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Association of enteric parasitic infections with intestinal inflammation and permeability in asymptomatic infants of São Tomé Island

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ABSTRACT

The cumulative effect of repeated asymptomatic enteric infections on intestinal barrier is not fully understood in infants. We aimed to evaluate the association between previous enteric parasitic infections and intestinal inflammation and permeability at 24-months of age, in asymptomatic infants of São Tomé Island. A subset of infants from a birth cohort, with intestinal parasite evaluations in at least four points of assessment, was eligible. Intestinal inflammatory response and permeability were assessed using fecal S100A12 and alpha-1-antitrypsin (A1AT), respectively. The cutoff <–1SD for weight-for-length and length-for-age was used to define wasting and stunting. Multivariable linear regression analysis explored if cumulative enteric parasitic infections explained variability of fecal biomarkers, after adjusting for potential confounders. Eighty infants were included. *Giardia duodenalis* and soil-transmitted helminths (STH) were the most frequent parasites. The median (interquartile range) levels were 2.87 μg/g (2.41–3.92) for S100A12 and 165.1 μg/g (66.0–275.6) for A1AT. Weak evidence of association was found between S100A12 levels and *G. duodenalis* (p = 0.080) and STH infections (p = 0.089), and between A1AT levels and parasitic infection of any etiology (p = 0.089), at 24-months of age. Significant associations between A1AT levels and wasting (p = 0.006) and stunting (p = 0.044) were found. Previous parasitic infections were not associated with fecal biomarkers at 24 months of age. To summarize, previous asymptomatic parasitic infections showed no association with intestinal barrier dysfunction. Notwithstanding, a tendency toward increased levels of the inflammatory biomarker was observed for current *G. duodenalis* and STH infections, and increased levels of the permeability biomarker were significantly associated with stunting and wasting.

KEY WORDS
Asymptomatic infection; enteric parasite; fecal alpha-1-antitrypsin; fecal S100A12; infants; stunting; wasting

Introduction

Enteric infections, including those caused by parasites, are defined as pathogen-associated disrupted intestinal absorptive and/or barrier function, with or without overt diarrhea [1] and may have devastating consequences on the infant growth [2,3], and neurodevelopment [4,5]. Two recent comprehensive multinational studies Global Enteric Multicenter Study [6] (GEMS) and Malnutrition and Enteric Disease (Mal-ED) Study [7], carried out in developing countries, highlight the role of protozoan as etiologic agents of enteric infections in the first two years of life, particularly *Cryptosporidium* spp. associated to moderate-to-severe diarrhea and *Giardia duodenalis* in asymptomatic infected infants. The soil transmitted helminths (STH) *Ascaris lumbricoides, Trichuris trichiura,* and hookworms, highly prevalent in infants from poor settings, also may be a cause of enteric infections [8].

Increased intestinal permeability and local inflammatory response are mechanisms by which enteric infections can cause epithelial damage [9,10]. The assessment of these phenomena is important for the understanding of gut-infection interaction. Parasitic enteric pathogens can disrupt the intestinal barrier directly, by binding to cell surface molecules, causing cell damage and apoptosis, or by disrupting tight junctions and cell cytoskeleton [9,10] as described in *Cryptosporidium* spp. [11,12], *G. duodenalis* [13,14] and STH infections [15,16]. The severity of the intestinal inflammatory response is variable and dependent on the immune status of the host, parasite invasive potential, and ecological niche [17]. At intestinal level, parasite may induce a robust innate mucosal immune response that includes activation of neutrophils and other cells which may participate in the intestinal lesion [18], as described in *Cryptosporidium* spp. [19], *G. duodenalis* [20,21], and STH infections [22].

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The aforementioned mechanisms were investigated using *in vitro* and animal models, which may not accurately reflect the intricate *in vivo* dynamics of the mucosal immune system in humans [10]. Several clinical studies have been carried out in children from developing countries to explore intestinal injury associated with enteric parasitic infections. The intestinal inflammatory response has been studied using systemic biomarkers (*e.g.*, alpha1-antichymotrypsin, alpha-1-acid glycoprotein, and cytokines) [23–25], or fecal biomarkers (*e.g.*, cytokines, lactoferrin, calprotectin, neopterin, myeloperoxidase) [26–30]. Fecal S100A12 is a calcium-binding pro-inflammatory protein restrictelly secreted by activated neutrophils, used as a noninvasive specific biomarker of intestinal inflammation [31]. In children, it is reported to have higher sensitivity and specificity in inflammatory bowel disease than other markers [32]. To the best of our knowledge fecal S100A12 was never used as biomarker of inflammation in enteric parasitic infections. For intestinal permeability, biomarkers such as urinary lactulose/mannitol absorption test, fecal A1AT, serum endotoxin core antibody, and zonuline test are the most frequently used biomarkers [30,33–35]. Fecal A1AT is classically used as a simple diagnostic method for protein-losing enteropathies [36]. In children, fecal A1AT was shown to be a convenient, cheap and sensitive method to indirectly assess the intestinal permeability in several gastrointestinal diseases, particularly in enteric infections [30,37,38], and environment enteropathy dysfunction [30,39,40].

Infancy is a period of rapid gastrointestinal development and the mucosal barrier function may not be fully established until after the second year of life [41]. The first 2 years of age is a critical period in which impaired intestinal absorptive function may result from repeated enteric infections, even in those without overt liquid diarrhea [42]. The less explored cumulative effect of exposure to repeated asymptomatic parasitic infections on the intestinal barrier motivated our study.

We aimed to evaluate the association between previous exposure to enteric parasitic infections and intestinal inflammation and permeability at 24-months of age, in asymptomatic infants from São Tomé Island. Fecal S100A12 and A1AT were used as biomarkers for intestinal inflammation and permeability, respectively. We hypothesize that previous exposure to asymptomatic enteric parasitic infections is associated with local inflammation and increased intestinal permeability at 24 months of age.

Methods

This study is nested within a birth cohort study aimed to determine the association between enteric parasitic infections and nutritional status, intestinal barrier function, and neurodevelopment in infants living in São Tomé, Island that belongs to the Republic of São Tomé and Príncipe, a low-middle income country of sub-Saharan Africa. The birth cohort included 500 appropriate-for-gestational age infants (>10th and <90th percentiles) recruited within the first 28 postnatal days, from March to June 2013, and followed-up to 24 months of age. Neonates with low birth weight (<2500 g), born preterm (<37 weeks of gestation), without gestational age information, or with major congenital malformations were excluded. Infants were recruited at the mother-infant health care center in the main district Água Grande, and in the local hospitals in Lembá and Caué districts.

In this cohort study, follow-up was scheduled for anthropometric measurements, neurodevelopment assessment, and intestinal parasites examination, approximately at 3, 6, 9, 12, 16, 18, and 24 months of age. To assess the cumulative exposure to enteric parasitic infections, a minimum of four points of assessment (at 6, 12, 18 and 24 months) were required, which coincided with semestral appointments for feeding advice and scheduled immunizations. The subset of infants complying with this criterion was selected for a single measurement of fecal biomarkers of intestinal barrier function at 24 months of age.

Written informed consent in the national official language (Portuguese) was obtained from parents or caregivers. A local nurse in each health care setting represented the parents or caregivers and signed the consent in case of subjects having language/literacy difficulties. The study was approved by the Ministry of Health of São Tomé and Principe and by the Institute of Tropical Medicine and Hygiene ethics committee.

Clinical data and anthropometry

Socio-demographic data regarding mothers' educational level and household data were recorded, including improved drinking water source and sanitation availability [43]. In each visit, feeding practices (breastfeeding, formula feeding, and complementary feeding), and clinical events were recorded if they occurred or were still occurring in the week prior to visit, including acute diarrhea (lasting < 14 days), persistent diarrhea (lasting > 14 days), respiratory symptoms, and malaria (confirmed by Rapid Diagnostic Test and/or blood smear microscopic identification).

Anthropometry was performed in duplicate by the same trained observer (MG) in each point assessment. Infants were weighed using an electronic baby scale to the nearest decigram and crown-heel length measured using an infantometer, to the nearest millimeter. The *z-scores* for weight-for-age (WAZ), weight-for-length (WLZ) and length-for-age (LAZ) were calculated using the WHO Anthro software v.3.2.2. Wasting and stunting were defined by WLZ and LAZ, respectively; in this study, the cut-off <-1SD was chosen by convenience to allow

the inclusion of infants with mild-to-moderate undernutrition [44].

Parasite examination techniques

For each point assessment, parents collected a single stool sample at home on the day before or on the same day of the evaluation visit, using a sterile container provided by the research team. Collected samples were stored at 4 °C in the local laboratory and processed on the same day of reception.

Microscopic ova and parasite examination was performed in iodine-stained wet mounts of feces dissolved in saline and after formol-ether concentration procedure [45]. A cold acid-fast Kinyoun stain (Biomerieux®) was used for *Cryptosporidium* spp. and coccidian species (*Cystoisospora* and *Cyclospora*) detection. The same trained observer (MG) performed these microscopic examinations. A Rapid test for *G. duodenalis* detection (STICK Giardia/simple Giardia Operon, Immune and Molecular diagnostics) was used for liquid stool samples. Examinations for bacterial and viral enteropathogens were not performed due to logistical and economic constraints.

Additionally, three aliquots of each stool sample were transported to the Institute of Tropical Medicine and Hygiene Laboratory in Lisbon. Two aliquots (one preserved in Protofix TM® Alphatec, and another obtained from the formol–ether sedimentation) were stored at 4 °C for a second microscopic exam by an independent experienced observer (AR) at the Institute of Tropical Medicine and Hygiene Laboratory. The third aliquot was stored for up to 6 months at −20 °C without preservative, for molecular characterization of *G. duodenalis* and detection of fecal markers by enzyme linked immunosorbent assay (ELISA). The aforementioned time of storage and temperature are reported to have low impact on protein (S100A12 and A1AT) concentrations [46].

Microscopically positive stool samples for *G. duodenalis* at 24 months were processed for molecular characterization**.** DNA was extracted from the stools stored at −20 °C, using the QIAamp DNA Stool Mini Kit (Qiagen). Amplification of the fragments from ssurRNA (175 bp) and *β-giardin* (511 bp) genes was performed according to previously described protocols [47]. PCR products were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE HealthCare Life Sciences) and sequenced from both strands. The obtained sequences were aligned with published sequences of *G. duodenalis* isolates available in the GenBank database, using Clustal Omega and BioEdit 7.0.9 software for subassemblage determination.

It should be noted that after delivery of stool samples at each point evaluation, infants older than 1 year received mebendazole every four months, in compliance with the WHO preventive chemotherapy strategy for STH [48] implemented by the Health Ministry of São Tomé and Principe. Additionally, infants were treated for *G. duodenalis* with metronidazole suspension (provided by the research team) in case of microscopic detection of trophozoite (independently of symptoms) or in case of detection of cysts or a positive rapid test only in symptomatic infants, according to the current recommendations [49].

Fecal biomarkers

The analysis of fecal biomarkers was performed in stool samples stored at −20 °C. Temperatures during transportation never exceed 18 to 25 °C as recommended. Stool samples were processed for S100A12 (Inflamark F-INFL-ELISA Cisbio Bioassays) and for A1AT (RIDASCREEN α_1-Antitrypsin R-Biopharm) determination by using the ELISA technique following the manufacturers' instructions. Samples out of the range of standard curve were run at higher or lower concentrations as appropriate. For S100A12, the absorbance was read at wavelength of 450 nm; final concentrations, expressed in µg/g, were derived from a calibration curve using a 3rd-degree polynomial extrapolation. For A1AT, the absorbance was read at 450 nm with a reference wavelength of 620 nm; final concentrations expressed in µg/g were obtained using a four-parameter logistic-log model. As the aforementioned tests measure protein (A1AT and S100A12) concentrations, these are more accurately determined using dry weight or standardized dilution of specimens [30]. Therefore, watery or diarrheal stool samples were excluded from the analysis.

Statistical analysis

Socio-demographic characteristics, feeding practices, clinical events, anthropometric measures, and laboratory findings were described with frequencies (percentages) and with mean (SD: standard deviation) or with median and interquartile range ($P_{25} - P_{75}$), as appropriate. Linear regression analysis was used to explore if cumulative enteric parasitic infections (including previous and current infections, etiology, and single or multiple infections) explained the variability of fecal markers, measured at 24 months. Potential confounders such as sex, feeding practices, and nutritional status were considered in this analysis. In the univariate regression analysis, all the variables with a p-value < 0.25 were selected for the multivariable models. Normality assumption of the residuals was verified using Kolmogorov–Smirnov goodness-of-fit test with Lilliefors correction. A logarithmic transformation of S100A12 and A1AT values was performed as this assumption has been violated. A level of significance of $\alpha = 0.05$ was used, although p-values greater than 0.05 and lower than 0.1 (weak evidence of the difference/association) were still considered [50].

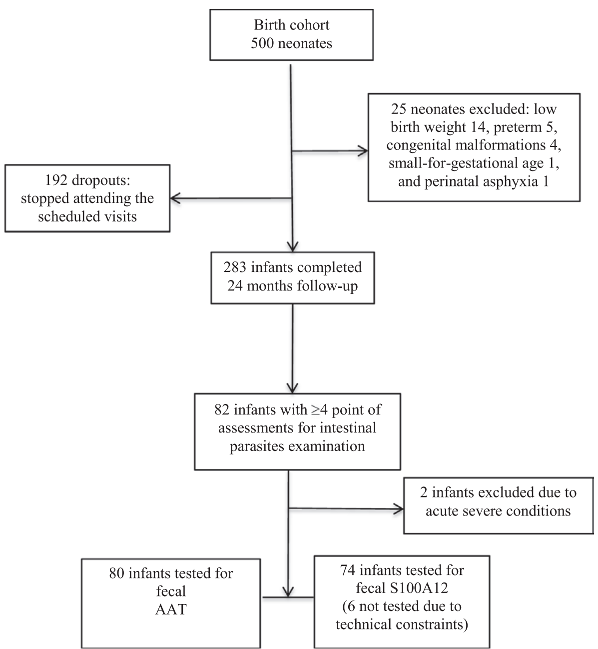

Figure 1. Flow-chart of infants enrolled in the study.

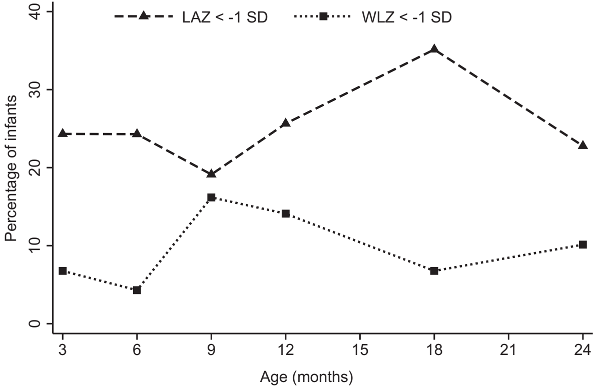

Figure 2. Wasting (WLZ) and stunting (LAZ) patterns up to 24 months of age. WLZ weight-for-length *z-score;* LAZ length-for-age *z-score*.

Data were analyzed using the software SPSS 22.0 (SPSS for Windows, Rel. 22.0.1. 2013. SPSS Inc., Chicago, IL, EUA) and Stata (Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

Results

From the birth cohort, 283 infants completed 24 months of follow–up; from these, 82 were eligible for fecal biomarkers determinations, but two were subsequently excluded due to acute severe conditions. Thus, a final sample of 80 infants (Figure 1) was included, in which 57.5% were females. Sex distribution and anthropometric data did not significantly differ between included and excluded infants (data not shown). In the final sample, 81.3% of mothers had more than five years of school education, 98.8% of the households had improved drinking water source, and 72.5% improved sanitation. In relation to feeding practices, 98.8% of infants were breastfed at some time point, 77.5% were exclusively breastfed at 6 months, and 10% maintained breastfeeding at 24 months; the mean (SD) duration of breastfeeding was 16.7 (2.9) months, and the mean (SD) age of introduction of complementary feeding was 5.7 (1.0) months. The frequency of clinical events varied along the study period, the respiratory symptoms (most frequent) varied between 10.5 and 37.2% of cases, and acute diarrhea between 1.3 and 9.3%; one case of persistent diarrhea and one of malaria were registered. In most infants the anthropometric measures were within the normal range at neonatal period, with a mean (SD) weight of 3.46 (0.46) kg and length of 50.2 (1.95) cm. After the neonatal period and up to 24 months of age, the rate of wasted infants varied between 4.3% and 16.2%, and of stunted infants between 19.1 and 35.1% (Figure 2). Noteworthy, the WLZ variation preceded the LAZ variation (Figure 2). At the end of the study at 24 months of age, 23% of infants were stunted and 10% wasted; from these, only five infants were moderately-to-severely (<–2SD) stunted and none was moderately-to-severely (<–2SD) wasted.

Parasitic infection

During the study period 497 stool samples were examined for intestinal parasites. The frequencies of intestinal pathogenic parasites at each point assessment are shown in Table 1. Parasites were not detected at 3 months of age; subsequently, the frequency of parasitic infection increased progressively with age, from 7.9% at 6 months to 57.5% at 24 months of age. Single parasitic infections predominated. Co-infections were detected from 12 months of age and their frequency increased up to the end of the study. The three most frequent pathogenic parasites, either as single or multiple infection, were by decreasing order *G. duodenalis*, STH, and *Cryptosporidium* spp. (Table 1). *Entamoeba histolytica/ dispar* complex was not detected. Other pathogenic parasites detected are shown in Table 1.

In the entire study period, 1 to 2 episodes of parasitic infection occurred in 39 (48.8%) infants, 3 to 4 episodes in 26 (32.5%) infants, and more than 4 episodes in 5 (6.3%) infants, independently of the etiology, either as single or multiple agent infection. In 11 (13%) infants no parasite was found.

From the 30 stool samples with a positive microscopy for *G. duodenalis* at 24 months of age, only 28 had enough material to be processed for molecular characterization. Twelve samples were successfully amplified for β-*giardin*, 14 for *ssu-rRNA* fragment gene, and two failed to amplify. Eighty percent (20/25) of samples belonged to Assemblage B and 20% (5/25) to Assemblage A. Additionally, *β-giardin* sequences were analyzed for sub-assemblage discrimination according to the described genetic polymorphisms. Nine isolates belonged to subassemblage B3, and 3 to subassemblage A3.

Table 1. Frequency of intestinal pathogenic parasites by age.

Stool samples = 497	3 months $n = 74$	6 months $n = 76$	9 months[a] $n = 52$	12 months $n = 78$	16 months[a] $n = 71$	18 months $n = 70$	24 months $n = 80$
Infected (%) (*n*)	0 (0)	7.9 (6)	15.4 (8)	25.6 (20)	45.1 (32)	42.8 (30)	57.5 (46)
Single Infections (%)(*n*)	0 (0)	7.9 (6)	15.4 (8)	21.8 (17)	40.9 (29)	32.8 (23)	42.5 (34)
Protozoa (%)(*n*)							
Giardia duodenalis	0 (0)	6.6 (5)	9.6 (5)	11.5 (9)	22.5 (16)	22.9 (16)	26.3 (21)
Cryptosporidium spp.	NA	1.3 (1)	5.8 (3)	3.8 (3)	2.8 (2)	1.4 (1)	3.75 (3)
Entamoeba histolytica/dispar complex	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cystoisospora belli	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Soil transmitted helminths (STH) (%)(*n*)							
Ascaris lumbricoides	0 (0)	0 (0)	0 (0)	5.1 (4)	15.5 (11)	4.3 (3)	10.0 (8)
Trichuris trichiura	0 (0)	0 (0)	0 (0)	1.3 (1)	0 (0)	2.9 (2)	2.50 (2)
Necator americanus/Ancylostoma duodenale	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Strongyloides stercoralis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.4 (1)	0 (0)
Multiple Infections (%)(*n*)	0 (0)	0 (0)	0 (0)	3.9 (3)	4.2 (3)	10.0 (7)	15.0 (12)
G. duodenalis + *Cryptosporidium* spp.	0 (0)	0 (0)	0 (0)	0 (0)	1.4 (1)	1.4 (1)	1.25 (1)
G. duodenalis + *A. lumbricoides*	0 (0)	0 (0)	0 (0)	1.3 (1)	1.4 (1)	0 (0)	5.0 (4)
G. duodenalis + *T. trichiura*	0 (0)	0 (0)	0 (0)	1.3 (1)	0 (0)	5.7 (4)	3.75 (3)
Cryptosporidium spp. + *A. lumbricoides*	0 (0)	0 (0)	0 (0)	1.3 (1)	0 (0)	0 (0)	2.50 (2)
Cryptosporidium spp. + *T. trichiura*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.25 (1)
A. lumbricoides + *T. trichiura*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2.9 (2)	0 (0)
G. duodenalis + *Cryptosporidium* spp. + *T. trichiura*	0 (0)	0 (0)	0 (0)	0 (0)	1.4 (1)	0 (0)	0 (0)
G. duodenalis + *A. lumbricoides* + *T. Trichiura*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.25 (1)
Total *Giardia duodenalis* (single or multiple) (%)(*n*)	0 (0)	6.6 (5)	9.6(5)	14.1 (11)	26.8 (19)	30 (21)	37.5 (30)
Total *Cryptosporidium* spp. (single or multiple) (%)(*n*)	0 (0)	1.3 (1)	5.8 (3)	3.8 (3)	5.6 (4)	2.8 (2)	8.7 (7)
Total STH (single or multiple) (%)(*n*)	0 (0)	0 (0)	0 (0)	10.2 (8)	18.3 (13)	20 (14)	27.5 (22)

Note: NA not available.
[a]Parasite examination was included in the analysis of the cumulative effect of enteric parasitic infections before 24 months of age.

Fecal biomarkers

Fecal biomarkers were tested in 80 infants for A1AT, and in 74 for S100A12 due to unsolvable technical constraints. The fecal S100A12 median (interquartile range) level was 2.87 (2.41–3.92) µg/g and of A1AT was 165.1 (66.0–275.6) µg/g (Table 2). A descriptive statistics of both biomarkers by the categories of several variables and distribution of their values is shown in Table 2 and Figure 3, respectively.

Variables with a p-value < 0.250 obtained in the univariable analysis (Table 3) were selected for the multivariable analysis. Results from the multivariable models (Table 4) showed a weak evidence of an association between fecal S100A12 levels and current *G. duodenalis* infection ($p = 0.080$) and between fecal S100A12 levels and current STH infection ($p = 0.089$). This suggests an increasing tendency of 23.6% and 24.1% in fecal S100A12 levels in infants infected with *G. duodenalis* and STH, respectively. Similarly, a weak evidence of an association was found between fecal A1AT levels and current parasitic infection of any etiology ($p = 0.089$), suggesting an increasing tendency of 33.6% higher A1AT levels in infected infants. Remarkably, significant associations were found between fecal A1AT levels and wasting ($p = 0.006$) and stunting ($p = 0.044$) at 24 months of age; specifically, fecal A1AT levels were twice higher in wasted infants and 50% higher in stunted infants. No significant associations between either fecal S100A12 or fecal A1AT and parasitic infection before 24 months of age (including number and etiology), were found.

Discussion

This prospective study, conducted in a low-middle income African country, showed that 87% of infants were infected by at least one pathogenic parasite in the first two years of life. Enteric parasitic infection was diagnosed as early as at 6 months of age, with frequency increasing with age and affecting around half of the infants at 24 months. *G. duodenalis* was the most frequently isolated parasite followed by STH, only detected after 12 months of age. Low frequencies were found for *Cryptosporidium* spp. In Africa, few longitudinal studies have assessed the prevalence of enteric parasitic infections in infants and recognized a high burden of parasitism during the first years of life [8,23], as in our study. During the study, most of the infants were asymptomatic, suggesting colonization and the development of a possible mutualistic or commensal relationship with the host. Moreover, the pathogenic role of parasites isolated in asymptomatic infants is difficult to interpret, because it may represent convalescent excretion of enteropathogens after acute diarrhea rather than true asymptomatic infection [51].

Regarding intestinal inflammation, a weak evidence of association was found between fecal S100A12 levels and current *G. duodenalis* and STH infections at 24 months of age. This association may be clinically meaningful, since this tendency toward increased fecal S100A12 levels was noticeable with the most frequent parasites. Classically, *G. duodenalis* infection is characterized by little or no inflammatory intestinal response [52]. In young children

Table 2. Fecal values of alpha1-anti-trypsin (A1AT) and S100A12 at 24 months of age, considering sex, parasite agent, and nutritional status categories.

	A1AT (μg/g)			S100A12 (μg/g)		
	n	median ($P_{25} - P_{75}$)	*p* value[a]	*n*	median ($P_{25} - P_{75}$)	*p* value[a]
Whole sample	80	165.1 (66.0–275.6)		74	2.9 (2.4–3.9)	
Girls	46	151.4 (66.0–272.4)	0.669	42	3.0 (2.6–4.4)	0.340
Boys	34	175.9 (66.0–291.4)		32	2.8 (2.3–3.5)	
Infected by any intestinal parasite						
No	34	129.6 (66.0–242.6)	0.131	33	2.7 (2.2–3.7)	0.075
Yes	46	184.3 (66.0–325.5)		41	3.1 (2.7–4.4)	
Giardia duodenalis						
No	50	143.0 (66.0–245.8)	0.143	49	2.8 (2.3–3.7)	0.068
Yes	29	200.6 (66.0–389.9)		25	3.2 (2.5–5.3)	
Cryptosporidium spp.						
No	72	153.2 (66.0–278.7)	0.656	67	2.9 (2.4–3.9)	0.589
Yes	7	167.1 (115.7–314.9)		7	3.0 (2.7–3.5)	
Soil transmitted helminths						
No	58	159.3 (66.0–289.4)	0.675	53	2.8 (2.2–3.9)	0.075
Yes	21	162.9 (66.0–261.7)		21	3.1 (2.6–4.7)	
Multiple infections						
No	68	159.3 (66.0–269.1)	0.446	62	2.8 (2.2–3.9)	0.093
Yes	12	169.5 (78.4–404.1)		12	3.2 (2.9–5.4)	
Stunting						
No	61	151.4 (66.0–248.6)	0.053	56	2.9 (2.4–3.9)	0.750
Yes	18	236.6 (104.3–510.8)		18	3.0 (2.3–4.3)	
Wasting						
No	71	151.4 (66.0–256.9)	0.008	66	2.9 (2.3–3.9)	0.574
Yes	8	281.3 (149.9–689.7)		8	3.5 (2.9–4.8)	

[a] *p* values obtained by Student's *t*-test after logarithmic transformation of A1AT and S100A12.

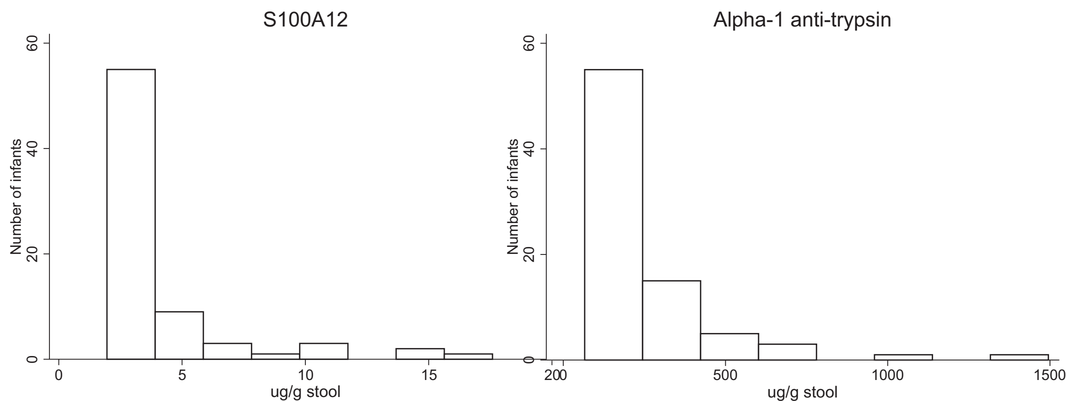

Figure 3. Distribution of fecal S100A12 ($n = 74$) and fecal alpha-1 anti-trypsin ($n = 80$) at 24 months of age.

from developing countries *G. duodenalis* is usually asymptomatic, probably as a result of modulation of the innate immune system [53] or a decrease of inflammatory response in subsequent infections [20,29]. Previous studies reported the association between *G. duodenalis* and biomarkers of intestinal inflammation in children [28,29], while others did not [30]. In the recent MAL-ED study, fecal myeloperoxidase, a marker of neutrophil inflammation, was surprisingly lower in children with *Giardia* [30]. In our study, despite the fact that most infants with *Giardia* infection were asymptomatic, the tendency toward increased fecal S100A12 levels may suggest local inflammatory response with participation of neutrophils. Histopathological studies using murine models showed intestinal infiltration of neutrophils that persisted several days after *G. duodenalis* infection [21,54]. One plausible explanation for our findings may be the predominant existences of assemblage B (80.0%) in our sample, as described in other African countries [55,56]. This genotype has been associated to extensive damage of the mucosal architecture and infiltration of inflammatory cells [21,57]. Very few studies have explored the effects of STH infections on mucosal immunity in infants [58]. In early STH infections, Zanzibari infants showed a regulatory Th2 pattern of peripheral cytokine responses to *Ascaris* and hookworm antigens [59], without association with acute phase proteins [60]. In our study, the tendency toward increased fecal S100A12 levels in current STH infections may suggest the presence of local inflammatory response mediated by neutrophils. In murine models using *Heligmosomoides polygyrus*, an early and pronounced infiltration of neutrophils and macrophages

Table 3. Univariable analysis for fecal alpha1-anti-trypsin (A1AT) and S100A12, considering sex, parasitic infection before and at 24 months of age, nutritional status, and feeding practices.

Variables	ln A1AT		ln S100A12	
	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value[d]
Females	0.08 (−0.28; 0.44)	0.669	−0.113 (−0.35; 0.12)	0.340
Parasitic infection before 24 months of age				
nr. parasites	0.11 (−0.05; 0.272)	0.181	0.10 (−0.00; 0.20)	0.054
nr. *G. duodenalis*	0.14 (−0.07; 0.36)	0.198	0.11 (−0.02; 0.25)	0.106
nr. *Cryptosporidium* spp.	0.11 (−0.34; 0.55)	0.638	0.07 (−0.22; 0.37)	0.624
nr. STH	0.03 (−0.25; 0.30)	0.840	0.08 (−0.09; 0.25)	0.373
Parasitic infection at 24 months of age				
Infected at 24 months	0.27 (−0.08; 0.62)	0.131	0.21 (−0.02; 0.43)	0.075
nr. parasites	0.21 (−0.03; 0.44)	0.087	0.15 (0.00; 0.30)	0.047
G. duodenalis	0.27 (−0.09; 0.63)	0.143	0.22 (−0.01; 0.46)	0.068
Cryptosporidium spp.	0.14 (−0.45; 0.77)	0.656	−0.11 (−0.50; 0.29)	0.589
STH	0.09 (−0.31; 0.49)	0.675	0.23 (−0.02; 0.48)	0.075
Multiple infections	0.19 (−0.30; 0.69)	0.446	0.27 (−0.04; 0.57)	0.093
Nutritional status				
Mean ΣWAZ[a]	−0.22 (−0.45; −0.00)	0.054	−0.03 (−0.18; 0.12)	0.692
Mean ΣWLZ[b]	−0.07 (−0.33; 0.19)	0.589	−0.03 (−0.20; 0.13)	0.682
Mean ΣLAZ[c]	−0.28 (−0,50; −0,07)	0.011	−0.03 (−0.17; 0.12)	0.732
Underweight at 24 months	0.21 (−0.21; 0.64)	0.321	0.07 (−0.20; 0.34)	0.636
Wasting at 24 months	0.77 (0.21; 1.34)	0.008	0.10 (−0.27; 0.48)	0.574
Stunting at 24 months	0.41 (−0.01; 0.83)	0.053	0.04 (−0.23; 0.32)	0.750
Feeding practices				
Ever breastfeeding	−0.82 (−2.40; 0.76)	0.306	−0.07 (−1.01; 0.93)	0.880
Exclusive breastfeeding at 6 months	0.14 (−0.32; 0.61)	0.543	0.13 (−0.17; 0.44)	0.391
Age (months) of introduction complementary foods	0.11 (−0.07; 0.28)	0.238	0.02 (−0.01; 0.14)	0.750

Notes: ln logarithmic transformation, STH soil transmitted helminths.
[a]ΣWAZ [b]ΣWLZ [c]ΣLAZ sum up of weight-for-age, weight-for-length, and length-for-age *z*-scores, respectively, at all point assessments [d]*p* values obtained by linear regression models after logarithmic transformation of A1AT (ln A1AT) and S100A12 (ln S100A12**)** values.

in regions immediately adjacent to the parasite has been described [61]. Furthermore, primary infections by STH, as occurring in younger children, may stimulate a strong inflammatory response in the mucosa [58]. Young children in developing countries frequently respond to *Cryptosporidium* infection with intestinal inflammation [27]. In our study *Cryptosporidium* spp. was not associated with the fecal inflammatory biomarker, probably explained by its low frequency and less severe infections in asymptomatic infants.

The fecal biomarkers have the advantage of measuring proteins originating in the intestinal mucosa, more accurately reflecting local inflammation [62]. Several fecal biomarkers of neutrophil activity (e.g., lactoferrin, myeloperoxidase, and calprotectin) or of activated cell mediated immunity (e.g., neopterin) have been used to assess intestinal inflammation in infants [63]. High levels of these biomarkers were reported in infants from developing countries associated either to enteric parasite infections [24,27–30] or to environmental enteric dysfunction [30,39,40,64]. In accordance, we found high median fecal levels of S100A12 in all infants at 24 months of age, five times above those described for healthy children (0.5 mg/kg), but below the threshold 10 mg/kg used for inflammatory bowel disease [65,66]. This comparison should be interpreted with caution, since the reported values were obtained from a limited number of healthy children from a developed country [65,66], and measured using different ELISA method [67]. To the best of our knowledge, this is the first time that S100A12 is used to assess intestinal inflammatory response in enteric parasitic infection in children. This biomarker was chosen taking into account its advantages in field studies involving children. This protein is restrictively secreted by activated neutrophils and is strongly correlated with histologically intestinal inflammation and neutrophil infiltration [68]. Fecal S100A12 has a sensitivity of 96% and a specificity of 92% in distinguishing healthy children from those with inflammatory bowel disease, using the threshold 10 mg/kg [32,67]. It is evenly distributed throughout feces and is stable at a wide range of temperatures (4 to 20 °C) for several days [67]. These characteristics make fecal S100A12 a convenient biomarker, facilitating samples collection and transportation to a laboratory for measurement, avoiding resource-consuming storage needs [67]. Several commercial ELISA kits are available for fecal S100A12 analysis requiring a small stool sample (approximately 100 mg). Other fecal neutrophil-derived proteins used as biomarkers of intestinal inflammation may have limitations compared with S100A12, particularly in infants. Calprotectin, another calcium and zinc-binding protein, is present in neutrophils, but also in monocytes and macrophages [69]. Fecal calprotectin correlates well with endoscopic and histological inflammatory bowel

Table 4. Multivariable regression models for alpha1-anti-trypsin (A1AT) and S100A12.

Variables	β-estimate (95%CI)	*p* value [a]
	ln A1AT	
Parasitic infection at 24 months of age	0.29 (−0.05; 0.62)	0.089
Wasting at 24 months of age	0.78 (0.23; 1.33)	0.006
Stunting at 24 months of age	0.41 (0.01; 0.80)	0.044
	ln S100A12	
Giardia duodenalis at 24 months of age	0.21 (−0.03; 0.45)	0.080
Soil transmitted helminths at 24 months of age	0.22 (−0.03; 0.47)	0.089

Notes: β regression coefficient, CI confidence interval.
[a]*p* values obtained by linear regression models after logarithmic transformation of A1AT(ln A1AT) and S100A12 (ln S100A12) values.

disease activity [70] but a meta-analysis showed a relatively low pooled specificity (0.76) in children [71]. It seems to be a less accurate biomarker for inflammatory bowel disease than fecal S100A12 in pediatric population [32]. In healthy infants, fecal calprotectin levels are higher than in older children [72], and in those breastfed [73], Lactoferrin is likewise not specific, as it is produced by neutrophils and epithelial cells [74], It may be inaccurate to determine intestinal inflammation in infants using this biomarker, since breast milk may contribute to increase its fecal levels [75]. Additionally, fecal lactoferrin may be less sensitive in malnourished children [76]. Myeloperoxidase is produced by neutrophils, but it is found at lower concentrations in monocytes and macrophages [77]. In spite of a good correlation of fecal myeloperoxidase with laboratory and endoscopic parameters of inflammation [77], recent breastfeeding is also associated with increased fecal levels [30]. Furthermore, gender differences, with lower intracellular neutrophil myeloperoxidase levels in boys, have been reported [78]. Neopterin, an indicator of T-helper cell 1 activity, is used as a biomarker of intestinal inflammation [28,30,39,40]. Its fecal levels were found to be much higher (26 times) in infants from developing countries than from non-tropical countries [30,40].

Regarding intestinal permeability, a weak evidence of association was found between fecal A1AT levels and current parasitic infection of any etiologic at 24 months of age. This association may have limited clinical relevance, since the tendency toward increased fecal A1AT levels was associated with unspecific etiology. Several studies (most using lactulose:mannitol test) described the association between enteric parasitic infection and increased intestinal permeability [23,30,33,34], while others did not [25,28]. Significant associations were found between increased fecal A1AT levels and both acute (wasting) and chronic (stunting) undernutrition at 24 months of age, independently of intestinal parasitic infection. The association between increased intestinal permeability and undernutrition has been previously described in infants from developing countries [25,28,35,39,40]. It has been suggested that increased intestinal permeability explains at least 40% of growth faltering [79]. Recently, fecal A1AT was combined with two inflammatory biomarkers (myeloperoxidase and neopterin) in a score to predict growth deficit in infants [40]; specifically, fecal A1AT levels at or above 75th were reported to predict a loss of 0.152 LAZ in the subsequent six months [40]. Similar results were described in Brazilian infants [39]. In our study, the convenience threshold of 1SD for WLZ and LAZ was used to define wasting and stunting. A higher number of undernourished infants due to the inclusion of mild-to-moderate degrees may be responsible for the significant association we found. In fact, the hazardous effects of undernutrition happen along a continuum spectrum, in which mild-to-moderate degrees may be associated with otherwise-unobserved changes in disease exposure [44,80]. Marginally nourished children may have inadequate or rate-limiting stores of key nutrients to repair the mucosal damage [42]. Despite the aforementioned association of fecal A1AT levels with stunting and wasting, the median A1AT level of 165.1 μg/g found in our sample was below the mean 299 μg/g described in infants from developing countries [30]. Most of the studies have used urinary lactulose:mannitol ratio to assess intestinal permeability [81]. In our study, fecal A1AT was preferred instead of lactulose:mannitol test considering some advantages. The AAT is neither degraded by intestinal proteases nor reabsorbed and it is notably stable in stool samples [82]. Furthermore, large size molecules such as A1AT (50,000 Da), transported through the paracellular route, may better reflect structural damage of tight junctions than small size molecules such as lactulose (342 Da) and mannitol (182 Da) [83]. Moreover, the lactulose:mannitol test has inconveniences that may limit its use in infants, particularly in field settings, such as the requirement of fasting before testing, the need for several hours for urine collection, the lack of standardized procedures, and of reference values for children [81].

The suspected effect of repeated enteric infections on intestinal function [42] was recently confirmed by a better correlation of fecal biomarkers with cumulative pathogen burden than with a single ongoing infection [30]. In spite of this evidence, we found no associations between previous exposure to asymptomatic parasitic infections and fecal biomarkers of intestinal inflammation and permeability at 24 months of age. Since these biomarkers were not measured longitudinally, a reversible process of intestinal epithelial injury [33] cannot be excluded; otherwise, asymptomatic infections may induce less severe intestinal barrier dysfunction than symptomatic infections [26,76].

The following constitute strong points of our study. The measurement of intestinal barrier biomarkers at 24 months, and not before, reflects more accurately the impact of parasite infection, not biased by a potential effect of an incomplete development of mucosal barrier function [30,41]. The longitudinal search for intestinal parasites included not only protozoa but also STH, providing a broader view of the association between

these enteric parasites and intestinal barrier dysfunction in younger children. Fecal S100A12 was herein firstly used as a biomarker of intestinal inflammatory response in enteric parasitic infection, and it appears to be a convenient and accurate tool for field studies in infants. Finally, analyzing mild-to-moderate degrees of undernutrition allowed early detection of its association with intestinal barrier dysfunction related with parasitic infection, before severe undernutrition was established [42,44].

Limitations of this study should be acknowledged. Firstly, this study may be underpowered once the sample size was not calculated. By the time the study started no reference values were found in the literature to provide an idea about the mean and variability of the two biomarkers under study specifically in enteric parasitic infection in children. In spite of the convenience size of the sample, most of the associations obtained have relatively narrow confidence intervals. Secondly, microscopic parasite examination using a single stool specimen have low sensitivity [84]; which may however be increased when it is performed by a trained laboratory technician in high prevalence settings [85], as in our study. Thirdly, since bacterial and virus were not analyzed, changes in fecal markers attributable to these enteropathogens cannot be discarded. In children, transient high intestinal permeability may occur in rotavirus infection [33] and to a lesser degree in bacterial infections [86]. Intestinal inflammatory response is common in invasive bacterial enteric infections [30,87], but not in viral infections [87]. Noteworthy, all these studies assessed enteric infections in children with diarrhea, and we only selected asymptomatic infants with non-diarrheic stools to minimize the probability of coexisting viral and bacterial enteric infections. Fourthly, anthelminthic and anti-Giardia treatments might have biased the results, although it is described that treatment does not have a significant impact on small intestine function [88]. Finally, the determination of environmental enteric dysfunction [89] was beyond the scope of our study. Thus, it is not possible to exclude that parasitic enteric infections may have contributed to the development or exacerbation of a potential environmental enteric dysfunction [90] or that both conditions coexisted.

To conclude, the hypothesized association between previous exposure to asymptomatic enteric parasitic infections and intestinal barrier dysfunction at 24 months was not confirmed. Notwithstanding, an observed tendency toward increased fecal levels of inflammatory biomarker associated with the most prevalent parasitic infections in asymptomatic infants may have clinical relevance. Although our study could be underpowered to assess the aforementioned associations, it was powered enough to demonstrate a significant association of increased intestinal permeability with wasting and stunting, including of mild-to-moderate degrees, in 24-months aged infants.

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