The Association between Intestinal Fatty Acid Binding Protein and Enteric Pathogens

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Abstract

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Introduction: Enteric pathogens account for an unacceptable number of pediatric deaths in Sub-Saharan Africa (SSA) every year. Intestinal fatty acid binding protein (I-FABP) is a small intracellular protein released into the bloodstream when intestinal epithelial cells are damaged and can be used to measure gut damage and recovery. This study aimed to compare I-FABP concentration in children with at least one of the following four pathogens of interest: *Shigella, Campylobacter,* EAEC, and *Giardia* to children without a pathogen of interest identified at enrollment as well as determine whether children randomized to a 5-day course of azithromycin have lower I-FABP levels 3 months after discharge than those randomized to placebo.

Methods: In this retrospective cohort study, we utilized linear regression models to assess the connection between I-FABP and each pathogen of interest in 1361 enrollment samples from children under 5 years old recently discharged from the hospital collected from the Toto Bora Trial. A subset of 971 children had

additional plasma specimens available for I-FABP assessment at month 3. Linear regression was used for the secondary analysis with a likelihood ratio test of nested models.

Results: A total of 60.8% of the children had at least one pathogen of interest at enrollment. Of those children with a pathogen of interest, 11.9% had a *Shigella* infection, 33.2% had *Giardia*, 9.0% had *Campylobacter*, and 74.2% had enteroaggregative *Escherichia* coli. Mean I-FABP concentration at enrollment for children without a pathogen of interest was similar when compared to the mean of all pathogens of interest combined (no pathogen of interest mean= 1653.1 pg/mL, all pathogens of interest mean =1663.4). Increased I-FABP was, however, found to be significantly associated with younger age, being underweight, and being stunted (all p-values<0.01), while decreased I-FABP was observed in children who received an antibiotic during their hospital stay (p=0.038). No significant difference was found in the mean I-FABP levels of children with or without a pathogen of interest both individually by pathogen and collectively (each comparison, p>0.05). Unexpectedly, I-FABP levels significantly increased from enrollment to 3 months post discharge in all groups (+337.61 pg/mL, p<0.05). The I-FABP level difference between enrollment and month 3 and was found to be 74.17 pg/mL less in the AZM randomization group compared to placebo [95% CI = (-253.23, 104.88 pg/mL)] but was not statistically significant (p=0.416).

Conclusions: Higher I-FABP levels were found in malnourished children and lower I-FABP levels were found in children who had received antibiotics at their hospital stay prior to study initiation. We did not find an association between any of the pathogens we examined and I-FABP level, except for an inverse association with *Shigella* cycle thresholds. In contrast to our hypothesis that I-FABP levels would decrease during the post-discharge period, we found that I-FABP increased in all groups after hospitalization discharge in our cohort and was not associated with randomization arm. Further research is needed to understand why intestinal inflammation may increase following discharge, and whether this increase has clinical significance.

INTRODUCTION

Nearly 500,000 children die each year in Sub-Saharan Africa (SSA) due to diarrheal disease.[1] When accounting for stunted growth that often follows diarrheal illness, enteric pathogens are estimated to cause an additional 10-20% more deaths than those directly attributed to diarrheal illness. [1] Enteric pathogens are thought to cause growth stunting through a syndrome named environmental enteric dysfunction or EED.[2–4] This syndrome is thought to be the consequence of frequent enteric pathogen exposure leading to blunting of intestinal villi, increased permeability of the small intestine, chronic gut inflammation, and a disruption of the gut microbiome.[2] Mechanisms underlying pathogen-mediated EED and therapeutic targets are urgently needed to address child morbidity, undernutrition, and mortality in SSA.

Intestinal fatty acid binding protein (I-FABP or FABP2) is used to assess gut-related disease severity and recovery in various clinical settings. I-FABP is a small intracellular protein which regulates the fatty acid metabolism cycle within the small intestine.[5] During instances of inflammation or trauma to the gut, intestinal villi enterocytes become damaged and release I-FABP into the body's circulatory system.[5] As I-FABP is released into the body, it can be detected and quantified in non-invasive peripheral blood serum samples as opposed to a standard intestinal biopsy (see Figure 1). Recently, I-FABP has been used in various studies to predict disease severity, identification, and morbidity and mortality in many settings due to its correlation with intestinal ischemia and damage.[6,7] Studies have shown that I-FABP has been able to accurately predict disease severity and mortality in cohorts of individuals with compromised kidney function[8], COVID-19 [9,10], septic shock[11], celiac disease[12–14], malaria[15], and enteric pathogens such as *Giardia* infection[16] and enterotoxigenic *E. coli.*[17] Additionally, I-FABP has been shown to accurately describe disease recovery, as the concentration varies over time and as damage to the gut changes or is resolved. One study showed that patients who had intestinal jejunum ischemia followed by intestinal reperfusion during surgery had decreased I-FABP levels when compared to patients who did not have reperfusion, indicating that it is possible to quantitatively record patient gut recovery with I-FABP.[6] Furthermore, studies have shown that I-FABP can indicate gut recovery in children with celiac disease after initiation of a gluten-free diet as compared to those who did not adhere to the diet.[14]

Enteric pathogens differ in their association with growth stunting, possibly due to a differential relationship to EED. *Shigella, Campylobacter*, enteroaggregative *Escherichia coli*, and *Giardia*, each have been found to be associated with growth stunting and have been shown to cause damage to the intestinal villi of their hosts as measured by intestinal biopsy, an invasive measure not scalable for identifying children with EED.[4] I-FABP has been looked at in relation to only one of the pathogens of interest: *Giardia*. Among Brazilian children aged 10 months to 4 years, I-FABP levels were significantly higher in children with *Giardia* infections (mean I-FABP=1274 pg/mL) than children without (mean I-FABP=741 pg/mL; p=0.05).[16] With I-FABP's use in identifying intestinal damage in other disease models, we sought to determine whether I-FABP is associated with specific enteric pathogens known to be associated with growth stunting.

The central hypothesis of our study was that enteric pathogens contribute to increased intestinal inflammation in children at post-hospital discharge and can affect responses to antimicrobial therapy. We evaluated intestinal pathogens and the intestinal injury marker, I-FABP, in 1361 children enrolled in the Toto Bora trial, a well characterized cohort of Kenyan children followed longitudinally for six months post hospital discharge, half of whom received a 5-day course of azithromycin starting on the day of discharge.[18] In this nested study, we assessed the association between plasma I-FABP levels and the presence and quantity detected in rectal swabs of four enteric pathogens which cause substantial morbidity and are highly inflammatory: *Shigella*[1], *Campylobacter*[2], EAEC,[2] and *Giardia*[3]. Further, we evaluated the effect of randomization to a five-day post-hospital discharge course of azithromycin compared to placebo on 3-month I-FABP concentrations in subgroups of children defined by the four enteric pathogens of interest.

Aim 1: To compare enrollment I-FABP concentrations in children with each of the following four pathogens of interest: *Shigella, Campylobacter*, EAEC, and *Giardia* to children without an enteric pathogen identified at enrollment in the study.

Hypothesis: Children with *Shigella, Campylobacter*, EAEC, and *Giardia* will have higher blood serum concentrations of I-FABP at enrollment than children without any of the pathogens of interest.

Aim 2: To determine whether children randomized to a 5-day course of azithromycin (AZM) have lower I-FABP levels 3 months after discharge than those randomized to placebo and to test whether the effect is greatest in children with *Shigella, Campylobacter, EAEC,* and or *Giardia* at baseline.

Hypothesis: Children randomized to azithromycin will have lower I-FABP levels than children randomized to placebo 3 months after discharge; the effect will be greatest among children with at least one of the inflammatory enteric pathogens of interest at discharge.

METHODS

Study Design

This project utilized a retrospective cohort study design. We evaluated the association between four specific enteric pathogens of interest (*Shigella*, *Campylobacter*, EAEC, and *Giardia)* and I-FABP levels at enrollment (hospital discharge) (Aim 1), whether randomization to AZM was associated with lower I-FABP levels at three months, and whether azithromycin's effect on I-FABP was modified by infection with enteric pathogens of interest (Aim 2).

Setting

The cohort of children for this study were screened for eligibility from four hospital inpatient wards in Western Kenya: Kisii Teaching and Referral Hospital, Homa Bay Teaching and Referral Hospital, St. Paul Mission Hospital, and Kendu Adventist Mission Hospital. Children were screened for eligibility between June 28th, 2016, and November 4th, 2019.

Study Participants

Participants within the parent study included a cohort of 1,400 male and female children between the ages of 1 and 59 months who have recently been discharged from an inpatient hospital stay and planned to stay in the study area for the next 6 months. For aim one, 1,361 children had enrollment plasma samples available that were tested for I-FABP levels. For aim two, a subset of 971 additional I-FABP measurements were included at month 3; this smaller subset represents samples available and tested at the time of this analysis. Children were eligible to participate in the parent study if they were admitted to the hospital for any medical reason excluding trauma, poisoning, or congenital anomaly. Additionally, children who had

been prescribed a macrolide antibiotic at discharge, were taking a protease inhibitor for HIV, or had a documented allergy to a macrolide antibiotic were also excluded from the parent trial as were children with a twin already enrolled in the study. For this retrospective nested study, all children with stool and plasma samples available at enrollment were eligible for I-FABP testing.

Data Source & Collection

Data and specimens from the Toto Bora trial were used in this study.[19] Participants were seen by the study team and clinical providers at enrollment (hospital discharge), 3 months post-discharge, and 6 months post-discharge, and this paper includes measurements from the enrollment and 3-month timepoints. Initial interviews with caregivers were administered at study enrollment to gather household demographics, medical history, information about the current hospitalization, HIV status, and treatment information. At each study visit, blood samples and rectal swab specimens were collected, and a standardized questionnaire was administered to collect information on clinical status, illnesses, and treatments. Height, weight, and mid-arm circumference were collected, and z-scores were calculated based on the WHO standard reference population.[20] Consent for each child was collected from their caregivers through informed written consent in their preferred language as well as through witnessed thumbprints if caregivers were unable to read the consent documents. The study was approved by the UW IRB and the Kenya Medical Research Institute IRB.

Pathogen Detection

Flocked rectal swabs and/or whole stool samples from enrollment were collected from each child and were tested by quantitative PCR using a customized TACMan Array card, as described elsewhere.[18] While the TAC assay measures approximately 61 serotypes and sub-serotypes of enteric pathogens, *Shigella*, *Campylobacter*, EAEC, and *Giardia* were chosen as primary pathogens of interest, as they are the most well-characterized as being highly inflammatory, and associated with clinical disease severity. [1,3]

I-FABP Measurements

Two labs conducted the I-FABP analysis, Fred Hutch Cancer Center in Seattle, WA and the Centre for Microbiological Research (CMR) Kenya Medical Research Institute in Nairobi, Kenya. I-FABP

concentrations were ascertained through a commercial enzyme-linked immunosorbent assay (Quantikine ELISA, R&D Systems, Inc.), using plasma samples collected from enrollment and 3-month blood draws for each participant. All kits used for this analysis were purchased from the same manufacturer and had standardized lower and upper limits of detection (78 pg/mL, 5000 pg/mL respectively).

Statistical Analysis

For our first aim, we considered both pathogen presence (at or below the lower limit of detection [cycle threshold values {Ct} value of 35]) and quantity of DNA (as measured by Ct values which are inversely related to pathogen DNA concentration). I-FABP concentrations were considered as continuous variables, as most samples were detectable, and to enable evaluation of possible dose-response relationships. I-FABP concentrations had a relatively normal distribution, thus absolute concentrations were reported to optimize interpretability. To contextualize I-FABP concentrations, we conducted a correlates analysis using linear regression assessing key baseline demographic and clinical features of children enrolled in the Toto Bora trial and baseline I-FABP levels adjusted for age and hospital site.

In aim one, mean I-FABP concentrations were compared in children with *Shigella, Campylobacter,* EAEC, *and Giardia* at enrollment to children without the pathogens of interest at enrollment, individually, and collectively. All linear regression models adjusted for continuous age and enrollment hospital. Additionally, we ran secondary linear regression models with the pathogen Ct values as a continuous variable to assess the dose response relationship between pathogen quantity and I-FABP concentrations at enrollment.

For the second aim of this project, we used linear regression to assess mean change in I-FABP levels between baseline and 3 months between those who received a 5-day course of azithromycin and those who received placebo adjusted for enrollment site and enrollment site. Effect modification was tested by including an interaction term between presence/absence of *Shigella*, *Campylobacter,* EAEC, and *Giardia* at baseline with randomization arm. The interaction term was tested using a likelihood ratio test of nested models with and without the interaction of the pathogens combined and individually by randomization arm.

RESULTS

Participant characteristics and pathogen detection

Of the 1400 children enrolled in the parent trial, two children, one from each of the randomization groups, were removed from the analysis due to ineligibility after randomization. Additionally, 37 children did not have blood samples, or their samples had low volumes that were unable to be tested, leaving a total enrollment sample size of 1361 children. Of the 1361 children included in the study, 683 (50.2%) were randomized to receive a 5-day course of azithromycin (oral suspension 10 mg/kg on day one, followed by 5 mg/kg per day on days 2–5) and 678 (49.8%) to an identical appearance and taste placebo. Among the 1361 participants, 808 (59.4%) were males, and 519 (38.1%) were two years or older. At enrollment, 308 (22.7%) of the children were stunted (height for age z-score < -2), and 171 (12.6%) were underweight (weight for age z-score <-2). Most common diagnoses were lower respiratory tract infections, malaria, diarrhea, anemia, sickle cell, and malnutrition (Table 1). Additionally, many children were prescribed an antibiotic during their hospital stay (1220 [89.6%] as well as many being prescribed an antibiotic (non-macrolide, per eligibility criteria) at discharge (847 [62.2%]).

I-FABP Detection and Correlates

1343 children of the 1361 had detectable I-FABP levels (98%), and the mean level overall was 1659.4 pg/mL (SD= 1126.0 pg/mL). Children who were stunted (β=206.2 pg/mL (95% CI: 54.72, 357.61). p=0.008) or underweight (β =401.1 pg/mL, (95% CI: 197.5, 604.5), p<0.001) had increased I-FABP when compared to their healthy height and weight peers (Supp. Table 1). Additionally, children under the age of 24 months had increased levels of I-FABP (β-=234.93, (95% CI: 19.5, 450.4) p=0.03). Children who received antibiotics as part of their in-patient stay preceding enrollment in the trial had lower I-FABP levels on average (β=-246.93, (95% CI: -479.6, 14.25), p=0.038).

Enteric Pathogens

The inflammatory pathogens were common among all children: 7.2% had a *Shigella* infection, 20.2% had *Giardia*, 5.4% had *Campylobacter*, and 45.1% had enteroaggregative *Escherichia* coli. A total of 60.8% of the children had at least one pathogen of interest at enrollment (Table 2).

I-FABP levels and pathogens of interest

Children with one or more of the enteric pathogens of interest did not have higher I-FABP levels than children without (adjusted mean difference 21.5 pg/mL, 95% CI: (-101.7, 144.6), p=0.732). Similarly, presence of any individual pathogen of interest was not associated with IFAB-P (Table 3). *Shigella,* however, did appear to have a negative dose response relationship with IFAB-P, such that each one-unit increase in cycle threshold (corresponding to lower bacterial load) was associated with a 17.6 pg/mL higher I-FABP level (95% CI 4.1, 31.0, p-value =0.01). No other pathogens appeared to have a quantitative relationship with IFAB-P levels at hospital discharge.

Randomization and changes in I-FABP levels post-discharge

The mean I-FABP level increased by 337.61 pg/mL (SD= 1386.27 pg/mL, $p<0.05$) between hospital discharge and 3 months later among all children combined. The change in IFAB-P levels between enrollment and discharge did not differ between azithromycin and placebo randomization arms (difference in difference [DD] -74.12 (95%CI: -253.23, 104.88) p=0.42) (Table 4). There was modest evidence of effect modification by presence of an inflammatory enteric pathogen (likelihood ratio p-value= p=0.074.); children with a pathogen of interest randomized to azithromycin compared to placebo appeared to have a slight increase in mean IFAB-P levels (DD: 125.18 (95%CI: -360.31, 109.95) at 3 months whereas children without a pathogen of interest did not (DD: -4.36 (95%CI: -273.04, 281.76) (Table 5) .

DISCUSSION

In this cohort of children who were hospitalized and subsequently discharged, we found a high prevalence of infection with enteric pathogens associated with growth stunting. These pathogens did not appear to be differentially associated with I-FABP levels other than higher quantity *Shigella* infections, which was be associated with lower IFAB-P levels. There did not appear to be an effect of AZM on I-FABP changes in the three months post-randomization overall. However, there did appear to be modest evidence that presence of an inflammatory pathogen at discharge led to a slightly larger increase in IFABP levels among children randomized to azithromycin compared to placebo, a finding that warrants further exploration.

At discharge from hospital, children had a relatively high level of IFABP (median= 1306.9 pg/mL) as compared to reported values in pediatric populations in the United States and the Netherlands, which medians range from 20 pg/mL to 485 pg/mL.[14] EED has been documented across settings where access to clean water and sanitation is limited, such as in Kenya, and it's likely that children enrolled in the Toto Bora trial are no more or less likely to have EED than other populations in similar LMIC settings.[4] When compared to a healthy cohort of 2-year old children in Nairobi, the Linda Kizazi cohort, the age matched individuals from Toto Bora (18-30 months) showed similar median I-FABP levels at enrollment (Supplemental Figure 1). The fact that IFAB-P levels did not appear to go down in the 3 months following discharge from hospital further substantiates this hypothesis that EED is common among children living in Western Kenya, not only those who have been hospitalized.

Azithromycin did not appear to impact I-FABP levels. This broad-spectrum macrolide antibiotic has both antibacterial and anti-inflammatory properties thus it is biologically plausible that this antibiotic effect varied by pathogen status.[21] Intriguingly, we found modest evidence of effect modification by baseline enteric pathogen status, such that the subgroup of children with an inflammatory pathogen of interest at baseline appeared to have a greater change in I-FABP levels between enrollment associated with azithromycin than children without the enteric pathogens of interest. Given we found increased I-FABP levels to be associated with age, and antibiotic use cross-sectionally, it could be that children with inflammatory enteric pathogens were less likely to have been exposed to antibiotics during their hospital stay and thus experienced higher I-FABP levels in the months following discharge.

As discussed earlier, I-FABP has also been shown to be high in cohorts of children who live in LMIC even without direct enteric pathogen infection or hospital stay exposure as explained by the I-FABP levels of the Linda Kizazi cohort of healthy children. Therefore, we suspect that children's I-FABP increased after hospital stays due to them returning to their homes and environments (often with unstable food access, leading to possible undernutrition, ceasing antibiotics from their hospital stay, and returning to areas with high burden of enteric pathogens), similar to circumstances that can cause EED which is characterized by gut inflammation and damage. This concern may have also skewed our ability to pinpoint

the direct cause of I-FABP level changes. EED may also cause persistent high I-FABP levels, however this syndrome and diagnosis was not analyzed directly in this study and cannot be assessed as a defined variable, only hypothesized.

Limitations

This study has several limitations. First, I-FABP levels have been shown to change quickly, with some measurements taken in minute or hour increments to assess recovery of intestinal jejunum after ischemia and reperfusion.[6] It is therefore possible that the extended time period of three months may be too long to identify acute changes in I-FABP in our sample. The time of sample collection at three months may also dampen the observable effect of azithromycin or placebo on I-FABP as the treatment was only five days in duration. The effect of antibiotics on I-FABP levels may be better assessed with plasma measurements closer to the completion of the antibiotic regimen. We also recognize the limitation of possible co-infection with other inflammatory pathogens and disease. Although best attempts were made to reduce intra-lab variability in I-FABP samples, there is a possibility of variability in samples tested in Seattle, Washington versus those tested in Nairobi, Kenya. However, standardized manufacturer assumptions and procedures were employed at both labs and no noticeable difference in the distribution of I-FABP levels were identified throughout the analysis process. We also acknowledge that due to the cohort we selected having been hospitalized, survival bias is present within the sample as acutely ill children who would be most likely to have extremely high I-FABP, could have died during hospitalization.

Conclusion

Enteric pathogen infection and diarrheal disease are still a substantial concern for children living in LMIC. Although our results suggest that I-FABP levels were not associated with the presence or absence of *Shigella, Campylobacter,* EAEC, or *Giardia* infection in this cohort, our results do demonstrate that overall, I-FABP levels in children recently discharged from the hospital increase after 3 months, regardless of antibiotic usage after discharge. Investigation into the timeline best suited for I-FABP measurement after antibiotic interventions should be considered. Further research identifying the relationship between I-FABP

and specific species or groups of enteric pathogens, overall gut inflammation, and EED in children living in LMIC is warranted.

Appendix

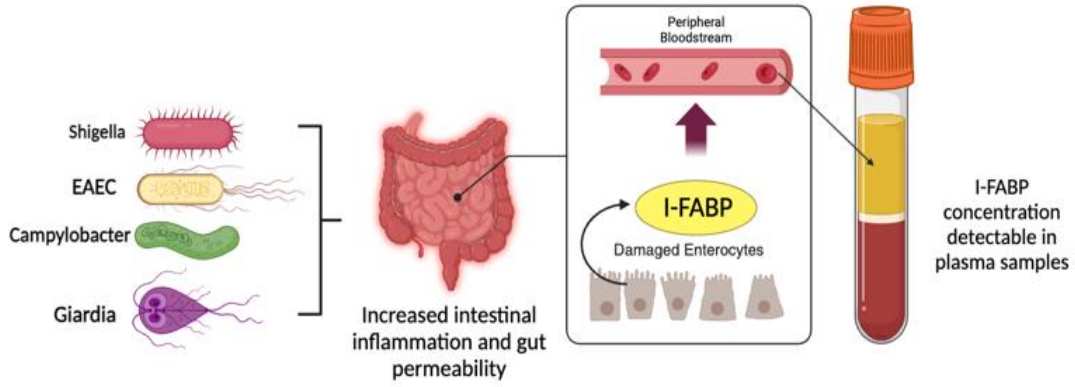


Figure 1. Process of detecting I-FABP levels after infection with enteric pathogens in plasma samples.

Table 1. Demographics of participants with plasma samples at enrollment from the Toto Bora Trial, N=1361

Characteristic	Whole Cohort (%) N=1361
Enrollment site	
Kisii	799 (58.7)
Homa Bay (combined with St. Paul & Kendu)	562 (41.3)
Age categories (in months)	
0-5	180 (13.2)
6-11	265 (19.5)
12-23	397 (29.2)
24-59	519 (38.1)
Sex assigned at birth	
Male	808 (59.4)
Female	553(40.6)
Breastfeeding	
Exclusively breastfed	646 (47.5)
Partially breastfed	613 (45.0)
Never breastfed	24 (1.8)
Unknown	78 (5.7)
Growth Characteristics	
Underweight (WAZ< -2)*	171 (12.6)
Stunting (HAZ< -2)*	308 (22.7)
Wasting (WHZ < -2)*	77 (5.7)
AZM randomization status	
Azithromycin	683 (50.2)
Placebo	678 (49.8)
HIV Infection Status	
HIV unexposed	1161 (85.3)
HIV exposed, uninfected or status unknown	143 (10.5)
HIV infected	17 (1.3)
HIV uninfected, unknown exposure	40 (2.9)
Discharge Diagnoses	
Anemia	173 (13.4)
Diarrhea	241 (18.6)
Lower respiratory tract infection	427 (33.0)
Malaria	328 (25.3)
Malnutrition	85 (6.6)
Sickle Cell	106 (8.2)
None of the above diagnoses	291 (21.4)
Antibiotic Background	
Antibiotics during hospital stay	1220 (89.6)
Antibiotics prescribed at discharge	847 (62.2)

Antibiotics reported during follow up	306 (22.5)

**WAZ= Weight for age z-score, HAZ= Height for age z-score, WHZ= Weight for height z-score, all based on WHO nutrition and growth standards*

Table 2. Frequency table of pathogen infection status of participants at enrollment.

Pathogen Infection Status*	**Whole Cohort (%**) N=1361**
Pathogens of Interest	
Shigella	98 (7.2)
Giardia	275 (20.2)
Campylobacter	74 (5.4)
EAEC	614 (45.1)
Any pathogen(s) of interest	**827 (60.8)**
Other Common Pathogens	
Cryptosporidium	110 (8.1)
Typical Enteropathogenic *Escherichia Coli (EPEC)*	71 (5.2)
Atypical EPEC	180 (13.2)
Rotavirus	118 (8.7)
Norovirus	89 (6.5)

**Infection is measured dichotomously as positive (cycle threshold under 35) or negative (cycle threshold over 35).*
***Due to co-infection, it is possible for children to have one or many of the pathogens, so percentages may add to over 100%*

Table 3. Mean I-FABP concentration of participants with various pathogens of interest detected at enrollment and difference of mean I-FABP from children without a pathogen of interest (n=1361).

	Enrollment I-FABP mean (pg/mL) (SD)	Mean Difference (pg/mL), 95% CI (Adjusted)*, **	p-value for adjusted model
Enrollment Pathogen Presence			
Any one of *Shigella*, *Giardia*, *Campylobacter*, EAEC (n= 818)	1663.4 (1122.8)	21.5 (-101.7, 144.6)	0.732
S*higella* (n=98)	1448.0 (971.8)	-146.6 (-363.9, 70.6)	0.186
Giardia (n=275)	1635.5 (1075.9)	61.8 (-100.5, 224.0)	0.455
Campylobacter (n=74)	1864.9 (1158.5)	175.7 (-100.3, 451.6)	0.212
EAEC (n=614)	1690.6 (1145.2)	28.6 (-104.4, 161.5)	0.673
No pathogen of interest (n=534)	1653.1(1132.1)	---	---
Enrollment Pathogen Cycle Threshold*			
Shigella (n=98)	---	17.6 (4.1, 31.0)	**0.010**
Giardia (n=275)	---	-4.4 (-20.7, 11.8)	0.594
Campylobacter (n=74)	---	-25.0 (-67.8, 17.7)	0.251
EAEC (n=614)	---	-3.1 (-14.6, 8.44)	0.599

* Adjusted for site and age

** Mean difference is the difference in I-FABP between the pathogen group and no pathogen of interest

*** Mean difference in I-FABP for every one unit increase in pathogen cycle threshold value. Cycle threshold values increase as pathogen quantity decreases.

Table 4. Effect of a 5-day course of azithromycin versus placebo on enrollment and month 3 I-FABP levels not stratified by pathogen of interest.

	Unstratified Effect of AZM						
	AZM		Placebo		Effect Estimate*		
Outcome	Mean	SD	Mean	SD	Difference	95% CI	p-value
Mean I-FABP (pg/mL)							
Month 0	1572.71	1005.26	1679.64	1194.48	--	--	--
Month 3	1947.16	955.62	1977.8	998.29	--	--	--
Difference (M3-M0)	+374.45	1317.52	+299.96	1453.66	+74.17	(-104.88, 253.23)	0.416

*Adjusted for continuous age and site.

Table 5. Effect estimates of a 5-day azithromycin course versus placebo on I-FABP change from enrollment to month 3 stratified by pathogen of interest presence or absence at baseline.

	Pathogen of interest				**No pathogen of interest**			
	AZM	Placebo	Effect Estimate*		AZM	Placebo	Effect Estimate*	
Outcome	**Mean (SD)**	**Mean (SD)**	**Difference (95% CI)**	**p-value**	**Mean (SD)**	**Mean (SD)**	**Difference (95% CI)**	**p-value**
Mean I-FABP (pg/mL)								
Month 0	1541.27 (994.06)	1680.38 (1181.87)	--	--	1621.36 (1023.16)	1678.45 (1218.08)	--	--
Month 3	2007.05 (996.54)	2024.51 (1018.57)	--	--	1854.56 (883.34)	1906.82 (962.97)	--	--
Difference (M3-M0)	+465.78 (1358.78)	+344.13 (1481.21)	+125.18 (-109.95, 360.31)	0.296	+233.20 (1241.48)	+228.38 (1409.16)	-4.36 (-281.76, 273.04)	0.975

*Adjusted for site and age

Supplemental Materials

Supplemental Table 1. Correlates of I-FABP level at enrollment of all participants with I-FABP samples (n=1361).

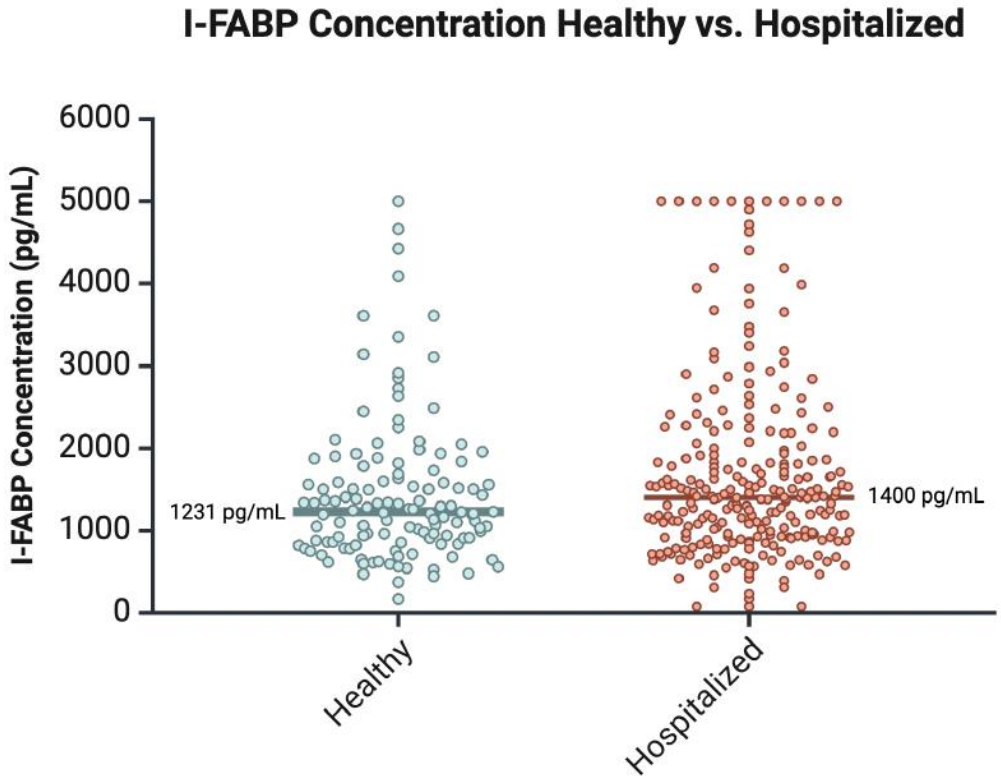
Characteristic	Mean IFAB-P (pg/mL)	Beta from linear regression model (95% CI) *	p-values (0.05 significance)
Enrollment site			
Kisii	1715.44	Ref	N/A
Homa Bay (combined with St. Paul+Kendu)	1620.12	-122.52 (-247.02, 1.97)	0.054
Age categories (in months)			
0-5	1771.67	321.74 (126.7, 516.8)	**0.001**
6-11	1758.53	298.62 (126.1, 471.2)	**0.001**
12-23	1795.63	329.54 (185.5, 473.6)	**<0.000**
24-59	1467.7	Ref	N/A
Under 24 months	1778.93	234.93 (19.45, 450.41)	**0.033**
Continuous, by month	NA	-9.5 (-13.1, -5.9)	**0.000**
Sex assigned at birth			
Male	1669.5	Ref	N/A
Female	1644.47	-22.21 (-142.6, 98.2)	0.72
Breastfeeding			
Exclusively breastfed	1671.67	Ref	
Partially breastfed	1667.60	30.55 (-101.1, 162.2)	0.65
Never breastfed	1491.04	-180.81 (-596.72, 231.1)	0.39
Unknown	1546.78	-40.66 (-301.2, 219.9)	0.76
Growth Characteristics			
Underweight (WAZ< -2)**	2025.77	401.0 (197.49, 604.5)	**0.000**
Not underweight	1608.07	Ref	
Stunting (HAZ< -2)**	1799.81	206.16 (54.72, 357.61)	**0.008**
Not stunted	1617.95	Ref	
AZM randomization status			
Azithromycin	1629.49	-67.13 (-186.9, 52.62)	0.20
Placebo	1689.29	Ref	
HIV Infection Status			
HIV unexposed	1646.96	Ref	
HIV exposed, uninfected or status unk.	1775.14	100.85 (-117.98, 319.68)	0.36
HIV infected	2046.73	446.06 (151.63, 1043.75)	0.14
HIV uninfected, unk. exposure	1438.49	-170.41 (-493.32, 152.5)	0.3
Antibiotic Background (No AZM)	**(SD)**		
Antibiotics during hospital stay	1632.52 (1097.9)	-246.93 (-479.6, -14.25)	**0.038**
Antibiotics prescribed at discharge	1587.78 (1040.4)	-204.52 (-333.4, -75.6)	**0.002**
Antibiotics reported during follow up	1688.99(1177.93)	-2.93 (-152.3, 146.4)	0.97
Enteric Pathogen			
Shigella	1447.09	-166.36 (-367.6, 34.86)	0.12

EAEC	1690.63	30.97 (-90.49, 152.44)	0.61
Campylobacter	1864.93	171.38 (-92.79, 435.54)	0.2
Giardia	1635.48	60.05 (-86.55, 206.65)	0.4
Any of the above infections	1663.40	21.49 (-101.66, 144.60)	0.73

*Adjusted for site and age

** *WAZ= Weight for age z-score, HAZ= Height for age z-score, WHZ= Weight for height z-score, all based on WHO nutrition and growth standards*

Supplemental Figure 1. Median I-FABP concentration levels in a subset of children 18-30 months from Toto Bora and a healthy community of 24 month old Kenyan children in Nairobi.



References

1. Anderson JD, Bagamian KH, Muhib F, et al. Burden of enterotoxigenic Escherichia coli and shigella non-fatal diarrhoeal infections in 79 low-income and lower middle-income countries: a modelling analysis. *The Lancet Global Health*. 2019;7(3):e321-e330. doi:10.1016/S2214-109X(18)30483-2

2. Keusch GT, Denno DM, Black RE, et al. Environmental Enteric Dysfunction: Pathogenesis, Diagnosis, and Clinical Consequences. *Clinical Infectious Diseases*. 2014;59(suppl_4):S207-S212. doi:10.1093/cid/ciu485

3. Kosek MN, Ahmed T, Bhutta Z, et al. Causal Pathways from Enteropathogens to Environmental Enteropathy: Findings from the MAL-ED Birth Cohort Study. *EBioMedicine*. 2017;18:109-117. doi:10.1016/j.ebiom.2017.02.024

4. Tickell KD, Atlas HE, Walson JL. Environmental enteric dysfunction: a review of potential mechanisms, consequences and management strategies. *BMC Med*. 2019;17(1):181. doi:10.1186/s12916-019-1417-3

5. Huang X, Zhou Y, Sun Y, Wang Q. Intestinal fatty acid binding protein: A rising therapeutic target in lipid metabolism. *Progress in Lipid Research*. 2022;87:101178. doi:10.1016/j.plipres.2022.101178

6. Schellekens D. Plasma Intestinal Fatty Acid–Binding Protein Levels Correlate With Morphologic Epithelial Intestinal Damage in a Human Translational Ischemia-reperfusion Model. *Journal of Clinical Gastroenterology*. 2014;48(3):253-260.

7. Kanda, Tatsuo, Tsukahara, Akihiro. Diagnosis of ischemic small bowel disease by measurement of serum intestinal fatty acid-binding protein in patients with acute abdomen: a multicenter, observer-blinded validation study. *Journal of Gastroenterology*. 2011;46:492-500. doi:10.1007/s00535-011-0373-2

8. Okada K, Sekino M, Funaoka H, et al. Intestinal fatty acid–binding protein levels in patients with chronic renal failure. *Journal of Surgical Research*. 2018;230:94-100. doi:10.1016/j.jss.2018.04.057

9. Saia RS, Giusti H, Luis-Silva F, et al. Clinical investigation of intestinal fatty acid-binding protein (I-FABP) as a biomarker of SARS-CoV-2 infection. *International Journal of Infectious Diseases*. 2021;113:82-86. doi:10.1016/j.ijid.2021.09.051

10. Tyszko M, Lipińska-Gediga M, Lemańska-Perek A, Kobylińska K, Gozdzik W, Adamik B. Intestinal Fatty Acid Binding Protein (I-FABP) as a Prognostic Marker in Critically Ill COVID-19 Patients. *Pathogens*. 2022;11(12):1526. doi:10.3390/pathogens11121526

11. Sekino M, Funaoka H, Sato S, et al. Intestinal fatty acid-binding protein level as a predictor of 28-day mortality and bowel ischemia in patients with septic shock: A preliminary study. *Journal of Critical Care*. 2017;42:92-100. doi:10.1016/j.jcrc.2017.07.012

12. Adriaanse MPM, Tack GJ, Passos VL, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Aliment Pharmacol Ther*. 2013;37(4):482-490. doi:10.1111/apt.12194

13. Logan M, MacKinder M, Clark CM, et al. Intestinal fatty acid binding protein is a disease biomarker in paediatric coeliac disease and Crohn's disease. *BMC Gastroenterol*. 2022;22(1):260. doi:10.1186/s12876-022-02334-6

14. Vreugdenhil AC, Wolters VM, Adriaanse MP, et al. Additional value of serum I-FABP levels for evaluating celiac disease activity in children. *Scandinavian Journal of Gastroenterology*. 2011;46(12):1435-1441. doi:10.3109/00365521.2011.627447

15. Ngai M, Hawkes MT, Erice C, et al. Intestinal Injury in Ugandan Children Hospitalized With Malaria. *The Journal of Infectious Diseases*. 2022;226(11):2010-2020. doi:10.1093/infdis/jiac340

16. Cascais-Figueiredo T, Austriaco-Teixeira P, Fantinatti M, et al. Giardiasis Alters Intestinal Fatty Acid Binding Protein (I-FABP) and Plasma Cytokines Levels in Children in Brazil. *Pathogens*. 2019;9(1):7. doi:10.3390/pathogens9010007

17. Brubaker J, Zhang X, Bourgeois AL, Harro C, Sack DA, Chakraborty S. Intestinal and systemic inflammation induced by symptomatic and asymptomatic enterotoxigenic *E. coli* infection and impact on intestinal colonization and ETEC specific immune responses in an experimental human challenge model. *Gut Microbes*. 2021;13(1):1891852. doi:10.1080/19490976.2021.1891852

18. Pavlinac PB, Singa BO, John-Stewart GC, et al. Azithromycin to prevent post-discharge morbidity and mortality in Kenyan children: a protocol for a randomised, double-blind, placebo-controlled trial (the Toto Bora trial). *BMJ Open*. 2017;7(12):e019170. doi:10.1136/bmjopen-2017-019170

19. Pavlinac PB, Singa BO, Tickell KD, et al. Azithromycin for the prevention of rehospitalisation and death among Kenyan children being discharged from hospital: a double-blind, placebo-controlled, randomised controlled trial. *The Lancet Global Health*. 2021;9(11):e1569-e1578. doi:10.1016/S2214-109X(21)00347-8

20. World Health Organization. Child Growth Standards. Accessed February 15, 2024. https://www.who.int/tools/child-growth-standards/software

21. Parnham MJ, Haber VE, Giamarellos-Bourboulis EJ, Perletti G, Verleden GM, Vos R. Azithromycin: Mechanisms of action and their relevance for clinical applications. *Pharmacology & Therapeutics*. 2014;143(2):225-245. doi:10.1016/j.pharmthera.2014.03.003