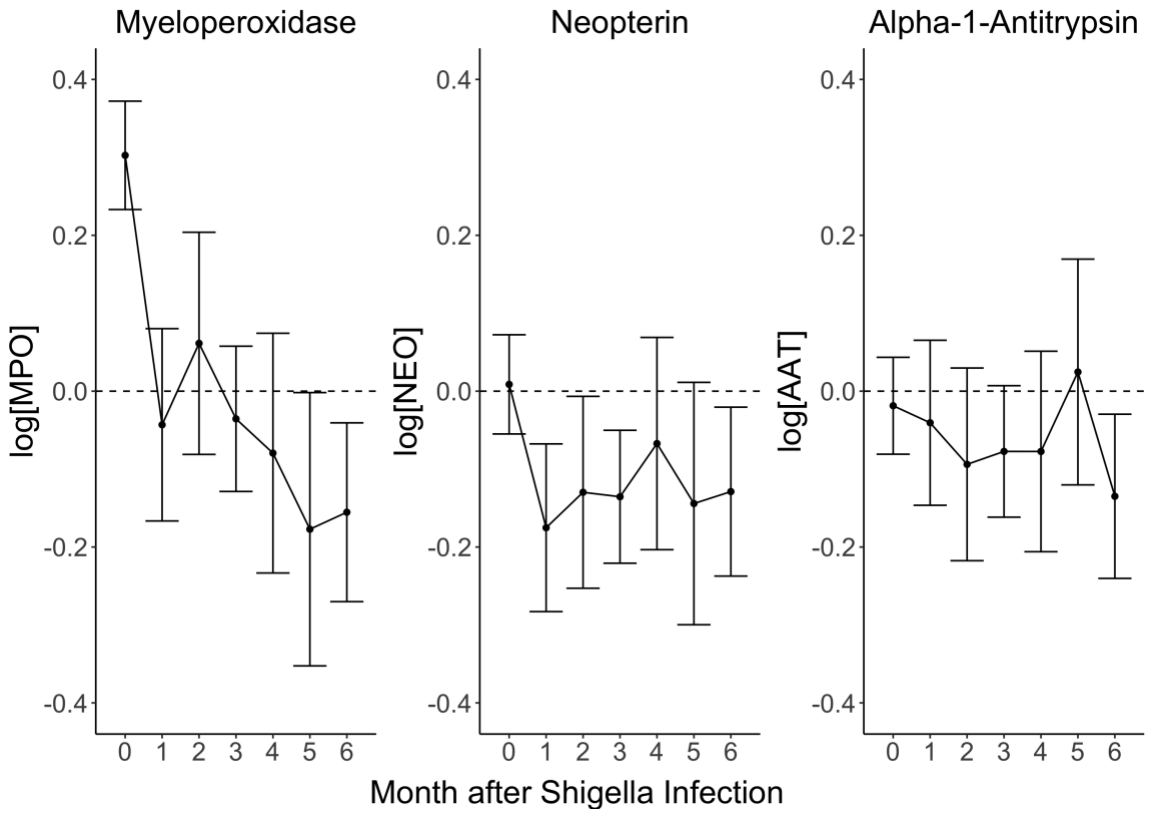

Figure 1. *Shigella* infection impact on EE biomarker concentration over time. Each plot is a depiction of respective EE biomarker concentration differences on the log scale of non-diarrheal stool samples with *Shigella* detection. Concentrations were measured monthly from the initial *Shigella*-positive detection through 6-months post-detection.

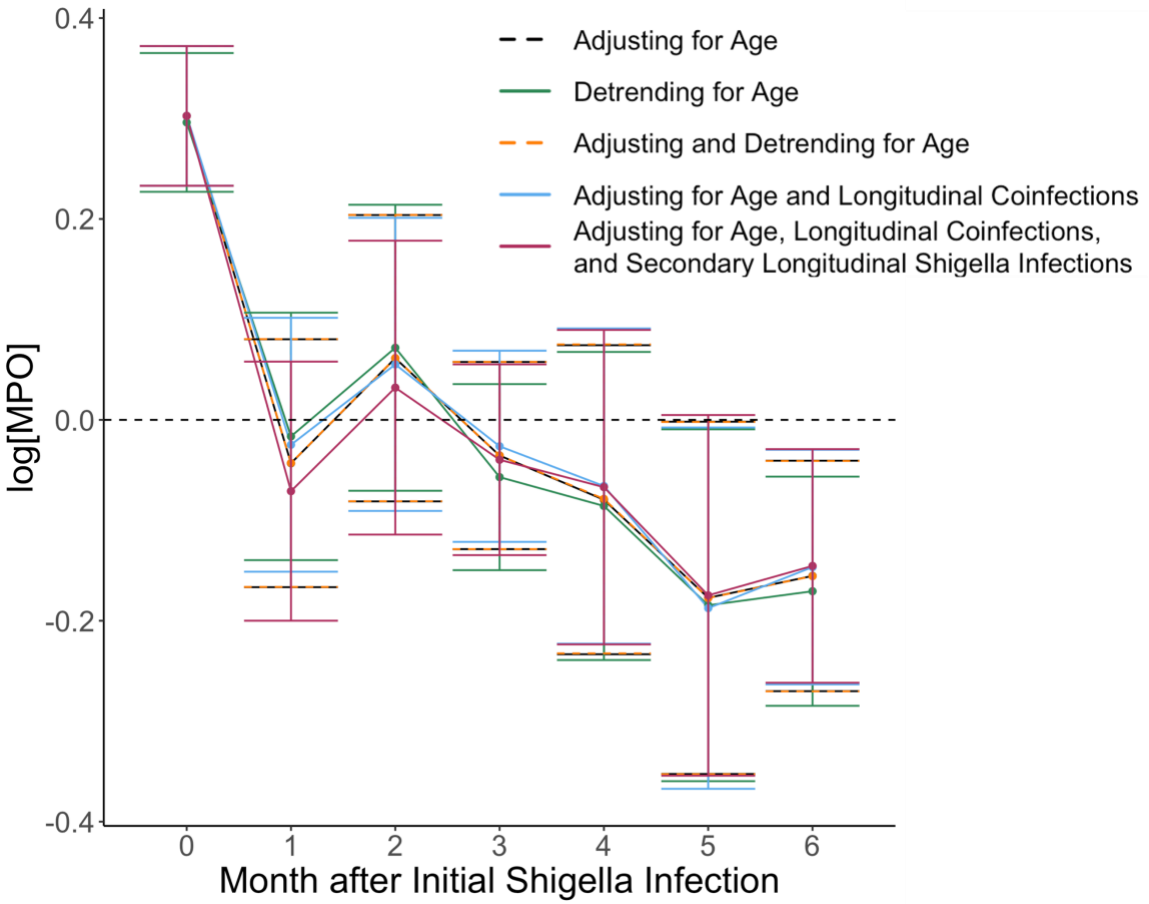

Figure 2. Longitudinal impact of *Shigella* infections on MPO concentrations by varying confounder definitions of age and coinfection covariate adjustments. The dashed black line represents the modeling while adjusting for age which completely overlaps with the dashed orange line that represents the model adjusting for age and detrending for age. The green line is the model only detrending for age. The blue line is the model adjusting for age and longitudinal coinfections. The purple line is the model adjusting for age, longitudinal coinfections, and longitudinal secondary *Shigella* infections.

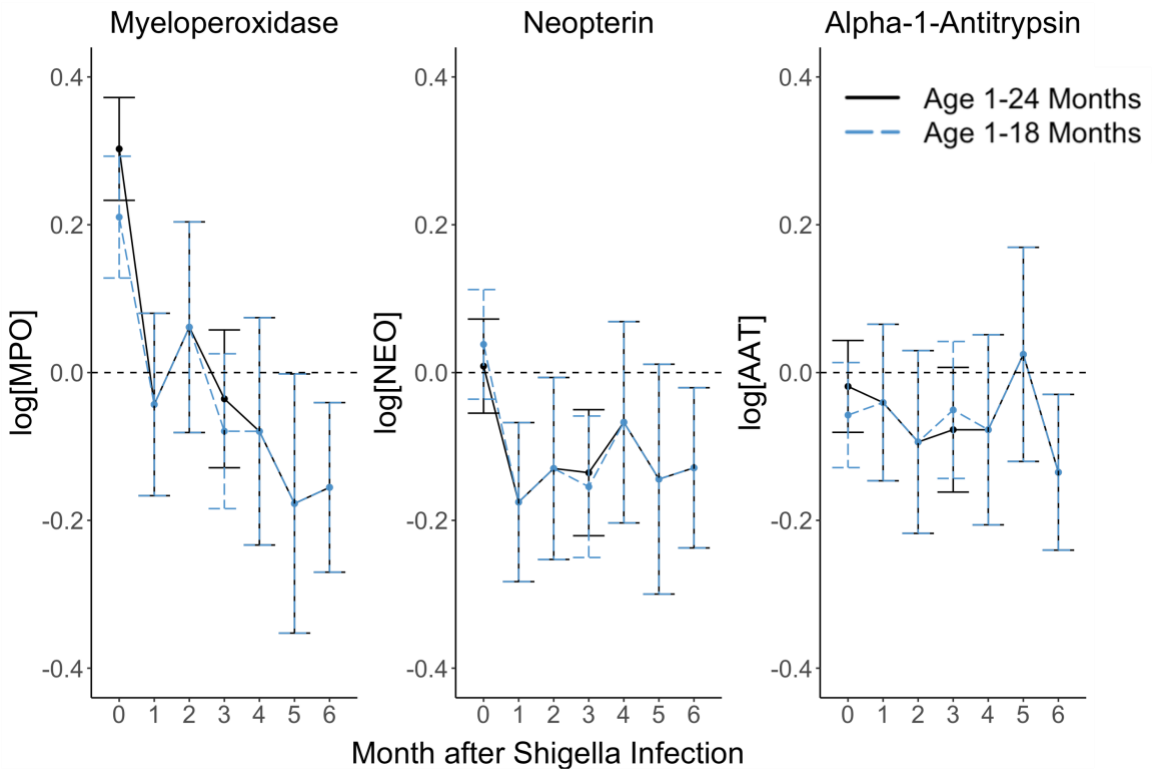

Figure 3. Longitudinal impact of *Shigella* infections on biomarker concentrations among children 1-24 months of age and among children 1-18 months of age. The association of subclinical *Shigella* infection on fecal biomarkers among children younger the age of 24 months is represented by the black line. The association of subclinical *Shigella* infection on fecal biomarkers among children younger than the age of 18 months is represented by the dashed blue line.

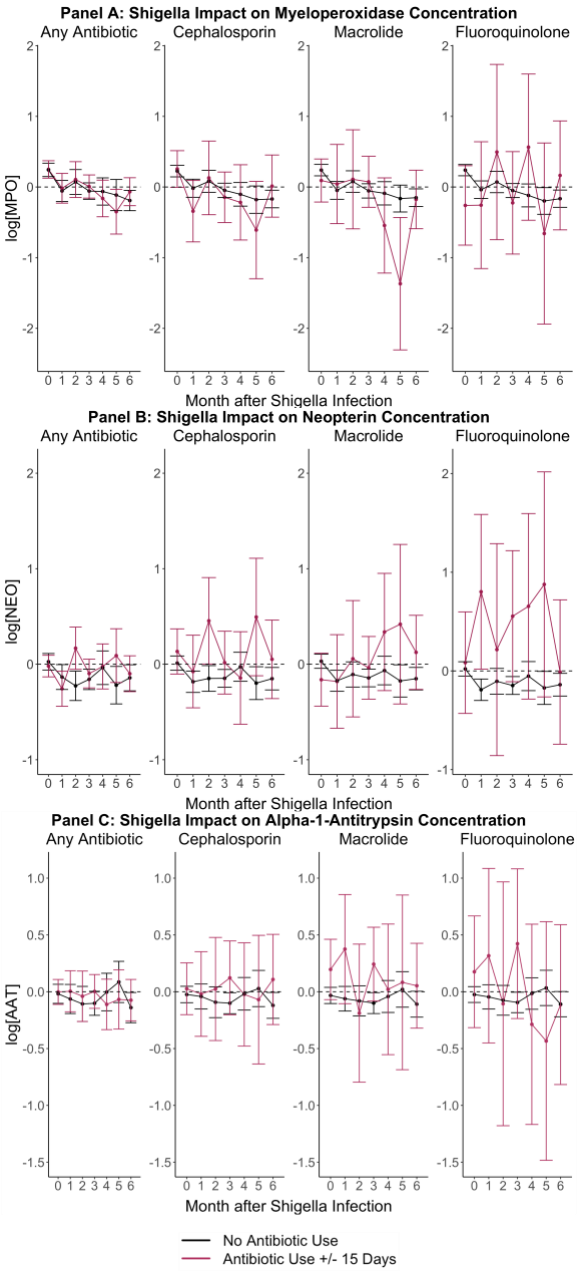

Figure 4. Monthly EE biomarker concentration associations from time of initial subclinical *Shigella* detection through 6-months post-detection by antibiotic-class use within the 15 days before or after *Shigella* infection. Each panel shows the respective EE biomarker concentration differences on the log scale of non-diarrheal stool samples with *Shigella* detection. Concentrations were measured monthly from the initial *Shigella*-positive detection through 6-months post-detection. The black line represents no antibiotic use, and the red line represents antibiotic use within the 30-day range around a *Shigella* infection.

DISCUSSION

The MAL-ED study design allowed for the unique longitudinal analysis of subclinical *Shigella* infections on EE fecal biomarker concentrations for 6 months post-infection among children across 8 global sites. At time of initial subclinical infection, MPO concentrations were elevated, but this elevation was not sustained in the following 6 months. Subclinical *Shigella* infections were not associated with elevated NEO or AAT concentrations within the initial month of infection nor the following 6 months. All 3 biomarker concentrations tended to be lower among stools with an initial *Shigella* infection compared to those without initial infections by, at the latest, 6-months post-infection. There was no evidence that there was a difference in long-term EE biomarker concentrations by the types of antibiotics taken around time of infection. The sensitivity analysis demonstrated that there is not much difference in the effects of *Shigella* on EE biomarker concentrations when detrending for age or adjusting for longitudinal coinfections and secondary *Shigella* infections, as well as when subsetting 24-month age to 18-month age.

Fecal biomarkers have shown to provide insight into gut function and EE in a non-invasive way. Some of the most common and easily measured biomarkers are MPO, NEO, and AAT which measure gut inflammation, immunity, and permeability respectively.[14] Elevated concentrations of these biomarkers have been associated with a risk for growth stunting among young children.[17] Children naturally have high levels of these biomarkers which does decrease with age, but compared to high-income countries, children living in LMICs have elevated levels.[12,23] While evidence has shown that the biomarkers are best indicative of stunting when measuring multiple pathogens, studies have found that specific enteric pathogens, particularly *Shigella*, can play a role in the effects of EE.[10,12,17,23] The effect of *Shigella* infection on EE fecal

biomarkers during the initial month of infection had similar findings as previous research. Among those with a subclinical infection in the MAL-ED study, MPO concentrations were consistently elevated at time of infection while there was not a clear difference in NEO and AAT concentrations compared to those not infected.[8,12] A secondary analysis of enteric pathogens on biomarker concentration using the MAL-ED cohort illustrated that *Shigella* infection at any age did not impact biomarker concentration, though it is important to note that this study used a less sensitive diagnostic for detecting *Shigella* which would explain the null finding in MPO concentrations.[23] Similar findings to ours came out of a Bangladesh study measuring *Shigella* and *E. coli* pathogen-types and the effect on biomarker concentrations.[24]

Our results found similar null findings longitudinally among all 3 biomarkers. Even though MPO concentrations were elevated among *Shigella* infections in the initial month, these concentrations declined in subsequent months. MPO, NEO, and AAT concentrations continued to decline until those with *Shigella* infections had lower levels of the biomarkers than those without *Shigella* infections. This clear difference appeared 6 months post-infection, the furthest our study measured. Even with the sensitivity analyses of detrending for age, measuring different longitudinal confounders and accounting for missing data, the data were minimally effected. The benefits of using a fecal biomarker score comprised of all 3 biomarkers have been proposed to address a holistic and more descriptive approach to defining EE, yet a study investigating *Shigella's* effect on an overall biomarker score returned null.[11] Those studies that did find an association between *Shigella* infection and EE fecal biomarkers noted that the effect was smaller than anticipated and more predominant in the first year of life.[12] Previous research that gathered results from cross-sectional data and our longitudinal results suggest that *Shigella* infections do

contribute to the short-term impacts of EE, but do not solely contribute to the long-term impacts of EE.

Shigella infections are commonly treated with fluoroquinolones, cephalosporins, and macrolides.[29] Despite site variability, a majority of *Shigella*-attributed diarrheal episodes in the MAL-ED study were treated with antibiotics which consisted of mostly the aforementioned specific classes.[8] Though, it is important to note that these antibiotics are suggested to be prescribed with the presence of dysentery which make up a small percentage of *Shigella* cases.[25] In the MAL-ED study, dysentery made up only 14% of *Shigella*-attributed diarrheal episodes.[6] A study observing a birth cohort in LMICs in similar global regions found that for every appropriate prescription of antibiotics, 13 inappropriate antibiotic prescriptions were wrote.[25] This mistreatment along with the evidence for *Shigella's* emerging resistance to fluoroquinolones, cephalosporins, and macrolides question their effectiveness of treating *Shigella* infections.[8,25,30] Our findings provide evidence that the biomarker concentrations alter only slightly if at all in *Shigella* infections with antibiotic treatment of fluoroquinolones, cephalosporins, and macrolides, and if they do, antibiotic treatment is typically seen with an increase in biomarker elevation throughout the 6 months post-infection. This suggests that antibiotic treatment does not influence the inflammation brought on by subclinical *Shigella* infections.

One major strength in this study, unlike previous research, is that we evaluate *Shigella's* longitudinal impact on EE biomarkers. Data was collected consistently from the same individuals over the course of 2 years.[26] While clinical cases impact EE, subclinical infections contribute persistently and unknowingly. We analyzed non-diarrheal stools for this purpose along with the knowledge that diarrhea dilutes biomarker concentration and cannot be analyzed

the same.[23] Associations between fecal biomarkers and age, breastmilk consumption, and other enteric infections have been noted, so appropriate adjustments were made in our analysis.[23] The advantage of using a dataset from a diverse and large population measuring common biomarkers with precise methods is that these results are more likely to be generalizable and reproducible. This study was limited by not all non-diarrheal stool samples being measured for biomarker concentrations. Monthly samples in the second year of life were measured quarterly for biomarker levels which gave the infections in the first year of life a heavier weight. There is lack of literature on healthy biomarker levels among children in both high-income countries and LMICs. While the elevated levels between those with and without *Shigella* infections can be measured, the interpretation of which elevated levels contribute to EE are hindered.

EE is a condition described by chronic inflammation and permeability of the intestines usually from colonization of enteric pathogens. EE results in and is exacerbated by malnutrition which has documented long-term effects of growth stunting, poor cognitive development, and reduced vaccine effectiveness.[13,14] Those most impacted are young children living in LMICs who have higher rates of exposure to enteric pathogens and poor access to nutritional diets.[13–15] Many studies have investigated which enteric pathogens contribute to EE, and *Shigella* has consistently found to be a high burden pathogen in LMICs with similar long-term effects to EE.[1,3] Recent findings have even observed that newer technology and more sensitive methods suggest that *Shigella* has a greater burden than previously documented and causes many subclinical infections.[5–8] It has been theorized that symptomatic and asymptomatic *Shigella* infections contribute to these adverse long-term effects through the cycle of EE. This study concludes that if *Shigella's* long-term effects are brought upon through a sustained impact on EE, these effects are not captured by the biomarkers studied here. Other mechanisms may also be at play.

Research has suggested that stronger associations between enteric pathogens and EE biomarkers arise when multiple pathogens are considered. Many children in these regions experience co-infections which may have a bigger impact on the long-term health of those infected. Our research also suggests that which antibiotic use for treating enteric pathogens and preventing EE needs to be reevaluated in these settings. It is important to continue researching the interaction of these pathogens and how interventions could decrease the burden of EE.

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