Universidade NOVA de Lisboa

Instituto de Higiene e Medicina Tropical

Cohort study of associations between intestinal protozoa infection and intestinal barrier function, nutritional status, and neurodevelopment in infants from Republic of São Tomé

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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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Dedicatory

To my love, my family, and my friends.

"All children should have the same opportunities to survive, develop and attain their full potential"

The State of the World's Children 2016 - UNICEF

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Abstract

Cohort study of associations between intestinal protozoa infection and intestinal barrier function, nutritional status, and neurodevelopment in infants in Republic of São Tomé.

Marisol Garzón Lozano

Key words: infant growth, intestinal inflammation, intestinal parasites, intestinal permeability, neurodevelopment

Background

Giardia lamblia, Cryptosporidium and *Entamoeba hystolitica* are prevalent etiologic agents of enteric infections in infants from low- and middle-income countries. Host-parasite interactions may lead to mucosal inflammatory response and increased intestinal permeability. Clinically this can result in a negative impact on growth and neurodevelopment. The effects of these subclinical enteric protozoa infections on infant health are poorly explored.

Aim

To analyze the associations between enteric parasitic infections and intestinal barrier function, nutritional status and neurodevelopment in asymptomatic infants in São Tomé.

Methods

A birth cohort study with a follow-up until 24 months of age was implemented. Anthropometry was assessed monthly and included attained growth (weight-for-length z-score, length-for-age z-score – LAZ, and length-for-age difference – LAD), growth velocity (weight velocity z-score – WAVZ, and length velocity z-score – LAVZ), and risk for undernutrition (wasting and stunting, using the <-1SD cut-off). Neurodevelopment was screened at key ages using the "Bayley Infant Neurodevelopmental Screening" score. Fecal biomarkers for intestinal inflammation (S100A12) and permeability (alpha 1 anti-trypsin - A1AT) were measured at 24

months of age. Enteric protozoa and intestinal helminths were examined quarterly in stool samples using microscopic techniques. Different statistical models were used to explore associations between enteric parasitic infections and the three outcomes: intestinal barrier function, nutritional status, and neurodevelopment.

Results

A total of 475 neonates were enrolled, representing 8.6% of live-births in São Tomé; 280 (58.9%) infants completed 24 months of follow-up. *Giardia lamblia* and helminths were the most prevalent parasites. The multivariable analysis showed that: 1) infants with *Giardia lamblia* and helminths infections had a tendency toward an increase of 23.6 % and of 24.1 % in the inflammatory biomarker, respectively; those infected by any enteric parasite had a tendency toward an increase of 33.6% in the permeability biomarker; additionally, this biomarker was 100% higher in wasted infants and 50% higher in those stunted; 2) infants with *Giardia lamblia* and helminths infections showed a significant association with a decrease in linear growth (by - 0.10 and -0.16 of LAZ and by -0.32 and -0,48 of LAD, respectively); those with *Cryptosporidium* spp. infection displayed a significant association with a decrease in weight and length velocities (-0.43 WAVZ and -0.55 LAVZ); 3) *Giardia lamblia* infection and stunting were independently and significantly associated with a 1.69 and 2.37 increased risk of poor development, respectively.

Conclusions

This first birth cohort ever performed study in São Tomé is innovative in exploring associations between enteric parasitic infections and the intestinal barrier function, nutritional status and neurodevelopment in infants. The underestimated role of protozoa and helminths as etiologic agents of subclinical enteric infections was confirmed. These parasitic infections showed a tendency of association with intestinal barrier dysfunction and significant associations with decreased linear growth and risk of poor neurodevelopment. In the context of São Tomé, an endemic area for *Giardia lamblia* and helminths with a non-negligible proportion of marginally undernourished infants such associations are problematic. Affected infants may have a limited capacity to repair mucosal damage, with a negative impact on growth and neurodevelopment, thus jeopardizing the achievement of their full potential.

Resumo

Estudo de coorte sobre as associações entre infeções por protozoários intestinais e a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento em lactentes da República de São Tomé.

Marisol Garzón Lozano

Key words: crescimento infantil, helmintas intestinais, inflamação intestinal, neurodesenvolvimento, permeabilidade intestinal, protozoários intestinais

Enquadramento

Em lactentes de países de baixo e médio rendimento, *Giardia lamblia*, *Cryptosporidium* e *Entamoeba hystolitica* são agentes prevalentes em infeções intestinais. As interações hospedeiro-parasita podem levar a uma resposta inflamatória da mucosa e aumento da permeabilidade intestinal. Clinicamente, isto pode refletir-se por impacto negativo no crescimento e neurodesenvolvimento. Os efeitos destas infeções intestinais subclínicas na saúde infantil têm sido pouco estudado.

Objetivo

Analisar, em crianças assintomáticas de São Tomé, as associações entre infeções por parasitas intestinais e a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento.

Métodos

Foi realizado um estudo coorte de nascimento com seguimento até aos 24 meses de idade. A antropometria foi avaliada mensalmente e incluiu o crescimento atingido (*z-scores* para peso/comprimento, comprimento/idade – CIzs e diferença do comprimento-para-idade – DCI), a velocidade do crescimento (*z-scores* para velocidade ponderal – VPzs e linear – VLzs) e o risco de desnutrição (aguda e crónica, definida como <-1DP). O neurodesenvolvimento foi rastreado em idades-chave usando o "Bayley Infant Neurodevelopmental Screening". Os biomarcadores fecais para inflamação (S100A12) e permeabilidade intestinais (alfa-1-antitripsina - A1AT) foram medidos aos 24 meses. A presença de protozoários e helmintas intestinais foi

avaliada trimestralmente por técnicas microscópicas Foram usados diferentes modelos estatísticos para estudar associações entre infecções por parasitas intestinais e os resultados das avaliações da função da barreira intestinal, estado nutricional e neurodesenvolvimento.

Resultados

Foram incluídos 475 recém-nascidos, representado 8,6% dos nados-vivos em São Tomé; 280 (58,9%) completaram os 24 meses de seguimento. *Giardia lamblia* e helmintas foram os parasitas mais prevalentes. A análise multivariável revelou que: 1) lactentes infetados com *Giardia lamblia* e helmintas tiveram tendência para aumento de 23,6% e 24,1% no marcador de inflamação intestinal, respectivamente; os infetados por qualquer parasita tiveram tendência para aumento de 33,6% no marcador de permeabilidade; além disso, os níveis de A1AT foram 100% superiores em lactentes com desnutrição aguda e 50% superiores nos com desnutrição crónica; 2) lactentes infetados com *Giardia lamblia* e helmintas tiveram associação significativa com diminuição no crescimento linear (-0,10 e -0,16 CIzs; e -0,32 e -0,48 de DCI, respectivamente); os infetados com *Cryptosporidium* spp. tiveram associação significativa com diminuição na velocidade de crescimento ponderal e linear (-0,43 VPzs e -0,55 VLzs); 3) a infecção por *Giardia lamblia* e a desnutrição crónica associaram-se independentemente e significativamente com 1,69 e 2,37 maior probabilidade de atraso no desenvolvimento, respectivamente.

Conclusões

Este é o primeiro estudo de coorte de nascimento em São Tomé, pioneiro em estudar associações entre infeções por parasitas intestinais e a função da barreira intestinal, estado nutricional e neurodesenvolvimento. Foi confirmado o papel subestimado dos protozoários e helmintas como agentes etiológicos de infecções intestinais subclínicas. Estas infecções revelaram uma tendência para associação com a disfunção da barreira intestinal e associações significativas com restrição do crescimento linear e neurodesenvolvimento. Estas associações são problemáticas em São Tomé, endémico para *Giardia lamblia* e helmintas, em contexto de proporção não negligenciável de lactentes marginalmente desnutridos. Estes poderão ter capacidade limitada para

reparar lesões da mucosa, com impacto negativo no crescimento e neurodesenvolvimento, ficando comprometido o atingimento do seu pleno potencial.

TABLE OF CONTENT

Appendices

LIST OF TABLES

LISTOF FIGURES

ABBREVIATIONS

A1AT: alpha-1-antitrypsin

AD: acute diarrhea

AF: attributable fraction

APOE: apolipoprotein E

ARI: acute respiratory infection

BINS: Bayley Infant Neurodevelopmental Screening

BMI: body mass index

BSID: Bayley scales of infant development

DDST: Denver development screening test

EIA: enzyme immunosorbent assay

ELISA: Enzyme-Linked Immunosorbent Assay

GBD: Global Burden Diseases

GEMS: Global Enteric Multicenter Study

HAD: height-for-age differences

HAZ: height-for-age

HC: head circumference

HIV: human immunodeficiency virus

HLA: Human leukocyte antigen

HR: hazard ratio

I-FABP: Intestinal fatty acid binding protein

IFN-γ: Interferon gamma

Ig: immunoglobulin

IL: interleukin

IQ: intelligent quotient

JAMs: junctional adhesion molecule

LAD: length-for-age difference

L:M: lactulose mannitol test

LAVZ: length velocity *z-score*

LAZ: length-for-age *z-score*

LMIC: low-income and middle-income

LOWESS: locally weighted scatterplot smoothing

LPS: lipopolysaccharide

MAL-ED: study of Malnutrition and Enteric Diseases

MBL: mannose-binding lectin

MGRS: multicenter Growth Reference Study

MHC: major histocompatibility complex

MPI: multidimensional poverty index

MPO: myeloperoxidase

MUAC: mid-upper arm circumference

NADPH: nicotinamide adenine dinucleotide phosphate

NEO: neopterin

NET: neutrophil extracellular traps

NF-κB: factor nuclear kappa B

OR: odds Ratio

PCR: polymerase chain reaction

PGE2: prostaglandin E2

RAGE: advanced glycation end products

RR: relative Risk

SD: standard deviation

SGA: small-for-gestational age

SSA: sub-Saharan Africa

STH: soil-transmitted helminths

STP: São Tomé and Principe

TGF β: Transforming growth factor-β

Th: T helper cells.

TJs: tight junctions

TLR: toll like receptors

TNF-α: tumor necrosis factor –alpha

WAD: weight-for-age difference

WAVZ: weight velocity *z-score*

WAZ: weight for age *z-score*

WHO: world health organization

WLZ: weight-for-length *z-score*

WHZ: weight-for-length *z-score*

ZN: ziehl-neelsen

ZO: zonuline protein

1. Introduction

Parasites found in the human gastrointestinal tract can be largely categorized into two groups, protozoa and helminths. Enteric protozoa *Giardia lamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica* are the protozoa of worldwide importance (WHO 2002a).

Infections by protozoa *Giardia lamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica* are considered neglected tropical diseases, since they belong to a heterogeneous group of infectious diseases that occur mostly in developing countries where climate, poverty, and lack of access to services are common (Savioli 2006). These diseases exhibit a considerable and increasing global burden and impair the ability of those infected to achieve their full potential, both developmentally and socio-economically (Savioli 2006). Whole populations will be geographically at risk, but children are observed to disproportionally carry the greatest burden of infection, this is particularly concerning, since the population structure of endemic regions is predominantly younger (Harnay 2010). Infection rates are highest in children living in Sub-Saharan Africa (SSA), followed by Asia, Latin America, and Caribbean (Harnay 2010).

Based on the conceptual model of the "cycle of poverty" (Guerrant 2008), enteric infections have a central role leading to malnutrition, with long-term impact in conginitve development (Guerrant 2008). The classical burden of enteric infection measured in terms of diarrhea-associated mortality does not capture the true impact that enteric infections have on human development, neither the subtle morbidities on endemic communities (Guerrant 2002, Harnay 2010, McCormick 2016). For example, when long-term health effects of subclinical enteric infections are quantifying, the burden of stunting is substantially greater than reflected by diarrhea *per se* (Guerrant 2002, McCormick 2016). Furthermore, metrics also ignore the impact of enteric infections on cognitive development and human potential (Guerrant 2011). Thereby, enteric infections may have social and economic impact on human development, destabilizing endemic communities and reinforcing local poverty, in a much greater extent than non-communicable diseases (Harnay 2010).

1.1. Prevalence of enteric parasites in infants in Africa and others low- and middle income countries

In Africa, several challenges such as the remoteness of communities, lack of transport, shortage of skilled health care workers, and lack of laboratory facilities make difficult to have reliable studies on prevalence of enteric protozoa (Squire 2017). Studies are mostly cross-sectional or case-control, addressed to children aged 0 to 16 years, at hospital or community settings, and using different diagnostic methods (Table 1). As a result, prevalence obtained from those studies is quite variable (Table 1).
Longitudinal studies conducted in infants from low- and middle-income countries (LMIC) are shown in Table 2.

1.1.1. *Giardia lamblia*

Giardia lamblia is ubiquitous and the initial infection is acquired in the first few weeks of life (Muhsen 2012). In Guatemala, a birth cohort of infants was followed-up 3 years, with weekly stool samples. *Giardia* infected at very early ages (first week of life) and every child has at least one episode in the first three years of life. The mean number of *Giardia* infections *per* child increased from 0.7 in the first year to 3.6 in the third year. Prevalence reached 20.2% by the end of the third year (Mata 1978, Farthing 1986). In northeast Brazilian, infants were followed three times weekly for diarrheal surveillance up to 4 years (Newman 2001). Of 157 children followed, 27.4% were infected with *Giardia lamblia* with similar frequency in non-diarrhoeal (7.4%) and diarrhoeal stools (9.7%)(Newman 2001). In Peru, a birth cohort of 220 infants was followed until 35 months of age, with weekly stool collection (Hollm-Delgado 2008). The overall prevalence of *Giardia lamblia* was 18.8% *per* child-week, and 85% of children became infected during their follow-up (Hollm-Delgado 2008). In Bangladesh, Mondal *et al.* (2012) followed 147 infants since birth until the first year of life with monthly stool surveillance for enteropathogens. *Giardia lamblia* was the more frequent pathogen, infected 34% of all infants (Mondal 2012). The cumulative percentage of *Giardia lamblia* infection, increased from 30% in the first month to almost 90% at 12 months of life (Mondal 2012).

Table 1. Prevalence of intestinal protozoa in children in African countries

Country, author	Study	N	Method	Prevalence		
				Giardia lamblia	*Cryptosporidium*	*E. histolytica*
Angola Gasparinho 2016	Etiology of Diarrhea in Children Younger Than 5 Years Attending the Bengo General Hospital in Angola	344 (0-5 y)	Microscopy	21.6%	30.0%	0.9% E. histolityca/dispar
Ethiopia Wegayehu, 2013	Prevalence of *Giardia* and *Cryptosporidium* among children and cattle in Ethiopia	384 (1-14y)	Microscopy	24.5 (1-5y)	12.3% (1-5y)	3.6% E his/dis
Ethiopia Mulatu, 2015	Intestinal parasitic infections among children < 5 y with diarrhoeal diseases in Health setting - Ethiopia.	158 (0-5 y)	Microscopy	7% (2-7% 0- 24m)	3.8% (0-3% 0- 24m)	11.4% E. hist/ disp. (7% 0-24m)
Gambia Sullivan, 1991	Prevalence and treatment of giardiasis in chronic diarrhea and malnutrition	64 (6-36 m)	Microscopy IgM	45% cases 12 % control		
Ghana Addy, 2004	Prevalence of pathogenic E. coli and parasites in infants with diarrhea in Kumasi, Ghana	284 (0-5 y)	Microscopy	3.7% cases 0% control	8% cases 0.8% control	
Ghana Reither, 2007	Acute childhood diarrhea in Ghana: epidemiological, clinical and microbiological characteristics	365 (0-12 y)	EIA-PCR	3.7% cases 9.7% control	0.4 % cases 0.8% control	
Guinea-Bissau Centeno-Lima, 2013	Giardia duodenalis and chronic malnutrition in children under five from a rural area of Guinea-Bissau.	109 (0 to 5 y)	Microscopy	29.0% cases 35.9% controls.		
Kenya Mbae, 2013	Parasitic infections in children with diarrhea in outpatient and inpatient in Nairobi, Kenya	2112 (0 - 5 y)	Microscopy	5.8% outpatient 1.3% inpatient	6.7 % out 15.7% in-patient.	13.8 % outpatient 1.3 % in-patient

Table 1. Prevalence of intestinal protozoa in children in African countries (*continued*)

Country, author	Study	N	Method	Prevalence		
				Giardia lamblia	*Cryptosporidium*	*E. histolytica*
Kenya Thiongo, 2011	Spatial distribution of Giardia intestinalis in children up to 5 years old attending out-patient clinic at Provincial General hospital, Embu, Kenya	376 (0-5 y)	Microscopy	12.8%		5.3%
Mozambique Fonseca, 2014	Intestinal parasites in children hospitalized at the Central Hospital in Mozambique	93 (0- 2 y)	Microscopy	6.5%	6.5 %	
Mozambique Nhampossa, 2015	Diarrheal Mozambique: burden, and Etiology of diarrhea 0–59 Months at Health Facilities	2329 (0-5 y)	EIA	0-12m: 10% cases; 18% control 12-24m: 28% cases; 46% control.	0-12m: 20% cases; 10% control 12-24m: 19% cases; 9% control	*E. his/dispar* 0-12m: 9% cases-control. 12-24m: 11% cases; 10% control
Rwanda Ignatius, 2012	High Prevalence of *G.duodenalis Assemblage B* and association with Underweight in Rwandan Children.	583 (0-5 y)	Microscopy PCR	60%	4.9%	1.1%
Senegal Roger, 2013	Parasitic Infections among children <5y Senegal: Prevalence and Effect on Anemia and Nutritional Status	636 (0- 5 y)	Microscopy	15.4%		
São Tomé Lobo 2014	*Cryptosporidium spp., G. duodenalis, E. bieneusi* in young Children in STP	314 (1 -10 y)	Microscopy PCR	7.5% community. 1.9% hospital	0% community 8.9% hospital	E. histolytica /dispar 1.5%
São Tomé Ferreira 2015	*G. duodenalis* and soil-transmitted helminths infections in children in São Tomé and Príncipe	444 (3-5 y)	Microscopy	41.7%		

Table 1. Prevalence of intestinal protozoa in children in African countries (*continued*)

Country, author	Study	N	Method	Prevalence		
				Giardia lamblia	*Cryptosporidium*	*E. histolytica*
São Tomé Liao, 2016	Prevalence of intestinal parasitic infections among school children in Democratic Republic of São Tomé and Príncipe, West Africa.	252 School children	Microscopy	28.6% of protozoan.		
Tanzania Moyo, 2011	Age specific of diarrhea in Hospitalized children < 5y In Salaam, Tanzania	280 (0-5 y)	EIA	1.9%	18. %	
Tanzania Ngosso 2015	Pathogenic Intestinal Parasitic Protozoa in diarrhea < 5y Tanzania	720 (0- 5 y)	Microscopy PCR	16.4% micro 35.6% PCR	4.4% Micro 7.8% PCR	20% microscopy 12% PCR
Tanzania Tellevick, 2015	Prevalence of *Cryptosporidium* , *Entamoeba histolytica and Giardia* among Young Children with and without Diarrhea	1259 (0 - 2 y)	PCR	3.4% cases 6 .1% control	16% cases 3.1% control	0%
Uganda Tumwine, 2003	*Cryptosporidium parvum* in children with diarrhea in Mulago hospital, Kampala, Uganda.	2446 (0-5y)	Microscopy		25% cases 8.5% control	
Zambia, Siwila, 2010	Intestinal helminths and protozoa in children in pre-schools in Kafue district, Zambia.	403 (3-5y)	Microscopy	29%	28%	

Also in Bangladesh, a birth cohort of 445 infants were followed until 2 years of the study with stool collected 3 times per week (Donowitz 2016). Seven percent of infants had a *Giardia lamblia* positive in the first 6 months of life, increasing to 74% by 2 years of age (Donowitz 2016). In Africa, only two longitudinal studies addressing *Giardia lamblia* infection in infants have been conducted. In Gambia, 60 infants were studied longitudinally between 2 and 8 months of age and followed-up with parasite-specific plasma immunoglobulins for *Giardia* (Lunn 1999). The median age for the first *Giardia lamblia* exposure was between 3 and 4 months, and by 8 months of age 95% of infants had a positive titer in at least one occasion (Lunn 1999). In Guinea-Bissau, a birth cohort 200 infants was followed-up to 2 years with weekly stool specimen collection from enteropathogens (Valentiner-Branth 2003). In this study, *Giardia lamblia* (cyst or trophozoites) was the most frequently detected protozoa, with an incidence rate of 3.7 infections *per* child-year at risk, without association to diarrhea (Odds ratio-OR 0.64) (Valentiner-Branth 2003). In São Tomé e Principe (STP), evidence came from two cross sectional studies. Lobo *et al*. (2014) studied 348 children between 2 months to 10 years using molecular techniques for protozoa detection. They found a prevalence of 7.5% for *Giardia lamblia* at community setting, being the Assemblage A (60%) more common. Ferreira *et al*. (2015) studied 444 preschools children using microscopic technique for parasite detection. *Giardia lamblia* was the second more frequent parasite, infecting 41.7% (185 of 444) of the children. A systematic review and meta-analysis of the association between *Giardia lamblia* and diarrhea from several case-control and cohort studies in children under 5 years in developing countries, concluded that *Giardia* did not cause acute diarrhea among children in developing countries (OR 0.60; $p = .03$), although limited data suggest that infants in the first trimester of life may experience acute diarrhea in response to initial *Giardia lamblia* infections (Muhsen 2012). Additionally, *Giardia* was positively associated with persistent diarrhea among children in developing countries (OR 3.18; $p < .001$). Thereby, there is an apparently paradoxical association with protection from acute diarrhea, despite of an increased risk of persistent diarrhea (Muhsen 2012). Data from a multisite cohort study conducted in infants from developing countries, confirmed that *Giardia lamblia* was not a risk factor nor

protective for diarrhea (Relative Risk (RR) 0.95; 95% CI 0.90–1.00) suggesting that asymptomatic *Giardia lamblia* infection is common in children from developing countries (Rogawski 2017). Regarding *Giardia* genotypes, assemblage B was more common in African countries such as Tanzania (Forsell 2016), Uganda (Ankarklev 2012), Guinea Bissau (Ferreira 2012), Rwanda (Ignatius 2012) and Kenya (Mbae 2016). Assemblage A was far less frequent, reported only in STP (Lobo 2014) and Ethiopia (Gelanew 2007).

1.1.2. *Cryptosporidium* spp.

Cryptosporidium spp. is recognized globally as an important cause of diarrhea in children either in self-limiting diarrhea in otherwise healthy children, or in chronic life-threatening illness in immunocompromised patients, most in those with human immunodeficiency virus -HIV or in malnourished young children (Mor 2008). In Peru, birth cohort of 185 infants was followed-up to 2 years with weekly stool samples. Forty eight percent of children aged 0-23 months became infected with *Cryptosporidium parvum* during the study period, 60% of them asymptomatic (Checkley 1998). In the same cohort, others authors reported prevalence near to 30%, being *Cryptosporidium hominis* the species most frequently detected (70%)(Cama 2008). Infections with *Cryptosporidium hominis* lasted longer and had higher parasite excretion scores than other species (Cama 2008). In a prospective 4-year cohort study of 157 children in Brazil, (Newman 1999) *Cryptosporidium* spp. oocysts were identified in 7.4% of all stools, more frequently in children with persistent diarrhea (16.5%) than in those with acute (8.4%) or no diarrhea (4.0%) ($p < .001$) (Newman1999). In the same cohort *Cryptosporidium hominis* was identified in 57% of cases, and *Cryptosporidium parvum* in 43 %. *Cryptosporidium hominis* infections were associated with higher duration of diarrhea and heavier infections than stools from children with *Cryptosporidium parvum* (Bushen 2007). In a semi-urban community in south India, a birth cohort of 452 infants was followed-up to 3 years in a twice-weekly basis (Ajjampur 2007) *Cryptosporidium* spp. alone accounted for 7.61% of episodes of diarrhea. The two most common species were *Cryptosporidium hominis* (81%) and *Cryptosporidium parvum* (12%)(Ajjampur 2007). *Cryptosporidium hominis* infected children had a significantly higher severity of diarrhea (Ajjampur

2007). In the same region, a weekly surveillance of 176 infants was conducted up to their second birthday. A total of 186 episodes of cryptosporidiosis, mostly asymptomatic, were observed in 67% of children during the follow-up period (Sarkar 2013). In Bangladesh, a birth cohort of 392 rural infants was followed-up to 2 years with monthly stool samples (Korpe 2016). In the first two years of life, 77% of children experienced at least one infection with *Cryptosporidium* spp. Non-diarrheal infection (67%) was more common than diarrheal infection (6.3%)(Korpe 2016). *Cryptosporidium hominis* was isolated from over 90% of samples (Korpe 2016). In India, 97% of children acquired cryptosporidiosis by 3 years of age (Kattula 2017). In Africa, hospital and community-based studies document a high prevalence of cryptosporidiosis in younger children, particularly among those who are malnourished or positive for human immunodeficiency virus (HIV) infection and during rainy seasons (Mor 2008). In most SSA countries, cryptosporidiosis prevalence peaks among children aged 6–12 months and decreases thereafter and the majority of infections are caused by *Cryptosporidium hominis* (Mor 2008). In an open cohort followed-up to 3 years in Guinea Bissau, *Cryptosporidium* spp. was found in 7.4% of 3215 episodes of diarrhea (Mølbak 1993). The parasite was most common in younger children (median age 12 months) at the beginning of the rainy seasons (Mølbak 1993). In Kenya, a prospective survey was conducted over a two-year study period, with analysis of 4899 stool samples in children (Gatei 2006). The overall prevalence of cryptosporidiosis was 4%, highest among children 13–24 months of age (5.2%), 87% of the *Cryptosporidium* isolates were *Cryptosporidium hominis*, and 9% *Cryptosporidium parvum* (Gatei 2006). A prospective cohort of 108 women and their infants in rural/semi-rural Tanzania were followed from delivery through six months of age (Pedersen 2014). *Cryptosporidium* spp. infection in infancy remained undetected until 2 months of age and by 6 months, 33% of infants were infected. Maternal *Cryptosporidium* infection was associated with increased odds of infant infection (unadjusted OR 3.18) (Pedersen 2014). In São Tomé, a prevalence of 8.9% was reported for *Cryptosporidium* spp. at hospital setting. From 19 isolates, 14 corresponded to *Cryptosporidium hominis* (6.5%) and 5 to *Cryptosporidium parvum* (2.3%)(Lobo 2014). Several subtypes were identified for both species (Lobo 2014).

Regarding *Cryptosporidium* species, a geographically assessment of data derived from a multisite study in infants from developing countries validated the dominance of *Cryptosporidium hominis* (5.9 million) in infants and toddlers, in comparison with *Cryptosporidium parvum* (0.76 million) at SSA and South Asia countries (Sow 2016).

1.1.3. *Entamoeba hystolitica*

The true prevalence and incidence of infection and disease caused by *Entamoeba histolytica* is unknown for most areas of the world (Stauffer 2006). This can be attributed to the fact that differentiation of *Entamoeba histolytica* from the identical appearing non-pathogenic amebic species *Entamoeba dispar* is not possible based on microscopic exam (Stauffer 2006). Most of the evidence came from studies from Bangladesh. In a study in 221 children aged 2-5 years who were followed-up for 3 years due to diarrheal illness (Mondal 2006), it was found that 53% of them had serum antibodies against the *Entamoeba histolytica* Gal/GalNac lectin (anti-lectin IgG) at the time of enrollment (Mondal 2006). A cohort study of acute diarrhea included 289 preschool children in an urban slum of Dhaka, Bangladesh, *Entamoeba histolytica* was identified in 8% of the diarrheal stool specimens based on antigen detection (Haque 2003). The incidence of *Entamoeba histolytica* associated diarrhea was 0.08 *per* child-year, while the incidence of asymptomatic infection was 0.44 episodes *per* child-year. Asymptomatic carrier state of *Entamoeba* histolytica infection increased by five-fold the risk of *Entamoeba histolytica*-associated diarrhea (Haque 2003). In the same cohort, 80% of children who completed 4.2 years of follow-up were infected with *Entamoeba histolytica* at least once (Haque 2006). Also in Bangladesh, a birth cohort study of 147 infants was followed-up to the first year of life (Mondal 2012). *Entamoeba histolytica* was isolated in 3.8% of specimens using molecular techniques, and children who were underweighted and stunted at birth were more colonized by *Entamoeba histolytica* (Mondal 2012). In another cohort study among infants from Bangladesh, approximately 80% of children were infected with *Entamoeba histolytica* by the age of 2 years and fecal anti-galactose/Nacetylgalactosamine lectin immunoglobulin A was associated with protection from reinfection (Gilchrist 2016). Additionally, a new specie *Entamoeba bangladeshi* isolates were identified in samples

derived from this study (Gilchrist 2016). To the best of our knowledge, there are not prevalence data of *Entamoeba histolytica* in children in African countries

1.1.4 *Soil transmitted helmitnhs*

Several studies addressing the prevalence of STH have been conducted in LMIC mostly cross-sectional studies in preschool and school children. Longitudinal studies of prevalence of STH in infants are scarce (Menzies 2014, LaBeaud 2015, MAL-ED 2015). In Peru, a survey of children of 7-9 and 12-14 months of age found that prevalence of any helminth infection increased linearly to approximately 37.0% by 14 months of age (Gyorkos 2011). In Kenya, STH infections were the most common among infants with a prevalence of 19% (Lebaud 2015). In MAL-ED study, only prevalence of *Ascaris lumbricoides* was mentioned: in diarrheal stools, it was of 5.3% only detected in Brazil in infants aged 0-11 months, and ranged from 5.9% in Bangladesh to 6.3% in Brazil in those aged 12-24 months; in non-diarrheal stools, it was not detected in infants aged 0-11 months, and ranged from 4.7% in Brazil to 6.6% in India in those aged 12-24 month (Platts-Hills 2015).

Despite the aforementioned studies, to date, the best evidence of enteropathogens prevalence in younger children came from two major well-designed longitudinal studies carried out in developing countries. The Global Enteric Multicenter Study - GEMS of diarrheal disease, conducted in in developing countries, enrolled children aged 0-59 months with moderate-severe diarrhea. This study involved seven developing countries, Kenya, Mali, Mozambique and Gambia in sub-Saharan Africa, and Bangladesh, India and Pakistan in South Asia (Kotloff 2013). Potential pathogens were identified in 83% of children with diarrhea and 72% in controls. *Cryptosporidium* spp. had the second highest attributed fraction during infancy at five sites, persisting in importance, regardless of HIV prevalence. Furthermore, *Cryptosporidium* spp. was associated with death during the ensuing 2–3 months in toddlers aged 12–23 months (HR 2.3). *Giardia lamblia* was not significantly positively associated with moderate to- severe diarrhea; to the contrary, in children aged 12–59 months *Giardia lamblia* was significantly more frequent in controls than in those with moderate-to-severe diarrhea (univariate analyses). *Entamoeba histolytica*

was only reported in two sites (Bangladesh and Mali) and soil transmitted helminths (STH) were not found (Kotloff 2013).

The Global Network for the Study of Malnutrition and Enteric Diseases (MAL-ED) (Platts-Mills 2015) included a birth cohort at eight community sites in Africa (South Africa and Tanzania), Asia (Bangladesh, India, Nepal and Pakistan), and South America (Peru and Brazil). They assessed pathogen-specific burdens in diarrheal and non-diarrheal stool specimens from children aged 0–24 months. At least one pathogen was detected in 76.9% of diarrheal stools and 64.9% of non-diarrheal stools. *Cryptosporidium* spp. (2.0%) exhibited one of the highest attributable burdens of diarrhea in the first year of life and was associated with a higher severity score. *Giardia lamblia* was not significantly associated with diarrhea for any age group, or site, but it was common, around 30% among asymptomatic children aged 12–24 months. *Entamoeba histolytica* was not found. STH showed low prevalence in few sites (Platts-Mills 2015).

These studies highlight the role of protozoan as etiologic agents of enteric infections in the first two years of age, particularly *Cryptosporidium* spp. associated to moderate-to-severe diarrhea and *Giardia lamblia* in asymptomatic infected infants (Kotloff 2013, Platts-Mills 2015). Moreover, the causality between the pathogens detected in the absence of diarrhea is a challenge (McCormick 2016). Hence, pathogens appear less causally linked to diarrhea than previously expected (McCormick 2016).

Table 2. Longitudinal studies of prevalence of *Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica* in infants from low-and middle income countries

Country	(N) Study design	Stool analysis	Prevalence	Reference
			Giardia lamblia	
Guatemala	45 Birth cohort up to 3 y	Weekly stool Microscopy	*Giardia* episodes *per* child were 0.71 at 1 year to 3.60 at 3 year. Prevalence 20 % by the 3 years of age.	Farting 1986
Kenya	84 Longitudinal 10-28	Weekly stool Microscopy	*Giardia* has a prevalence of 44.7%. New *Giardia* episodes *per* year per child were 2.77.	Chunge 1991
Gambia	60 Longitudinal 2-8m	IgM anti-Giardia	95% with IgM positive at first year of life.	Lunn 1999
Brazil	157 Birth cohort up to 4 y	3/weekly stool Microscopy	27.4% of infants were infected with *Giardia. Giardia* was more common in persistent (20.6%) than acute diarrhea (7.6%)(p= .002)	Newman 2001
Guinea Bissau	200 Birth cohort up to 2 y	Weekly stool Microscopy	*Giardia* incidence rate of 3.7 infections *per* child-year, without association to diarrhea (OR 0.64)	Valentiner-Branth 2003
Peru	220 Birth cohort up to 3 y	Weekly stool Microscopy	Prevalence of *Giardia* was 18.8% per child-week, and 85% of children became infected. High rate of reinfection (87%)	Hollm-Delgado 2008
Bangladesh	147 Birth cohort up to 1 y	Monthly stool PCR	*Giardia* infection increased from 30% in the 1 month to 90% at 12 months of life.	Mondal 2012
Bangladesh	445 Birth cohort up to 2 y	3/weekly stool EIA	7% of *Giardia* at first 6 months of life, increasing to 74% by 2 years of age.	Donowitz 2016
Multisite MAL-ED	2089 Birth cohort up to 2y	Monthly EIA	The incidence of at least one *Giardia* episode ranged from 37.7% (Brazil) to 96.4% (Pakistan) and was higher in the second year of life. Repeated detections in 40% of the children.	Rogawski 2017

Table 2. Longitudinal studies of prevalence of *Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica* in infants from low-and middle income countries *(continued)*

Country	(N) Study design	Stool analysis	Prevalence	Reference
			Cryptosporidium spp.	
Guinea Bissau	1315 Open cohort up to 3y	Microscopy Ziehl-Neelsen	*Cryptosporidium* spp were found in 7.4% of episodes of diarrhea.	Mølbak 1993
Peru	185 Birth cohort up to 2 y	Weekly /stool Microscopy	*Cryptosporidium* in 48% of infants, 60% asymptomatic.	Checkley 1998
Brazil	157 Birth cohort up to 5 y	Microscopy Ziehl-Neelsen	*Cryptosporidium* spp. was identified in 7.4%. Persistent diarrhea in 16.5% and acute diarrhea 8.4 % ($p < 0.001$).	Newman1999
Kenya	4889 Longitudinal 24 m	Microscopy Ziehl-Neelsen PCR	Prevalence was 4%, highest among children 13-24 months of age (5.2%). *C. hominis* in 87% - *C. parvum* 9%.	Gatei 2006
Peru	74 Birth cohort up to 2 y	Weekly /stool Microscopy -PCR	77% of children had one or more episodes of cryptosporidiosis.	Priest 2006
Brazil	157 Birth cohort up to 5 y	Microscopy PCR	Cryptosporidium was identified in 37% of children. *C. hominis* 57% - *C. parvum* 43%	Bushen 2007
India	452 Birth cohort up to 3 y	Weekly /stools Microscopy-PCR	*Cryptosporidium* spp. accounted for 7.61% of episodes of diarrhea. *C. hominis* in 81% - *C. parvum* in 12%.	Ajjampur 2007
Peru	533 Birth cohort up to 4 y	Microscopy PCR	Cryptosporidiosis was detected in 20%. *C. hominis* in 70% - *C. parvum* in 13% and *C. meleagridis* in 8%.	Cama 2008
India	2579 Open cohort 0-5 y	Microscopy- PCR	2.7% of children had cryptosporidial diarrhea (75% in children <2 years). *C. hominis* in 80% of cases	Ajjampur 2010
India	176 Birth cohort up to 2 y	Monthly /stool PCR	Cryptosporidiosis in 67% of children; 64.4% asymptomatic and 10.2% symptomatic. Incidence rate was 0.59 episodes *per* child year	Sarkar 2013

Table 2. Longitudinal studies of prevalence of *Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica* in infants from low-and middle income countries *(continued)*

Country	(N) Study design	Stool analysis	Prevalence	Reference
			Cryptosporidium spp	
Tanzania	108 Birth cohort up 6 m	Microscopy Ziehl-Neelsen	33% of infants were infected by *Cryptosporidium* spp.	Pedersen 2014
India	160 Birth cohort up to 2 y	PCR	62% of children had cryptosporidial infection during the follow-up.	Lazarus 2015
India	497 Birth cohort up to 3 y	Two weeks/ stool PCR	Infection at 6 months 40.2%, by 3 years in 97%. Incidence of 0.86 infections per child-year. Reinfection in 81% of infants.	Kattula 2016
Bangladesh	392 Birth cohort up to 2 y	Monthly/ stool PCR	77% of children had at least one *Cryptosporidium* infection. 67% asymptomatic and 6.3% with diarrheal.	Korpe 2016
			Entamoeba histolytica	
Bangladesh	289 Longitudinal 2-5 y	Monthly stool Stool antigen	*E. histolytica* was identified in 8% of the diarrheal stool. The incidence was 0.08 *per* child-year in diarrhea, and 0.44 episodes *per* child-year in asymptomatic infection.	Haque 2003
Bangladesh	202 Longitudinal 2- 5 y	Stool antigen	*E. histolytica* was detected in 80% of infants, and repeat infection in 53%. Incidence of *E. histolytica*-associated diarrhea was 0.09 episodes/child.	Haque 2006
Bangladesh	221 Longitudinal 2-5 y	Stool antigen	*E. histolytica*-associated diarrheal illness was 17%. High percentage 53% of anti-lectin IgG at time of enrollment.	Mondal 2006
Bangladesh	147 Birth cohort up to 1y	Monthly stool PCR	*E. histolytica* was isolated in 3.8%. Cumulative percentage was 40% at the end of 12 months of age.	Mondal 2012

Table 2. Longitudinal studies of prevalence of *Giardia lamblia, Cryptosporidium spp. and Entamoeba histolytica* in infants from low-and middle income countries *(continued)*

Country	(N) Study design	Stool analysis	Prevalence	Reference
Bangladesh	147 Birth cohort up to 1 y	2 week stool PCR	*E. histolytica* was associated to diarrhea (OR 2.4)	Taniuchi 2013
Bangladesh	226 Birth cohort up to 1y	Monthly stool Antigen stool	*E. histolytica* occurred in 50% of infants.	Korpe 2013
Bangladesh	392 Birth cohort up to 2y	Monthly stool ELISA -PCR	80% of children were infected with *E. histolytica* by the age of 2 years; 17% were associated to diarrhea.	Gilchrist 2016
			Soil transmited diseases	
Ecuador	1697 Birth cohort up to 3y	Every three months- Microscopy	42.3% of children were infected east one STH infection during the first 3 years of life. . A. Lumbricoides (33.2%)	Menzies 2014
Kenya	545 Birth cohort up to 3y	Every six months Microscopy	STHs were the most common infection with 106 infections (19%) by age three years.	LaBeaud 2015

EIA /ELISA enzyme immunosorbent assay, PCR polymerase chain reaction.

1.2. Taxonomy and life cycle of enteric parasites

Parasite is defined as an organism that obtains its nutrients from one or a very few host individuals, normally causing harm but not causing death immediately (Tyler Miller 2009). The classification described by Anderson & May in 1991 enabled to elucidate the principles governing the dynamics, epidemiology and courses of infection of pathogens that severely impair human health (WHO 2010a).

Microparasites: have simple life cycles and a tendency to replicate within the host. Transmission may be either direct through environmental contamination, or indirect through a vector that may or may not be an intermediate host, or through blood transfusions or organ transplants. The infections by microparasites may vary from acute and recurrent to subclinical forms. The enteric protozoa such as *Giardia lamblia, Cryptosporidium* spp., and *Entamoeba histolytica* are examples of microparasites (WHO 2010a).

Macroparasites: usually have complex life cycles involving intermediate and reservoir hosts, and they grow but do not multiply in their host. The parasite produce specialized infective stages that are released to infect new hosts. Transmission may be either direct through ingestion from a contaminated environment or through skin penetration, or indirect, through ingestion of an infected intermediate host or tissues of a reservoir host. The infections caused by macroparasites tend to be chronic rather than acute, and mortality rates are considered low. The helminth worms such as soil-transmitted helminths (STH), are the major macroparasites (WHO 2010a).

Taxonomy and life cycle of *Giardia lamblia, Cryptosporidium spp and Entamoeba histolytica* are described in Table 3.

Giardia lamblia (also named *Giardia duodenalis,* or *Giardia intestinalis)* is a diplomonad, not invasive protozoan (Adam 2001). Organisms in the genus Giardia are classified in the phylum Metamonada, subphylum Trichozoa, superclass Eopharyngia, class Trepomonadea, subclass Diplozoa and order Giardiida (Thompson 2012). It has special characteristics, including the presence of two transcriptionally active diploid nuclei, the absence of mitochondria and peroxisomes, and the unique attachment organelle, the ventral sucking disc (Thompson 2009). To date, six species are

recognized in the genus, one in amphibians (*G. agilis*), two in birds (*G. ardeae* and *G. psittaci*), two in rodents (*G. muris* and *G. microti*), and one in mammals (*G. lamblia*) (Certad 2017). Genetic studies have confirmed the division of *Giardia lamblia* human isolates into two major genotypes, assemblages A and B (Adams 2001). There is evidence of phenotypic difference between assemblages A and B, which may be reflected in the duration, drug sensitivity and virulence of infections (Thompson 2009). Genetic studies showed amino acid identity of only 78% in coding regions between assemblages A and B parasites, sufficient to recognize them as different species (Jerlström-Hultqvist 2010). This highly infected protozoan has two life cycle stages: the flagellated trophozoite that attaches to the intestinal microvilli and an infectious cyst that persists in the environment (Nosala 2015). The cysts are non-motile with a hardy cyst wall (consisting of 60% carbohydrate and 40% protein) that protects it from hypotonic lysis in the environment, being able to survive for several weeks outside the host (Ankarkev 2010). The trophozoite is the disease-causing stage. Infection occurs as a three-step process: excystation, attachment and multiplication, and encystation (Farthing 1997) (Table 2). Risk factors for *Giardia* are the high intro-/peri-domicilliary concentration of domestic animals (Sackey 2003, Rogawski 2017), and living in crowed conditions or living in a house without access to a sewerage system (Silva 2009).

Cryptosporidium is a small obligate intracellular (but extracytoplasmic) protozoan that infects epithelial cells and requires a host to multiply (Current 1991). *Cryptosporidium* has been placed in the phylum Apicomplexa since it posses an apical complex in some invasive life cycle stage (sporozoites and merozoites) (Ryan 2003). *Cryptosporidium* has several peculiarities that separate it from any other coccidian, including the location within the host cell, confined to the apical surfaces of the host cell (intracellular, but extracytoplasmic); the attachment of the parasite with a feeder organelle to facilitate the uptake of nutrients from the host cell; and the presence of two types of oocysts, thick-walled and thin-walled, with the latter responsible for the initiation of the auto-infective cycle in the infected host (Ryan 2003). Since *Cryptosporidium* invades and resides in epithelial cells but does not usually invade deeper mucosal layers, it can be viewed as a “minimally invasive” mucosal pathogen (Laurent 1999). To date, based on morphological, biological, and molecular data, 31

valid species of *Cryptosporidium* are recognized in fish, amphibians, reptiles, birds, and mammals (Ryan 2014). *Cryptosporidium hominis* and *Cryptosporidium parvum* are the species responsible for the majority of human infections (Xiao 2010). The genetic determinants that dictate the virulence and the host range of *Cryptosporidium* species and genotypes are not fully understood (Bouzid 2013). It is widely accepted that successful parasite species should evolve to become less virulent over time, and maladapted novel parasites are initially more harmful and subsequently attenuating (Eber 1994). This highly infected protozoa parasite has a multistage life: oocyst is ingested, then it travels to the small intestine where the oocyst wall opens (excystation) and four motile sporozoites are released, then motile sporozoites attach to the intestinal epithelium and are enveloped by the host cell apical membrane. In these cells, the parasites undergo asexual multiplication (schizogony or merogony) and then sexual multiplication (gametogony) (Leitch 2012). After fertilization, the zygote can develop into two types of oocysts, a thick-walled oocyst that is excreted into the environment (80%), or a thin-walled oocyst that can auto-infects the host (20%)(Leitch 2012). The broad host range for *Cryptosporidium* together with the high output of oocysts ensures a high level of contamination in the environment (Putignani 2010). Transmission of *Cryptosporidium* mainly occurs by ingestion of contaminated water, food sources or by person-to-person contact. In SSA, transmission appears to occur predominantly through an anthroponotic cycle (Putignani 2010). Seasonal peaks of cryptosporidiosis have been reported in all regions of SSA, being more common in raining season (Newman 1999, Mølbak 1993).

Entamoeba histolytica is an invasive enteric protozoan parasite that causes amebiasis (Ralston 2011). Molecular phylogeny places *Entamoeba* on one of the lowermost branches of the eukaryotic tree (Haque 2003). Features of *Entamoeba* include the presence of polyploid chromosomes, transposons and repetitive DNA that may facilitate genome rearrangements, and a novel GAAC - core promoter containing guanine (G), adenine (A), and cytosine (C) element important in transcriptional control in *Entamoeba* (Weedall 2011). Two morphologically identical species colonize the human colon, *Entamoeba dispar* and *Entamoeba histolytica*, the latter being the amoeba pathogenic for humans (Faust 2012). *Entamoeba moshkovskii* was

found to be pathogenic in mice, and has been associated temporally with diarrhea in children (Shimokawa 2012). *Entamoeba moshkovskii* shared with *Entamoeba histolytica* the ability to infect mice indicating that they share virulence mechanisms, which are not present in *Entamoeba dispar* (Shimokawa 2012). New specie *Entamoeba Bangladeshi*, isolated in Bangladesh community, appeared also to be closer to *Entamoeba histolytica* (Gilchrist 2016). *Entamoeba histolytica* exhibits a typical fecal–oral life cycle, consisting of infectious cysts passed into the feces and trophozoites that replicate within the large intestine. In many cases, the trophozoites remain confined to the intestinal lumen (noninvasive infection-asymptomatic carriers) or can invade the intestinal mucosa (invasive disease), or, through the bloodstream to extraintestinal sites such as liver, brain, and lungs (extra-intestinal disease) (Stanley 2003, Stauffer 2003). Transmission occurs mainly through the ingestion of fecally contaminated food and water containing cysts. Illness is spread by fecal oral route, person-to-person contact or fecally contaminated food and water (Stanley 2003).

Soil-transmitted helminthiases (STH) is a term referring to a group of parasitic diseases caused by nematode worms that are transmitted to humans by faecally-contaminated soil. The soil-transmitted helminths of major concern to humans are the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*) and hookworms (*Necator americanus and Ancylostoma duodenale*) (WHO 2012). The soil-transmitted helminths cause human infection through contact with parasite eggs or larvae that thrive in the warm and moist soil of the world's tropical and subtropical countries (Bethony 2006). Adult hookwormsparasitise the upper part of the human small intestine, whereas ascaris roundworms parasitise the entire small intestine and adult trichuris whipworms live in the large intestine, especially the caecum (Bethony 2006). *A. lumbricoides* infects individuals through faecal–oral transmission. After embryonated eggs are swallowed, first-stage larvae (L1) hatch, moult into second-stage larvae (L2), penetrate the intestinal mucosa, and migrate to the pulmonary circulation. Third-stage larvae (L3) migrate across the alveolar wall and traverse the tracheobronchial tree to the larynx and into the small intestine, to moult into fourth-stage larvae (L4) and adult worms. Adult female *A. lumbricoides* worms produce thousands of eggs daily that pass in the stool. Egg production occurs 2–3 months after infection, and can live for a few years. Eggs can remain viable in warm, moist soil for

years. *T. trichiura* is transmitted through a faecal–oral cycle, with embryonated eggs ingested via food or hands, and hatching into larvae that moult in the small intestine. Unlike ascaris, trichuris does not migrate through the lungs. The larvae attach to the intestinal villi and develop into adult worms, which reside in the caecum and ascending colon. Female worms lay thousands of eggs daily for several years. The eggs pass in the stool can survive for months (Bethony 2006). *A.duodenale* and *N americanus* larvae are free-living in the soil, and infect people by penetration of the skin, typically bare feet. Larvae are transported to the pulmonary capillaries, where they penetrate the alveolar wall, pass to the larynx, and are swallowed. Larvae moult and develop into mature worms in thesmall intestine over 1–2 months, and can survive for months (*A duodenale*) or years (*N americanus*). A female worm releases thousands of eggs daily into the stool, which after 5–10 days, hatch in warm, moist, sandy soil, or in faeces. Rhabditiform larvae (L1) become infective after moulting to L2 and L3 larvae that survive for several weeks (Jourdan 2018). Soil-transmitted helminth infections are particularly widespread in the tropical and subtropical regions of the world with warm and moist climate, which allow the development of helminth eggs and larvae, coupled with inappropriate hygiene, sanitation, and water (Jourdan 2018).

Table 3. Taxonomy, genotypes and life cycle of enteric protozoa *Giardia lamblia, Cryptosporidium spp. Entamoeba histolytica*

	Giardia lamblia	*Cryptosporidium spp.*	*Entamoeba histolytica*
Taxonomy	Genus *Giardia* is classified in the phylum Metamonada, subphylum Trichozoa, super class Eopharyngia, class Trepomonadea, subclass Diplozoa and order Giardiida (Thompson 2009).	Genus *Cryptosporidium* is classified in the phylum Apicomplexa, class Conoidasida and order Eucoccidiorida (Clode 2015). Recently, a closer affinity to gregarines, representing an early branch at the base of the phylum, has been demonstrated (Clode 2015).	Genus Entamoeba is classified in the phylum protozoa, superclass Rhizopoda and order Amoebida (Khan 2008).
Genotypes	Based on the morphology criteria and host specificity 6 species are recognized, one in amphibians (*G. agilis*), two in birds (*G. ardeae* and *G. psittaci*), two in rodents (*G. muris* and *G. microti*), and one in mammals (*G.lamblia*) (Adam 2001) Eight groups of genetically related strains (assemblages A to H) have been identified within the *Giardia lamblia* species complex, of which two (A and B) infect humans (Caccio 2008). Analysis highlighted large differences between assemblage A and B suggesting that are different *Giardia* species (Ankarklev 2010).	Based on morphological, biological, and molecular data, 31 valid species are recognized in fish, amphibians, reptiles, birds, and mammals (Ryan 2003). In humans, *C. hominis* and *C. parvum* are the major pathogens, causing >90% of cases (Xiao 2010).	The genus *Entamoeba* contains many species, six of which *E. histolytica, E. dispar, E. moshkovskii, E. polecki, E. coli and E. hartmanni* reside in the human intestinal lumen (Fotedar 2007). New specie *E. Bangladeshi* appears to be closer to *E. histolytica* (Royer 2012).
Morphology	The parasite has two stages: the cyst and the trophozoite. Cysts: non-motile and oval shaped. Size: 7–10 μm wide. Trophozoite: motile- and shape resembling a pear bisected. Size: 12–15 μm long and 5–9 μm wide.	Oocysts: Morphology: round, oval. Size: 4-6 μm.	The parasite has two stages: the cyst and the trophozoite. Cyst: spherical. Size: 10-20 μm. Trophozoite: highly motile, pleomorphic shape Size: 10 to 60 μm

Table 3. Taxonomy, genotypes and life cycle of enteric protozoa *Giardia lamblia, Cryptosporidium spp. Entamoeba histolytica (continued)*

	Giardia lamblia	*Cryptosporidium spp.*	*Entamoeba histolytica*
Localization	Trophozoites colonize the upper small intestine. Trophozoites reside and replicate at the intestinal epithelial surface (noninvasive) (Farting 1997)	Oocyst invades host epithelial cells, most commonly in the small intestine, and resides within a parasitophorous vacuole (extra-cytoplasmatic) (Laurent 1999)	Trophozoites colonize the large intestine (cecal and sigmoidorectal regions). Trophozoites can remain at intestinal lumen (noninvasive) or can invade the intestinal mucosa (invasive disease) (Stanley 2003)
Life cycle	Infection begins by ingestion of cysts, cysts begin excystation releasing two trophozoites; trophozoites adhere to the cells through an adhesive ventral disk and multiply by binary fission; trophozoites transform into cysts (encystation process) that are excreted and passed with the feces, completing their lifecycle (Farthing 1997).	The parasite has a multistage life cycle. Infection begins by ingestion of oocysts; from which sporozoites emerge in the small intestine; sporozoites invade epithelial cells and form trophozoites in the apical domain of the cell; the parasites has asexual multiplication (schizogony or merogony) and then sexual multiplication (gametogony): after fertilization, the zygote develop a thick-walled oocyst excreted into the environment (80%) or a thin-walled oocyst that auto-infect the host (20%) (Leitch 2012)	Infection begins with the ingestion of the cysts; at terminal ileum or colon, excyst to trophozoite stage; the trophozoite adhere to the epithelial cell and reproduce by binary fission, encyst within the colon, completing the lifecycle (Stauffer 2003).
Infective dose	Highly infectious: as few as 10 cysts may cause infection (Farthing 1997)	Highly infectious: a single oocyst may cause infection (Guerrant 1999)	Not known; theoretically, the ingestion of one viable cyst can cause infection (Stanley 2003)
Transmission routes	Fecal-oral Assemblage A: zoonotic and anthroponotic transmission Assemblage B: zoonotic and anthroponotic transmission	Fecal-oral *C. hominis*: anthroponotic transmission *C. parvum*: zoonotic transmission, especially young live stock, anthroponotic transmission is also documented.	Fecal-oral through the ingestion of fecally contaminated food and water containing cysts.

1.3. Host-parasite interaction: host damage

A redefined concept of host-parasite interaction proposes that host–pathogen interactions should be analyzed using host damage as the common denominator for characterizing microbial pathogenicity. Thus, pathogenesis should be focused on damage instead of pathogen or host (Casedevall 2000).

Giardia lamblia, Cryptosporidium and Entamoeba hystolitica can cause host-damage through mechanisms of apoptosis (cell death), disruption of epithelial brush border, cytoskeleton remodeling and disassembly of tight junctions (TJs), and inflammation (Berkes 2003, De Genova 2016, Certad 2017). Particularly, mechanisms involved in permeability, and inflammation are focused in this study (Figure1).

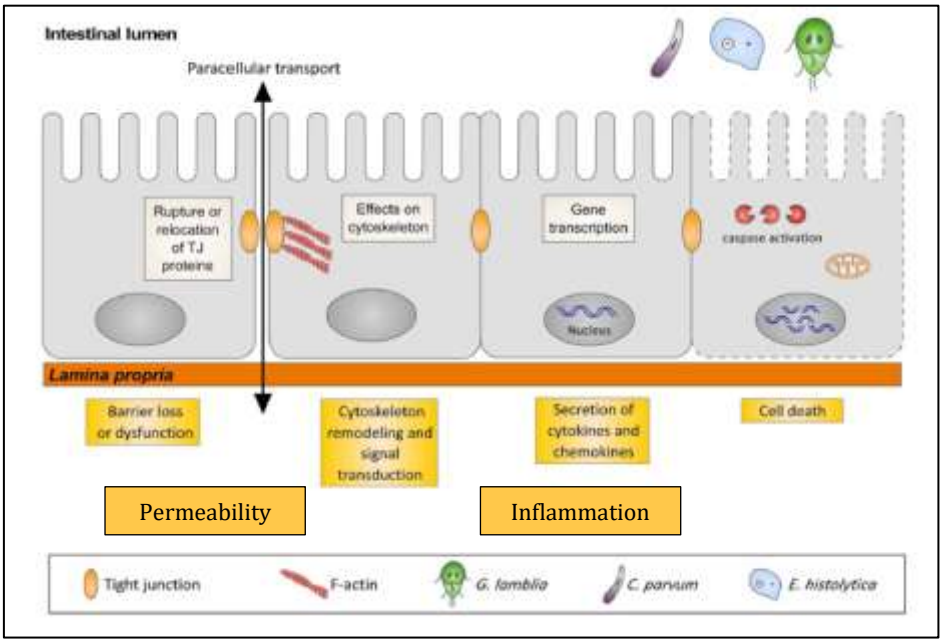


Figure 1. Host-parasite interactions in intestinal barrier (Adapted from De Genova 2016)

1.3.1. *Apoptosis*

Apoptosis connotes a controlled physiologic process of removing individual components of an organism without destruction or damage to the organism (Fink 2005). In the gastrointestinal tract, epithelial cells are constantly renewed by extrusion

to the lumen with a turn over of cells every 5 to 7 days, crucial for the maintenance of normal organ morphology and function. This process is highly regulated and maintained by balancing between cell death and proliferation (De Genova 2016).

In giardiasis, the physical attachment of trophozoites to epithelial cells may target specific signaling networks, provoking down stream events that impair normal organ function, and lead to associated signs and symptoms of giardiasis. The most striking outcome of *Giardia*–host interaction is the activation of cell death or apoptosis. *In vitro* studies, infection with *Giardia* induces host cell apoptosis reflected by signs of chromatin condensation within the nuclei of human carcinoma cell line HCT-8 (Chin 2002, Koh 2013). Moreover, different assemblages differed in their ability to induce enterocyte apoptosis, suggesting that strain-dependent induction of enterocyte apoptosis may contribute to the pathogenesis of giardiasis (Chin 2002). The apoptotic response elicited by *Giardia* can be mediated by caspase-3 activation (Chin 2002) and activation of intrinsic and extrinsic pathways (caspase-8 and caspase-9) (Panaro 2007). Another mechanism by which *Giardia* can induce apoptosis is through the up-regulation of genes implicated in apoptotic cascade (proteins Bax and Bak in Bcl-2 family) and formation of reactive oxygen species (Roxström-Lindquist 2006, Panaro 2007). In addition, *Giardia* can prevent the formation of nitric oxide, by consuming local arginine (Eckmann 2000). This mechanism may contribute to *Giardia*-induced enterocyte apoptosis, as arginine starvation in these cells is known to cause programmed cell death (Buret 2007). *In vivo* studies, important increase of epithelial apoptotic rate was observed in patients with chronic giardiasis, triggered by direct contact of the parasite or of parasite products with the epithelium (Troeguer 2007). Following induction of apoptosis, *Giardia* trophozoites can also induce alterations in the enterocyte tight junctions by breaking down or relocalization of proteins associated with these structures. Thus, there is a direct cause–effect relationship between *Giardia*-induced apoptosis and small-intestine barrier function (Chin 2002).

In *Cryptosporidium* infection, the parasite requires viable host cells to survive. The induction of apoptosis appear to play a host protective role by limiting parasite numbers and clearing the infection (Leitch 2012). Nuclear condensation and DNA fragmentation during *C. parvum* infection were shown to be caspase-dependent and

induced by Fas/Fas ligand (Chen 1999). In particular, *Cryptosporidium* is able to modulate apoptosis of the host cell at all parasite stages showing early inhibition of apoptosis at the trophozoite stage, and late induction of apoptosis at the sporozoite and merozoite stages (Mele 2004; Liu 2008). The inhibition of apoptosis was restricted to infected cells, while *C. parvum*-induced apoptosis was limited to by-stander uninfected cells (McCole 2000). This regulation of apoptosis by *Cryptosporidium* contributes to the parasite growth and development at initial stages of infection, and promotes its propagation later on (Male 2004).

In *Entamoeba histolytica* infection, host cell apoptosis is one of the critical steps of invasion (De Genova 2016). Human cells touched by amoebic trophozoites become immobile within minutes, and lose their cytoplasmic granules and structures, and eventually their nucleus (Stanley 2003). *In vitro* studies, DNA fragmentation, sign of apoptosis, was observed after trophozoite adhesion to a murine myeloid cell line (Ragland 1994). *Entamoeba histolytica* - Jurkat cell interactions demonstrated that contact dependent cell killing requires activation of host cell caspase 3-like caspases via an apparently caspase 8- and 9-independent pathway (Huston 2000) .The adhesion of *Entamoeba histolytica* trophozoites to host cells also induced a contact-dependent protein dephosphorylation by protein tyrosine phosphatases, through activation of host cell calpain (Teixera 2002). Oxidative stress could also cause cellular apoptosis. In amoebiasis, incubation of human neutrophils with *Entamoeba histolytica* trophozoites triggered NADPH oxidase-dependent production of reactive oxygen species and cell apoptosis (Sim 2005). Epithelial cell apoptosis in the intestine facilitates amebic infection once cells serve as fuel to ameba, and the barrier dysfunction that follows to apoptosis, allow ameba access to the tissue, promoting parasite survival (Becker 2010). More recently, a novel mechanism of partial apoptosis or trogocytosis was described (Ralston 2014). It was demonstrated that *Entamoeba histolytica* ingests small fragments of the cell membrane, or cellular components like cell cytoplasm and mitochondria (Ralston 2014). Thereby, *Entamoeba histolytica* infections might result in cell death both by apoptosis and trogocytosis, and that these events might contribute to tissue invasion by the parasite (De Genova 2016).

In *Soil-transmitted helminthiases* experimental studies showed an increase in epithelial cell apoptosis associated with chronic intestinal nematode infection (Cliffe 2007). Apoptosis was detected in chronic infection by *T. muris*, which coincide with high levels of proinflammatory cytokines sucha as TNF-α (Cliffe 2007). TNF-α may promote apoptosis in an indirect manner by enhancing the production of IFN-γ and by the recruitment of inflammatory cells into the intestinal microenvironment (Cliffe 2007). Although the induction of both proinflammatory cytokines and apoptosis are typically thought to be detrimental during intestinal colitis, they may in fact act in a host-protective manner during chronic *T. muris* infection by controlling the level of homeostatic dysregulation in the gut (Cliffe 2007).

1.3.2. *Disruption of brush border*

Enteric protozoan parasites can induce structural and functional dysfunction of epithelium being the enterocytes the major target cell (De Genova 2016).

Giardia lamblia colonizes the upper small intestine, without invading or causing severe inflammation (Ankarklev 2010). Studies using *in vivo* and *in vitro* models have established that *Giardia lamblia* causes diffuse shortening of epithelial microvilli with malabsorption of glucose, sodium, and water, and reduced disaccharidase activity (Buret 1992, Troeguer 2007). In gerbils model infected with a *Giardia lamblia*, infection resulted in crypt hyperplasia associated with an increased enterocyte migration rate; markedly shortened of epithelial microvilli, and decrease in brush border surface area (Buret 1992). In addition, infection resulted in decreased glucose-stimulated electrolyte, and water absorption (Buret 1992). *In vivo, Giardia lamblia* H3 cyst infection in a weaned mouse model demonstrated that persistent giardiasis leads to epithelial cell apoptosis, crypt hyperplasia and decreased villus height/crypt depth ratio and hyper cellularity in the lamina propria (Barlet 2013). Those histopathological changes were worsened in undernutrition conditions (Barlet 2013). It was suggested that CD8+ T cells might mediate brush border injury (Scott 2000). In giardiasis, the relative increase in immature enterocytes at the villus base can also account for microvillus cytoskeletal abnormalities and disaccharidase deficiency (Solaymani-Mohammadi 2013). In humans, biopsies of patients with chronic *Giardia* infection

showed alteration of mucosal architecture with reduction in villous surface area by 50%, epithelial barrier dysfunction owing to down regulation claudin 1, and increased epithelial apoptoses (Troeguer 2007). In addition, impaired of Na^+-dependent D-glucose absorption and hypersecretion of chloride was also present in chronic giardiasis (Troeguer 2007).

Cryptosporidium does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium (Bouzid 2013). Despite *Cryptosporidium* be a minimally invasive parasite, it can cause significant abnormalities in the absorptive and secretory functions of the gut (Bouzid 2013). In the gastrointestinal tract the most pronounced morphologic alteration is villous atrophy, although villous blunting and fusion might also occur (Kosek 2001). *In vitro*, analysis of infection by *C. parvum* in neonatal pig showed reduction of villous height, deeper crypt and reduced epithelial area on day 3 postinfection (Argenzio 1990). Epithelial cell damage was also associated to impaired glucose-stimulated sodium and water absorption mediated by prostaglandin PGE_2 (Argenzio 1990). In an organoid model of cryptosporidial infection, *C. parvum* resulted to patchy disruption of the epithelium interrupted by areas of intact epithelium, with irregular stunting of microvilli, and areas of loose paracellular spaces in infected cells (Alcantara 2008). The histopathologic changes seen in cryptosporidiosis are worsened in undernutrition conditions (Costa 2011). In humans, duodenal biopsy specimens from HIV patients with intestinal *Cryptosporidium* infection showed partial or total villus atrophy with a marked increase of neutrophil infiltrate in patients with more intensive infections. Vitamin B12 and D-xylose absorption were also negatively correlated with intensity of infection (Goodgame 1995).

Invasion by *Entamoeba histolytica* is preceded by the interaction of trophozoites with intestinal epithelial cells, and this interaction impacts cell morphology, and intercellular contacts in the intestinal epithelium (De Genova 2016). *In vitro* studies showed apical injury in regions of *Entamoeba histolytica* contact, with changes on the brush border as the result of a modification of F-actin cytoskeleton in Caco-2 cells (Mounier 2000). Furthermore, *Entamoeba histolytica* results in the disorganization and

disappearance of microvilli accompanied by changes in the distribution of ezrin and villin proteins (Lauwaet 2003). Also in epithelial Caco-2 cells, *E. histolytica* caused damage at the apical surface and drop of transepithelial resistance post incubation with trophozoites (Li 1994). Once *Entamoeba histolytica* invades tissue, there are histologic changes characterized by a non-specific lesion and mucopenic depression in the pre-invasive stages (with significant infiltrate of neutrophils), following by early invasive lesion with superficial erosion of the mucosa, and interglandular foci of microinvasion. Lastly, in a late invasive phase, the ulceration extends through the mucosa and muscularis mucosae into the submucosa, forming the granulating ulcer (Prathap 1970, Espinosa-Cantellano 2000).

In STH infections, jejunal biopsies of Ascaris-infected children showed histological changes in villi, crypts, and lamina propria, affecting the intestinal absorptive capacity (Tripathy

1.3.3. *Cytoskeleton remodeling and disassembly of tight junctions*

Enteric pathogens can disrupt the intestinal barrier either directly, or by binding to cell surface molecules or inducing changes in TJs protein expression. Alternatively, pathogens generate toxins and proteases, which beside to promote cell damage and apoptosis, they also disrupt TJs and the cytoskeleton (Berkes 2003, Groschwitz 2009).

In *Giardia lamblia* infection, the disassembly of TJs might be due to reduced expression of junctional components but also as a consequence of their relocation/reorganization within cells (De Genova 2016). *In vitro* studies in different cell lines infected with *Giardia* trophozoite, result in the disruption of TJs protein zonuline (ZO)-1, in a caspase-3-dependent fashion (Chin 2002, Buret 2007, Koh 2013). Degradation of the TJs proteins occludin and claudin-4 have been reported associated to bacteria translocation in giardiasis (Halliez 2016). Reduced expression of claudin-1 was observed in duodenal biopsy specimens from patients with chronic giardiasis (Troeguer 2007). Infections of colonic cells with *Giardia* induced localized condensation of F-actin, loss of perijunctional α-actinin and increased cell permeability (Teoh 2000). Relocation of ZO-1 and claudin-1 from the cell–cell contact region to the cytoplasm were also observed during co-incubation of caco-2 cells with

trophozoites. Moreover, F-actin was retracted and concentrated near cellular contacts, resulting in microvillous atrophy (Maia-Brigagão 2012). The reorganization of cytoskeletal F-actin and ZO-1 in seems to be mediated by myosin light chain myosin phosphorylation (Scott 2002).

In Cryptosporidium spp. infection, dysfunction of the epithelial barrier has been documented in humans and animals. *Cryptosporidium parvum* infection of caco-2 monolayers increased transmonolayer permeability in a dose- and time-dependent way (Griffiths 1994). *In vitro Cryptosporidium andersoni*-infected cells reported disruption of ZO-1 (Buret 2003). Incubation of human epithelial HCT8 cells with *Cryptosporidium parvum* resulted in patchy disruption of the epithelium with foci of indistinct TJs and areas of loose paracellular spaces (Alcantara 2008). Furthermore, the attachment of *Cryptosporidium parvum* sporozoites to the apical membrane of epithelial cells induces reorganization of the host cell actin cytoskeleton, by altering the actin polymerization processes, in order to invade and form the parasitophorous membrane (Elliott 2001). More recently, epithelial cell monolayers infected with *Cryptosporidium parvum* exhibit a drop in transepithelial resistance associated with a delocalization of E-cadherin and β-catenin mediated by inflammatory response (de Sablet 2016). Tumor necrosis factor–alpha (TNFα) and interleukin (IL)-1β produced by inflammatory monocytes recruited in the subepithelial space in *Cryptosporidium parvum* infection were associated to increase on permeability (de Sablet 2016).

In Entamoeba histolytica infection, studies showed apical injury in regions of contact with epithelial cells, accompanying by a rapid decrease of transepithelial electrical resistance, suggesting a selective disturbance of TJs complexes (Li 1994). In human enteric T84 cells co-cultured with amoebae, decreased transepithelial electrical resistance was associated with degradation of in TJs proteins (ZO-1, ZO-2) (Leroy 2000). Besides *Entamoeba histolytica*-host cell contact damage, amoebic products have been shown to be crucial for cellular barrier dysfunction. For instance, prostaglandin E2 secreted by *Entamoeba* was shown to alter the spatial localization of claudin-4 that resulted in increased sodium ion permeability (Lejeune 2011). It is also proposed that barrier disruption is likely be mediated by specific interactions with *Entamoeba histolytica*-secreted cysteine protease (Betanzos 2013). When complexed

with *Entamoeba* adhesin, it interacts with TJs proteins occludin and claudin-1, followed by degradation and epithelial damage (Betanzos 2013). Proinflammatory cytokines TNF-α, and interferon gamma (IFN-γ) released in *Entamoeba* infection also promote barrier dysfunction by decreasing the expression of claudins 1, 5, and 7, as well as occludin (Kissoon 2013). In addition, *Entamoeba histolytica* expressed an "occludin-like" protein. Apical administration of this protein to human colonic epithelial cells resulted in epithelial disruption and decreased transepithelial electrical resistance, suggesting the involvement of this protein in the pathophysiology of amoebiasis (De Genova 2016).

The mechanisms by which STH infection alters barrier function are not well understood (Su 2011). The transcellular pathway seems to be a route by which helminth-derived antigen could enter the body (McKay 2017). Experimental studies showed that excretory/secretory products from the nematode *Trichuris suis* decreased the electrical impedance across monolayers of the cecal epithelial line and reduced the expression of claudin-4 and a claudin-like protein (McKay 2017). Mast cells have been implicated in the increased intestinal permeability in infections by *T. spiralis*, associated to a reduced expression of occludin protein (McKay 2017). Experimental models with *H. polygyrus* infection produced a significant increase in colonic epithelial associated with changes in the tight junctions of colonic epithelial cells and an alteration in the expression and distribution of the junctional protein E-cadherin (Su 2011). All of this experimental evidence suggests that helminths can increase gut permeability by abrading the epithelium directly, or throughout the helminth-derived products, which may affect the structure of the TJs, or throughout the immune activity (McKay 2017). This increase in epithelial permeability may facilitate the movement of lumenal contents across the mucosa, explaining how helminth infection can alter the immune response to enteric antigens (Su 2011)

1.3.4. *Inflammation*

The intestinal inflammatory response to intestinal parasites appears to be driven by the initial interaction of the organism with the epithelium and the ecological niche that the parasite occupies (Farthing 2003).

Giardia lamblia is considered non-invasive protozoa. Giardiasis occurs in the absence of invasion of the intestinal tissues by the trophozoite and in the absence of any overt inflammatory cell infiltration, with the exception of a modest increase in intraepithelial lymphocytes and mast cells (Buret 2015). The reasons for this attenuation of host intestinal inflammation remain unclear (Buret 2015). In calves, *Giardia* infection was characterized by down-regulation of several proinflammatory cytokines without cell recruitment (Dressen 2012). *Giardia* cathepsin B cysteine attenuates secretion of the potent neutrophil chemo attractant IL-8 (Cotton 2014). In *Giardia*-infected individuals histological analysis of small intestinal mucosal showed no signs of inflammation (Oberhuber 1997). These findings suggest that *Giardia lamblia* possess immunomodulatory factors avoiding strong inflammatory responses in infected individuals (Cotton 2014). In fact, Tanzanian children infected with *Giardia lamblia* showed lower levels of acute phase proteins, when compared to *Giardia*-negative children (Venemans 2011). On the other hand, there is evidence that *Giardia* may invade (Reynoso-Robles 2015) and induce inflammatory response (Cotton 2014). It was showed that trophozoite invades intestinal mucosa and submocosal in Gerbil models (Reynoso-Robles 2015). Trophozites were found between epithelail cells, at the base of empthy global cells, and whtin submocosal days after infection (Robles 2015). In a mouse model of *Giardia lamblia* infection, mucosal influx of bacteria coincided with increases in neutrophil infiltration even after parasite clearance, accompanying of elevated intestinal IFNγ, TNFα, and IL-1β levels (Chen 2013). In experimental model in rats, significant increase of inflammatory cells (neutrophils, eosinophils, monocytes) were observed in whole small intestine several days after infection with *Giardia lamblia* (Abd-Al-Zahra 2012). *Giardia* Assemblage B infection in gerbil model elicited more extensive damage to mucosal architecture, with infiltration of inflammatory cells (Benere 2011). In humans, microscopic duodenal inflammation has been reported in some adults with either acute (Hanevik 2014) or chronic (Troeguer 2007) *Giardia* infection. In children, duodenal biopsies revealed increased eosinophilic infiltration of the lamina propria associated to *Giardia* infection (Koot 2009). This dichotomy of pro-inflammatory and immunomodulatory response can explain the variability in clinical outcome and severity of infection. It will depend on host factors (*e.g.*, immune status,

nutritional status and age), as well as differences in virulence and pathogenicity of *Giardia* strains (Bartelt 2015).

Cryptosporidium invades and resides in epithelial cells, most commonly in the small intestine, but does not invade deeper mucosal layers. In this regard, *Cryptosporidium*, it can be viewed as a 'minimally invasive' mucosal pathogen (Laurent 1999). Intestinal *Cryptosporidium parvum* infection is often associated with infiltration of inflammatory cells in epithelium and underling lamina propria (Lacroix-Lamandé 2002). Human colon epithelial cell lines infected with *Cryptosporidium parvum* showed upregulation of expression and secretion of chemokines (CCL-5, CXCL-8 and CXCL-10, IL-8) after infection (Laurent 1997, Borad 2010). *Cryptosporidium parvum*–infected polarized intestinal epithelial cells and/or human intestinal xenografts also stimulate intestinal epithelial cell secretion of proinflammatory cytokines, including TNF- α and IL-8 (Lauren 1997). These cytokines are considered to be important initiators of polymorphonuclear cell recruitment to the intestinal mucosa and contributing to increased intestinal permeability and subsequent tissue damage (Laurent 1999, Lacroix 2002). In children, mucosal inflammation, as measured by the proinflammatory cytokines TNF-α and IL-8, was increased among younger children with cryptosporidiosis, compared with healthy control subjects (Kirkpatrick 2006). These increased systemic and intestinal cytokines levels persisted at 6 months after enrollment (Kirkpatrick 2006). In earlier studies of symptomatic Haitian and Brazilian children, levels of fecal TNF-α and IL-8 levels were also elevated in cryptosporidiosis (Alcantara 2003, Kirkpatrick 2002). In Brazil, fecal lactoferrin was positive in 75% of children with cryptosporidiosis suggesting that young children in the developing world respond to *Cryptosporidium parvum* infection with intestinal inflammation (Alcantara 2003). Interestingly, *Cryptosporidium* infection also triggers expression of anti-inflammatory cytokines (Kirkpatrick 2002). Haitian infants with acute cryptosporidiosis mounted a proinflammatory, Th2 response, accompanying of counterregulatory mucosal intestinal response with higher fecal IL10 levels (Kirkpatrick 2002). IL-10 plays a central role in the immune balance between pathology and protection, through its immunoregulatory and immunosuppressive functions (Kirkpatrick 2002). The production of IL-10 can be

considered an attempt to restrain the immune system against over exuberant inflammatory responses and has been shown to have an important role in regulation of mucosal immunity, minimizing damage from inflammatory response (Kirkpatrick 2002).

In Entamoeba histolytica infection, the host inflammatory response contributes to tissue damage (Moonah 2013). In the early stages of invasion, *E. histolytica* is mainly surrounded by neutrophils. If properly activated, these immune cells are successful in eliminating amebas through oxidative and non-oxidative mechanisms (Campos-Rodríguez 2016). Moreover, epithelial infected cells produce proinflammatory cytokines (IL-8, IL-la, IL-1b, and IL-6), which contribute to the recruitment of more neutrophils and macrophages to the site of amoebic invasion (Seydel 1997). While TNF-α stimulates neutrophils and macrophages to release reactive oxygen species and nitric oxide to fight the parasite, an excess amount of TNF-α can result in direct damage to host tissue (Moonah 2013). Together, these data suggest that the neutrophil can be protective but can also play a detrimental role in the disease process, by perpetrating further mucosal damage (Mortimer 2010). Inflammatory acute reaction in *Entamoeba* infection is followed by an anti-inflammatory response by expression of monocyte locomotion inhibitory factor (Utrera- Barillas 2003). In addition, proteolysis of chemokines and IL-18 by *Entamoeba histolytica* cysteine protease has been reported to reduce the migration of monocytes and eosinophils (Garcia-Zepeda 2007). Studies aforementioned provide evidence that *Giardia lamblia, Cryptosporidium* spp. and *Entamoeba histolytica* can lead to intestinal epithelial damage in different ways. Since host-microbe interaction should be based in host-damage, the quantification of this damage contributes to the analysis of host-parasite interactions (Casadevall 2000). Specifically, our study addressed the measurement of fecal biomarkers of intestinal permeability and inflammatory response associated to enteric parasitic infections.

In STH infections are clasically associated with a immunomodulatory mechanisms to ensure their persistence within the host (Maizels 2016). Typically induce Th2 immune response that includes the production of cytokines (IL-4, IL-5, IL-10, and IL-13) that is linked to an anti-inflammatory phenotype (Maizels 2016). Experimental animal models, showed that *Ancylostoma caninum* secretes neutrophil inhibitory factor, which

blocks adhesion of activated human neutrophils to vascular endothelial cells as well as the release of hydrogen peroxide from activated neutrophils (Bethony 2006). Specific secreted molecules in *T. suis* can induce the anti-inflammatory response such as the macrophage migration inhibitory factor (Bethony 2006). In contrast, the inflamamatoy effects of STH are less explored response (Cooper 2009). In murine models using *Heligmosomoides polygyrus*, an early and pronounced infiltration of neutrophils and macrophages in regions immediately adjacent to the parasite has been described (Marimoto 2004). *Ascaris suum*-derived products can induce human neutrophil activation via a G protein-coupled receptor that interacts with the interleukin-8 receptor pathway (Falcone 2001).

1.4. Clinical picture

Giardia lamblia

Giardiasis can present as asymptomatic infection or acute diarrhea, which is often self-limiting; or chronic diarrhea (Farting 1997). In LMIC, the first *Giardia lamblia* infections may result in diarrhea but immunity is rapidly acquired, conferring protection against symptomatic disease when subsequently exposed (Muhsen 2012). Common symptoms of giardiasis include nausea, vomit, diarrhea with foul-smelling stools that are greasy, frothy, or bulky, abdominal cramps and bloating, and decreased appetite (Adam 2002). Diarrhea in giardiasis is mostly malabsorptive in nature, often with steatorrhea, and malabsorption of other nutrients such as vitamin A and vitamin B, and lactase deficiency (Farthing 1997, Roberston 2010). Chronic giardiasis could be associated with an exaggerated immune response leading to persistence of inflammatory changes in intestinal mucosa (Troeguer 2007, Hanevik 2007).

Giardia strains have been suggested to be important determinants for the severity of infection and symptoms (Bartelt 2015). In Rwandan children, infection with assemblage A showed more abdominal pain and vomiting, while assemblage B infections were associated with underweight and severe malnutrition (Ignatius 2012). In Bangladeshi children, higher odds ratios for diarrhea were observed for assemblage A and A2 infections, whereas higher parasite loads were observed for assemblage B infections (Haque 2005). High endemicity of assemblage B infection might thereby provide some genotype-limited protection against assemblage B–associated diarrhea (Haque 2005). In Brazilian children, there was not significant difference in diarrhoeal symptoms experienced during assemblage A, B or mixed infections although children with assemblage B showed greater *Giardia lamblia* cyst shedding than children infected with assemblage A (Kolhi 2008). This higher rate of cyst shedding in children with assemblage B may promote its spread, accounting for its increased incidence (Kolhi 2008).

In endemic areas, reinfection is common. In Peru, a hyperendemic country for *Giardia lamblia,* 98% of the children treated for *Giardia lamblia* with tinidazole became re-infected within 6 months, and after reinfection, stool excretion of the parasite lasted 3 months (Gilman 1988). Also in Peru, reinfection has been described in 87% of infants

(Hollm-Delgado 2008). Reinfections could be explained by an incomplete acquired immunity against *Giardia lamblia* either due to insufficient immune defences or antigenic variation of the parasite (Kolhi 2008). However, re-infection is less severe in second and third infections, reflected by decreased in fecal lactoferrin, suggesting some protection against severity (Kolhi 2008, Goto 2009).

Co-infection with multiple species of parasite or pathogens is likely to be the rule rather than the exception in most biological systems (Blackwell 2013). In a community-based study, simultaneous infection with rotavirus and *Giardia lamblia* resulted in a greater risk of having diarrhea (Bhavnani 2012). The heightened pathogenicity of *Giardia lamblia* in the presence of rotavirus may be related to a more successful attachment of the trophozoite ventral disk to the infected epithelium (Bhavnani 2012). Association of *Helicobacter pylori* and giardiasis has also been described in children with recurrent abdominal pain (Ankarkle 2012). Hypochlorhydria, common in malnourished child, increased the risk for both giardiasis and *Helicobacter pylori* infection (Sullivan 1991). Few studies have examined interactions between *Giardia* and STH. In a longitudinal study, *Giardia* infection was less likely in STH-infected individuals (hazard ratio (HR) 0.46), and vice-versa, infection with STH was less likely for *Giardia*-infected individuals (HR 0.71), suggesting an antagonistic relationship between STH and *Giardia lamblia* (Blackwell 2013).

Long-term and extra-intestinal consequences in giardiasis include post-infectious irritable bowel syndrome, chronic fatigue syndrome, ocular pathologies, polyarthritis, allergies, and hypokalemic myopathy (Halliez 2013). In children, stunting (Farthing 1986, Newman 2001, Goto 2008) and cognitive impairment are long-term paramount consequences (Berkman 2002), which will be discussed in next sections. To summarize, *Giardia* has distinct population-dependent epidemiological pattern (Barlet 2016). The parasite can cause malabsorptive diarrhea as well as chronic intestinal sequelae when exposure is infrequent, but can be asymptomatic when exposure is frequent (Barlet 2016). The mechanisms driving these protean clinical outcomes remain elusive, but recent advances suggest that variability in *Giardia* strains, host nutritional status, composition of microbiota, co-infecting enteropathogens, host

genetically determined mucosal immune responses, and immune modulation by *Giardia* are relevant factors influencing disease manifestations after *Giardia* infection (Buret 2015).

Cryptosporidium spp.

Cryptosporidium spp. is recognized as a leading cause of endemic childhood diarrhea particularly during the first 24 months of age, with a negative impact of on linear growth, and increased risk for severe or fatal outcomes, independently of HIV status (Kotloff 2013, Platt-Mills 2015, Sow 2016). The protozoan has three main epidemiological scenarios: sporadic, often water-related, self-limiting diarrhea in otherwise healthy persons; chronic, life-threatening illness in immunocompromised patients; and diarrhea and malnutrition in young children in developing countries (Mor 2008). In developing countries, children become infected at early ages (Kattula 2017, Korpe 2016). In infants, cryptosporidiosis was characterized by profuse, watery diarrhea associated with abdominal pain and, in some cases, fever malaise, nausea, vomiting, and loss of appetite (Newman 1999, Gatei 2006). In Kenya, cryptosporidiosis was associated with abdominal pain (51.1%), vomiting (51.6%), and abdominal swelling (11%)(Gatei 2006). A longitudinal study in infants from slum dwellers in southern India, found that 71.4% of episodes were associated with diarrhea and the remaining were asymptomatic; 40% of children had multiple episodes of cryptosporidiosis, and 50% were shedding oocysts well before or after episodes of cryptosporidial diarrhea, with important implications for transmission of disease (Ajjampur 2010). In these studies mentioned, cryptosporidiosis was significantly associated with persistent diarrhea (Newman 1999, Gatei 2006). Nevertheless, recent studies provide evidence that asymptomatic cryptosporidial infection is quite common. In a birth cohort in Bangladesh, infants experienced infection with *Cryptosporidium* spp. showed non-diarrheal infection in 67%, more common than diarrheal infection (6.3%)(Korpe 2016). Also in a birth cohort in Indian children, *Cryptosporidium* infection were mainly asymptomatic (66%) (Kattula2016). In this cohort, the proportion of reinfected children was high (81%) but multiple reinfections conferred some protection against subsequent infection (Kattula 2016). It was suggested that that parasite genetics might play an important role in the clinical manifestations of human cryptosporidiosis (Cama 2008). *Cryptosporidium hominis* is far more common

specie in developing countries (Gatei 2006, Ajjampur 2007, Bushen 2007, Korpe 2016, Saw 2016). This specie seems be more pathogenic and might induce more severe diarrhea, heavier infections and more damage to intestinal barrier than *C. parvum* and in consequence greater growth shortfalls, even in the absence of symptoms (Bushen 2007). Among copathogens detected in *Cryptosporidium* infection, *Escherichia coli, Giardia lamblia*, and STH were commonly identified (Newman1999, Gatei 2006).

While cryptosporidiosis is most commonly disease of the small intestine in immunocompetent individuals, extraintestinal gastric, hepatobiliary, pancreatic and pulmonary infections are non infrequent in immunodeficient individuals (Leitch 2012). In a recent Ugandan study in human HIV-seronegative children presenting with diarrhea, 35.4% of the children who had stool samples positive for *Cryptosporidium* also had positive sputum samples (Mor 2008). More importantly, *Cryptosporidium* plays a causal role in childhood malnutrition in developing countries (Checkley 1998, Mølbak 1993) and has been linked to impaired physical fitness in late childhood (Guerrant 1999) that will be discussed in the next section.

Entamoeba hystolitica

In *Entamoeba histolytica* infection most of individuals are asymptomatic (90%) while the remaining develops clinically disease (Samie 2012). Asymptomatic patients are potential reservoirs for dissemination by shedding of millions of cysts (Samie 2012). Some patients develop amebic colitis, acute dysentery, or chronic diarrhea, and occasionally life-threatening amebic liver or brain abscess (Samie 2012). Patients with amebic colitis typically present with a several-week history of cramping abdominal pain, weight loss, and watery or bloody diarrhea (Haque 2003). Symptomatic infants showed more severe symptoms such as high-grade fever, vomiting, severe abdominal pain, electrolyte disturbance, and dehydration (Hegazi 2012). In Bangladeshi infants, abdominal pain, mild-to-moderate dehydration and fever were frequent symptoms in *Entamoeba histolytica*-associated diarrhea, being more common in malnourished children (Haque 2003). Dysentery was demonstrated in 12 to 25% of cases of *Entamoeba histolytica*-associated diarrhea (Haque 2003). Asymptomatic *Entamoeba histolytica* infection was related to anti-lectin IgG status of the children (Haque 2003).

Intestinal dysbiosis may affect the phenotype of *Entamoeba histolytica* infection, since symptomatic amebiasis was associated with the presence of *Prevotella corpi* in children (Gilchrist 2016).

Host genetic factors contribute to the outcome of infection with the parasite (Moonah 2013). The Human leukocyte antigen (HLA) class II allele DQB1*0601 and the haplotype DQB1*0601/DRB1*1501 were associated with decreased rates of *Entamoeba histolytica* infection in Bangladeshi children (Duggal 2004), while polymorphism (Q223R) in the leptin receptor was associated with increased susceptibility to *Entamoeba histolytica* infection (Duggal 2011). The possibility of genetically distinct strains of *Entamoeba histolytica*, genetic predisposition (human leukocyte antigen alleles) and underlying immunologic mechanism might be responsible factors for more prevalent and more severe outcomes of *Entamoeba* infection in infants from Bangladesh (Petri 2009, Haque 2006, Hegazi 2012). Infant undernutrition and stunting also can predispose young children to amebiasis, which in turn exacerbates preexisting nutritional imbalance by altering gut function, with a negative impact on growth (Mondal 2006, 2012). Cognitive effects of *Entamoeba histolytica* infection are less clear (Tarlenton 2008). These aspects will be discussed in the next section.

STH

The clinical features of STH can be classified into the acute manifestations associated with larval migration through the skin and viscera, and the acute and chronic manifestations resulting from parasitism of the gastrointestinal tract by adult worms (Bethony 2006). STH of moderate and high intensity produce clinical manifestations, most commoly in children (Bethony 2006). *Ascaris* larval antigens can cause an intense inflammatory response with eosinophilic infiltrates resulting in verminous pneumonia (Bethony 2006). In the small intestine adult ascaris can cause abdominal distension and pain, lactose intolerance and malabsorption of vitamin A and other nutrients, and can aggregate in the ileum causing partial obstruction (Bethony 2006). Adult worms can also enter and block the ampullary orifice of the common bile duct, leading to biliary colic, cholecystitis, cholangitis, pancreatitis and hepatic abscess, mainly in adults (Bethony 2006). *T. trichiura* lives preferentially in the caecum,

although in heavy infections, whipworms can be seen throughout the colon and rectum. The adult parasite has the anterior end embedded in epithelial tunnels within the intestinal mucosa and the posterior end located in the lumen causing inflammation at the site of attachment which results in colitis (Bethny 2006). Colitis symptoms resemble those of inflammatory bowel disease, with chronic abdominal pain and diarrhoea, anaemia of chronic disease, and finger clubbing. Trichuris dysentery syndrome is a serious manifestation of heavy whipworm infection, resulting in chronic dysentery and rectal prolapse (Bethony 2006). The major hookworm-related injury in children occurs when the adult parasites cause intestinal blood loss (Hotez 2004). The term "hookworm disease" refers primarily to the iron-deficiency anemia that results from moderate or heavy infection. Blood loss occurs when the worms use their cutting apparatus to attach themselves to the intestinal mucosa and submucosa and contract their muscular esophagi to create negative pressure, which sucks a plug of tissue into their buccal capsules (Hotez 2004). Thus, the clinical manifestations of hookworm disease are the consequences of chronic intestinal blood loss. Iron-deficiency anemia occurs and hypoalbuminemia develops when blood loss exceeds the intake and reserves of host iron and protein (Hotez 2004). For instance, in Zanzibar, children who were infected only with *N. americanus* showed hypoferritinemia in 33.1%, whereas in children who were also infected with *A. duodenale* hookworms, the prevalence was 58.9 % (Stoltzfus 1997).

To summarize, enteric parasitic infections are diseases of poverty continuing to impose a substantial burden on children (Evans 1994, Harnay 2010).

In the next sections, the interaction of enteric parasites, mainly enteric protozoa and intestinal barrier will be addressed as etiopathogenic outcome and further, the interaction of enteric parasites and nutritional status and neurodevelopment as clinical outcomes.

1.5. Enteric protozoa infection and intestinal barrier

1.5.1. *Intestinal barrier*

In humans, the intestinal barrier is the largest barrier tissue with a surface of $300m^2$ and it is also the most extensive interface between host and environment (Anderson 2012). The complexity of the intestinal barrier develops over time from early gestation through to childhood (Ning-Shi 2005). During intrauterine development, villus and microvillus formation results in a 10^5-fold increase in the intestinal surface area, the intestine elongates 1000-fold, to reach a mean length at birth of 275 cm and continues to lengthen until 3 to 4 years of age (Ning-Shi 2005, Anderson 2012). The physical barrier begins developing from conception; its basic structure is formed by the end of the first trimester, and by week 22 of gestation the absorptive epithelial cells resemble those of the adult intestine (Anderson 2012). All major components of the intestinal immune apparatus are identifiable by week 29 of gestation (Ning-Shi 2005, Anderson 2012). The intestinal permeability is increased in the immature intestine but "gut closure" occurs early during the first postnatal week (van Elburg 2003). In the postnatal period, the intestinal mucosal barrier continues to grow in a process that involves fission and deepening of crypts, increase in villus width and number, associated with profound tissue remodelling and modification of intestinal digestive and absorptive functions (Ning-Shi 2005, Anderson 2012). The mucosal barrier function may not be fully established until after 2 years of age (Brandtzaeg 2016).

The structure of intestinal barrier is made up of a layer of columnar epithelial cells that forms the first line of defence between the intestinal lumen and inner milieu. Of these cells, greater than 80% are enterocytes with absorptive and immune function (Snoeck 2005). After intestinal epithelial cells undergo mitotic cell division in the crypt (pluripotent stem cells), they migrate up the villus, where they undergo differentiation and become actively absorbing cells, which are sloughed from the villus tip into the intestinal lumen. The epithelium surface is continually renewed with a turnover time of 96 hours in infants (Snoeck 2005).

The terms "intestinal barrier" and "intestinal permeability" describe two different aspects of the same anatomical structure, the intestinal wall (Bischoff 2014). Intestinal

barrier is a functional entity separating the gut lumen from the inner host, and consisting of multi-layered system, hence it emphasizes the protective component of the gut against invasion of microorganisms and their toxins (Bischoff 2014). Intestinal permeability is a functional feature of the intestinal barrier, measurable by analyzing flux rates across the intestinal wall as a whole or across wall components (Bischoff 2014).

The intestinal epithelium mediates selective permeability via two major routes: transepithelial/transcellular and paracellular pathways (Groschwitz 2009). Transcellular pathway formed predominantly by special transporters or channels located on the apical and basolateral membranes, is involved in the absorption and transport of nutrients (Mayer 2015). Paracellular permeability is associated with transport in the space between epithelial cells, and is regulated by junctional complexes localized at the apical-lateral membrane junction and along the lateral membrane (Groschwitz 2009). The junctional complexes consist of the TJs, adherens junctions, desmosomes, and gap junctions (Figure 2) (Ulluwishewa 2011).

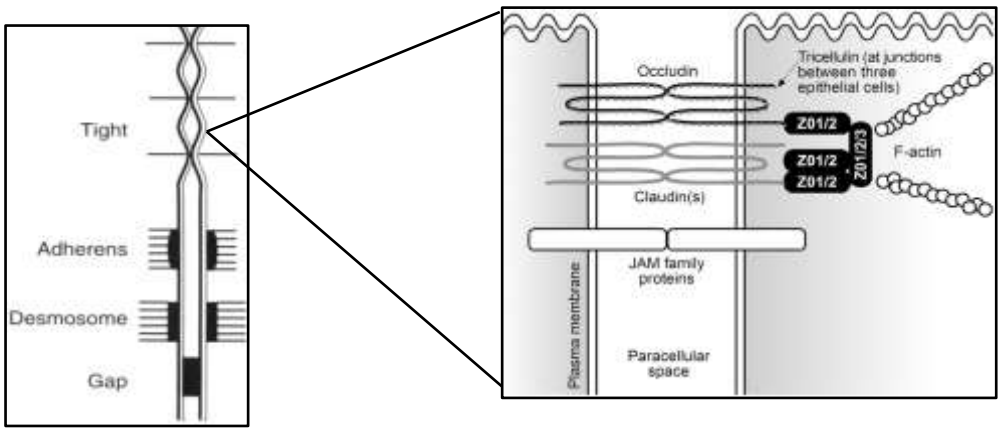


Figure 2. Tight junction complex (adapted from Ulluwishewa, 2011).

TJs are a dynamic and multi-protein complexes that function as a selective /semipermeable paracellular barrier, which facilitates the passage of ions and solutes through the paracellular space, while preventing the translocation of luminal antigens, microorganisms and their toxins (Hartsock 2008). TJs are composed of integral

membrane proteins (claudins, occludins, tricellulin and junctional adhesion molecule-JAMs) that directly regulate the gate function; cytoplasmic plaque zonuline proteins (ZO-1, ZO-2, and ZO-3), that link tight junctions to the actin-cytoskeleton; and by cytoskeletal proteins (Figure 2) (Hartsock 2008). The TJs are the responsible for sealing the intercellular space and regulating selective paracellular ionic solute transport (Groschwitz 2009).

Adherens junctions consist of transmembrane proteins (cadherin-catenin interactions) that link adjacent cells to the actin cytoskeleton via cytoplasmic scaffolding proteins (Groschwitz 2009). Desmosomes and gap junctions are involved in cell-cell adhesion and intracellular communication, respectively (Ulluwishewa 2011). The cytoskeleton is an intricate structure of protein filaments (actin) that extends throughout the cytosol that is essential for maintaining the structure of all eukaryotic cells. Disruption of the cytoskeleton is linked to the loss of intestinal barrier integrity (Ulluwishewa 2011).

1.5.2. *Mucosal inflammatory response*

Gastrointestinal tract is constantly in contact with commensal and pathogenic microbes thereby, it has to develop a defense system that will prevent microorganisms from establishing an infection, yet maintaining an interface between the body and necessary nutrients (Gill 2011). The first level of defense that pathogens encounter are microbiota and mucus layer. The microbiota has the ability to prevent enteric pathogens from establishing an infection by consumption of intestinal nutrients and occupation of attachment sites (Gill 2011). Mucus layer is a mucus coat lining the intestine, composed of a solution of glycoproteins (mucin) secreted by goblet cells and prevents potential pathogens and antigens from gaining access to the underlying epithelium (Mayer 2003). Enterocytes also play a role in immunity since they constitutively express major histocompatibility complex (MHC) class II molecules in order to stimulate CD4+ T cells in the context of mucosal inflammation and express pathogen-recognition receptors that enable them to act as dynamic sensors of the microbial environment and as active mucosal immune cell responses (Hershberg 2000). Paneth cells, localized on the base of crypts, secrete α-defensins, lysozyme and phospholipase A2 when they are exposed to pathogens (Gill 2011). Initiation of the innate immune response is triggered by recognition of pathogen associated molecular

patterns by pathogen-recognition receptors. The most studied are the toll-like receptors (TLR) (Santaolalla 2011). TLR are typically expressed in the intestinal epithelial cells as well as in other immunocompetent cells in the lamina propria, and they activate the innate immune response characterized by NF-κB activation, cytokine production, and chemokine-medicated recruitment of acute inflammatory cells (Santaolalla 2011).
Underneath the epithelium, there is the lamina propria where the immunocompetent cells are found. Neutrophils and macrophages have a key role in orchestrating the kinetics and magnitude of the inflammatory response (Blikslager 2007). Many of these early-phase reactive cells are essential for the protective response as well as account for much of the epithelial damage, particularly caused by the neutrophil (Kasper 2001). Neutrophils migrate in response to microbial chemoattractant peptides or by signals generated by epithelial cells invaded by pathogens (Blikslager 2007). This transepithelial migration of neutrophils is the key of inflammatory diseases of the gut (Kruger 2015). Neutrophils can damage intestinal tissue in several ways: reactive oxygen species, antibacterial proteins packaged into different granule subsets, secretion of inflammatory chemokines and cytokines to attract activated inflammatory cells, and neutrophil extracellular traps (NET) that bind the microorganism ensuring high local concentration of antimicrobials agents (Kruger 2015). Since the presence of neutrophil is a hallmark of local inflammation, the role of neutrophil against enteric protozoa infection is particularly addressed in our study.

1.5.3. *Assessment of intestinal barrier function*

Qualitative and quantitative measures of intestinal barrier disruption are necessary to determine the severity and magnitude of host response to enteric infection. Simple noninvasive markers are available to assess intestinal permeability and inflammation (Table 4).

Assessment of intestinal permeability

Intestinal permeability, expressed in cm s^{-1}, is an intrinsic property of the intestine and is defined as the facility with which intestinal epithelium allows molecules to pass through by non-mediated passive diffusion (Ménard 2010). Intestinal permeability and integrity can be measured in many ways. The techniques used for permeability and

integrity assessments vary depending on the setting (*in vitro* or *in vivo* measurements), the species (human or animal models), the molecules used for assessment (ions, carbohydrates of different sizes, macromolecules and antigens, bacterial products and bacteria), and the compartments used for measurement of the marker molecules (blood, urine, stools) (Bischoff 2014). All these tests have in common that defined molecules of different molecular weight are used for their capacity to enter and cross the epithelium or the mucosal layer, respectively and finally entering the submucosal site or the blood (Bischoff 2014) (Table 4). The permeability tests with clinical applicability in children are described below.

- Lactulose: mannitol test

The lactulose to mannitol ratio (L:M) has been the most commonly used marker of assesses gut leakiness, and also measures absorption in children (Denno 2014). This non-invasive test involves oral administration of a dose of both sugars (*i.e.*, lactulose and mannitol) followed by a timed urine collection (Denno 2014). Lactulose is a large sugar that is minimally absorbed from an intact small intestine. If permeability is altered, this disaccharide traverses paracellular spaces, is then cleared by glomerular filtration without renal tubular reabsorption, and is easily measured in urine (Denno 2014). The co-administered sugar alcohol, mannitol, is absorbed via transcellular pathways, proportional to small bowel absorptive capacity (*i.e.*, surface area)(Denno 2014). The L:M test has many attributes as a measure of gut dysfunction, including its safety, and correlation with gut pathophysiology (Denno 2014). Some disadvantages in children are mentioned. First, very small molecules (182-342 Da) such as mannitol and lactulose do not necessarily reflect structural damage of TJs (Vojdani 2013). Second, due to a repair mechanism, small openings in TJs can be repaired within hours, giving more false negative results (Ulluwishewa 2011, Vojdani 2013). Third, because gastric emptying may affect absorption kinetics, standard and adequate fasting should be employed which may be difficult to implement in infants who cannot easily tolerate fasts (Denno 2014). Fourth, five-hour bagged urine collections are considered standard, but are a considerable inconvenience in field studies involving infants. Finally, values may change with physiologic maturation, and reference values with age stratification or adjustment are poorly defined for children (Denno 2014).

Table 4. Assessment of intestinal barrier function

Measure	Assay	Description	Reference
		Intestinal permeability	
Permeability	α -1- antitrypsin*	Is used as a marker of exudation of serum protein from intestinal mucosa.	McCormick 2016
Permeability and absorptive capacity	Lactulose-mannitol Xylose Rahmose	Lactulose is a large sugar that is not normally absorbed and mannitol is a smaller sugar that is absorbed in proportion to absorptive surface. Urinary mannitol gives an index of absorptive capacity, while the presence of lactulose in the urine indicates impaired barrier function.	Denno 2014
Permeability	Endotoxin Endotoxin core- antibodies Soluble CD14	Reflecting translocation of bacteria due to mucosal barrier leakage.	Prendergast 2014
Epithelial cell integrity	Zonuline	Zonula occludens toxin (Zot), produced by Vibrio cholerae, is a recently identified molecule that opens tight junctions in the intestine.	Mondal 2012
Epithelial cell integrity	Citrulline	Amino acid produced by enterocytes. Proposed as measure of gross enterocyte mass.	Papadia 2010
Epithelial cell integrity	Intestinal fatty-acid binding protein (I-FABP)	I-FABP is expressed in epithelial cells of small intestine. In mucosal damage, I-FABP is released into the circulation and its plasma concentration increases.	Prendergast 2014

Table 4. Assessment of intestinal barrier function *(continued)*

Measure	Assay	Description	Reference
		Intestinal inflammation	
Neutrophil activity	S100A12*	Marker of neutrophil activation, elevated in IBD and bacterial enteritis, but not viral enteritis.	Day 2013
Neutrophil activity	Fecal Lactoferrin	Iron-binding glycoprotein found in granules of leukocytes. Used as a surrogate for fecal leukocytes and a measure of intestinal inflammation.	Guerrant 1992
Neutrophil activity	Calprotectin	This protein accounts for 60% of neutrophilic cytosol. It has been used for diagnosis of inflammatory bowel disease	Däbritz 2014
Neutrophil activity	Myeloperoxidase	Lysosomal protein in neutrophilic granulocytes. Marker of environmental enteropathy	McCormick 2016
Th1 response	Neopterin	Protein produced by monocytes and macrophages after stimulation with IFN-γ. Marker of environment enteropathy.	McCormick 2016

* Test used in our study

- Translocation of bacteria and their products

Breakdown of the mucosal barrier potentially leads to translocation of microbiota or their toxic products. Two plasma markers, reflecting translocation of bacteria or their products, are D-lactate and endotoxin lipopolysaccharide (LPS) (Derikx 2010). LPS in particular is the major constituent of the outer membrane of gram-negative bacteria Increased circulating LPS levels have been related to an impaired mucosal barrier. The presence of LPS can be measured either directly in blood or indirectly using LPS antibodies as measurement of LPS leakage into the circulation (Derikx 2010, Bischoff 2014). Because the endotoxin antibodies persist in the blood for much longer than the endotoxin itself, their measurement allows a better estimate of the overall level of endotoxin exposure than measurement of the antigen itself (Campbell 2004). However, in a pilot study this test failed to display acceptable lot-to-lot reproducibility in higher upon scale-up in the MAL-ED study (Kosek 2013). Other plasma bacterial DNA and/or plasma soluble CD14 could provide alternative markers for microbial translocation as a consequence of increased gut permeability (Guerrant 2013).

- Fecal alpha-1 antitrypsin

A1AT is a 52-kDa glycoprotein synthesized mostly in the liver and to a lesser extent by macrophages and neutrophils (Lisowska-Myjak 2005). A1AT is also expressed in human jejunal and ileal enterocytes (Molmenti 1993). It is an acute phase plasma protein, which its primary function is the inhibition of neutrophil elastase (Lisowska-Myjak 2005). It is present in normal serum at a concentration of 1.9-5.0 g/l (Crossley 1977). A1AT comprises approximately 4% of the total serum protein content and has a molecular weight of approximately 50,000 Da, similar to that of albumin; consequently, fecal A1AT excretion should parallel enteric loss of albumin (Thomas 1981). When A1AT is leaked into the intraluminal space, is excreted into the stool and is not degraded by intestinal proteases as the albumin does (Florent 1981). The value of fecal A1AT in the assessment of protein-loosing enteropathy is well established (Thomas 1981, Florent 1981). As A1AT is neither degraded by intestinal proteases nor reabsorbed, it a notably stable compound in stool samples (Magazzù 1985). The measurement of fecal A1AT in a random fecal sample provides a reliable index of excessive protein loss in the gastrointestinal tract (Crossley 1977, Thomas 1981).

Fecal losses of A1AT can be expressed in concentration of milligrams per gram (dry weight) or excretion rate (mg/day) or A1AT clearance (ml/day). Fecal A1AT has been examined in inflammatory bowel diseases (Meyers 1985) and pediatric patients with acute gastroenteritis syndrome (Amemoto 1996). Few studies reported fecal A1AT measurements in parasitic infection. Correlation between hypoalbuminemia and increased fecal A1AT excretion in *Strongyloides stercoralis* infection (Sullivan 1992) or *Giardia* infection (Korman 1990) was described. One disadvantage is the presence of A1AT in human milk. A1AT concentrations are high in colostrum (0.3 g/L) but decrease (0.1g/L) in mature milk at 30 days (McCormick 2017).

Assessment of intestinal inflammatory response

Accumulation of neutrophils is a hallmark of acute intestinal inflammatory response (Blikslager 2007). Several markers of neutrophil activity have been for diagnosis of intestinal inflammation (Derikx 2010, Kruger 2015) (Table 4). The inflammatory biomarkers with clinical applicability in children are described below.

- Fecal lactoferrin

Lactoferrin is a multifunctional iron binding glycoprotein that is found in the secretions of most mucosal surfaces including tears, saliva, human breast milk, synovial fluid and serum (Däbritz 2014). Lactoferrin is a major component of secondary granules released during the degranulation of neutrophils in response to inflammation (Däbritz 2014). This biomarker has disadvantages in children. The diagnostic accuracy of fecal lactoferrin in the differentiation of inflammatory bowel diseases *versus* inflammatory bowel syndrome is low, showed sensitivity and specificity between 56%-100% and 61%-100%, respectively (Wang 2015). Furthermore, it may be inaccurate to determine intestinal inflammation in infants, since human milk contains high concentrations of lactoferrin (280 mg/dl) in the second year postpartum (Perrin 2017). Fecal lactoferrin levels were lower in children who were malnourished and had diarrhea compared with those without malnutrition thereby, fecal lactoferrin may be less sensitive for diagnosis intestinal inflammation in malnourished children (Opitan 2010).

- Fecal calprotectin

Calprotectin, also named calgranulin A, is member of the S100 calcium-binding protein family. This protein is linked to the innate immune system and expressed in granulocytes, monocytes/macrophages and epithelial cells (Däbritz 2014). Fecal calprotectin correlates well with endoscopic and histological grade of mucosal inflammation (Canani 2008) and is a stable protein in stool samples for up to seven days at room temperature (van Rheenen 2010). Nevertheless, this biomarker has several disadvantages in pediatric age. A meta-analysis showed a pooled sensibility of 0.92 and low specificity of 0.76 in studies in children and teenagers (van Rheenen 2010). Median fecal calprotectin levels in healthy children aged 1-18 months were 174.3 μg/g and exhibited a downward trend with increasing age. In children aged 6-18 months fecal calprotectin concentrations were still higher than those of children aged more than 4 years (Li 2014). Furthermore, fecal calprotectin concentration in breast fed infants was higher (377μg/g) than that in non-breastfed ones (233μg/g) (p=0.001) (Li 2014).

- Fecal myeloperoxidase

Myeloperoxidase (MPO) is a major component of the primary granules especially in neutrophils, but it is found at lower concentrations in monocytes and macrophages (Saiki 1998). Fecal MPO has a good correlation with laboratory and endoscopic parameters of inflammation (Saiki 1998) and is stable for at least four days in feces at room temperature (Däbritz 2014). Recently, MAL-ED study showed high average concentrations (4.815 ng/mL) in infants from developing countries in relation to published references (2.000 ng/mL) for healthy individuals in high-income country (McCormick 2017, Kosek 2017). The trend observed for MPO changed over the first 24 months of life with higher values in the first year of life, declining thereafter (McCormick 2017). One limitation of this fecal biomarker in infants is the increased levels in breastfed infants (McCormick 2017). Moreover, significant gender differences were found in intracellular MPO content, being lower in boys from birth to adulthood (Nikulskin 2015).

- Fecal neopterin

Neopterin (NEO), an indicator of T-helper cell 1 activity, is used as a biomarker of intestinal inflammation (Campbell 2004, Naylor 2015, McCormick 2017). Fecal neopterin concentrations correlated closer with endoscopic scores, as alternative marker to predict and monitor the severity of mucosal damages in patients with inflammatory bowel disease (Nancey 2013). Its fecal levels were found to be much higher (26 times) in infants from developing countries than from developed countries (Kosek 2013, McCormick 2017). In MAL-ED study, mean concentration of fecal neopterin was much higher 1372 nmol/L than values reported in the literature (70 nmol/L) (McCormick 2017). Enterohepatic circulation of neopterin and its elevated concentrations in the bile seem to be the source of a large amount of neopterin in feces (Kosek 2013). Despite intestinal inflammation significantly increases neopterin levels, it is unlikely to be the sole cause of such elevated levels (Kosek 2013). Similarly to MPO and A1AT, a trend for neopterin change over the first 24 months of life with higher values in the first year of life, declining thereafter was found (McCormick, 2017). In infants from developing countries, high neopterin, combined with high MPO and A1AT, was associated with poorest growth (Kosek 2013, Guerrant 2016).

- Fecal S100A12

S100A12, also named calgranulin C, is a calcium-binding proinflammatory protein predominantly secreted by granulocytes (Meijer 2012). Gene expression of S100A12 in humans is almost completely restricted to neutrophil granulocytes (Meijer 2012). Fecal S100A12 has strong correlation with histologically and endoscopically confirmed intestinal inflammation (Foell 2003, Kaiser 2007). This protein is evenly distributed throughout feces and is stable at a wide range of temperatures (4ºC to 20ºC) for several days (de Jong 2006). Median S100A12 levels in the healthy children were 0.5 mg/kg (ranging from 0.39 to 10), without gender variation (Day 2013). Fecal S100A12 levels in healthy infants are lower than the standard cut-off and elevated fecal levels are likely to represent organic gut disease (Day 2013). Fecal S100A12 have been used in children for diagnosis of inflammatory bowel disease (Sidler 2008, de Jong 2006, Däbritz 2014). The sensitivity and specificity of fecal S100A12 (cut-off 10 mg/kg) for the detection of inflammatory bowel disease were both 97% (Sidler

2008). Several commercial ELISA kits are available for fecal S100A12 analysis requiring a small stool sample (approximately 100 mg).
In our study fecal A1AT was chosen as a biomarker of intestinal permeability and S100A12 as a biomarker of local intestinal inflammation.

1.5.4. *Enteric protozoa infection and intestinal barrier*

Giardia lamblia, Cryptosporidium spp. and *Entamoeba histolytica* can lead to intestinal epithelial damage in different ways. Mechanisms developed by enteric parasites to disrupt the intestinal barrier and breaches the lines of defense were already outlined in the previous section. Since most evidence came from *in vivo* or *in vitro* experimental models, these studies did not accurately reflect the intricate *in vivo* dynamic of interaction host-pathogen. Evidence from clinical studies of host-pathogen interaction focusing on intestinal permeability and inflammation is addressed in this section.

Enteric protozoa infection and intestinal permeability

Measures of intestinal permeability provide an important metric of the impact of an enteric infection. Several studies in infants from developing countries have assessed the intestinal permeability in the context of enteric infection (Lunn 1999, Zhan 2000, Goto 2002, Campbell 2004, Goto 2009, McCormick 2016) (Table 5).

In *Giardia lamblia* infection, longitudinal studies have investigated the potential association between intestinal permeability, mostly by using lactulose: mannitol (L:M) test and *Giardia* infection. Three found association (Lunn 1999, Goto 2002, McCormick 2017) and three found no (Campbell 2004, Goto 2009) or limited (Northrop-Clewes 2001) association (Table 5). Gambian infants, raised *Giardia*-IgM titers were associated with elevated L:M values (Lunn 1999). Similarly, in Nepalese children, mean L:M values in *Giardia*-infected infants was higher than in those non-infected (0.43 vs. 0.25) (Goto, 2002). Bangladeshi and Gambian infants reported higher L:M values associated with chronic malnutrition, but no correlation was found with *Giardia* infection (Campbell 2004, Goto 2009). More recently, in the multisite MAL-ED study, the presence of *Giardia* was associated with an elevated L:M values across all sites. Fecal A1AT despite showed positive association with exposure to

enteropathogens did not show association with *Giardia* (Rogawski 2017, McCormick 2017). These results confirmed that increased intestinal permeability, as a component of impaired gut function, is present in giardiasis in infants from developing countries (Rogawski 2017).

In *Cryptosporidium* spp. infection, increased intestinal permeability was also reported (Zhan 2000) (Table 5). Infants with *Cryptosporidium parvum*-associated diarrhea showed higher L:M excretion ratios during the acute phase (0.76) in comparison with controls (0.26). However, in the convalescent phase (day 20) those values decreased to normal excretion ratio (Zhan 2000). These results indicate that increased intestinal permeability in *Cryptosporidium* infection is a significant but reversible phenomenon (Zhan 2000).

In *Entamoeba histolytica* infection no studies were found that link infection with intestinal permeability. However, in Bangladesh, increased levels of serum antibodies against bacterial endotoxin (endocab antibodies) in children with diarrhea and stunting were found (Mandel 2012). Since *Entamoeba* is a common cause of diarrhea in this setting, it could suggest some association with intestinal barrier dysfunction (Mandel 2012)

Few studies have addressed intestinal permeability in STH infections. In Bangladeshi children infected with *A. lumbricoides* had poorer permeability values using L:M than did those without *Ascaris* infection, but the difference was not significant. The permeability values for children with and without *Trichuris* and hookworm infections were very similar (Northrop-Clewes 2001). Although no changes in intestinal permeability or plasma albumin were observed after deworming, significant decreases in total protein and alpha 1-antichymotrypsin were observed in the treatment group, indicating reductions in inflammation after deworming (Northrop-Clewes 2001).

Table 5. Clinical studies of association between enteric protozoa infection and intestinal permeability in infants from developing countries

Country	N (age)	Test	Results	Reference
			Giardia lamblia	
Gambia	60 2- 8 months	Lactulose mannitol	High Giardia-IgM titers associated with elevated Lactulose: mannitol ratio (r=.25 p <0.0001)	Lunn, 1999
Nepal	201 0-60 months	Lactulose mannitol	Giardia has higher Lactulose: mannitol ratio 0·43 vs. non-Giardia 0·25 respectively (p< 0.01)	Goto 2002
Gambia	73 2-15 months	Lactulose mannitol	Lactulose: mannitol in the Gambian children (0.31) vs. UK children (0.1) .Not association with *Giardia*	Campbell 2004
Bangladesh	298 3-15 months	Lactulose mannitol	Higher lactulose: mannitol ratio was associated with chronic malnutrition but not associated with Giardia.	Goto 2009
Multisite MAL-ED	2.076 0-24 months	Lactulose mannitol	*Giardia* was associated with an increase in lactulose: mannitol ratio z-score 0.22 (0.12 to 0.32)	Rogawski 2017
			***Cryptosporidium* spp.**	
Peru	30 0- 36 months	Lactulose mannitol	Higher Lactulose: mannitol ratio in acute Cryptosporidiosis (0.67) in comparison with convalescent phase (0.19)	Zhang 2000

Enteric protozoa infection and intestinal inflammatory response

Several studies reported high levels of inflammatory biomarkers associated to enteric parasite infections in infants from developing countries (Kirkpatrick 2006, 2002, Campbell 2004, Kolhi 2008) (Table 6).

In *Giardia lamblia* clinical studies addressed to evaluate inflammatory response showed contradictories results. By using different biomarkers, some authors found association (Lunn 1999, Newman 2001, Kolhi 2008) while other did not (Campbell 2004, McCormick 2017)(Table 6). Longitudinal studies in Gambian infants showed giardia-IgM antibody titers positively associated with plasma concentrations of α-1antichymotrypsin (Lunn 1999). In contrast, recent evidence from MAL-ED study, *Giardia lamblia* showed no association with fecal inflammatory markers (MPO, A1AT and NEO) or systemic inflammatory markers (α-1-acid glycoprotein) (McCormick 2017, Kosek 2017). These results confirm that *Giardia* might disrupt epithelial cells through mechanisms independent of those of chronic immune activation (Bartelt 2016, Rogawski 2017).

In *Cryptosporidiosis* the outcome and severity of infection is critically dependent on the immune status of the host. However, the nature of the immune response in particularly in infants is poorly understood (Borad 2010). Three cohort studies in infants from Brazil and Haiti support association of *Cryptosporidium* and inflammatory markers (Newman 1999, Kirkpatrick 2002, 2006) (Table 6). Significant association was observed between positive lactoferrin test and symptomatic *Cryptosporidium parvum* versus asymptomatic cases (p= 0.004) (Newman 1999, Bushen 2006). In malnourished infants, both systemic and stool proinflammatory cytokines were significantly elevated in acute cryptosporidiosis, even persistent 6 months after infection (Kirkpatrick 2002, 2006). However, in the same cohort (Kirkpatrick 2002) the contra- regulatory IL-10 was elevated in the infected children. That suggest that infants with acute cryptosporidiosis mount both inflammatory, and counterregulatory intestinal immune responses (Kirkpatrick 2002). It may prevent excessive, potentially host-threatening immune responses during the course of infection (Pantenburg 2008).

Only one study had addressed the study of inflammatory response in *Entamoeba* infection in infants (Table 6). In a cohort of Bangladeshi infants, high TNF-α levels were associated to higher risk of *E. histolytica* diarrhea (HR= 1.18), and children with *E. histolytica* diarrhea had higher TNF-α levels than those with asymptomatic infection (p =0.027) and those with no infection (p =0.017) (Peterson 2010). Those findings suggest a role of inflammatory response, namely TNF-α, in the susceptibility to and pathogenesis of *E. histolytica* diarrhea (Peterson 2010).

Very few studies have explored the effects of STH infections on mucosal inflammatory response in infants (Cooper 2009). In Zanzibari infants, early STH infections showed a regulatory Th2 pattern of peripheral cytokine responses to *Ascaris* and hookworm antigens, without association with acute phase proteins (Wright 2009), Simmilarly, it shool children from Cuba and Cambodia, STH infections were not associated with either local intestinal (calprotectin) or systemic inflammation (PCR) (de Gier 2018). In fact, a trend towards an inverse association between elevated CRP and STH infections was observed (de Gier 2018).

To summarize, enteric parasites are pathogens with the ability to disrupt intestinal absorptive and/or barrier function, with or without overt diarrhea (Petri 2008). The epithelial damage may be reflected by an increase intestinal permeability and/or mucosal inflammation (Berkes 2003, De Genova 2016). The assessment of these phenomena is important for the understanding of host-pathogen interaction, particularly in the two first formative years, when any alteration of nutrient absorption is critical for growth and development (Guerrant 2008). Our study the aspects of host-protozoa interaction was recently published by Garzón *et al.* (2017).

Table 6. Clinical studies of association between enteric protozoa infection and intestinal inflammatory response in infants from developing countries.

Country	N	Test	Result	Reference
			Giardia lamblia	
Gambia	60 2 - 8 months	Plasma α-1 antichymotrypsin	*Giardia*-IgM antibody titers were positively associated with α-1 antichymotrypsin.	Lunn 1999
Brazil	189 0- 5 years	Fecal lactoferrin	Faecal lactoferrin was positive in asymptomatic and symptomatic Giardiasis	Newman 2001
Gambia	73 2 – 15 months	Fecal Neopterin	Neopterin in children with *Giardia* was 20.2μmol/l vs.14.8μmol/l in those non-infected ($p > 0.05$).	Campbell 2004
Brazil	189 0- 3 years	Fecal lactoferrin	Lactoferrin was similar between assemblages. Higher lactoferrin in the first infection.	Kolhi 2008
Bangladesh	298 2-15 months	Plasma α-1 acid glycoprotein	Higher a-1-acid glycoprotein and higher L:M ratios were both associated with chronic malnutrition but not with *Giardia*	Goto 2008
Multisite MAL-ED	2076 0-24 months	Myeloperoxidase Neopterin Plasma α1- acid-glycoprotein	*Giardia* was not associated with inflammatory markers such a as myeloperoxidase, A1AT, neopterin and systemic α-1 acid-glycoprotein.	McCormick 2017 Rogawski 2017

Table 6. Clinical studies of association between enteric protozoa infection and intestinal inflammatory response in infants from developing countries *(continued)*

Country	N	Test	Result	Reference
			***Cryptosporidium* spp.**	
Brazil	189 0- 5 years	Fecal Lactoferrin	Fecal lactoferrin was detected only in symptomatic *Cryptosporidium* infection.	Newman 1999
Haiti	50 0-18 months	Fecal lactoferrin Stool cytokines	Stool IL8 and TNFα and lactoferrin were elevated in Cryptosporidium cases.	Kirkpatrick 2002
Brazil	18 3-43 months	Fecal lactoferrin Stool cytokines	IL8 were detectable only in few children with diarrhea, and TNFα was not elevated in cryptosporidial infection.	Alcantara 2003
Haiti	50 0-18 months	Blood Cytokines	Increased levels of IL8 and TNFα in cryptosporidiosis that persisted at 6 months after enrollment.	Kirkpatrick 2006
Brazil	157 2-46 months	Fecal lactoferrin	Symptomatic *C. parvum*-infected children have lactoferrin-positive than asymptomatic (p= 0.004).	Bushen 2007
			Entamoeba histolytica	
Bangladesh	138 0-5 years	TNFα	Children who develop *Entamoeba histolytica* diarrhea had higher TNF-α than those asymptomatic (p= 0.027).	Peterson 2010

IL interleukin, TNFα tumor necrosis alpha.

1.6. Enteric protozoa infection and nutritional status

1.6.1. *Infant growth*

The first 1.000 days of life - the time spanning roughly between conception and the end of second birthday - is a unique period of opportunity when the foundations of optimum health, growth, and neurodevelopment across the lifespan are established (Cusick 2016). The growth velocity in height over this period is faster than at any other time, including adolescence. Early growth plays a vital role in setting the trajectory of growth in childhood and adolescence and stature in adult life (Matorell 2017). In addition, the most rapid period of brain growth and highest plasticity occurs from the last trimester of gestation through the first two postnatal years (Grantham-McGregor 2007).

In LMIC it is widely assumed that growth faltering often begins in utero and continues for at least the first 2 years of post-natal life (Figure 3) (Victora 2010). Those analyses confirm that the worldwide timing of growth faltering is concentrated within the first 1000 days, considered a "window of vulnerability", but also a window of opportunity for prevention and intervention (Victora 2010).

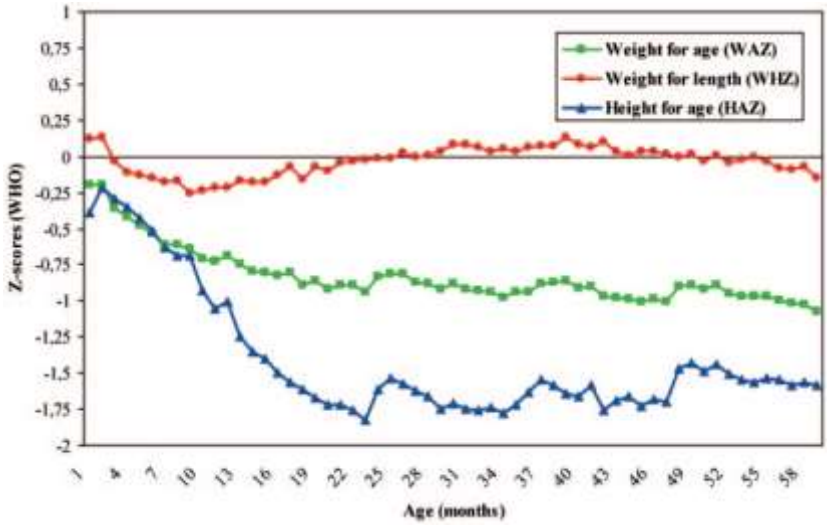


Figure 3. Trends of anthropometric *z-scores* according to age relative to WHO standards (source: Victora 2010).

Consequences of early growth failure

In LMIC, poor fetal growth or stunting in the first 2 years of life leads to irreversible damage, including shorter adult height, lower attained schooling, reduced adult income, and decreased off spring birthweight (Victora 2008)

- Early growth failure leads to short adult stature unless there is compensatory growth (catch-up) in childhood, which is partly dependent on the extent of maturational delay (Victora 2008). Because maturational delay in LIMC is usually shorter, only a small part of the growth failure is compensated for (Victora 2008). Data from five cohort studies (Brazil, Guatemala, India, the Philippines, and South Africa) concluded that Height-for-age *z-score* (HAZ) at 2 years was strongly correlated to adult height (Victora 2008, Adair 2013). Each 1 *z-score* of HAZ at 2 years equals 3.1 cm for boys and 3.2 cm for girls in adult height (Victora 2008). In these populations, projected adult heights were estimated by doubling the children's length at 2 years of age (Garza 2013). Birth weight was also found to predict adult height, for each 1 standard deviation (SD) of birth weight predicted 1.5 cm higher adult height (Adair 2013).

The genetic regulation of growth is well documented (Lettre 2009). Around 200 genes are associated with stature; nevertheless, those genes collectively explain only 10% of observed variability in populations (Lettre 2009). This relatively low proportion of variability explained by that large number of genes reflects the influences of disease, adverse environments, and/or inadequate nutrition and care on stature, rather than genetics or parental experiences (Garza 2013). This finding is consistent with the secular trend of height increase in all societies where child undernutrition was reduced and environment conditions were improved (Cole 2000).

- Early growth failure can predict later cognition, school progress, or both (Grantham-McGregor 2007). Longitudinal data from several countries (Philippines, Jamaica, Peru, Indonesia, Brazil and South Africa) assessed the size of the deficit in later function associated with a loss of 1 SD in height in early childhood (Grantham-McGregor 2007). These studies showed that stunting between 12 and 36 months of age was associated with a negative effect size varying from 0.4 to 1.05 SD in the scores for cognition (Grantham-McGregor 2007). A reanalysis of the five-birth cohorts from LMIC confirmed that 1 SD higher birth weight predicted 0.2 years more

schooling and 1 SD higher height at 2 years predicted 0.5 years more schooling (Adair 2013).

- Early growth failure can predict adult diseases. Although birth weight had little relation with adult diseases, faster weight gain at mid-childhood was strongly associated with fat mass and increased risk of elevated blood pressure and dysglycemia in adulthood (Adair 2013). These results indicate that chronic diseases associated with high glucose concentrations, high blood pressure, and harmful lipid profiles are especially common in undernourished children who experienced rapid weight gain after infancy (Victora 2008, Adair 2013). The not well defined 'thrift' genes have a role in promoting fat storage to protect against starvation and signalling pathways responsible for catch-up growth after resolution of infections, and might increase an individual risk of obesity and associated comorbidities (Guerrant 2013).

- Early growth failure is also associated with lower birth weight in the next generations, as maternal birthweight is a strong predictor of offspring birth weight (Ramakrishnan 1999). Since maternal stature is a composite indicator representing genetic and environmental effects on the growing period of childhood, undernourished girls tend to become short adults (Ramakrishnan 1999, Victora 2008). Meta-analysis of data from 12 population-based cohort studies and the WHO Global Survey on Maternal and Perinatal Health from LMIC found that maternal stunting (height <145 cm) are risk factors for offspring small-for-gestational age (SGA)(adjusted risk ratios 2.03, 95% CI: 1.76, 2.35)(Kosuki 2015) as described previously (Christian 2013). In turn, these SGA infants have higher risk for neonatal (relative risk (RR) 1.83) and post-neonatal (RR 1.90) mortalities (Katz 2013) as well as increase risk of growth faltering in the first 2 years of life (Christian 2013). Furthermore, the risk for childhood stunting was higher (0.682) in infants born to shortest mothers than to tallest ones (0.194) (Ozaltin 2010). The damage suffered in early life leads to permanent impairment, and may also affect future generations (Victora 2008).

Thereby, height at 2 years of age is a good predictor of human development, since early growth failure is associated with less schooling, shorter adult height, less productivity, and lower offspring birth weight, affecting future generations (Victora 2008).

1.6.2. *Assessment of nutritional status/anthropometry*

Anthropometry is a simple, noninvasive, inexpensive and convenient method for monitoring individual physiological process and for population surveillance, with implications in the planning of health policies (Liu 2012).

Growth is a form of motion that can be measured by the distance achieved in a certain time, and by the rate of growth expressed in units *per* time period (Tanner 1952) (Figure 4).

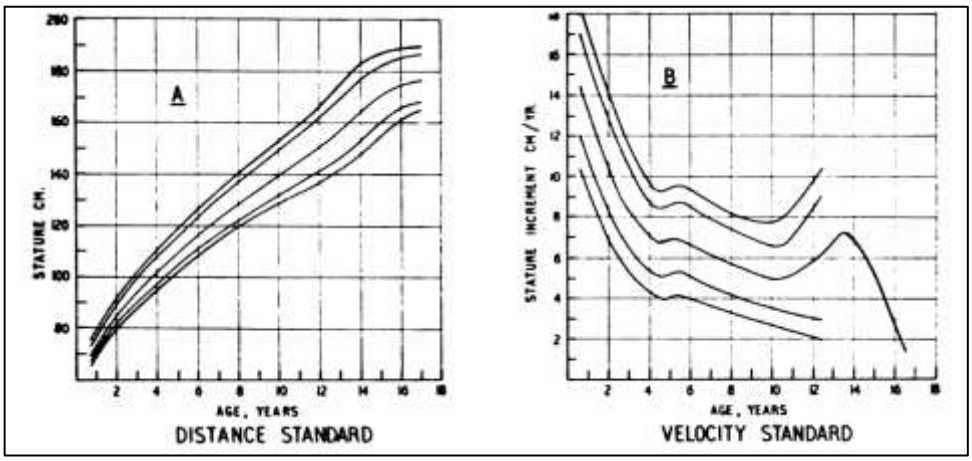


Figure 4. Growth curves of attained growth and growth velocity (source: Tanner 1951)

Growth monitoring is the process of following the growth of a child in comparison to a standard by periodic, frequent anthropometric measurements, useful to assess early growth faltering (Griffiths 2007).

Assessment of growth has been controversial for many decades. Since early papers of Dr. Tanner in 1951 multiple approaches have been proposed to find a "gold standard" to monitor growth, that is, "percentiles *vs.* standard deviations", "attained *vs.* velocity", or a combination of both (Tanner 1952). In 2006, WHO published the first multicenter WHO Child Growth Standards, conducted in Brazil, Ghana, India, Norway, Oman, and the USA, between 1997 and 2003 (WHO 2006a). The Multicenter Growth Reference Study (MGRS) combined a longitudinal follow-up from birth to 24 months and a cross-sectional survey of children aged 18 to 71 months. The MGRS was purposely designed to produce a standard by selecting healthy children living under conditions favoring the achievement of their full genetic growth

potential. Only children that were single term birth, on exclusive or predominant breastfeeding for at least 4 months, have initiated complementary feeding around 6 months of age, and without significant morbidity were included (de Onis 2004). Differences in linear growth among the child populations of the MGRS show similarity among sites, reflected by about 3% of variability (de Onis 2004).

- Attained growth

WHO Child Growth Standards include two anthropometric measures: attained growth (WHO 2006a) and growth velocity (WHO 2009a). Attained growth is a static approach that measures the distance from the median of individual's anthropometric measurement at particular time, reflecting the cumulative growth (Schwinger 2017). The MGRS provide attained growth charts that are sex specific and expressed both in percentiles and *z-scores*, including for length/height-for-age (LAZ/HAZ), weight-for-age (WAZ), weight-for-length/height (WLZ/WHZ), and body mass index (BMIZ)-for-age, from 0 to 60 months (WHO 2009a).

- Height-for-age difference

Height-for-age difference is an alternative or complementary measure for attained growth. The *z-scores* of anthropometric measures have been used for comparisons of children of different age and sex across populations, involving an assessment at one point in time (Leroy 2014, 2015). However, for HAZ calculation, the standard deviation (SD) of the growth standard is used as denominator. As the SD increases with age, the usefulness of HAZ to assess changes in height over time or across ages may be uncertain (Leroy 2014, 2015). For this reason, changes in mean growth deficits over time, as the child ages, should be assessed using the absolute height-for-age difference (HAD) which calculation (observed height *minus* median height growth standard) does not depend on the SD, contrarily to HAZ (Leroy 2014, 2015). Expressing growth faltering in absolute terms (HAD) provides an alternative, complementary approach to the relative measures (HAZ), and both are worth presenting (Lundeen 2014). The HAD was found particularly useful to refine the assessment of growth faltering in children from LMIC (Leroy 2014, 2015).

- Growth velocity

Growth velocity is a dynamic measure of changes of anthropometric measurements at

a period of time (Tanner 1952). While attained growth is a cumulative measure of an altered growth rate, velocity has the advantage of reflecting current, not past, events (Schwinger 2017). Thereby, velocity measures have more sensitivity to capture influencing factors and greater potential to predict short-term consequences (Griffiths 2007, Schwinger 2017). The MGRS growth velocity standards were developed for weight, length and head circumference (HC) (WHO 2009a). The intrinsic biological complexity of human growth dynamics makes the use and interpretation of velocity standards more challenging than that of the attained growth standards since growth velocities are highly variable in consecutive growth intervals (WHO 2009a).

- Undernutrition

Undernutrition encompasses underweight, stunting, wasting, and deficiencies of micronutrients (Black 2008). Waterlow in 1974 proposed a functional classification for child malnutrition in acute and chronic malnutrition. The acutely malnourished children were those with inadequate weight-for-height or wasted, and the chronically malnourished children those that had inadequate height-for-age or stunted (Waterlow1974).

Undernutrition is well recognized as a public health problem with short-term and more often long-term consequences (Black 2008, Victora 2008). Child mortality is the main short-term consequence of undernutrition either directly or indirectly in consequence of weakened defenses against infectious diseases (Black 2008). In 2011, it was estimated that undernutrition in an aggregate including fetal growth restriction, stunting, wasting, deficiencies of vitamin A and zinc, and suboptimum breastfeeding, caused 45% of all child deaths under-5 (Black 2013). Particularly, high disease burden was attributable to undernutrition in the first 2 years of life (Black 2013). Globally, in 2015, 159 million (24%) of children under-5 were stunted, around 100 million (16%) were underweighted, and 49.8 million (8%) were wasted (Black 2013, UNICEF 2016a). More than one third of all stunted children under-5 and more than one quarter of all wasted children under-5 lived in Africa (UNICEF 2016a). In fact, the number of stunted children in Africa rose from 50.4 million in 2000 to 58.6 million in 2015 (UNICEF 2016a).

Underweight defined as ≤-2 SD of WAZ, is the indicator of child undernutrition

officially used to monitor progress towards achieving one of the targets of Millennium Development Goals. However, WAZ may reflect low height-for-age, low weight-for-height, or a combination of both. Therefore, this indicator has the disadvantage of not distinguishing between these forms of undernutrition (Lutter 2011).

Wasting is defined as ≤-2 SD of WLZ/WHZ. Wasting appears to be short-term and reversible in nature (Richard 2012). However, repeated bouts of wasting may preclude long-term linear catch-up growth (Richard 2012). Serial episodes of decreased weight-for-height are thought to limit the catch-up, contributing to linear growth restriction (Walker 1996, Richard 2012). In fact, data from four longitudinal studies confirmed that linear growth in infants is partly regulated by early body mass or fatness (Dewey 2005). Ponderal and linear growth faltering are both rooted in poverty, and are the result from a mixture of environmental, infectious exposures, dietary, cultural, and socioeconomic factors (Richard 2012). Both decreased weight-for-height and height-for-age are important risk factors for illness and death during childhood (Richard 2012).

Stunting, defined as ≤-2 SD of LAZ/HAZ, is a measure of the cumulative effects of undernutrition during the critical 1.000 first days of life (de Onis 2016). Stunting begins *in utero* and continues for at least the first 2 years of post-natal life as mentioned before (Victora 2010). Stunting is the most common form of undernutrition globally, considered as a marker of healthy growth given it association with risk of short-term morbidity and mortality, non-communicable diseases in later life and learning capacity and productivity (Black 2008, Stewart 2013, Prendergast 2014). Hence, height is considered the best overall indicator of well-being and human capital (de Onis 2016). In some LMIC the monitoring of growth is based only on weight, because length measurement is time-consuming and not an easy procedure for local health workers (de Onis 2016). Thereby, the stature, used to define stunting, is still lacking in individual growth records.

The WHO Global Database on child growth and undernutrition uses the cut-offs ≤ -2 SD to define moderate-to-severe undernutrition and >-2 SD ≤ -1 to define mild undernutrition (UNICEF 2012a). Since undernutrition has a potentiating effect on mortality in a population, rather than an additive effect (Pelletier 1995), all degrees of undernutrition are risks for mortality, which increases as *z-scores* decrease (Black

2013). The risk of undesirable health outcomes (including mortality) does not change dramatically by simply crossing the cut-off line, and the hazardous effects of nutrition deficits happen along a continuum of mild, moderate, and severe undernutrition (Stevens 2012). Mild-to-moderate cases serve as a large "reservoir" from which severe cases are derived and thereby subjected to an even higher risk of death (Pelletier 1994). From a public health perspective, mild-to-moderate undernutrition is yet neglected. A high proportion of nutrition-related deaths is still missed by policies and programs focusing exclusively on the severely malnourished children (Pelletier 1994, Stevens 2012). By using narrow or finer categories provides a better understanding of the dose response relationship between suboptimal growth and adverse outcomes (Olofin 2013).

To capture an unapparent negative impact of pediatric subclinical conditions on nutrition status, a comprehensive approach combining several anthropometric measurements, including attained measurements (*z-scores* and length-for-age difference-LAD), and velocity measurements may be needed. Mild-to-moderate degrees of undernutrition should be also part of the complete analysis. This approach may be useful to assess an unapparent undernutrition associated to subclinical enteric infections in infants.

1.6.3. *Enteric protozoa infection and nutritional status*

The conceptual model of the “vicious cycle of poverty” links enteric infections to gut dysfunction, impaired nutrient absorption, malnutrition and restricted physical and cognitive development (UNICEF 1990a, Guerrant 2008). Despite nutrient deprivation theoretically result in poor childhood growth, enteric infections are also likely to contribute to growth fathering (Guerrant 2013). In general, the nature of interaction between undernutrition and infection has been recognized be synergistic and bi-directional (Scrimshaw 1968).

Undernutrition can worsen enteric infections since *per se* it can disrupt the intestinal epithelium, with atrophy of brush border, altered apical tight junctions, villus blunting, crypt derangement, and altered gut immune function (Guerrant 2008). Clinically, undernutrition leads to increased frequency and duration of infectious diarrheal

episodes, accounting for a doubling of the diarrhea burden in malnourished children (Guerrant 1992, Black 1984). Possible explanations are the poor immune response, delayed recovery of the intestinal mucosa, and deficiency of micronutrients (zinc and vitamin A) (Guerrant 2000). On the other hand, numerous reports documented the clear impact of repeated infectious diarrheal episodes on children's growth (Mata 1970, Rowland 1977, Martorell 1975, Guerrant 1983). Higher cumulative burden of infectious diarrhea adversely affects the nutritional status during early childhood, increasing the risk of stunting (Checkley 2008, Black 2008, Petri 2008). It was found that adjusted odds of stunting increased by 1.13 for every five episodes (95% CI 1.07–1.19), in other words, the proportion of stunting attributed to ≥5 diarrheal episodes before 24 months is around 25% (Checkley 2008). Enteric infections cause mucosal damage altering intestinal absorptive function that is critical in malnourished children (Guerrant 2008). Intestinal barrier function is dependent on the constant intestinal epithelial cell turnover and the balance between cell proliferation, differentiation, and cell death, processes that can be affected by depletion of nutrients and microelements such as glutamine, arginine, retinol, carotenoids, vitamin A and zinc (Guerrant 2008). Moreover, this absorptive intestinal disruption may occur not only in diarrhea but also in asymptomatic enteric infections (Guerrant 2008, Petri 2008).

Infections by *Giardia lamblia*, *Cryptosporidium* and *Entamoeba histolytica* are among the enteric parasitic infections increasingly recognized as predisposing to growth impairment, as shown in Table 7.

Table 7. Longitudinal studies of association between enteric protozoa infection and poor nutritional status in infants from developing countries

Country	N (age)	Results	Reference
		Giardia lamblia	
Guatemala	45 Birth up to 3y	Weight velocity was lower at 2 y in *Giardia*-infected. Diarrhea-associated *Giardia* infection was associated to reduction in height velocity.	Farthing 1986
Gambia	60 2 to 8 m	Infant growth was not related to *Giardia*-infection or the time of first exposure to the parasite.	Lunn 1999
Brazil	157 Birth up to 4 y	Children with symptomatic infections had lower WAZ and HAZ than asymptomatic children.	Newman 2001
Gambia	72 2-15 m	There was no difference in growth between children with or without *Giardia* infection.	Campbell 2004
Brazil	597 6-45 m	Less gain in HAZ in infected than uninfected children, even if in asymptomatic infection.	Prado 2005
Peru	220 Birth up to 2 y	Giardiasis did not affect growth at 1 or 2 months following the first infection.	Delgado 2008
Bangladesh	222 3-15 m	GS-IgM was associated with poor WAZ and WHZ (p=0.015 and p=0.039 respectively).	Goto 2009
Bangladesh	445 Birth up to 2 y	Giardia positive first 6 months of life decreased LAZ at 2 years of age (p= .05)	Donowitz 2016
Multisite MAL-ED	2089 Birth up to 2y	Persistence *Giardia* before 6 months was associated with a -0.29 deficit in WAZ and -0.29 of HAZ at 2 years	Rogawski 2017

Table 7. Longitudinal studies of association between enteric protozoa infection and poor nutritional status in infants from developing countries *(continued)*

Country	N (age)	Results	Author
		Cryptosporidium spp.	
Guiné Bissau	1064 0-3 year	Cryptosporidiosis was accompanied by weight loss (3.7% in boys and 2.9% in girls) at 2 y of age. A similar effect in linear growth was shown (p= 0.02).	Molback 1997
Peru	185 Birth up to 2 years	Children infected with *C. parvum* experienced growth faltering, both in weight and in height, for several months after the onset of infection, followed by a period of catch-up growth.	Checkley 1998
Brazil	157 Birth up to 4 year	HAZ decreased after infection for *C. hominis or C. parvum*. Children with *C. hominis* infection continued to decline HAZ score, even asymptomatic infections.	Bushen 2007
India,	20 Birth up to 2 year	Children with multiple infections had significantly lower WAZ and HAZ at 24 months.	Ajjampur 2010
Bangladesh	147 Birth up to 1year	Children stunted at birth had more *Cryptosporidium* diarrhea during their first year of life.	Modal 2012
Bangladesh,	392 Birth up to 2 year	Children with *Cryptosporidium* spp. infection had a greater than 2-fold increased risk of severe stunting at 2 y compared to uninfected children (OR 2.69).	Korpe 2016
		Entamoeba histolytica	
Bangladesh	221 2-5 year	Children with E. histolytica-associated diarrheal illness were 2.93 times (p = 0.047) more likely to be malnourished and 4.69 times (p = 0.006) more prone to be stunted.	Mondal 2006
Bangladesh	147 Birth cohort up to 12months	Malnutrition at birth is a risk factor for Entamoeba diarrhea infection in the first year of life	Mondal 2012

Giardia lamblia infections are syndemic with undernutrition, diarrhea, and growth delay (Barlet 2013). In malnourished mice, *Giardia* infection is associated with further decreased of growth and impairment of immune mucosal responses (Barlet 2013). This finding confirms that *Giardia* is associated with growth failure, an outcome that is influenced by host nutritional status (Barlet 2013). Despite this evidence from experimental models, longitudinal studies in children showed contradictories results (Table 7). Some of them confirm the association between *Giardia* infection and growth failure (Newman 2001, Goto 2009, Donowitz 2016) while others did not (Lunn 1999, Campbell 2004, Hollm-Delgado 2008). Mata *et al*. (1978) showed that the duration of *Giardia* episodes appeared to be the most important factor associated with growth failure (Farting 1986). To date, two longitudinal studies, an independent cohort study conducted in Bangladesh (Donowitz 2016) and the multisite MAL-ED study (Rogawski 2017) provide the best evidence of the impact of *Giardia lamblia* on infant growth. In MAL-ED study infants with high exposure to *Giardia* had adjusted LAZ and WAZ decrements at 2 years of age (Rogawski 2017). Both studies confirmed that even in the absence of diarrheal symptoms, *Giardia* infection, especially early persistent infection, was associated with reduced weight and height attained at 2 years of age (Donowitz 2016, Rogawski 2017).

Cryptosporidiosis in early childhood can lead to undernutrition, which put them at higher risk for recurrent diarrheal diseases (Checkley 1997, Agnew 1998, Guerrant 1999). In mice, cryptosporidial infections cause an approximate 40% decrement in weight gain, but when infection and undernutrition coexist the addition of undernutrition increases the effect of deepening mucosal crypts, more severe mucosal damage, and oocysts shedding (Costa 2011). Hence, cryptosporidial infections not only cause undernutrition, but also undernutrition worsens cryptosporidial infections (Costa 2011). Association between cryptosporidiosis and growth failure have been confirmed by several longitudinal studies (Table 4). One of the first studies conducted in Peru reported that infants with *Cryptosporidium parvum* infection experienced growth faltering, both in weight and in height, for several months after the onset of infection (Checkley 1998). A birth cohort in Bangladesh found that infants with *Cryptosporidium* spp. infection had greater than 2-fold increased risk of severe

stunting at 2 years of age (Korpe 2016). *Cryptosporidium* infection at any point in the first two years of life, whether diarrheal or non-diarrheal, may result in impaired growth at age two (Korpe 2016).

In *Entamoeba histolytica* infection, undernutrition is hypothesized to be one of the host factors increasing the risk of infection by this protozoon (Petri 2009). Two studies in Bangladesh addressed the association between *Entamoeba* infection and infant growth (Table 7). Mondal *et al.* (2006) found that preschoolers with *Entamoeba histolytica*-associated diarrhea were 2.93 times more likely to be underweighted and 4.69 times to be stunted (Mondal 2006). Undernourished children experienced more *Entamoeba histolytica*-associated diarrheal episodes than those well nourished (Mondal 2009). In a birth cohort study Mondal *et al.* (2012) reported that infants who were underweighted and stunted at birth had more risk of *Entamoeba histolytica* colonization during their first year of life. The increased intensity of infection with *Entamoeba histolytica* in undernourished children can be explained by the role of leptin in mucosal immunity against amebiasis and by the relatively poor ability to produce IFN-γ in response to amebic antigen (Petri 2009). Undernutrition predisposes young children to amebiasis, which in turn exacerbates preexisting nutritional imbalance by altering gut function, with a negative impact on growth (Mondal 2006, 2012).

Few studies have evaluated the impact of STH infection on infant growth (Moore 2001, Gyorkos 2011, LeBeaud 2015). In Peru, reduced LAZ was observed in children with moderate to heavy helminth infections than those non-infected or with light infections (Gyorkos 2011). In a Kenyan birth cohort, *Ascaris* infection at 24 months of age was significantly associated with decrease in LAZ (LeBeaud 2015). In Brazil, STH infection was associated with a linear growth faltering of 4.6 cm at 7 years of age (Moore 2001).

To summarize, the first 1.000 days of life is a “window of vulnerability”, but also a window of opportunity for prevention and intervention (Victora 2010). Evidence suggests that multiple enteric parasitic infections within the first postnatal months, even sublcinical, could have impact on infant growth that extends long beyond the infection itself (Guerrant 2008). Both, eneric protozoa and helminth are recognized be associated with growth shortfalls (Bartelt 2013). A comprehensive growth assessment

is needed to evaluate the extension of the impact of enteric parasitic infections on the nutritional status, especially in marginally nourished children.

1.7. Enteric parasitic infection and neurodevelopment status

1.7.1. *Infant neurodevelopment*

Early human development is characterized by developmental spurts and plateaus and bio-behavioral changes, which are shaped by a dynamic and continuous interaction between biology and experiences (Shonkoff 2000, World Bank 2009a). The first few years of life are particularly important because the brain developing occurs during this period (Grantham-McGregor 2007). The brain is not a homogeneous organ, each anatomic region has a unique developmental trajectory that begins and accelerates in fetal life or shortly after birth (Keunen 2017). In the prenatal months occur the neurulation (*i.e.*, closure the neural tube), followed by generation, proliferation, migration and finally differentiation of neurons (Keunen 2017). The second trimester of pregnancy is characterized by synaptogenesis, dendritic sprouting, and neural circuit formation (Keunen 2017). A number of processes take place at later trimester and extends into the postnatal period, including myelination, synaptogenesis and the formation of dendrites and associated dendritic spines (Keunen 2017). The brain of the full-term newborn infant has many more synapses than adult brain. The period of synaptic overproduction (synaptogenesis) is normally followed by a period of synaptic retraction, or reduction that confers efficiency in brain functioning "blooming and pruning" (Keunen 2017). This process varies according to the brain region. It is estimated that the peak of synaptic overproduction in the visual cortex occurs at about 4^{th} postnatal month with a gradual retraction until the middle of preschool period. In the areas of audition and language a similar although somewhat latter time course is observed. In the prefrontal area, where higher level of cognition take place, the peak of synaptogenesis occur at around one year of life and only in middle to late adolescence the adult density of synapses is achieved (Figure 5) (Thompson 2001).

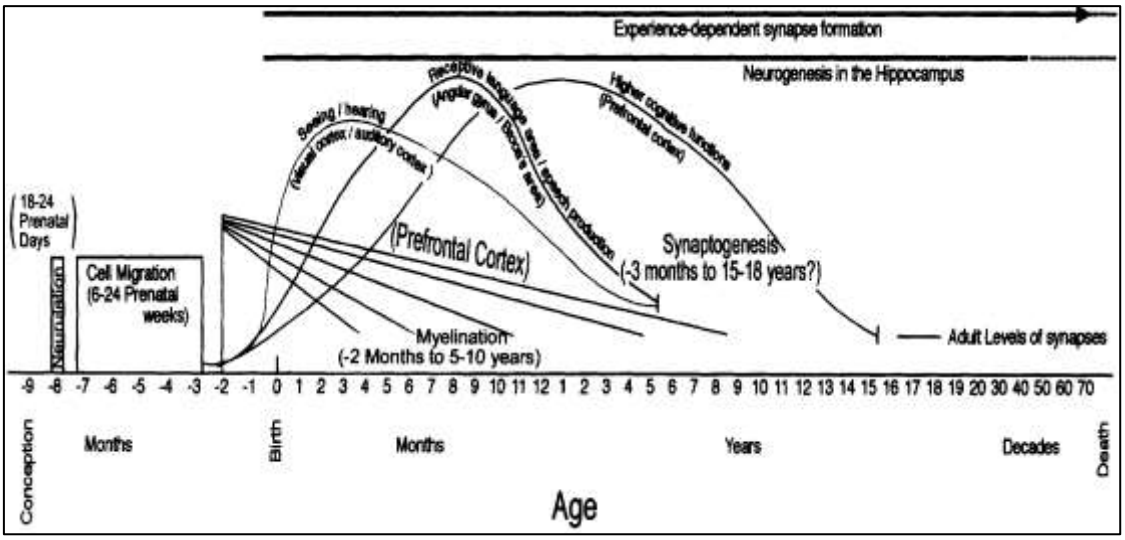


Figure 5. The developmental course of human brain (source: Thompson 2001).

Every aspect of early human development is affected by genetic, environment and cumulative experiences in the prenatal period, and extending throughout the early childhood years (Shonkoff 2000). Both genetic and environmental influences act synergistically and dynamically over the period of development, and can be favorable or detrimental for child development (Shonkoff 2000).

There is wide consensus that during early childhood the brain is taking shape with a speed that will never be again equaled (Grantham-McGregor 2007). Adverse effects occurring during the prenatal period and earliest postnatal months and years could have long-term consequences in cognitive development and mental health (Grantham-McGregor 2007). Children in developing countries are especially in disadvantage (World Bank 2009a). They are exposed to multiple risks of poor nutrition, poverty, and lack of psychosocial stimulation than those from more privileged backgrounds (Walker 2007). It was estimated that over 250 million children under-5 years worldwide are not fulfilling their potential for growth, cognition, or socio-emotional development (Grantham-McGregor 2007). They are disproportionately exposed to a wide range of co-occurring risk factors that impact development such as deficiencies in basic health and nutrition (Shonkoff 2000, World Bank 2009a).

The lack of a comprehensive concept of neurodevelopment assessment in infants in LMIC is part of the problem (Black 2017). While in high-income countries there is a

growing trend to use developmental/behavioral tools, few instruments have been standardized and validated for use in less developed countries (McCarthy 2012).

1.7.2. *Assessment of infant neurodevelopment*

A young child develops through advances in three interrelated domains: sensory-motor, socio-emotional, and cognitive and language abilities (World Bank 2009a). Since these domains are overlapping and mutually influencing, the assessment of development should be as comprehensively as possible (World Bank 2009a).

Cognitive skills include analytical skills, mental problem solving, memory, and early mathematical abilities (Johnson 1998). Language skills include babbling, pointing, and gesturing in early infancy; first words and sentences appears by two years of age (Johnson 1998). Motor skills include large (gross) motor skills, such as walk and run, and fine motor skills, that involve eye-hand coordination and muscle control such as picking up objects and holding eating utensils (Johnson 1998). In the social and emotional domain, infants learn whether they will be responded to by others, learn to explore, and also acquire early strategies for dealing with their negative feelings. Healthy infants will show preferential attachments to caregivers, and enjoy initiating and responding to social interactions (Saarni 1998).

The choice of the tool depends on the population to be assessed; the problems needed to be assessed; the context in which will be applied (*e.g.*, urban or rural settings, level of poverty, parent education, language spoken); and the method to obtain developmental data (*e.g.*, directly testing, reports of the child's skills from mothers, observation of the child in daily activities) (Council for infant development 2006, World Bank 2009a).

Data obtained by direct assessment, is considered to be a "gold standard" because there is no concern about recall bias. The data can be very high quality with a trained interviewer, than using a parental report (World Bank 2009a).

The child development can be assessed through screening tests or ability tests. Screening test is a brief assessment used to identify children who are at risk of having developmental problems in one or more domains. Screens usually include motor, cognitive and language domains, but often do not measure social-emotional

development. They are inexpensive, quick and relatively easy to administer, and require minimal time for training (Glascoe 2001, World Bank 2009a). The ability tests are designed to assess the maximum skill level for a child at any given age. They provide detailed, comprehensive information on children's developmental levels within domains and as a summary across domains (World Bank 2009a). Comparing with screening test, ability tests produce continuous scores that can be used to compare children's developmental levels with more precision. However, ability tests are time-consuming and require a high degree of training (World Bank 2009a).

Some constraints regarding the application of a development test in a LMIC should be considered: the budget, as many standardized tests are expensive for use in large-scale studies; copyright issues, as most of the tests developed and licensed in the developing world are strictly protected by copyrights; training, as some tests require considerable time to adequately train and standardize observers; language and cultural differences, as differences difficult the meaning of items, and materials such as pictures or objects which are unfamiliar to many children living in developing countries (World Bank 2009a).

There are few tools offering a description of the child development in LMIC.

- Motor milestones

WHO collected information on 6 motor milestones from well-nourished infants in 5 countries (Ghana, India, Norway, Oman, and USA) providing information on normative attainment of various skills (WHO 2006b). Six motor milestones were incorporated into growth monitoring charts: sitting without support (3.8 to 9.2 months), standing with assistance (4.8 to 11.4 months), hands and knees crawling (5.2 to 13.5 months), walking with assistance (5.9 to 13.7 months), standing alone (6.9 to 16.9 months), and walking alone (8.2 to 17.6 months). These milestones were selected because they are considered to be universal, fundamental to the acquisition of self-sufficient erect locomotion, and simple to test and evaluate (Wijnhoven 2004). This test has high reliability (WHO 2006b). Unfortunately, no other developmental dimensions beyond the motor skills are assessed.

- Early Childhood Development Index

It was developed cross-culturally by UNICEF to assess the developmental status of

children aged 36 to 59 months of age. This index includes a set of 10 items in four main domains: literacy-numeracy (3 items), social-emotional (3 items), physical (2 items), and learning (2 items)(UNICEF 2009a). Unfortunately, this test was developed for children aged 3 to 5 years.

Developmental tools for infants are showing in Table 8.

- The Bayley Infant Neurodevelopmental Screener

The most worldwide used assessment of infant development is the Bayley Scales of Infant Development (BSID) (World Bank 2009a). This test requires a trained professional, takes about an hour or more to administer, and tends to be expensive. To overcome these inconveniences, a simpler alternative is the The Bayley Infant Neurodevelopmental Screener (BINS) a screening tool derived from the BSID and the Early Neuropsychologic Optimality Rating Scales (Aylward 2000). It was designed to identify, in infants aged 3 to 24 months, who are developmentally delayed or in risk (Aylward 1995). Four conceptual areas are assessed: basic neurological functions/intactness (posture, muscle tone, movement, asymmetries, abnormal indicators); expressive functions (gross motor, fine motor, oral motor/verbal); receptive functions (visual, auditory, verbal); and cognitive processes (object permanence, goal-directedness, problem solving) (Aylward 1995). For standardization of BINS two populations with 600 healthy and 300 non-healthy infants from US were used, with percentages representative in each age and ethnicity (Aylward 1995). Psychometric properties of BINS showed sensitivity of 75-86% (moderate), a specificity of 75- 86% (moderate), good reliability (0.71 to 0.81) and moderate to strong internal consistency (Aylward 1995). The BINS has very acceptable concurrent validity when compared with the Bayley Scales of Infant Development-II (Aylward 1995). It is an instrument convenient to administer by a trained professional in about 10 to 15 minutes (Aylward 1995).

The American Academy of Pediatrics (Council on Children with Disabilities 2006) considered the BINS as a useful screening instrument. This test has been validated in particular populations, such as in at risk Brazilian preterm infants in whom it showed high sensitivity and moderate correlation with Denver development screening test (DDST)-II and Bayley Scales of Infant Development (BSID)-II tests (Guedes 2011).

In a multisite study in South American infants, BINS was feasible and appropriate for neurodevelopmental screening, regardless of their cultural, socioeconomic and languages background (McCarthy 2012).

- Ages and Stages Questionnaire

It is a parents report that measure skills in communication, gross motor, fine motor, personal-social and problem-solving (similar to cognitive) domains (World Bank 2009a). As with all maternal reports, bias is possible (World Bank 2009a).

- Denver Developmental Screening Test

It assesses fine motor/adaptive, gross motor, language, and personal/social domains. It has been used extensively within the developing world but it requires trained administrator (World Bank 2009a).

- The National test "*Evaluacion de Escala de Evaluacion del Desorrollo Psicomotor*" (EEDP)

It assesses areas of language, social, coordination, and gross motor. Children are divided into three categories: normal, risk, and delayed (World Bank 2009a). Althoug this screening can be administered in low resource settings, it has not validity and reliability data (Ridriguez 2001, World Bank 2009a).

- The Guide for Monitoring Child Development

It is parent a report assessment, provides a method for developmental monitoring and early detection of developmental difficulties in children of LMIC. The questions pertain to child's social, emotional, and cognitive development (Ertem 2008, World Bank 2009a). As with all maternal reports, bias is possible (World Bank 2009a).

- Developmental Milestones Checklist

It is a structured interview developed in Kenya to monitor development in infants aged 3–24 months, consisted of 66 items covering motor, language and personal–social development domains (Abubakar 2010).

- The Rapid Neurodevelopment Assessment Instrument

It was developed for children from birth to 5 years in Bangladesh (Khan 2010, 2013). This test measures developmental milestones, vision, hearing, behavior, seizures and sleep patterns (Khan 2010).

Table 8. Developmental screening tools for infants in low and middle income countries (Council for infant and young child development 2006)

Test	Description	Age	Nr of items	Admon. time	Psychometric properties	Reference
Denver-II Developmental Screening Test	Direct assessment of expressive and receptive language, gross motor, fine motor, and personal social skills.	0 to 72 months	125	10-20 min	Sensitivity: 0.56–0.83 Specificity: 0.43–0.80	Frankenburg 1971
Bayley Infant Neurodevelopmental Screener	Direct assessment of basic neurologic functions; receptive, expressive and cognitive processes.	3 to24 months	10-11	8- 10 min	Sensitivity:0.75–0.86 Specificity: 0.75–0.86	Aylward 1995
Ages & Stages Questionnaires	Maternal report of communication, gross motor, fine motor, problem-solving, and personal adaptive skills.	4 to 60 months	20	10–15 min	Sensitivity 0.70–0.90 Specificity 0.76–0.91	Squires 1999
Evaluacion de escala del desarrollo sicomotor EEDP	Direct assessment of language, social, coordination, and gross motor domains	0 to 24 mo	5-6	5-30 min	Not determined	Rodriguez 2001
Guide for Monitoring Child Development	Maternal report.	3 to 24 months			Sensitivity 0.88 -0.96 Specificity 0.93- 0.97	Ertem 2008
Developmental Milestone Checklist -Kenya	Interview including three broad domains: motor, language and social–emotional development	6 to 35 months	66	-	Not available	Abubakar 2009
Rapid neurodevelopmental assessment.	Direct assessment of primitive reflexes, gross motor, fine motor, vision, hearing, speech, cognition, behavior, and seizures	0 to 24 months	27		Sensitivity 80–90% Specificity 60–78%	Khan 2010

Despite all the tests currently available, there is no universally accepted screening tool appropriate for all populations and all ages (Council for infant development 2006). An ideal test for low-resource settings should have good reliability and validity, few and simple items in all domains, be easily applied in short time by local health professionals. In this context, the BINS seems to be a convenient tool for neurodevelopment assessment of infants in LMIC.

1.7.3. *Enteric protozoa infection and infant neurodevelopment*

Cognitive impairment is a key detrimental outcome in the "cycle of poverty" involving malnutrition and enteric infections (UNICEF 1990a). In children, intestinal infections may have harmful effects not only on stunting, but also on cognitive development; this effect seems to be independent of the effect of diarrhea on malnutrition (Guerrant 2011). The "parasite-stress hypothesis" was proposed by Eppig *et al.* (2010) to explain mechanisms of impairment of cognitive development related to parasitic infection (Eppig 2010). The brain is the most complex and costly-energy organ in the human body. If a child cannot meet adequate energetic demands during the rapid brain growth and development, the brain's growth and developmental will be compromised (Eppig 2010). The exposure to infectious agents may cause a developmental pathway that permanently invests more energy into immune function at the expense of brain growth (Eppig 2010). Although this analysis did not address specifically enteric infections or diarrhea, several studies strongly support that diarrheal diseases may lead infectious diseases causing lifelong detrimental effects on brain development (Guerrant 1999, Niehaus 2002, Guerrant 2011). In Brazil, multivariate models showed that early childhood diarrhea remained a significant predictor for Test of Nonverbal Intelligence and coding tasks (WISC-III) scores, after adjusting for the effect of 24 months LAZ and WAZ (Pinkerton 2016).

Studies of association between enteric protozoa and child neurodevelopment in developing countries are described in Table 9.

Table 9. Studies reporting association between enteric protozoa and neurodevelopment in infants from developing countries

Country	N (age)	Results	Reference
		Giardia lamblia	
Nicaragua	961 0-10 years	The presence of *Giardia* was not associated with suspect findings on the Denver II. Language tests were associated with Ascaris (p <0.044).	Oberhelman 2011
Peru	239 9 year	Children with more than one episode of *Giardia per* year scored 4·1 points lower Wechsler intelligence scale.	Berkman 2002
India	116 3 year	Children with a past history of giardial diarrhea showed a trend towards lower social quotients of maturity scale, (p=0.09) and had significantly lower intelligence quotients (p=0.04)	Ajjampur 2011
Turkey	100 6 years	Children infected with *Giardia* and other intestinal parasites had higher growth retardation and psychomotor development delay than non-infected children.	Yentur 2015
		***Cryptosporidium* spp.**	
Brazil	26 6-9 years	Early childhood cryptosporidial infections were also associated with reduced fitness at 6–9 year of age.	Guerrant 1999
Peru	239 9 years	*Cryptosporidium* infection was not associated with WISC-R scores.	Berkman 2002
India	116 3 years	Cryptosporidial diarrhea was not associated with poor cognitive test.	Ajjampur 2011
		Entamoeba histolytica	
Bangladesh	191 2-9 years	*Entamoeba histolytica*-associated dysentery was associated with lower cognitive test scores.	Tarlenton 2006

In *Giardia lamblia* infection, Peruvian children from a birth cohort were examined at 9 years of age for cognitive function using the Wechsler Intelligence Scale for Children (Berkman 2002). Children with severe stunting in the second year of life scored 10 points lower in the Wechsler Intelligence Scale -R test (95% CI 2.4–17.5), and children with more than one episode of *Giardia lamblia* infection per year scored 4.1 points lower than children with none episodes (Berkman 2002). The combined effect of stunting and *Giardia* infection accounted for an intelligence quotient deficit of almost 15 points (Berkman 2002). Indian children with a past history of giardial diarrhea showed lower social quotients and lower intelligence quotients (p=0.04), while cryptosporidial diarrhea did not; thus, *Giardia*-associated diarrhea predicts poor cognitive performance (Ajjampur 2011). Similarly, Turkish children infected with *Giardia lamblia* and other intestinal parasites showed development delay up to 1.9 times, language–cognitive delay up to 2.2 times, and fine motor development delay up to 2.9 times, in comparison with children without any parasitic infections (Yentur 2015). There is no a rationale for a specific aspect of the pathogenesis of *Giardia* infection to have negative effects on cognitive development (Berkman 2002). However, giardiasis can lead to zinc and other micronutrient deficiencies that have been associated with deficits in cognitive development. *Giardia* variant specific surface proteins bind zinc and other heavy metals in the intestine (Berkman 2002).

Early childhood diarrhea due to *Cryptosporidium* spp. infection has lasting effects on child growth and cognitive development (Checkley 1998, Guerrant 1999). In Brazilian children, early enteric infections with *Cryptosporidium* with diarrhea were correlated with later reductions in physical fitness at 6–9 year of age, even when controlling for current nutritional status (Guerrant 1999). On the other hand, the studies in children by Berkman *et al.* (2002) in Peru and Ajjampur (2010) in India did not found association of *Cryptosporidium* enteric infection with cognitive scores (Table 9).

In Bangladesh, cognitive function was assessed in school children with *Entamoeba histolytica* enteric infection, and in those with concurrent *Entamoeba histolytica*-dysentery was associated with lower Wechsler Abbreviated Scale of Intelligence definitions scores (-1.7; 95% CI -3.35–0) (Tarlenton 2006) (Table 9).

For STH infections, it was hypothesize that the effects on cognitive function associated with helminth infection can be partly explained by a secondary/ indirect effect of the gut microbiota dysbiosis induced by infection (the helminth-gut microbiome-CNS axis)(Guernier 2017). Furthermore, there is strong evidence linking parasite infections, particularly hookworms, to anemia, which can cause disturbances in social, emotional, and cognitive development (Guernier 2017). Further research is needed to elucidate the mechanisms through which gastrointestinal STH infections can induce cognitive developmental delay (Guernier 2017).

To summarize, the predominant brain and synapse development in humans occur in the first 2 years after birth. The absorption of key nutrients during this time is critical to assure the optimal development of brain that determines human capacity (Petri 2008). Repeated enteric parasitic infections, even asymptomatic, may alter intestinal absorption and put infants at risk of poor development (Guerrant 1999, Berkman 2002, Tarlenton 2006). In this context, early identification of developmental delays is critical to break the vicious cycle and contribute to infants reaching their developmental potential (Black 2017). Few instruments for neurodevelopment assessment have been standardized in LMIC (McCarthy 2012). Among the several tools, the BINS seem to be a convenient screening test for infants from these settings (Aylward 1995).

2. Objectives

This project is aimed to explore the association of enteric protozoa infection with intestinal barrier function, nutritional status, and neurodevelopment in infants from São Tomé.

It is hypothesized that repeated enteric protozoa infections during the first 24 months of age is associated with (Figure 6):

- Intestinal barrier dysfunction, specifically increased permeability and local inflammatory response;
- Wasting and stunting;
- Poor neurodevelopment.

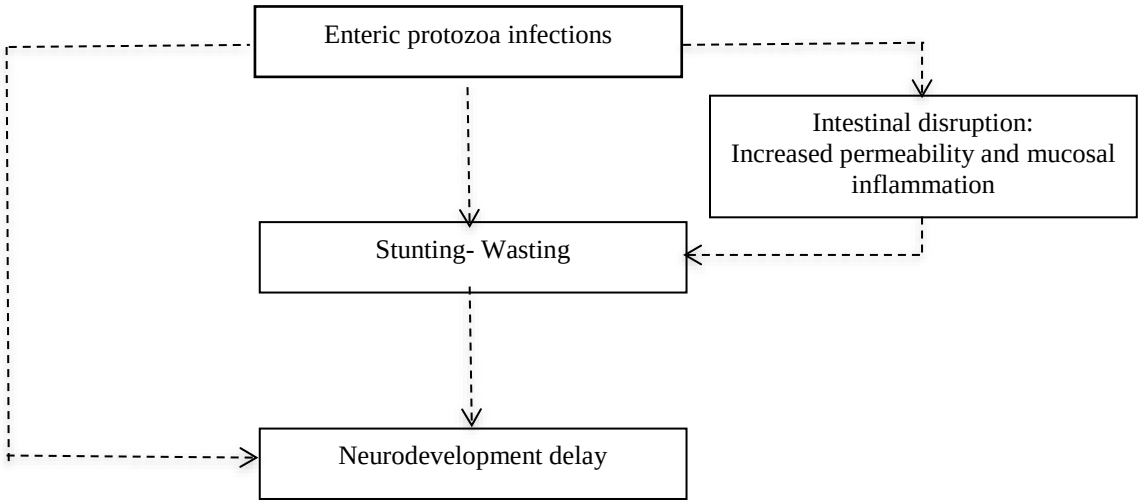


Figure 6. Conceptual framework (adapted from Guerrant RL, *et al.* 2008)

3. Methods

3.1. Study design

This is a prospective birth cohort study with 24 months of follow-up, conducted from March 2013 to July 2015.

Associations of enteric protozoa infection with intestinal barrier function, nutritional status, and neurodevelopment are explored.

3.2. Ethical and legal issues

This study was approved by the Ministry of Health of Democratic Republic of São Tomé and Príncipe (STP) and by the ethics committee of the Institute of Tropical Medicine and Hygiene.

Written informed consent in the national official language (Portuguese) of STP was obtained from parents or caregivers (Appendix 1). 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.

3.3. Setting

The project was conducted in São Tomé, an sub-Saharan archipelago consisting of two small islands, São Tomé and Principe, located in the Gulf of Guinea, 380 km away from the West African coast (00º latitude 04 'N was 010 41' N longitude 25'E06° and a 28'E07°. These islands occupy an area of 1.001 km² with 187.356 inhabitants in 2012, distributed in seven districts including the autonomous region of Principe (Figure 7) (Instituto Nacional de Estatística -INE STP 2012).

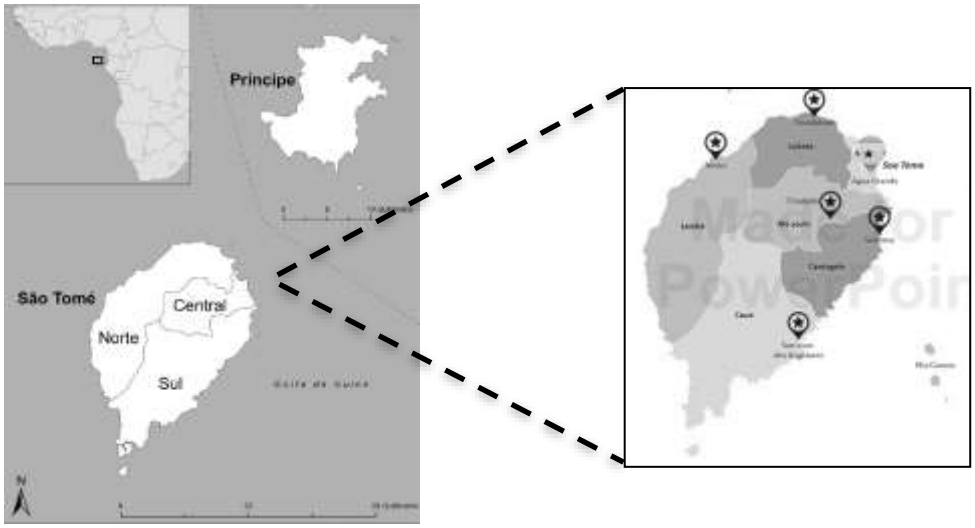


Figure 7. The Democratic Republic of São Tomé and Príncipe (Source: INE 2012)

For this project, infants were recruited from three districts: Agua Grande, Lembá, and Caué. Água Grande is the main district of São Tomé province, located at central region. In terms of area, it is the smallest of seven districts (17 Km^2), but the largest in population with 73.091 habitants, which correspond to 38.9% of total population, with a population density of 4299.3 habitants/Km^2. Lembá district, located at North region, has an extension of 230 Km^2 with 14.652 habitants, that correspond to 8.2% of total population and a population density of 63.7 habitants/Km^2. Caué district, located at South region, has an extension of 267 Km^2 with 6.031 habitants, which correspond to 3.4% of total population and the lowest population density of 22.6 habitants/Km^2 (INE STP 2012). Those districts were chosen by convenience, attempting to represent this LMIC under socio-economic point of view. Greater proportion of inhabitants in Agua Grande district is in middle, fourth and richest wealth index quintiles, while greater proportions of inhabitants in Caué and Lembá districts are in the poorest wealth index quintiles (Multiple Indicator Cluster Survey – MICS 2016).

Infants were recruited at the health settings where the infants born in those districts are attended in the first postnatal visit as outpatients: at the primary care center "Protecção Materno-Infantil" in Agua Grande district, and at the only available local hospitals in Lembá and Caué districts.

3.4. Inclusion criteria

In spite of this study is named birth cohort, the infants were recruited in the neonatal period, that is, within the first 28 postnatal days.

Consecutive appropriate-for-gestational age neonates (>10th and <90th percentiles) (Lee AC, Lancet 2013) were eligible. Non-inclusion criteria were neonates with low birth weight (<2500 g), born preterm (<37 weeks of gestation), without gestational age information, with major congenital malformations, or suffering perinatal asphyxia needing hospitalization.

3.5. Sample size

For sample size calculation, the proportion of children with undernutrition not exposed to enteric parasitic infections was firstly estimated (Aguiar 2007). Since that stunting is the most prevalent form of child undernutrition (de Onis 2016), the proportion of 29% of undernutrition was considered for this study, once this was the stunting prevalence in children under-5 reported in the STP survey contemporary with the initiation of the study (MICS 2016). Secondly, the risk of undernutrition in children after exposure to enteric protozoa infections was estimated. A speculated OR of 2.0 was calculated for children becoming stunted after exposure to enteric protozoa infections, based on the average OR reported for *Giardia lamblia* infection (OR 2.10) (Sackey 2003), *Cryptosporidium* spp. infection (OR 1.31) (Tumwine 2003), and *Entamoeba histolytica* infection (OR 2.93) (Mondal 2006). Assuming an alpha error of 0.05%, a power of test of 0.8, the percentage of 29% undernourished children not exposed to enteric protozoa infections, and the OR of 2.0, a total sample size of 310 infants was calculated using the OpenEpi version 3 software. As the surveillance in this study was passive, since the mothers came with their infants to local heath settings, a high attrition rate of 40% was considered. Considering this attrition rate, the estimated number of neonates to be recruited for this cohort was 510. The number of neonates recruited by district was proportional to the live births recorded in each district in the year 2011 (INE STP 2012) (Table 10):

Table 10. Newborns by district in São Tomé e Principe (Source: Instituto Nacional de Estatística - INE 2012)

District	Live births /year in 2011	Proportion	Cohort size by district
Agua Grande	2174	0.76	388
Lembá	448	0.16	81
Caué	234	0.08	41
Total	2856	1	510

The same investigator (MG) with collaboration of local health staff recruited the infants. Three days per week (Tuesday, Thursday and Friday) were scheduled for Agua Grande district due to the greater size of subsample, and once per week for Lembá (Monday) and Caué (Wednesday) districts with smaller size subsamples.

In the follow-up, infants recruited in March 2013 were classified as group 1, those in April as group 2, in May as group 3, in June as group 4, and in July as group 5 (Figure 8).

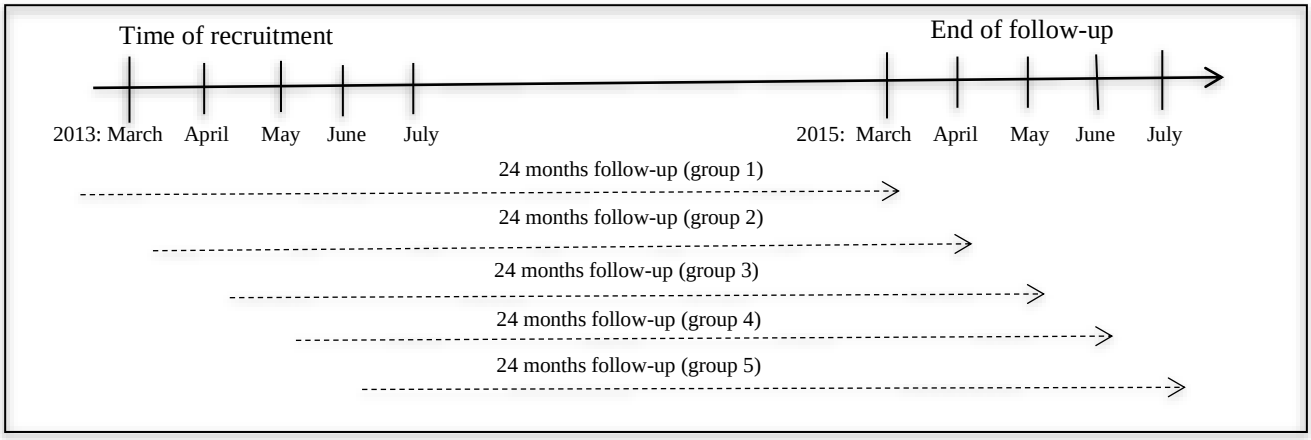


Figure 8. Time of recruitment and follow-up

3.6. Follow-up: points of assessment

Points of assessment were scheduled according to different outcome measurements (Table 11): for nutritional status, anthropometry was scheduled monthly from the neonatal period to 12 months of age, and then every two months until 24 months of age; for neurodevelopment assessment, tests were scheduled at 3, 6, 9, 12, 18 and 24 months of age; for enteric parasite assessment, collection of stool samples were

scheduled approximately every three months; and a single assessment of intestinal barrier function was scheduled at the end of follow-up, at 24 months of age. The aforementioned ages

Table 11. Follow-up point assessments

Postnatal age (months)	0	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
Anthropometry	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Neurodevelopment				x			x						x			x			x
Enteric parasites				x			x			x			x		x	x			x
Intestinal barrier																			x

3.7. Data collection

3.7.1. *Questionnaire for the first visit*

In the first scheduled visit, a questionnaire containing questions to mothers on socio-demographic and economic household data as well as perinatal data registered in the infants' booklets was fulfilled (Appendix 2). The questionnaire was in Portuguese (official language), and was applied by the same observer (MG). A local nurse in each health care setting collaborated in translation in case of mothers or caregivers did not understand Portuguese.

Socio-demographic and economic household data

The questions applied covered socio-demographic and economic household data, including parents 'age, educational level and employment status.

The multidimensional poverty index (MPI) for socio-economic household status (Alkire 2010) was used in this study. The MPI has three dimensions: health, education, and standard of livings. Education dimension includes years of schooling and child school attendance; health dimension includes child mortality and nutrition; and standard of living includes electricity, drinking water, sanitation, flooring, cooking fuel, and assets (Alkire 2010). Each dimension is equally weighted, and each indicator within a dimension is also equally weighted. A household is identified as

"multidimensionally poor" if it is deprived in some combination of indicators whose weighted sum is more than 30% of the dimensions (Alkire 2010).

Similarly to previous studies (Psaki 2014), an adaptation of the MPI was used for this study: a. The education dimension was adapted only considering the mother education status and deprived household was defined if mother had less than five years of school. This was based on the fact that having no formal education or only primary education are risk factors for child mortality (Ezeh 2015) and stunting (Psaki 2014). b. The health dimension was adapted considering only the child mortality. Child nutritional status, the second component of this dimension, was not included because it may be an outcome variable in some analyses, as proposed by others (Psaki 2014). C. Standard of living dimension was according to the original MPI. Household was considered deprived if had no electricity, no access to improved water source (WHO 2015a), no access to improved sanitation (WHO 2015a), if the floor was dirt, sand, or dung, if cooking was done by means of solid fuel, and if did not own more than one asset (radio, TV, telephone, bike, motorbike, refrigerator, car, or truck) (Alkire 2010).

MPI was not calculated when all the indicators within a dimension or when 50% or more out of the ten indicators were missing (United Nations Development Program-UNDP 2014a).

Perinatal data

Hereditary diseases, complications during pregnancy, mothers' age, parity (Bai 2002), single or multiple pregnancy, local of delivery, professional assistance of delivery, type of delivery (vaginal or caesarian section), post-delivery mother's measured weight and height.

3.7.2. *Feeding practices*

The feeding practices were enquired and recorded (Appendix 2) by same observer (MG) in each point of assessment. This enquiry included UNICEF indicators for assessing infant feeding practices (WHO 2009b): exclusive breastfeeding at 6 months (proportion of infants aged 0 to 5 months), continued breastfeeding at 1 year (proportion of infants aged 12 to15 months), continued breastfeeding at 2 years (proportion of infants aged 20 to 23 months), infants ever breastfed (proportion of

infants up to 24 months of age), and the introduction of solid, semi-solid or soft foods (proportion of infants aged 6 to 8 months). The prevalence of these indicators was calculated by dividing the number of infants who were breastfed by the total infants regardless of their feeding practices (WHO 2009b).

3.7.3. *Acute infectious and associated conditions*

Acute infectious events and other associated conditions were enquired and recorded by same observer (MG) in each point of assessment (Appendix 2). The acute events were considered if they occurred within two weeks preceding the visit, and included: acute diarrhea (AD) (either watery or bloody diarrhea, lasting <14 days), and persistent diarrhea (lasting > 14 days) (WHO 2005a); acute respiratory infections (ARI), including pneumonia (with fast breathing and/or chest indrawing, or severe pneumonia with any ominous sign), and other upper or lower respiratory infections (WHO 2014a); and malaria (confirmed by Rapid Diagnostic Test and/or blood smear microscopic identification) (Appendix 3).

3.7.4. *Nutritional status/ anthropometry*

Anthropometry was performed and recorded by same trained observer (MG) in each point of assessment (Appendix 2). The direct measurements were: body weight, recumbent crown-heel length, HC, and mid-upper arm circumference (MUAC). The length was measured in duplicate using a portable infantometer (with digit counter readings with precision of 1 mm) (Seca 207) and the average of the two measurements was considered; body weight was measured using a portable electronic scale with precision of 10 g (Seca 334, GmbH & Co. KG, Hamburg, Germany); head circumference was measured using a non-distensible tape for infants, with precision of 1 mm (Seca 211, GmbH & Co. KG, Hamburg, Germany) and mid-upper circumference was measured with standard UNICEF tapes, with precision of 1 mm.

Data cleaning procedures: infants were scheduled to be measured at ages in months (0 to 24 months) that coincide with WHO target ages in days (0, 28, 61, 91, 122, 152, 183, 213, 244, 274, 304, 335, 365, 426, 487, 548, 609, 670, 731 days) (WHO 2009a) The maximum tolerable differences in days between target age and actual

measurement age should be ±3 days (0-6 months), ±5 days (6-12 months) and ±7 days (12-24 months) (WHO 2009a). In each point of assessment, measurements of infants who did not comply with these maximum tolerable differences were excluded. To avoid the influence of "unhealthy" weights for length, measurements falling below -3 SD were also excluded (WHO 2006a). The below outcome measurements were obtained.

Mothers' height was measured using a portable stadiometer (Seca 217, GmbH & Co. KG, Hamburg, Germany).

- *Attained growth*

Attained growth included measurements of body weight (kg) and length (cm) that were converted into age- and sex specific *z-scores* for WAZ, LAZ, WLZ, and BMI (BMIZ), using the WHO Anthro software v.3.2.2, based in the WHO standards (WHO 2006a). The *z-scores* for MUAC (MUACZ) and HC (HCZ) were also obtained using the same software.

Length-for-age difference (LAD) was used as alternative metrics for attained growth. At each point of assessment, the LAD was computed by subtracting the measured length to the age- and sex-specific median of the reference population (WHO 2006a), and expressed in absolute values (cm) (Leroy 2014).

- *Growth velocity*

For this study, the interval between measurements chosen to calculate velocity was 2 months (WHO 2009a). The aforementioned maximum tolerable differences in days between target age and actual measurement were considered. The velocities for weight and length were converted into age- and sex specific *z-scores* WAVZ and LAVZ respectively, following WHO methodology (WHO 2009a):

- $WAVZ = \{[(y+\delta)/M(t)]^{L(t)} - 1\}/S(t)L(t)$
- $LAVZ = \{[y/M(t)]^{L(t)} - 1\}/S(t)L(t)$

In which: *y* corresponds to 2-months weight increment in grams or length increment in centimeters, δ is a constant value (600 g), *t* corresponds to the specific interval of age, and L, M and S are the estimates for each interval of age (*t*) (WHO 2009a).

- *Wasting and stunting*

As aforementioned, wasting is defined as low WLZ and stunting as low LAZ. The cut-offs ≤ -1 SD, ≤ -2 SD and ≤-3 SD for weight-for-length and length-for–age were used to define mild (or risk of), moderate and severe degrees of wasting and stunting, respectively.

3.7.5. *Neurodevelopment assessment*

The BINS was used for neurodevelopment assessment and applied at 3 months, 6 months, 12 months, 18 months, and 24 months, according to the respective manual recommendations (Aylward 1995) (Appendix 2). This screener has 10 to 11 items that include fourth conceptual areas:

- Neurological functions: include items that assess neurological intactness of the developing central nervous system. Evaluations of muscle tone, head control, asymmetries in movement, and absence of abnormal indicators (*e.g.*, excessive drooling or motor overflow) are included in this category.
- Receptive functions: involve the entry of information into the central processing system, namely, sensation and perception. Visual, auditory, and tactile input are assessed but the first two are more emphasized in BINS.
- Expressive functions: involve fine motor (prehension, manipulation of objects with fingers, eye-hand coordination), oral motor (vocalizations, verbalizations) and gross motor (sitting, crawling, and ambulating). Although expressive function directly evaluates motor ability, verbal-cognitive function also is assessed.
- Cognitive process: involves higher order functions, namely memory/learning, thinking/reasoning. Object permanence, goal directedness, attention and problem solving are in this group.

In this study, BINS scores were analyzed as a dichotomous variable as low-risk or high-risk, using the cut-off recommended in BINS manual having a good sensitivity and specificity for developmental delay (Aylward 2000). Those infants in moderate risk were closely monitored and those with high risk were appointed for further evaluations. This test was performed by the same trained observer (MG). Mothers collaborated whenever it was requested. If the infant was sick or did not cooperate the assessment was re-scheduled.

3.7.6. *Parasite examination techniques*

The methods for parasite examination techniques are described elsewhere (Garzon 2017). In summary, at each point of 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 4ºC 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 *Giardia 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 4ºC 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 -20º C without preservative, for molecular characterization of *Giardia duodenalis* and detection of fecal markers by enzyme linked immunosorbent assay (ELISA).

Molecular characterization was planned at 24 months of age for *Giardia duodenalis*, to explore the association between *Giardia* assemblage and fecal biomarkers of intestinal function. The molecular characterization was performed in microscopically positive stool samples**.** DNA was extracted from the stools stored at -20ºC, 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 (Caccio 2008). 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 *Giardia 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 (WHO 2006c) implemented by the Health Ministry of STP. Additionally, infants were treated for *Giardia 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 (Reed Book 2015).

3.7.7. *Fecal biomarkers of intestinal function*

The analysis of fecal biomarkers (A1AT and S100A12) was performed in a subset of infants complying with a minimum of four points of assessment with stool sampling (at 6, 12, 18 and 24 months), to explore the cumulative exposure to enteric parasitic infections on intestinal barrier function at 24 months of age (Garzon 2017). The collected stool samples were stored at -20ºC. Temperatures during transportation never exceed 18ºC to 25ºC as recommended. S100A12 (Inflamark F-INFL-ELISA Cisbio Bioassays) and A1AT (RIDASCREEN α_1-Antitrypsin R-Biopharm) were quantified 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 (McCormick 2016) Therefore, watery or diarrheal stool samples were excluded from the analysis.

3.8. Statistical analysis

Descriptive analysis of all variables including socio-demographic characteristics, socio-economic status, feeding practices, clinical events, anthropometric measures, neurodevelopment assessments, and laboratory findings (parasitic infection and stool biomarkers) are presented with frequencies (percentages) and with mean (SD) or with median and interquartile range (P_{25} – P_{75}), as appropriate. Description and definitions of all variables are presented in Appendix 3. Locally weighted scatterplot smoothers (LOWESS) were used to plot growth curves.

In the univariable regression analysis, all the variables with a p-value <0.25 were selected for the multivariable models. Different statistical models were used to explore the hypothesized associations (Figure 9).

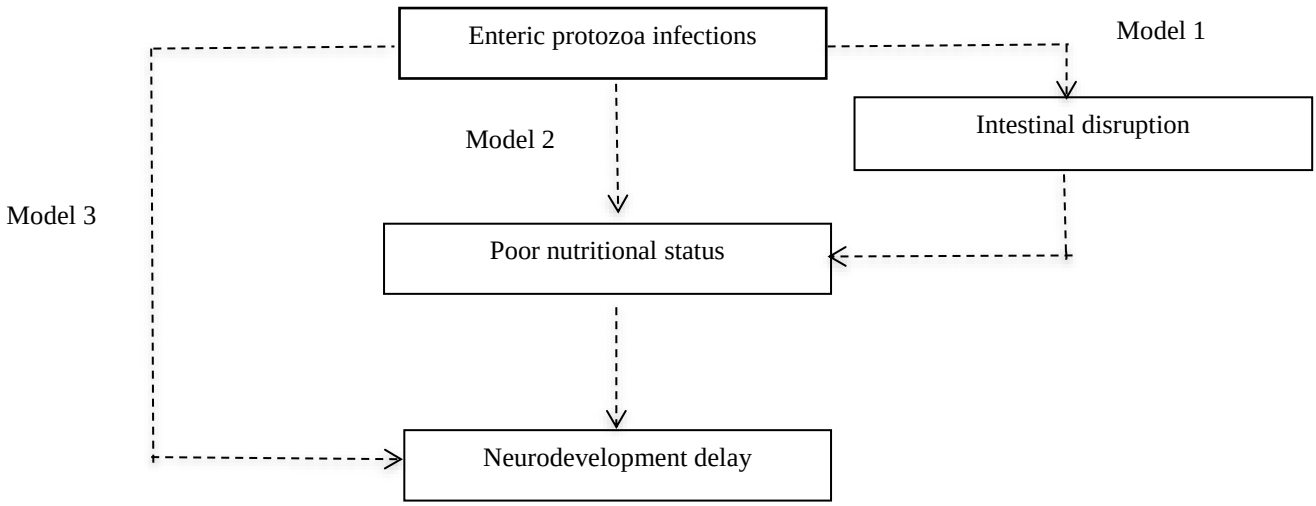


Figure 9. Conceptual framework: hypotheses explored

Model 1: to explore the association between previous exposure to enteric protozoa infection and intestinal barrier function.

Outcome variable: intestinal barrier function. This was assessed by fecal A1AT as marker of intestinal permeability and S100A12 as marker of mucosal inflammation.

Primary explanatory variable: cumulative enteric parasitic infections (previous *plus*

current infections).

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 the univariable 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 α=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 (Bland 2000).

Model 2: to explore the association between enteric protozoa infection and nutritional status.

Outcome variable: nutritional status. This was assessed by *z-scores* of attained growth (WLZ and LAZ); *z-scores* for growth velocity (WAVZ and LAVZ); measures of differences of length (LAD); and undernutrition (wasting and stunting)

Primary explanatory variable: enteric parasitic infections (*Giardia lamblia*, *Cryptosporidium* spp. and STH).

Generalized additive mixed regression models were used to take into account the correlation structure between measures in time and to explore the association between each anthropometric parameter and relevant data (socioeconomic, feeding practices, clinical, and intestinal parasites). For all multivariable models, age was modeled with splines because a non-linear association with each anthropometric parameter was identified. A level of significance α=0.05 was considered.

Model 3: to explore association between enteric protozoa infection and neurodevelopment

Outcome variable: neurodevelopment status, assessed by the BINS.

Primary explanatory variables: nutritional status (WLZ and LAZ, wasting and

stunting); enteric infection (*Giardia lamblia*, *Cryptosporidium* spp. and STH).

Generalized mixed effects regression models were used to explore if nutritional status/anthropometry and enteric parasitic infections are predictors of high risk of poor neurodevelopment. Potential confounders such as sex, and feeding practices were also considered. A level of significance $\alpha=0.05$ was considered, although p-values greater than 0.05 and lower than 0.1 (weak evidence of the difference/association) were still considered (Bland 2000).

Data were analyzed using STATA 13.0 (StataCorp. 2013. Stata Statistical Software: Release 13. StataCorp LP, College Station, TX).

4. Results

4.1. Sample description

In a period of five months (March 2013 to July 2013) 500 neonates were recruited for the birth cohort. From this sample, 25 neonates were excluded for the following reasons: 18 with preterm birth/ low birth weight, 5 with major congenital malformation (2 congenital heart disease, 1 cleft lip/ palate, 1 myelomeningocele, and 1 dwarfism), and 2 with perinatal asphyxia. Thus, 475 infants were enrolled in the birth cohort, corresponding to approximately 8.6% of live-births in São Tomé in 2012 (INE São Tome 2012). During the study period, different proportions of infants missed the scheduled visits, although some of the infants that were not present at one point of assessment but returned for the next, corresponding to an attrition rate of 41.05% at 24 months (Figure 10). The observations lost to follow-up neither depended on the team research nor on the exposure, confounders, or the outcome (Kristman 2004). Two hundred eighty-two (59.4%) infants completed the study, attending a median of 18 points of assessment. Significant differences were found between infants who completed 24 months of follow-up and infants who prematurely dropped-out (Table 12). Infants completing the study had higher weight and length at birth, belonged predominantly to Agua Grande district (higher wealth index), had better access to improved sanitation and water source, and their mothers were older and had more schooling.

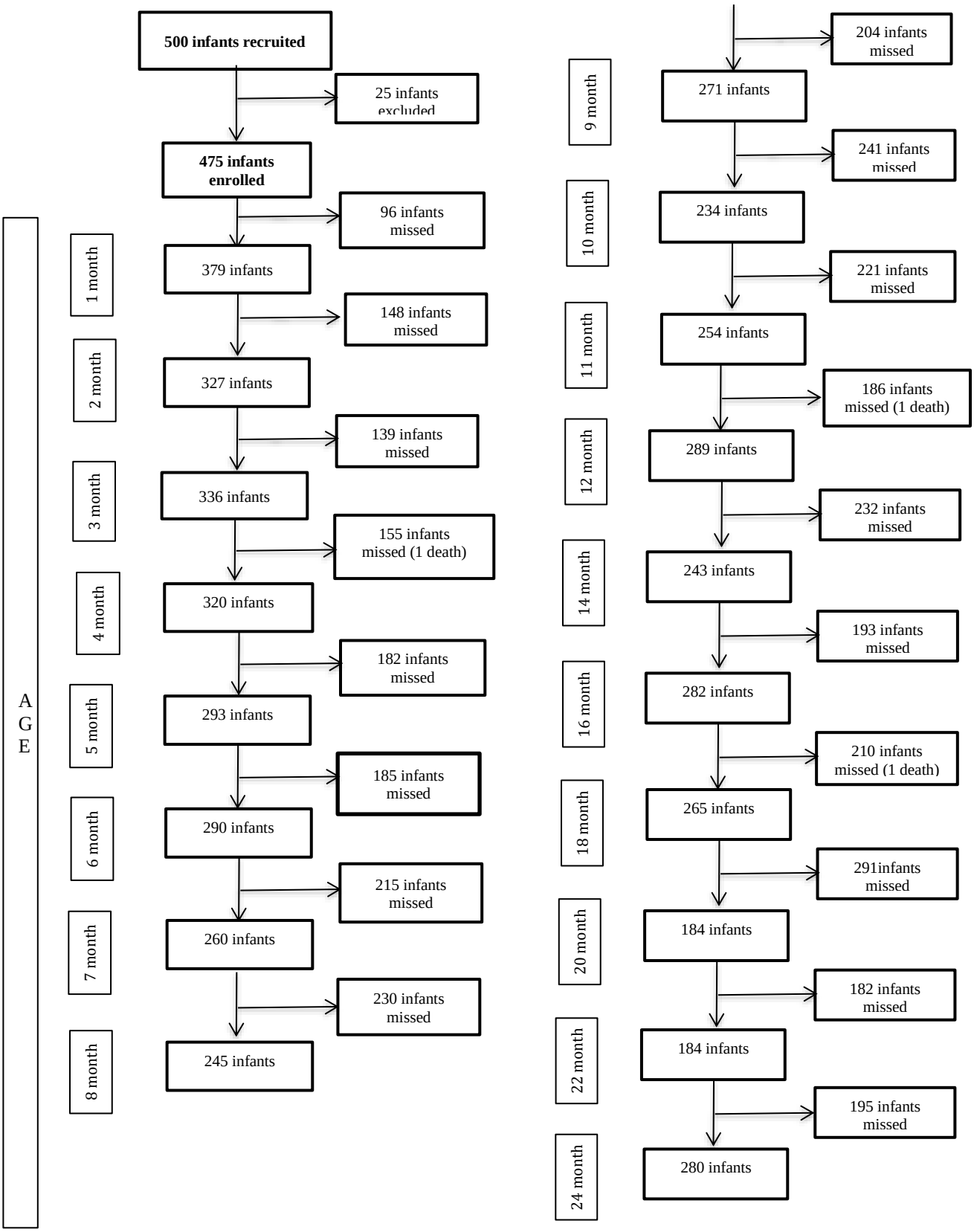


Figure 10. Flow chart

Table 12. Differences between infants who completed the study (at least 10 visits) and those who did not.

Variables	Complete follow-up	Incomplete follow-up	*p value*[a]
Sex, n (%)			
Female	142 (50.4)	100 (52.6)	0.627[a]
Male	140 (49.6)	90 (47.4)	
District, n (%)			
Agua Grande	240 (85.1)	124 (65.3)	0.001[a]
Caué	15 (5.3)	16 (8.4)	
Lembá	27 (9.6)	50 (26.3)	
Anthropometry at birth, mean (SD)			
Weight (Kg)	3.5 (0.48)	3.3 (0.46)	0.009[b]
Length (cm)	50.3 (1.90)	49.9 (1.98)	0.050[b]
Maternal data			
Mother's age, median (min-max)	26.0 (14-43)	24.0 (15-43)	0.009[b]
Maternal height, mean (SD)	158.95 (5.9)	159.7 (5.8)	0.188[C]
Mother's school years, median (min-max)	8.0 (0-15)	6.0 (0-15)	0.001[b]
Improved sanitation			
Not, n (%)	95 (33.7)	98 (52.1)	0.001[a]
Yes, n (%)	187 (66.3)	90 (47.9)	
Improved water source			
Not, n (%)	1 (0.4)	8 (4.3)	0.003[a]
Yes, n (%)	281 (99.6)	180 (95.7)	

[a]*Chi-Square test,* [b] *Mann-Whitney Test,* [c] *t-test*

4.1.1. *Socio-demographic and socio-economic status*

The characteristics of the studied cohort are shown in Table 13. The proportion of males and female did not differ significantly (p= 0.843). Approximately two thirds of infants were from Agua Grande district and the neighboring districts Cantagalo, Mé-Zóchi, and Lobata; the remaining infants were from Lembá and Caué districts (Table 13). The majority of the mothers (67.2%) had secondary school or higher education level. The percentage of working fathers was higher (90%) than working mothers (less than 50%), and most mothers had low-wage informal jobs. Most of the households had electricity and finished floor, but around one third used solid fuels for cooking. Almost all households (97.6%) had improved water source, mainly public tap/ standpipe, but only 58.7% had access to improved sanitation facilities, mostly latrines (Table 13).

Table 13. Socio-demographic and household characteristics of the cohort (N = 475).

	N	%
Sex		
Females	244	51.4
Males	231	48.6
District		
Agua Grande	**363**	**76.4**
São Tomé	264	
Almeirin	4	
Oque d'el Rei	13	
Pantufo	9	
Riboque	8	
Cantagalo	28	5.9
Lobata	5	1.0
Mé-Zóchi	32	6.8
Lembá	**75**	**15.8**
Neves	58	
Diogo Vaz	5	
Pontafigo	1	
Ribeira Alfonso	1	
Santa Catarina	10	
Caué	**37**	**7.8**
Angolares	27	
Angra Toldo	1	
Monte Mário	2	
Porto Alegre	5	
Ribeira Peixe	2	
Maternal education level		
None /primary school	152	32.2
Secondary /higher school	319	67.2
Missing information	1	
Mother's employment		
Unemployed	217	45.7
Student	19	4.0
Informal employment	143	30.1
Formal employment	85	17.9
Missing information	8	

Table 13. Socio-demographic and household characteristics of the cohort (N = 475) (*continued*)

	N	%
Father's age (years, mean)	31.1	
Father's employment		
Unemployed	6	1.2
Student	4	0.8
Informal employment	287	60.4
Formal employment	156	32.8
Missing information	19	
Inhabitants per house (mean)	4.7	
Housing characteristics	2.4	
Electricity		
Yes	261	85.3
No	45	14.7
Missing information	169	
Finished floor		
Unfinished	2	0.4
Finished	466	98.7
Missing information	7	
Cooking fuel		
Solid fuel	151	31.9
Non solid fuel	319	67.6
Missing information	5	
Assets ownership		
No asset	28	9.7%
At least one asset	260	90.2
Missing information	187	
Access to improved water source		
Not improved (river)	9	1.9
Improved	461	97.6
- Community standpipe	363	
- Connected to public potable supply	98	
- Missing informatio	5	

Table 13. Socio-demographic and household characteristics of the cohort (N = 475) (*continued*)

	N	%
Access to improved sanitation		
Not improved	193	40.9
Improved	277	58.7
- Latrine	139	
- Connected to public sanitary system	138	
Missing information	5	

From the 472 households, only 287 had complete data for MPI scoring (Table 14). Twenty four percent of households were classified as deprived (MPI score ≥33.3 %) and from these one third were severely deprived (MPI score >50%).

Table 14. Multidimensional poverty index of the cohort (N = 287).

	N	%
Health dimension		
- Child mortality	8	
Education dimension		
- Mothers with <5 years of school	152	
Standard of living dimension		
- Households without electricity	40	
- Households without improved sanitation	97	
- Households without improved water source	1	
- Households without finished floor	2	
- Households cooking with solid fuel	59	
- Households without assets	70	
Deprived household	**69**	**24.0**
Non-deprived household	**218**	**75.9**

4.1.2. *Obstetrical data and mothers' anthropometry*

Obstetrical data and mothers' anthropometry are shown in Table 15. Most of mothers (75.4 %) were aged between 20 and 34; only 12.7% were adolescents. Around one fifth of women were grand multiparous. The great majority of pregnancies were single. Most of deliveries were vaginal and 93.7% were assisted at hospital setting by skilled professionals (the majority nurses). Most (89.8%) of women had no pathology or complications during pregnancy.

Regarding mother's anthropometry, only 0.6% of them had short stature. Based on BMI, 0.2% of mothers were underweight, 25.5% overweight, and 8.6% obese. It should be noted that BMI could not reflect adiposity, since body weight was measured within the first month after delivery when postpartum weight retention usually occur (Martin 2014).

Table 15. Obstetrical data and mothers' anthropometry of the cohort (N=475)

	N	%
Age (years), mean (SD)	25.9 (6.1)	
< 15	1	0.2
15-19	59	12.4
20-24	167	35.15
25-29	132	27.8
30-34	58	12.2
35-39	41	8.6
≥40	13	2.7
Missing information	1	
Live births /woman, median (min.-max.)	2(1-8)	
1-3	382	80.4
≥4 (grand multiparity)	93	19.6
Pregnancy		
Single	463	97.5
Multiple	12	2.5
Delivery		
Vaginal	463	97.5
Cesarean section	17	3.6

Table 15. Obstetrical data and mothers' anthropometry of the cohort (N=475)(*continued*)

	N	%
Local and assistance of delivery		
Hospital	448	94.3
Home	27	5.7
Skilled professional	448	94.3
Not–skilled person	27	5.7
Maternal and obstetrical pathology		
No	428	90.1
Yes	47	9.95
- Hypertension related to pregnancy	9	
- Diabetes related to pregnancy	1	
- Placenta previa	1	
- Cephalopelvic disproportion	4	
- Sickle-cell anemia	2	
- Urinary tract infection	6	
- Malaria (last trimester)	8	
- HIV	1	
- Hepatitis B	8	
- Others	7	
Mothers' anthropometry		
Height		
≤ 145 cm	3	0.6
145-159.9 cm	250	52.6
≥ 160 cm	195	41.0
Not measured	27	5.7
Body mass index (kg/m^2)		
≤ 18.5	10	0.2
18.6- 24.9	261	54.9
25- 29.9	121	25.5
≥ 30	41	8.6
Not measured	42	8.8

4.1.3. *Infant feeding practices*

Indicators of infant feeding practices are presented in Table 16 and Figure 11. Practically all infants (99.8%) were ever breasted during the first 24 months of age; most of them (88.4%) were exclusively breastfed during the first 6 months; 64.8% and 13.9% were breastfed up to 1 year and 2 years of age, respectively. Complementary feeding was introduced at mean age of 6 months, and in 21.2% it was introduced before.

Table 16. Feeding practices of infants in the cohort (N=475)

	N	%
Exclusive breastfeeding during the first 6 months of age	420	88.4
Breastfeeding *plus* formula feeding	53	11.2
Formula feeding	1	0.2
Ever breastfed		
Yes	474	99.8
No	1	0.2
Continued breastfed up to 1 y		
Yes	308	64.8
No	7	1.5
Missing information	160	3.4
Continued breastfed up to 2 y		
Yes	66	13.9
No	249	52.4
Missing information	160	33.7
Age (months) of complementary feeding introduction, mean (SD)	5.9 (0.9)	
Missing information	132	28.0

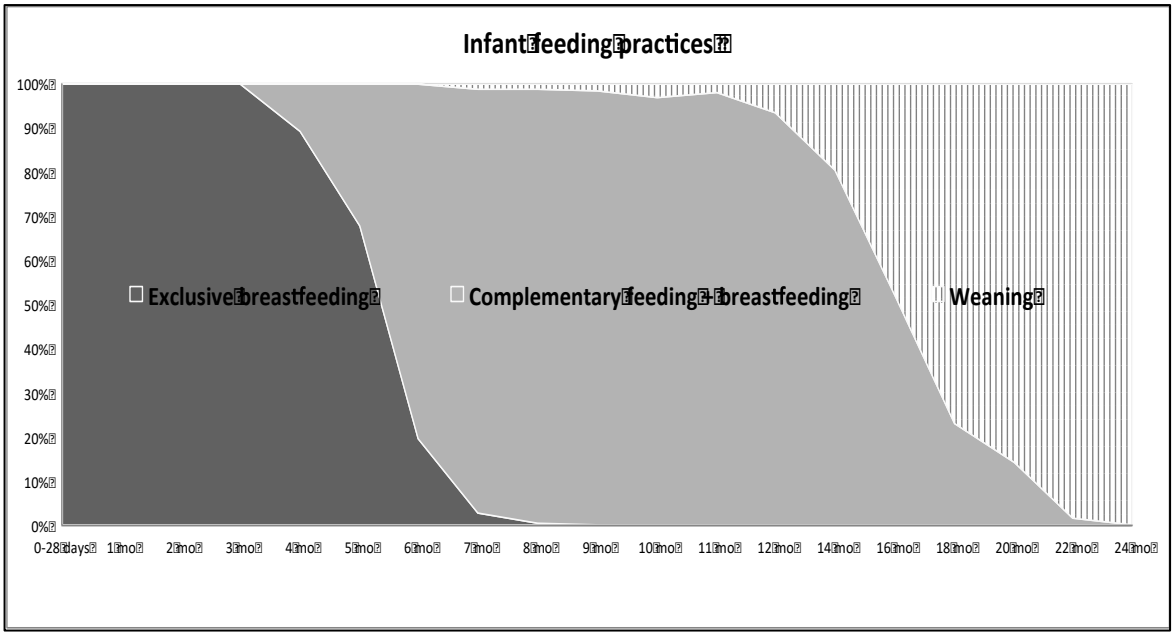


Figure 11. Feeding practices by age

4.1.4. *Acute infectious events and associated conditions*

The frequency of acute infectious events and associated conditions diagnosed at different ages are shown in Table 17. The acute infectious events registered include those occurring within the two weeks preceding each point of assessment.

The proportion of cases with acute diarrhea in the first 4 months of age was less than 5.2%; subsequently, the proportion increased (6.8% - 11.1%) and peaked at 20 months of age (15.1%) (Table 17)(Figure12). Most cases were watery diarrhea and very few were bloody diarrhea (dysentery), persistent diarrhea, or diarrhea complicated with dehydration.

Acute respiratory infections were infrequent in the first 2 months of age (0 – 3.2%); from 4 to 24 months of age the proportion increased, ranging from 19.9% to 32.8% (Table 17) (Figure 12). Most were upper respiratory infections (predominantly otitis media). The number of infants with severe pneumonia was low, and bronchiolitis/ wheezing were diagnosed after 2 months of age.

Malaria was infrequent (0.3% - 0.7%), only diagnosed after 12 months of age (Table 17).

From 187 infants with measured hemoglobin, 130 (69.5%) had anemia. Among these, 35.8% had mild anemia, 33.15% moderate anemia, and 0.5% severe anemia.

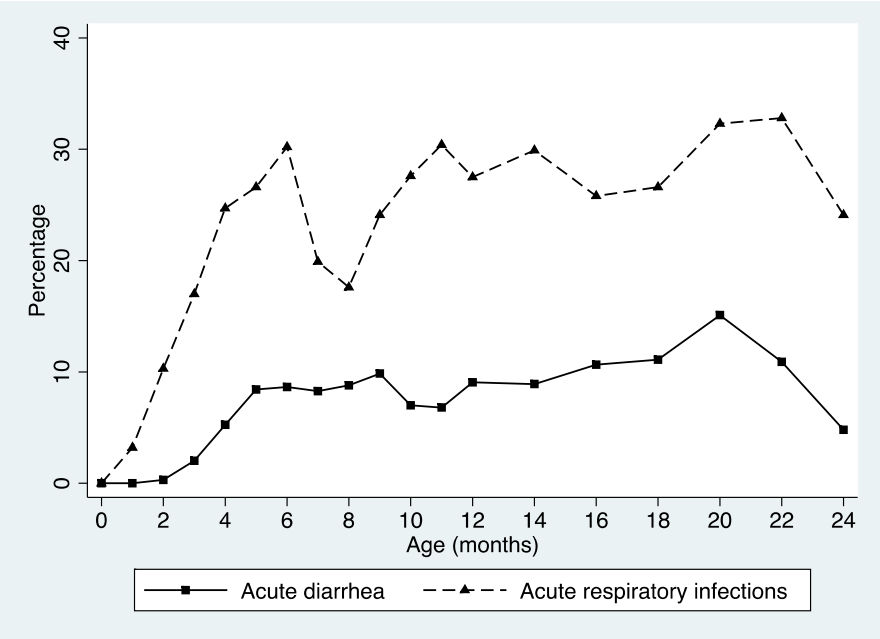


Figure 12. Proportion of infants with acute diarrhea and acute respiratory infant

Table 17. Acute infectious events and other conditions, by age (N = 475)

Age (months)	0	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
Number of observed infants	414	378	329	346	324	297	301	266	250	274	243	250	298	247	291	278	192	192	290
Acute diarrhea																			
n	0	0	1	7	17	25	26	22	22	27	17	17	27	22	31	31	29	21	14
%	0	0	0.3	2.0	5.2	8.4	8.6	8.3	8.8	9.8	7.0	6.8	9.0	8.9	10.6	11.1	15.1	10.9	4.8
Acute watery diarrhea (n)			1	7	17	25	22	20	21	24	14	17	25	21	28	26	26	19	12
Acute bloody diarrhea (n)							2			1	1			3	3	2	2	2	2
Persistent diarrhea (n)							1	1			1					2	1		
Diarrhea with dehydration (n)					3		1	1	1	2	1		2			1			
Acute respiratory infections																			
n	0	12	34	59	80	79	91	53	44	66	67	76	82	74	75	74	62	63	70
%	0	3.2	10.3	17.0	24.7	26.6	30.2	19.9	17.6	24.0	27.6	30.4	27.5	29.9	25.8	26,.6	32.3	32.8	24.1
Fast breathing pneumonia (n)			1	1	2	2	1	1	3	3	3	2	1	1		11	4	3	4
Severe pneumonia (n)					2		1	1				2	1		1	2		2	
Other respiratory conditions (n)																			
Otitis				5	8	14	15	17	17	22	31	33	27	26	17	6	21	27	12
Rhinitis								1		2	2	2	1	3	7	2	2	3	7
Sinusitis								1		1				1	1	1	1	1	1
Pharyngitis								1		1	2	1					2		
Bronchiolitis/wheezing			10	5	7	8	12	4	4	6	10	11	9	16	4		9	7	9
Whooping cough		3	5	4	1														
Wheezing																			
Malaria																			
n	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	2
%	0	0	0	0	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0.5	0.7
Fever (n)	0	0	0	0	1	1	2	5	2	2	2	0	0	3	2	0	2	1	0

Table 17. Acute infectious events and other conditions, by age (N = 475)(*continued*)

Age (months)	0	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
Other infectious events and conditions (n)																			
Conjunctivitis			1							1							1		
Oral infections		1						1							1	1			1
Urinary tract infection				1	1						1	2	1	3					1
Skin infection				1	1	2	3		4	3	2	2	5	5	2	3		2	5
Varicella				1		1										1		1	
HIV										1									
Sickle cell disease				1	1				1		2			1	1	1			
Anemia						1		1			2	1	1			1		1	130
Pica											1		2	1				1	
Vomiting										1	1			1	1	1		1	
Atopic dermatitis			8	16	2	6	4	7	1			1		1	1	1	1	2	1
GER symptoms			7	6	4	1	1						1						
Rectal prolapse																			1
Congenital heart disease					1					1					1				
Myocarditis (?)		1																	
Bone fracture	1				1														

4.1.5. *Nutritional status/anthropometry*

During the study period a total of 5888 anthropometry observations were made in the entire sample. From these, 550 observations were excluded because they were out of the range of scheduled target ages. Therefore, 5338 definitive anthropometry observations were used in the analysis. During the follow-up, each infant attended an average of 12.3 out of 19 scheduled points of assessment.

Anthropometric measurements at target ages are presented: attained weight, length, HC, and MUAC in Table 18, Table 19, and Figure 13; WAZ, LAZ, WLZ, BMIZ, HCZ, and MUACZ in Table 20 and Figure 14; LAD by sex in relation to WHO median reference in Table 21 and Figure 15, and WAVZ and LAVZ in Table 22 and Figure 16, Figure 17.

Attained growth

Infants during the neonatal period, evaluated at a mean of 10.1 days, had mean (SD) values close to WHO standards (WHO 2006a), with weight of 3.340 (0.459) kg in females and of 3.495 (0.489) kg in males, and length of 49.7 (1.89) cm in females and of 50.7 (1.86) cm in males (Table 18, Table 19, Figure 13); expressed as *z-scores*, these values correspond to -0.36 and -0.42 for WAZ, 0.03 and -0.04 for WLZ, and -0.73 and -0.68 for LAZ for females and males, respectively (Table 20).

After the neonatal period, WAZ, WLZ, and LAZ were above -1 SD with few fluctuations. At 24 months of age, females weighted 11.081 (1.121) kg and males 11.641 (1.134) kg, and measured 84.7 (3.25) cm and 86.37 (3.1) cm, respectively; expressed as *z-scores*, these values correspond to -0.36 and -0.41 for WAZ, 0.14 and -0.23 for WLZ, and -0.55 and -0.49 for LAZ, respectively (Table 20, Figure 14). During all study period, the means of WAZ, WHZ and LAZ averaged -0.29, 0.07, and -0.5, respectively.

Regarding LAD at birth, females were -1.52 cm and males -1.44 cm shorter than the median values of WHO standards (WHO 2006a), and this difference remained almost unchangeable until 24 months of age, with -1.52 cm for females and -1.33 cm for males (Table 21, Figure 15).

Table 18. Attained weight, length, head circumference, and mid-upper arm circumference at target ages, expressed in mean and standard deviation of their absolute values (N=475)

Age	Observations	Age at visit (months)		Weight (kg)		Length (cm)		HC (cm)		MUAC (cm)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0-28 days	410	0.33	0.18	3.40	.48	50.14	1.94	35.49	1.21	10.47	.94
1 month	378	1.02	0.17	4.26	.55	53.05	1.95	37.26	1.16	11.65	.89
2 months	327	2.06	0.18	5.33	.66	56.71	2.14	39.07	1.14	12.61	.95
3 months	336	3.06	0.17	6.13	.71	59.93	2.05	40.47	1.12	13.31	1.02
4 months	320	4.14	0.24	6.74	.81	62.37	2.18	41.67	1.22	13.56	1.05
5 months	293	5.12	0.21	7.19	.83	64.07	2.18	42.56	1.19	13.84	1.07
6 months	290	6.12	0.20	7.58	.89	65.87	2.19	43.38	1.27	14.03	1.16
7 months	260	7.08	0.17	7.86	.90	67.50	2.35	44.04	1.29	14.34	1.21
8 months	245	8.10	0.18	8.10	.97	68.97	2.40	44.50	1.32	14.44	1.21
9 months	271	9.10	0.16	8.29	.99	70.25	2.46	45.03	1.28	14.39	1.25
10 months	234	10.07	0.18	8.54	.98	71.59	2.45	45.33	1.30	14.50	1.26
11 months	254	11.06	0.18	8.62	1.01	72.52	2.55	45.41	1.21	14.38	1.24
12 months	289	12.08	0.20	8.90	1.06	73.73	2.65	45.75	1.20	14.52	1.23
14 months	243	14.05	0.20	9.47	1.08	75.88	2.66	46.21	1.27	14.83	1.18
16 months	282	16.06	0.18	9.89	1.03	78.01	2.74	46.45	1.25	14.82	.96
18 months	265	18.11	0.19	10.30	1.07	79.80	2.87	46.70	1.23	14.86	1.04
20 months	180	20.13	0.21	10.62	1.10	81.84	3.06	47.15	1.42	14.87	.98
22 months	180	22.10	0.22	10.91	1.13	83.91	3.22	47.54	1.18	14.79	.98
24 months	280	24.10	0.23	11.36	1.15	85.58	3.32	47.88	1.31	14.96	.97

HC head circumference, MUAC mid-upper arm circumference, SD standard deviation

Table 19. Attained weight, length, head circumference, by sex, at target ages, expressed in mean and standard deviation of their absolute values (N=475)

	Females			Males		
Age	Weight (kg)	Length (cm)	PC (cm)	Weight (kg)	Length	PC (cm)
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
0-28 days	3.3 (0.4)	49.7 (1.9)	35.2 (1.1)	3.5 (0.5)	50.7(1.8)	35.8 (1.2)
1 month	4.1 (0.5)	52.5 (1.8)	36.9 (1.2)	4.4 (0.5)	53.6 (1.9)	37.6 (1.1)
2 months	5.1 (0.6)	55.9 (1.9)	38.7 (1.1)	5.5 (0.6)	57.4 (2.0)	39. 4(1.0)
3 months	5.9 (0.6)	59.1 (1.9)	40.0 (1.0)	6.4 (0.7)	60.7 (1.9)	40.9 (1.0)
4 months	6.4 (0.7)	61.3 (1.9)	41.1 (1.1)	7.0 (0.7)	63.3 (1.9)	42.2 (1.1)
5 months	6.9 (0.8)	63.2 (1.9)	42.1 (1.1)	7.4 (0.0)	64.9 (2.0)	43.0 (1.1)
6 months	7.3 (0.8)	64.9 (2.0)	42.9 (1.2)	7.9 (0.8)	66.7 (1.9)	43.9 (1.2)
7 months	7.5 (0.8)	66.4 (1.9)	43.5 (1.1)	8.2 (0.9)	68.7 (2.2)	44.5 (1.2)
8 months	7.7 (0.9)	67.8 (2.1)	43.9 (1.2)	8.4 (0.9)	70.1 (2.0)	45.05 (1.2)
9 months	8.0 (0.9)	69.2 (2.1)	44.6 (1.1)	8.6 (0.9)	71.3 (2.2)	45.5 (1.3)
10 months	8.2 (0.9)	70.6 (2.2)	44.8 (1.2)	8.8 (0.9)	72.5 (2.2)	45.8 (1.2)
11 months	8.3 (0.9)	71.4 (2.2)	44.9 (1.2)	8.9 (1.0)	73.6 (2.4)	45.9 (1.0)
12 months	8.5 (0.9)	72.7 (2.4)	45.3 (1.1)	9.2 (1.0)	74.7 (2.4)	46.2 (1.1)
14 months	9.2 (1.0)	74.8 (2.4)	45.7 (1.2)	9.8 (1.0)	76.8 (2.4)	46.6 (1.2)
16 months	9.6 (0.9)	77.1 (2.6)	46.0 (1.2)	10.2 (1.0)	78.9 (2.6)	46.9 (1.1)
18 months	10.0 (1.0)	79.0 (2.9)	46.3 (1.2)	10.5 (1.0)	80.5 (2.7)	47.1 (1.1)
20 months	10.4 (1.0)	80.9 (3.0)	46.7 (1.1)	10.9 (1.1)	82.6 (2.8)	47. 6 (1.5)
22 months	10.6 (1.0)	83.2 (3.1)	47.1 (1.2)	11.2 (1.1)	84.6 (3.1)	47.9 (1.0)
24 months	11.1 (1.1)	84.7 (3.2)	47.4 (1.2)	11.6 (1.1)	86.4 (3.1)	48.4 (1.3)

Table 20. Attained z-scores of weight-for-age, length-for-age, weight-for-length, body mass index-for-age, head circumference-for-age, and mid-upper arm circumference-for-age at target ages, expressed in mean and standard deviation

	WAZ		LAZ		WLZ		BIMZ		HCZ		MUACZ	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0-28 days	-.39	.91	-.71	.92	-.01	.99	-.04	.96	.15	.93	a	a
1 month	-.27	.89	-.72	.93	.53	.95	.15	.91	.17	.89	a	a
2 months	-.19	.91	-.65	.95	.58	1.02	.21	.97	.19	.87	-.68	.01
3 months	-.12	.91	-.48	.90	.38	.96	.21	.96	.24	.84	-.00	.98
4 months	-.12	.95	-.47	.87	.32	1.03	.20	1.05	.30	.85	-.10	.96
5 months	-.15	.96	-.55	.91	.33	1.04	.21	1.05	.33	.86	-.06	.98
6 months	-.16	.97	-.49	.90	.25	1.04	.15	1.06	.39	.91	-.04	1.03
7 months	-.22	.95	-.44	.92	.10	1.05	.02	1.06	.42	.92	.11	1.06
8 months	-.29	.99	-.38	.94	-.03	1.03	-.10	1.04	.37	.94	.12	1.04
9 months	-.37	1.01	-.42	.96	-.15	1.04	-.18	1.05	.41	.93	.03	1.09
10 months	-.37	.96	-.41	.94	-.19	1.05	-.19	1.07	.33	.93	.07	1.09
11 months	-.50	.95	-.50	.97	-.29	.95	-.26	.96	.17	.85	-.04	1.09
12 months	-.45	.97	-.52	.99	-.27	.99	-.21	1.00	.17	.84	.03	1.06
14 months	-.32	.95	-.56	.96	-.09	.98	.01	.98	.11	.89	.24	.99
16 months	-.30	.87	-.55	.96	-.05	.89	.04	.90	-.00	.87	.20	.87
18 months	-.30	.88	-.64	.99	.00	.85	.12	.84	-.09	.85	.10	.82
20 months	-.37	.85	-.60	1.00	-.10	.85	.00	.87	-.01	.98	.05	.82
22 months	-.45	.86	-.51	1.05	-.30	.81	-.20	.84	.06	.80	-.11	.82
24 months	-.39	.84	-.51	1.03	-.20	.82	-.11	.85	.11	.88	-.07	.80

BIMZ body mass index –for-age z-score; HCZ head circumference-for-age z-score; LAZ length-for-age z-score, MUACZ mid-upper arm circumference-for-age z-score; WAZ weight-for-age z-score; WLZ weight-for-length z –score; [a] not calculated by the WHO Anthro version 3.2.2

Table 21. Length- for- age difference (LAD) by sex, at target ages, expressed in cm.

Age	Females LAD (cm)	Males LAD (cm)	
	Mean	Mean	*p* value
0-28 days	-1.52	-1.44	0.648
1 month	-1.38	-1.26	0.599
2 months	-1.28	-1.25	0.902
3 months	-0.74	-0.79	0.815
4 months	-0.98	-0.97	0.940
5 months	-0.96	-0.93	0.903
6 months	-0.95	-0.89	0.829
7 months	-0.89	-0.60	0.295
8 months	-0.87	-0.43	0.160
9 months	-0.81	-0.72	0.735
10 months	-0.98	-0.76	0.518
11 months	-1.23	-0.78	0.154
12 months	-1.47	-0.97	0.123
14 months	-1.40	-0.86	0.127
16 months	-1.37	-1.36	0.979
18 months	-1.70	-1.77	0.849
20 months	-1.62	-1.68	0.901
22 months	-0.62	-1.32	0.196
24 months	-1.52	-1.33	0.669

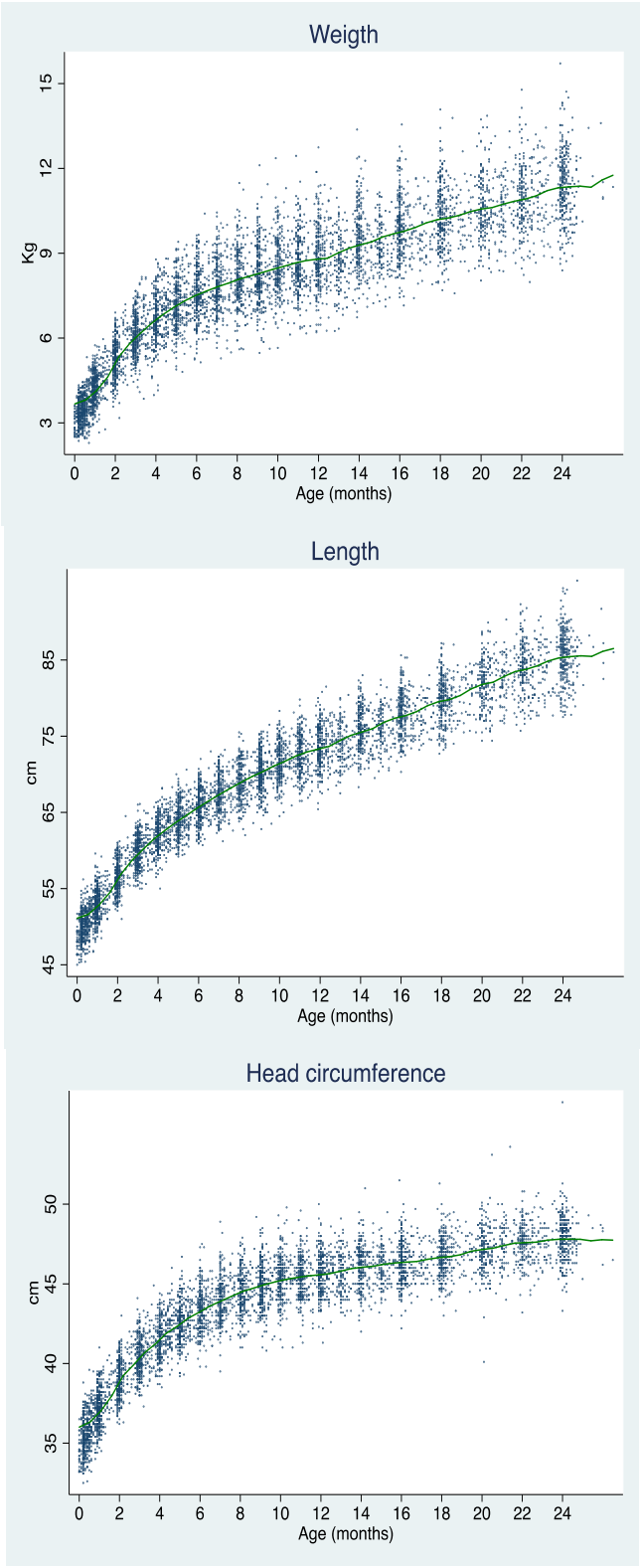


Figure 13. LOWESS-fitted curves applied to longitudinal data on weight, length and head circumference curves (N=5880 data points)

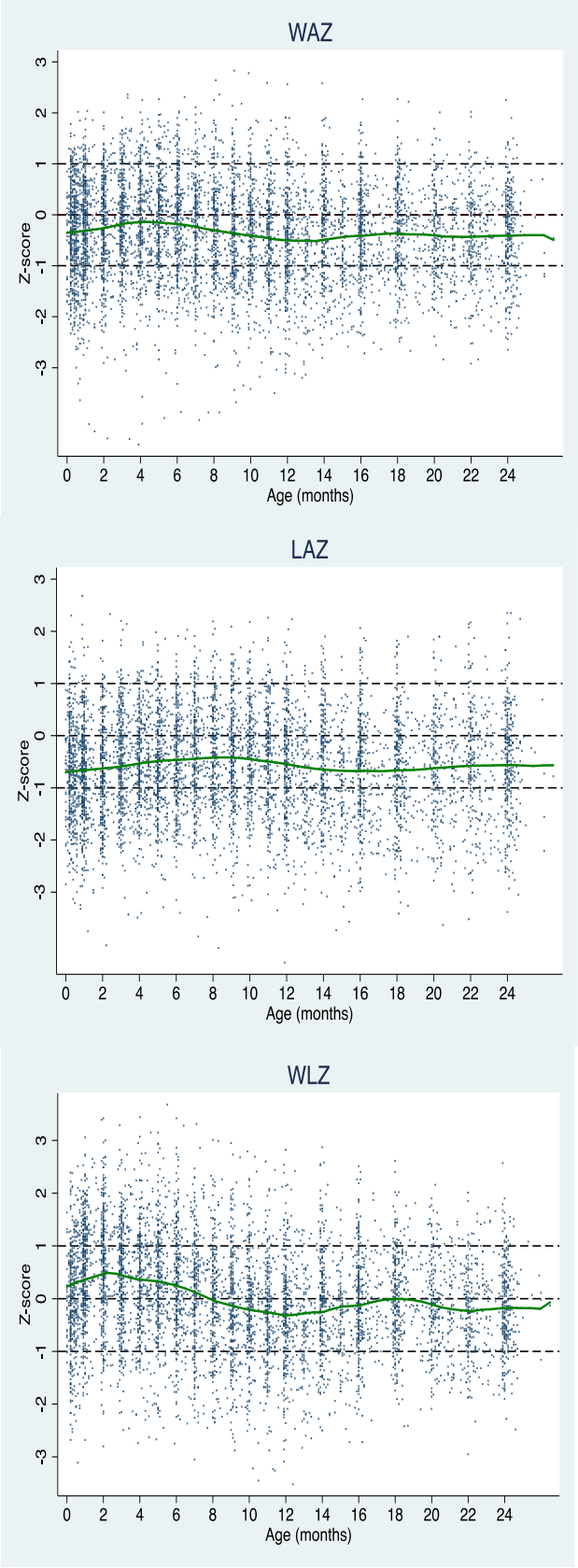


Figure 14. LOWESS-fitted curves applied to longitudinal data of of weight-for-age, length-for-age and weight-for-length *z-scores* (N=5880 data points)

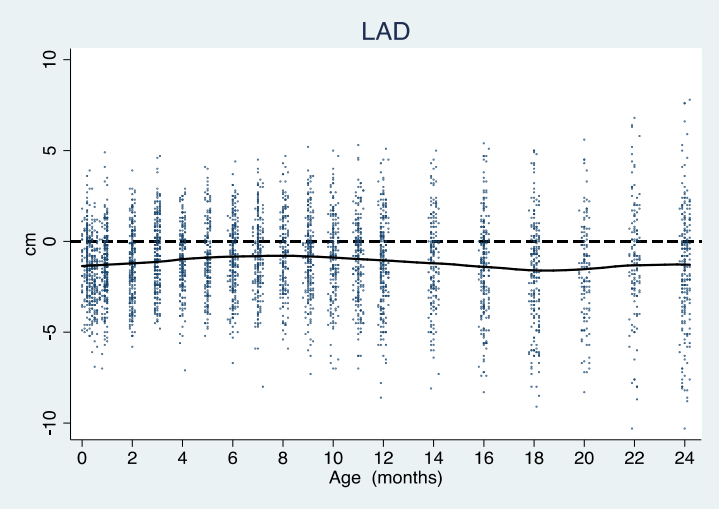


Figure 15. LOWESS-fitted curves applied to longitudinal data on length for age difference (N=5880 data points

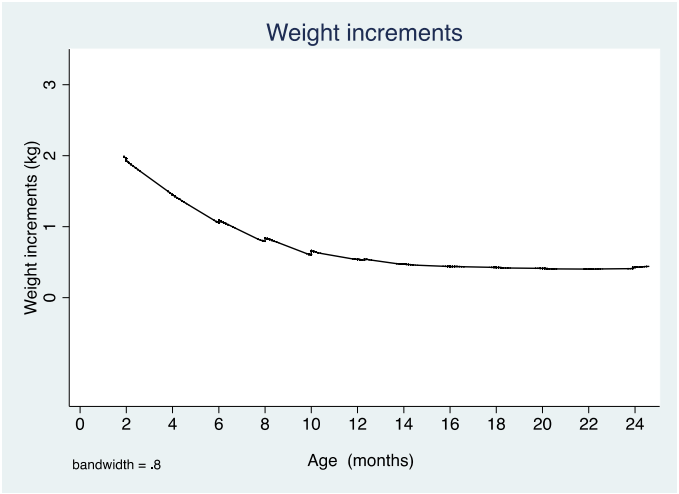
Growth velocity

A total of 2514 two-month observations with calculated velocities, along the 12 points of assessment, were analyzed. With exception of the first interval (52.2 days), the intervals were always very close to 60 days (Table 22).

Weight and length gain curves (Figure 16, Figure 17) showed a decelerating rate from neonatal period, reaching a near-plateau by the end of the first year, and continued to decelerate gradually through the second year, similar to standard velocity patterns (WHO 2009a).

The mean (SD) weight gain of every two-month interval was of 740 g (626). The mean WAVZ along the study period was close to the standard values (WHO 2009a) and between -0.66 and 0.33 (Figure 16).

The mean (SD) length gain of every two-month interval was of 3.15 cm (1.9). The mean LAVZ of -1.34 from 0 to 2 months of age was below the standard values (WHO 2009a); subsequently, mean LAVZ increased varying between -0.41 and 0.27 (Figure 17).



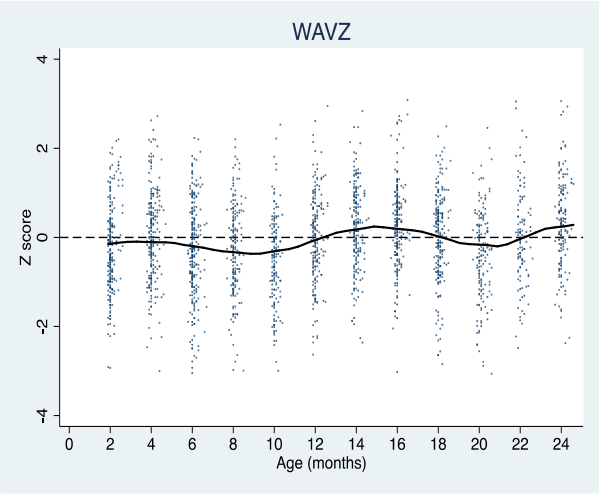
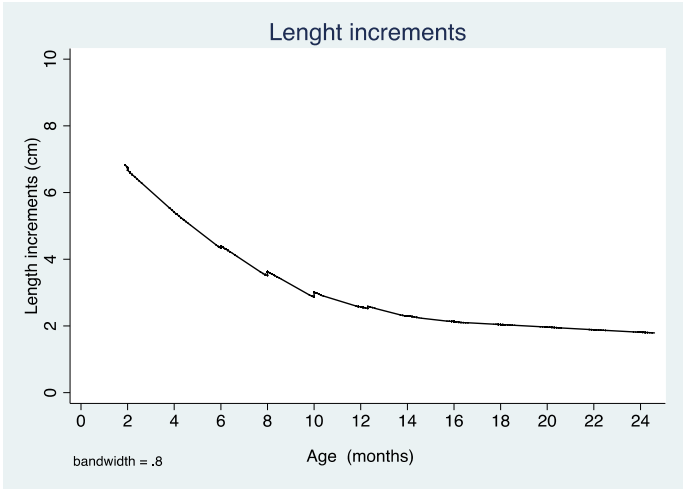


Figure 16. LOWESS-fitted curves applied to longitudinal data of weight increments (left) and weight velocity *z-scores* (right) (N=5880 data points)



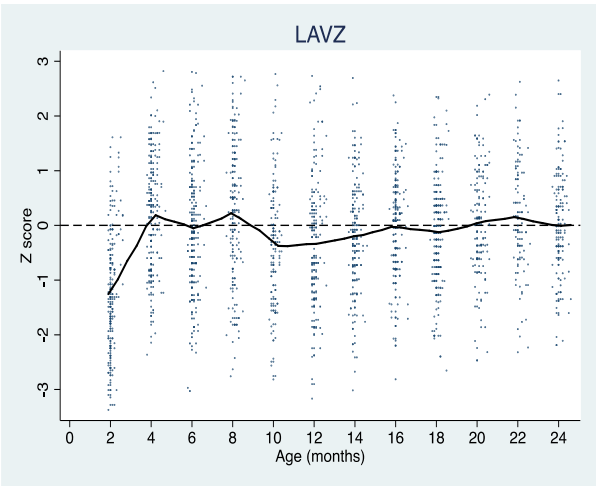


Figure 17. LOWESS-fitted curves applied to longitudinal data of length increments (left) and length velocity *z-scores* (right) (N=5880 data points).

Table 22. Weight and length increments in two-month intervals and respective velocities z-scores expressed in mean and standard deviations.

Age	Observations	Interval (days)		Weight increments (g)		WAVZ		Length increments (cm)		LAVZ	
	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0-2 months	280	52.2	7.8	1972.0	481	-.22	.98	6.69	1.34	-1.34	1.19
2-4 months	275	63.6	8.4	1416.7	393	.06	1.08	5.55	1.17	.18	1.23
4-6 months	258	60.3	8.9	835.0	320	-.32	1.11	3.60	1.05	-.06	1.20
6-8 months	211	60.4	8.1	576.8	338	-.39	1.34	3.22	1.08	.27	1.29
8-10 months	168	60.1	7.6	373.1	327	-.66	1.30	2.38	0.99	-.41	1.22
10-12 months	214	60.9	8.1	470.9	288	-.03	1.01	2.24	0.94	-.33	1.20
12-14 months	218	60.3	8.1	515.8	290	.24	.96	2.16	0.79	-.21	1.01
14-16 months	220	60.9	7.3	506.6	339	.28	1.07	2.19	0.87	.00	.97
16-18 months	238	61.9	7.2	417.8	329	-.01	1.07	1.95	0.77	-.12	.96
18-20 months	160	62.7	7.4	267.1	385	-.46	1.23	1.96	0.78	.03	.98
20 -22 months	112	58.9	6.3	364.4	403	-.09	1.20	1.97	0.82	.16	1.01
22-24 months	160	59.7	6.4	489.8	357	.33	1.06	1.77	0.80	.026	1.00

LAVZ length velocity z-score; SD standard deviation; WAVZ weight velocity z-score

Wasting and stunting

At neonatal period, 11.3% of infants were at risk of wasting (WLZ <-1SD >-2) and 3.3% were moderate-to-severe wasted (WLZ ≤-2 SD). After the neonatal period two peaks seemed to occur with slopes from 7 up to 12 months of age and from 18 up to 22 months of age. At 24 months of age, 15.1% of infants were at risk of wasting and 0.72% were moderate-to-severe wasted (Table 23 and Figure 18).

At neonatal period, 29.7% of infants were at risk of stunting (LAZ ≤-1SD >-2) and 7.8% were moderate-to-severe stunted (LAZ ≤-2 SD). After the neonatal period, stunting prevalence remained high along the study period. At 24 months of age, 19.9% of infants were at risk of stunting and 8.9% were moderate-to-severe stunted (Table 23 and Figure 18).

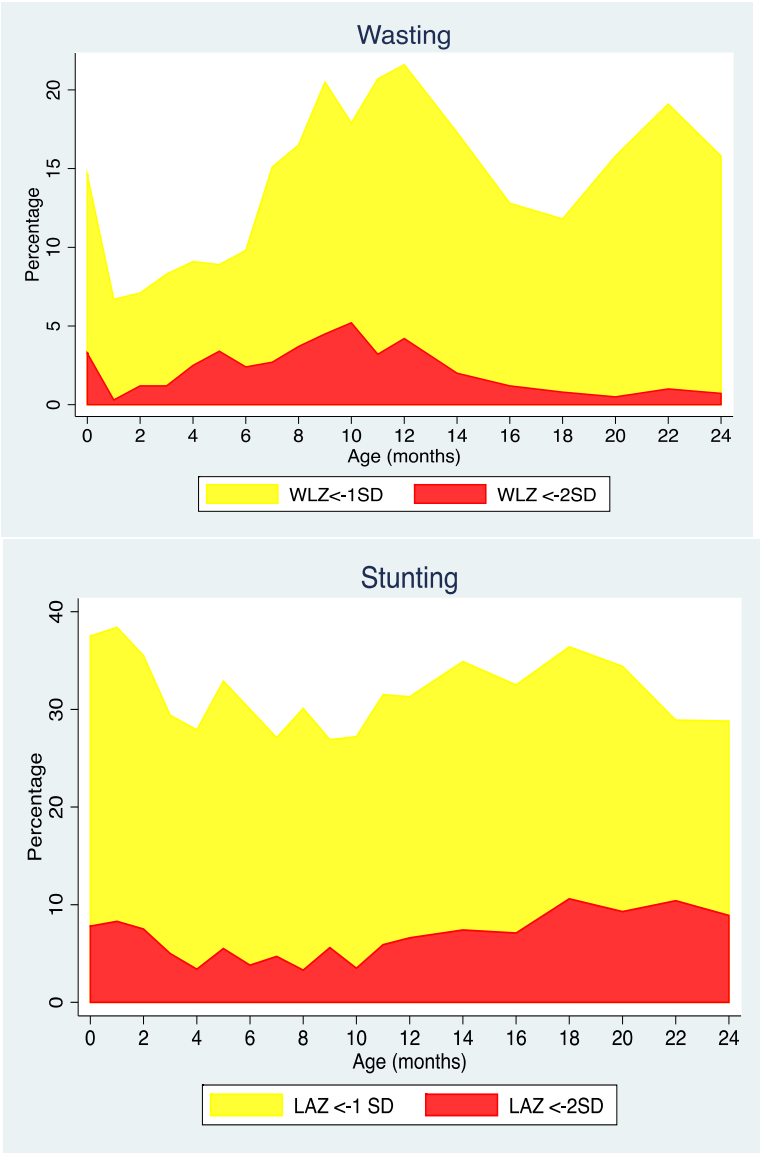


Figure 18. Frequency of wasting and stunting during the study period, considering mild (<-1 SD) and moderate/severe (<-2 SD) degrees

Table 23. Frequency of wasting and stunting during the study period, considering mild (<-1 SD) and moderate <-2 SD) and severe (<-3SD) degrees

	N	Wasting						Stunting					
		≤ -1SD		≤ -2 SD		≤ -3SD		≤ -1SD		≤ -2 SD		≤ -3SD	
		n	%	n	%	n	%	n	%	n	%	n	%
0-28 days	398	58	14.6	13	3.27	1	0.2	149	37.5	31	7.8	3	0.7
1 month	374	25	6.7	1	0.3	0	0	143	38.4	31	8.3	3	0.8
2 months	322	23	7.1	4	1.2	0	0	114	35.5	24	7.5	1	0.3
3 months	336	28	8.3	4	1.2	0	0	99	29.4	17	5.0	2	0.6
4 months	318	29	9.1	8	2.5	0	0	89	27.9	10	3.4	3	0.9
5 months	291	26	8.9	10	3.4	0	0	96	32.9	16	5.5	1	0.3
6 months	286	28	9.8	7	2.4	0	0	86	30.0	11	3.8	2	0.7
7 months	258	39	15.1	7	2.7	1	0.4	70	27.1	12	4.7	1	0.4
8 months	242	40	16.5	9	3.7	0	0	73	30.1	8	3.3	1	0.4
9 months	268	55	20.5	12	4.5	0	0	72	26.9	15	5.6	3	1.1
10 months	228	41	17.9	12	5.2	2	0.9	62	27.2	8	3.5	1	0.4
11 months	251	52	20.7	8	3.2	1	0.4	79	31.5	15	5.9	2	0.8
12 months	287	62	21.6	12	4.2	2	0.7	90	31.3	19	6.6	2	0.7
14 months	243	42	17.3	5	2.0	0	0	85	34.9	18	7.4	2	0.8
16 months	280	36	12.8	5	1.2	0	0	91	32.5	20	7.1	0	0
18 months	261	31	11.8	2	0.8	0	0	95	36.4	28	10.6	3	1.1
20 months	183	29	15.8	1	0.5	0	0	63	34.4	17	9.29	1	0.5
22 months	183	35	19.1	2	1.0	0	0	53	28.9	19	10.4	2	1.0
24 months	278	44	15.8	2	0.72	0	0	80	28.8	25	8.9	2	0.2

4.1.6. *Neurodevelopment status*

A total of 1440 BINS were applied during the study period at 5 points of assessment. From the entire sample (N=472), 344 (72.3%), 285 (60.4%), 271 (57.4%), 257 (54.5%), and 283 (60.0%) infants were screened at 3, 6, 12, 18, and 24 months of age, respectively.

Regarding BINS median scores values at each point of assessment (Table 24), infants showed a suboptimal performance.

Regarding risk of poor development at each point of assessment (Table 25), the propotion of infants at high risk increased with age.. At 3 months of age, no infant was classified at high risk; at 6 months of age, only 12.3% of infants were at high risk. Notably, from 12 months to 24 months of age, the proportions of infants at high risk markedly increased ranging from 21.4% to 27.7% (Table 25).

Regarding the developmental areas at each point of assessment (Table 26), almost all infants (96.5% to 100%) performed all neurologic tasks. The performance of receptive tasks was variable, from 92.4% of infants at 3 months to 49.8% at 18 months of age performing all tasks. The performance of expressive tasks was highly variable with less than half (between 1.4% and 43.5%) of infants performing all tasks. The performance of all cognitive tasks varied between 38.4% and 61.9% in infants between 6 and 18 months of age. At 24 months of age only 2.8% of infants performed the single task on cognitive domain (places three pieces in puzzle board). It should be noted that the lower scores in expressive and cognitive areas found at 24 months of age (Table 26) concern more complex tasks.

Table 24. BINS scores at each point of assessment. Pink shadow represents infants at poor risk for poor development and gray shadow represents infants at high risk for poor development.

Age	Score	N	%
3 months (N=346)	11 (maximum)	8	2.3
	10	66	19.1
Score median (min. max): 9 (7-11)	9	226	65.3
	8	38	10.9
	7	8	2.3
	≤6	0	0
6 months (N=287)	13 (maximum)	81	28.2
	12	116	40.4
Score median (min. max): 12 (6-11)	11	54	18.8
	10	25	8.7
	9	7	2.4
	8	3	1.0
	≤7	1	0.3
12 months (N=271)	11(maximum)	41	15.1
	10	81	29.9
Score median (min. max): 9 (6-11)	9	74	27.3
	8	47	17.3
	7	21	7.7
	≤6	7	2.6
18 months (N=257)	11(maximum)	13	5.0
	10	52	20.2
Score median (min. max): 9 (5-11)	9	70	27.2
	8	67	26.1
	7	43	16.7
	6	10	3.9
	≤5	2	0.8
24 months (N=283)	13(maximum)	1	0.35
	12	10	3.5
Score median (min. max):10 (5-13)	11	100	35.3
	10	63	22.2
	9	36	12.7
	8	40	14.1
	7	24	8.5
	6	6	2.1
	≤5	3	1.1

Table 25. Proportion of infants at low and high risk of poor development (BINS) at each point of assessment

	3 montsh (N=356)	6 months (N=285)	12 months (N=271)	18 months (N=257)	24 months (N=283)
	N (%)	N (%)	N (%)	N (%)	N (%)
Low risk	336 (97.9)	250 (87.7)	196 (72.3)	202 (78.2)	210 (74.1)
High risk	0	35 (12.3)	75 (27.7)	55 (21.4)	73 (25.8)

Table 26. Proportions of infants performing tasks in each BINS's developmental areas.

Areas	3 months (N=346)		6 months (N=285)		12 months (N=271)		18 months (N=257)		24 months (N=283)	
	Number of items	n (%)	Number of items	n (%)	Number of items	n (%)	Number of items	n (%)	Number of items	n (%)
Neurologic	1	0 (0)	1	0	1	0	1	257 (100)	1	283 (100)
	2	0 (0)	2	0	2	0				
	3	1 (0.3)	3	2 (0.7)	3	271 (100)				
	4	11 (3.2)	4	283 (99.3)						
	5	332 (96.5)								
Receptive	1	26 (7.6)	1	285 (100)	1	60 (22.1)	1	128 (49.8)	1	69 (24.4)
	2	318 (92.4)			2	203 (74.9)			2	201 (71.0)
Expressive	1	16 (4.65)	1	1 (0.35)	1	52 (19.2)	1	0	1	0
	2	246 (71.5)	2	8 (2.8)	2	110 (40.6)	2	0	2	0
	3	72 (20.9)	3	33 (11.6)	3	99 (36.5)	3	22 (8.6)	3	0
	4	8 (2.3)	4	119 (41.7)			4	105 (40.9)	4	16 (5.6)
			5	124 (43.5)			5	111 (43.2)	5	27 (9.5)
							6	19 (7.4)	6	45 (15.9)
									7	69 (24.4)
									8	122 (43.1)
									9	4 (1.4)
Cognitive			1	6 (2.1)	1	2 (0.7)	1	13 (5.0)	1	8 (2.8)
			2	100 (35.0)	2	165 (60.9)	2	85 (33.0)		
			3	174 (61.0)	3	104 (38.4)	3	159 (61.9)		

4.1.7. *Enteric parasites*

During the study period 1674 stool samples were examined for intestinal parasites. The frequencies of intestinal pathogenic parasites at each point of assessment are shown in Tables 27 and 28 and Figures 19 and 20.

Enteric parasites were not detected at 3 months of age; subsequently, the frequency of parasitic infection increased progressively with age, from 4.9 % at 6 months to 43.8% at 24 months. Single parasitic infections predominated. Co-infections were detected from 12 months of age on and their frequency increased up to the end of the study. The three most frequent pathogenic parasites, either as single or multiple infections, were by decreasing order *Giardia lamblia*, STH, and *Cryptosporidium* spp. *Entamoeba histolytica*/*dispar* complex was not detected (Table 27 and Figure 19).

Giardia lamblia was detected in 35.1% of infants, in at least one stool sample. *Giardia* detection increased with age, from 5% at 6 months to 23.9% at 24 months. The median of age of first *Giardia* detection was 16 months. Twenty two percent of infants had one episode of *Giardia* infection, 10.85% two episodes, and 1.8% three or more episodes, either as single or multiple agent infection (Table 28 and Figure 20). The molecular characterization of *Giardia lamblia* was performed in 25 stool samples (Table 29). Twenty (80%) of *Giardia* isolates belonged to Assemblage B; from these, 9 (45%) samples were subtype BIII. Five (20%) of *Giardia* isolates belonged to Assemblage A.

Cryptosporidium spp. was detected in 14.7% of infants in at least one stool sample. *Cryptosporidium* spp detection ranged from 2.5% to 7.1% during the study period. The median of age of first *Cryptosporidium* spp detection was 12 months, with an earlier peak of detection frequency in relation to *Giardia* and STH infections. Thirteen percent of infants had one episode of *Cryptosporidium* spp infection, and 1.05% had two or more episodes, either as single or multiple agent infection (Table 28 and Figure 20).

STH were detected in 30.4% of infants in at least one stool sample. More specifically, prevalence of *Ascaris lumbricoides* ranged from 4.3% to 20.3%, *Trichuris trichiura* from 1.2% to 3.1%; and hookworms were not detected. STH detection increased with

age, from 4.35% at 9 months to 27.5% at 24 months. The median age of first STH detection was 16 months. Eighteen percent of infants had one episode of STH infection, 8.3% two episodes and 3.8% three or more episodes, either as single or multiple agent infection (Table 28 and Figure 20). Other intestinal pathogenic parasites detected are shown in Table 27.

In 41.2% of infants no enteric parasite was detected at any point of assessment during the study period.

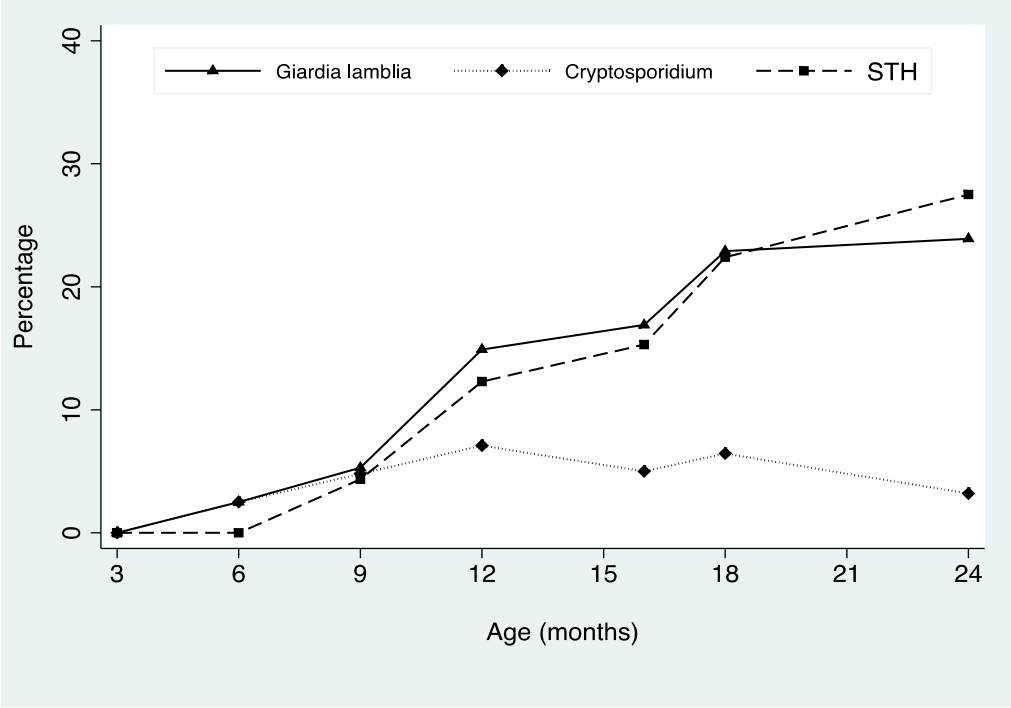


Figure 19. Prevalence of enteric parasite by age

Table 27. Frequency of intestinal pathogenic parasites by age (N=475)

	3 months N=295	6 months N=243	9 months N=207	12 months N=268	16 months N=249	18 months N=161	24 months N=251
Infected, % (n)	0 (0)	4.9 (12)	14.5(30)	30.2 (81)	33.3 (83)	41.6(67)	43.8(110)
Single infections, % (n)	0 (0)	4.9 (12)	14.5(30)	25.7(69)	30.1(75)	32.3(52)	33.9(85)
Protozoa, % (n)							
Giardia lamblia	0 (0)	2.5 (6)	5.3(11)	12.7 (34)	14.45 (36)	15.52 (25)	17.1 (43)
Cryptosporidium spp	NA	2.5 (6)	4.8(9)	3.7 (10)	3.2(8)	3.7 (6)	1.6 (4)
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 (1)	0.7 (2)	0 (0)	1.2 (2)	0 (0)
Soil transmitted helminths (STH), % (n)							
Ascaris lumbricoides	0 (0)	0 (0)	4.3(9)	7.1 (19)	12.4 (31)	8.1(13)	12.7 (32)
Trichuris trichiura	0 (0)	0 (0)	0 (0)	1.5 (4)	0(0)	3.1(5)	1.9 (5)
Necator americanus /Ancylostoma duodenale	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		
Strongyloides stercoralis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.6(1)	(0)
H. nana	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		0.4 (1)
Multiple infections, % (n)	0 (0)	0 (0)	0 (0)	4.5(12)	3.2(8)	9.3(15)	9.95(25)
Giardia lamblia + STH	0 (0)	0 (0)	0 (0)	1.5(4)	1.2 (3)	3.7(6)	5.6 (14)
Cryptosporidium spp. + STH	0 (0)	0 (0)	0 (0)	2.2(6)	0.4(1)	0.6(1)	1.2 (3)
Giardia lamblia + Cryptosporidium spp.	0 (0)	0 (0)	0 (0)	0.75(2)	0.8(2)	1.9(3)	0.4(1)
Ascaris lumbricoides + Trichuris trichiura	0 (0)	0 (0)	0 (0)	0 (0)	0.4(1)	1.2(2)	1.9(5)
Giardia lamblia + Cryptosporidium spp + STH	0 (0)	0 (0)	0 (0)	0 (0)	0.4(1)	0 (0)	0 (0)
Ascaris lumbricoides + Trichuris trichiura + one protozoa	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.9(3)	0.8(2)

Table 27. Frequency of intestinal pathogenic parasites by age (N=475) (*continued*)

	3 months	6 months	9 months	12 months	16 months	18 months	24 months
	N=295	N=243	N=207	N=268	N=249	N=161	N=251
Total *Giardia lamblia* (single or multiple), % (n)	0 (0)	2.5 (6)	5.3 (11)	14.9(40)	16.9(42)	22.9(37)	23.9 (60)
Total *Cryptosporidium* spp (single or multiple), % (n)	0 (0)	2.5 (6)	4.8 (9)	7.1 (18)	5.0 (11)	6.45 (10)	3.2 (8)
Total STH (single or multiple), % (n)	0 (0)	0 (0)	4.35 (9)	12.3 (33)	15.3(38)	22.4 (36)	27.5 (69)

STH soil transmitted helminths

Table 28. Cumulative data on enteric parasitic infections, including age at first detection, total number of infections, and number of episodes of infection

	Age at first detection	Total number of infections	Number of episodes of infection % (N)				
	Median (min-max)	% (N)	1	2	3	4	≥5
Never infected, % (n)		41.2 (160)					
Ever infected, % (n)		58.5 (227)					
Giardia lamblia infections (single or multiple)	16.0 (5.9-24.7)	35.1 (136)	22.4 (87)	10.8 (42)	1.0 (4)	0.5 (2)	0.25 (1)
Cryptosporidium spp. infections (single or multiple)	12.15 (5.9-24.1)	14.7 (57)	13.7 (53)	0.8 (3)	0.25 (1)	0.0(0)	0.0(0)
STH infections (single or multiple)	16.2 (8.8-24.7)	30.4 (118)	18.3 (71)	8.3 (32)	3.3 (13)	0.5 (2)	0.0(0)

STH soil transmitted helminths

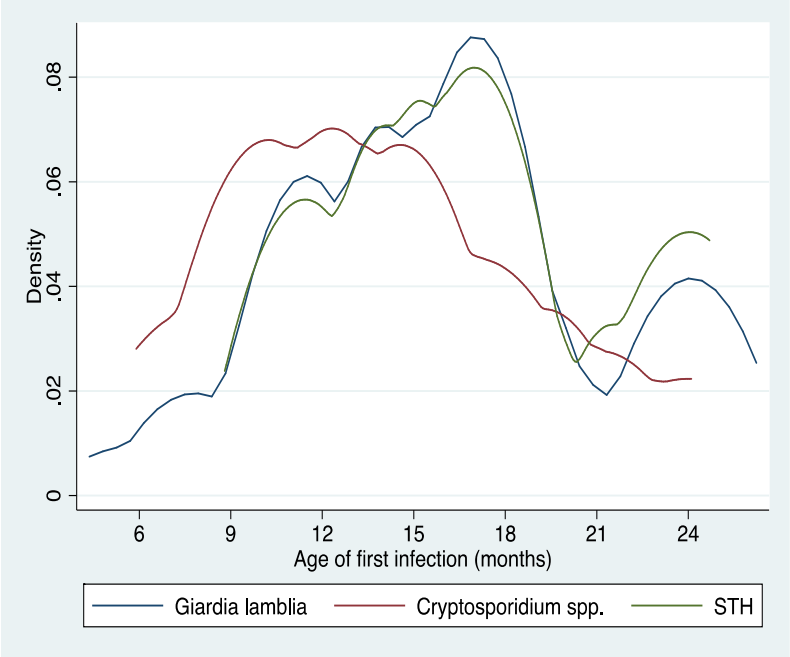


Figure 20. Time of first detection of parasitic infections

Table 29. Molecular characterization of *Giardia lamblia* (N= 25 samples)

	Gene	Genotype	Subgenotype	Genbank	Identities
26	SSU-rRNA	B		KM388530.1	100%
33	SSU-rRNA	B		KR048494.1	100%
78	SSU-rRNA	B		KR048494.1	100%
88	SSU-rRNA	B		KR048494.1	100%
100	β-Giardin	B	III	DQ090529.1	99%
110	β-Giardin	B	III	DQ090529.1	99%
113	β-Giardin	B	III	DQ090527.1	99%
117	SSU-rRNA	B		KR048494.1	100%
119	β-Giardin	A		KM190682.1	99%
157	SSU-rRNA	B		KR048494.1	100%
165	SSU-rRNA	B		KR048494.1	100%
196	β-Giardin	B	III	DQ090526.1	99%
205	β-Giardin	B	III	AB480877.1	99%
236	β-Giardin	B	III	DQ090524.1	100%
265	β-Giardin	B	III	DQ090527.1	99%
276	β-Giardin	B	III	DQ090527.1	99%
290	SSU-rRNA	B		KR048494.1	100%
335	SSU-rRNA	B		KM388530.1	99%
343	β-Giardin	A		KM190682.1	99%
359	β-Giardin	A		KM190682.1	99%
380	SSU-rRNA	A		LN875379.1	99%
390	SSU-rRNA	B		KR048494.1	100%
391	SSU-rRNA	A		LN811460.1	98%
428	SSU-rRNA	B		KR048494.1	100%
433	β-Giardin	B	III	DQ090523.1	99%

4.1.8. *Fecal biomarkers of intestinal function*

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 (Garzon 2017). Thus, a final sample of 80 infants for fecal biomarker analysis was included. Fecal A1AT was tested in 80 infants and S100A12 tested in 74 infants due to unsolvable technical constraints. The fecal A1AT median (interquartile range) level was 165.1 (66.0 - 275.6) μg/g and of S100A12 was 2.87 (2.41 - 3.92) μg/g. There were not differences of fecal biomarkers between sex (Table 30). Distribution of their values is shown in Figure 21.

Table 30. Alpha 1 antitrypsin (A1AT) and S100A12 stool biomarkers

	A1AT (μg/g)			S100A12 (μg/g)		
	N	median (P25-P75)	p value	N	median (P25-P75)	p value
All	80	165.1 (66.0-275.6)		74	2.9(2.4-3.9)	
Females	46	151.4 (66.0-272.4)	0.669	42	3.0 (2.6-4.4)	0.340
Males	34	175.9 (66.0-291.4)		32	2.8 (2.3- 3.5)	

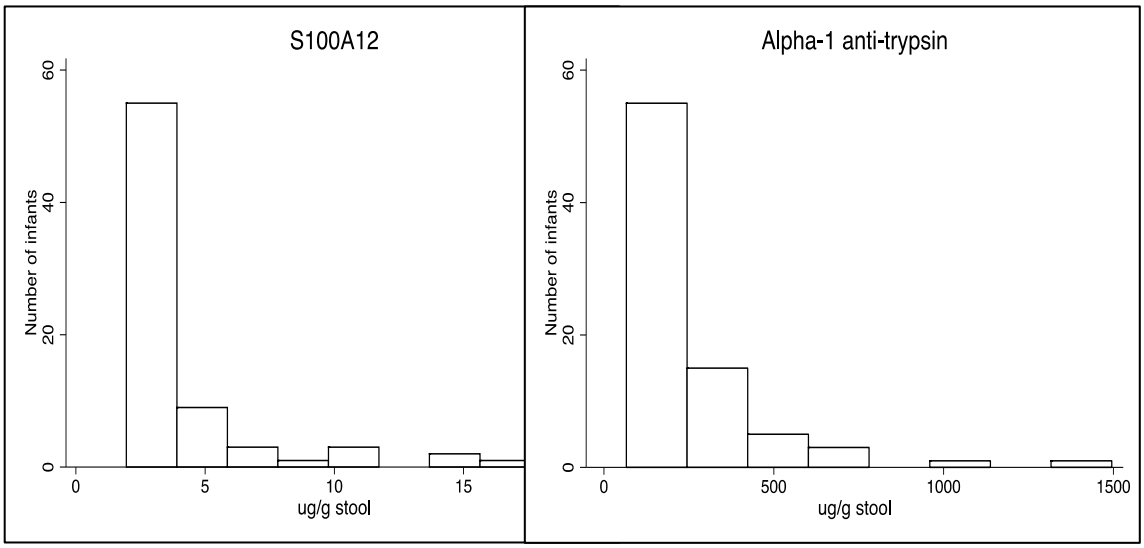


Figure 21. Distribution of fecal S100A12 and alpha 1 anti-trypsin

4.2. Associations between enteric parasites and outcomes

In the next sections the results of univariable and multivariable analyses of associations between enteric parasitic infections (explanatory variable) intestinal barrier function, nutritional status/anthropometry, and neurodevelopment (outcome variables) are presented. For the analyses, other explanatory variables including socio-economic status, feeding practices, mother's height, and acute diarrhea were considered.

4.2.1. *Enteric parasitic infection and intestinal barrier function*

A descriptive statistics of both biomarkers values by the categories of several variables is shown in Table 31. S100A12 values were significantly higher in infants infected by any enteric parasite, *Giardia lamblia* and STH infections. A1AT values were significantly higher in wasted and stunted infants (Table 31).

Table 31. 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)	
STH						
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)	

Table 31. Fecal values of alpha1-anti-trypsin (A1AT) and S100A12 at 24 months of age, considering sex, parasite agent, and nutritional status categories (*continued)*

	A1AT (µg/g)			S100A12 (µg/g)		
	n	Median (P_{25} – P_{75})	*p* value	n	Median (P_{25} – P_{75})	*p* value
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

Univariable analysis

Results of univariable analysis are shown in Table 32. Significant associations of fecal A1AT levels with mean LAZ values, wasting and stunting at 24 months were found. Significant association of fecal S100A12 level with number of parasites at 24 months was found. Variables with a p-value <0.250 were selected for the multivariable analysis.

Multivariable analysis

Results from the multivariable models are shown in Table 33. Weak evidence of associations of fecal S100A12 levels with current *Giardia duodenalis* infection (p = 0.080) and with current STH infection (p = 0.089) were found. This suggests an increasing tendency of 23.6% and 24.1% in fecal S100A12 levels in infants infected with *Giardia duodenalis* and STH, respectively.

A weak evidence of an association of fecal A1AT levels with current parasitic infection of any etiology (p = 0.089) was found, suggesting an increasing tendency of 33.6% higher A1AT levels in infected infants.

Significant associations of fecal A1AT levels with wasting (p = 0.006) and stunting (p = 0.044) at 24 months of age were found; specifically, fecal A1AT levels were twice higher in wasted infants and 50% higher in stunted infants.

Table 32. 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; 0.27)	0.181	0.10 (-0.00; 0,20)	0.054
nr. *Giardia lamblia*	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
Giardia lamblia	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

Table 32. 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 (*continued)*

Variables	ln A1AT		ln S100A12	
	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value[d]
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

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.

Table 33. 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 lamblia 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

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.

4.2.2. *Enteric parasitic infection and nutritional status/anthropometry*

Univariable analysis

- Attained growth

The WLZ was significantly associated with age, MPI, exclusive breastfeeding, acute diarrhea, ARI, malaria, enteric infections by any parasite, *Giardia lamblia* infection, and STH infections (Table 34).

The LAZ was significantly associated with living in Lembá district, improved sanitation, electricity, finished floor, cooking with solid fuel, MPI score, poor household, mother's height, acute diarrhea, enteric parasites infections, *Giardia lamblia* infection, and STH infections (Table 34).

The LAD was significantly associated with age, improved sanitation, electricity, finished floor, cooking with solid fuel, MPI score, poor household, mother's height, duration of breastfeeding, acute diarrhea, enteric parasites infections, *Giardia lamblia* infection, and STH infections (Table 34).

- Growth velocity

The WAVZ was significantly associated with age, improved sanitation, electricity, MPI, mother's height, acute diarrhea, and *Cryptosporidium* spp. infection (Table 34).

The LAVZ was significantly associated with age, improved sanitation, improved water source, electricity, MPI score, mother's height, *Cryptosporidium* spp. infection, and total number of parasites (Table 35).

- Wasting and stunting

The odds of wasting (WLZ $\leq$ -1) was significantly higher in infants with more age, living in Lembá district, in household with higher MPI score, acute diarrhea, ARI, and malaria; the odds of wasting was significantly lower in infants with exclusive breastfeeding and later introduction of complementary feeding (Table 36).

The odds of stunting (LAZ $\leq$ -1) was significantly higher in infants living in Lembá district and in household with higher MPI score; the odds of stunting was significantly lower in infants whose mothers were taller (Table 36).

The analysis of risk of wasting and stunting was not stratified by their degrees, because the prevalence of moderate and severe degrees was very low.

Table 34. Univariable analysis of attained measures (N=475)

	WLZ		LAZ		LAD	
Variable	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value[d]
Age (months)	-0.03 (-0.03; -0.03)	0.000	0.00 (-0.00; 0.00)	0.350	-0.01 (-0.02; -0.01)	0.000
Sex (male)	-0.01 (-1.61; 0.14)	0.892	0.05 (-0.10; 0.21)	0.521	0.08 (-0.28: 0.45)	0.661
Caué	0.16 (-0.13; 0.45)	0.275	-0.16 (-0.46; 0.14)	0.295	-0.44 (-1.13; 0.27)	0.225
Lembá	-0.13 (-0.34; 0.08)	0.229	-0.38 (-0.59; -0.16)	0.001	-0.89 (-1.41; -0.37)	0.225
Improved sanitation	0.06 (-0.09; 0.22)	0.408	0.37 (0.21; 0.53)	0.000	0.82 (0.45; 1.19)	0.000
Improved water	0.14 (-0.45; 0.74)	0.648	-0.16 (-0.76; 0.43)	0.592	-0.00 (-1.41; 1.40)	0.996
Electricity	0.10 (-0.15; 0.36)	0.442	0.59 (0.33; 0.85)	0.000	1.42 (0.80; 2.05)	0.000
Finished floor	0.07 (-0.85; 1.00)	0.879	1.50 (0.53; 2.47)	0.002	3.55 (1.31; 5.79)	0.002
Cooking with solid fuel	-0.02 (-0.19; 0.13)	0.732	-0.34 (-0.51; -0.17)	0.000	-0.71 (-1.11; -0.32)	0.000
MPI	-0.05 (-0.10; -0.00)	0.028	-0.10 (-0.16; -0.60)	0.000	-0.27 (-0.39; -0.16)	0.000
Poor household	-0.18 (-0.39; 0.04)	0.108	-0.32 (-0.54; -0.09)	0.005	-0.80 (-1.35; -0.27)	0.003
Mother's height	-0.00 (-0.02; 0.01)	0.543	0.04 (0.02; 0.05)	0.000	0.10 (0.07; 0.13)	0.000
Exclusive breastfeeding	0.42 (0.19; 0.65)	0.000	0.25 (0.01; 0.49)	0.040	0.59 (0.03; 1.14)	0.039
Duration of breastfeeding	0.00 (-0.03; 0.03)	0.881	-0.03 (-0.06; 0.00)	0.052	-0.07 (-0.14; -0.00)	0.044
Age of introduction CF	0.14 (0.05; 0.23)	0.003	0.00 (-0.09; 0.10)	0.864	0.03 (-0.19; 0.26)	0.766
Acute diarrhea	-0.31 (-0.38; -0.24)	0.000	-0.06 (-0.11; -0.01)	0.023	-0.17 (-0.33; -0.01)	0.033
ARI	-0.14 (-0.18; -0.09)	0.000	0.01 (-0.02; 0.04)	0.459	-0.05 (-0.13; 0.04)	0.260
Malaria	-0.34 (-0.59; -0.08)	0.010	0.04 (-0.15; 0.22)	0.700	-0.00 (-0.51: 0.51)	0.991

Table 34. Univariable analysis of attained measures *(continued)*

	WLZ		LAZ		LAD	
Variable	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value[d]	β-estimate (95%CI)	*p* value
Infected by any parasite	-0.18 (-0.25; -0.11)	0.000	-0.09 (-0.14; -0.03)	0.003	-0.42 (-0.59; -0.24)	0.000
Single parasite infection	0.07 (-0.09; 0.23)	0.401	0.04 (-0.09; 0.18)	0.551	-0.01 (-0.48; 0.45)	0.948
Giardia lamblia infection	-0.18 (-0.27; -0.09)	0.000	-0.11 (-0.19; -0.03)	0.004	-0.46 (-0.69; -0.22)	0.000
Cryptosporidium spp. infection	-0.12 (-0.28; 0.03)	0.124	-0.01 (-0.13; 0.10)	0.814	-0.09 (-0.48; 0.28)	0.617
STH infection	-0.13 (-0.23; -0.02)	0.017	-0.15 (-0.23; -0.07)	0.000	-0.59 (-0.85; -0.34)	0.000
Total number of infections	-0.5 (-0.12; 0.02)	0.177	-0.00 (-0.08; 0.08)	0.967	-0.05 (-0.23; 0.13)	0.607
Total number of parasites	-0.04 (-0.10; 0.02)	0.158	-0.01 (-0.07; 0.05)	0.787	-0.05 (-0.20; 0.09)	0.461
Total number of *Giardia lamblia* infections	-0.08 (-0.18; 0.02)	0.113	-0.04 (-0.14; 0.07)	0.503	-0.13 (-0.38; 0.12)	0.295
Total number of *Cryptosporidium* spp. infections	0.00 (-0.19; 0.20)	0.945	-0.14 (-0.35; 0.06)	0.176	-0.39 (-0.88; 0.09)	0.117
Total number of STH infections	-0.03 (-0.13; 0.07)	0.567	0.04 (-0.05; 0.15)	0.366	0.08 (-0.16; 0.32)	0.503

ARI Acute respiratory infection, CF complementary feeding, LAD length-for-age difference, LAZ length-for-age z-score MPI Multidimensional Poverty Index, STH soil transmitted helminths WAD weight-for-age difference, WLZ weight-for-length z-score

Table 35. Univariable analysis for anthropometric velocity measures (N= 475)

	WAVZ		LAVZ	
Variable	β-estimate (95%CI)	*p* value	β-estimate (95%CI)	*p* value
Age (months)	0.01 (0.00; 0.02)	0.000	0.03 (0.02; 0.03)	0.000
Sex (male)	-0.05 (-0.15; 0.04)	0.254	0.00 (-0.09; 0.10)	0.966
Caué	0.04 (-0.21; 0.29)	0.756	-0.12 (-0.47; 0.08)	0.162
Lembá	-0.03 (-0.22; 0.16)	0.787	-0.11 (-0.31; 0.09)	0.289
Improved sanitation	0.14 (0.03; 0.24)	0.010	0.20 (0.09; 0.31)	0.000
Improved water	0.38 (-0.24; 1.01)	0.230	0.66 (0.01; 1.31)	0.048
Electricity	0.21 (0.06; 0.37)	0.005	0.29 (0.14; 0.45)	0.000
Finished floor	0.08 (-0.52; 0.69)	0.790	0.24 (-0.38; 0.87)	0.441
Cooking with solid fuel	0.34 (-0.09; 0.16)	0.588	-0.07 (-0.20; 0.06)	0.306
MPI	-0.03 (-0.06; -0.00)	0.027	-0.03 (-0.06; -0.00)	0.025
Poor household	-0.11 (-0.24; 0.01)	0.082	-0.09 (-0.22; 0.03)	0.166
Mother’s height	0.01 (0.00; 0.02)	0.009	0.01 (0.00; 0.02)	0.001
Exclusive breastfeeding	-0.02 (-0.15; 0.10)	0.719	-0.08 (-0.21; 0.05)	0.231
Duration of breastfeeding	-0.01 (-0.02; 0.00)	0.183	-0.01 (-0.02; 0.00)	0.416
Age of introduction Complementary feeding	0.00 (-0.04; 0.05)	0.915	0.02 (-0.04; 0.07)	0.516
Acute diarrhea	-0.43 (-0.60; -0.25)	0.000	0.05 (-0.13; 0.23)	0.587
ARI	-0.05 (-0.15; 0.05)	0.364	0.06 (-0.04; 0.16)	0.291
Malaria	-0.04 (-0.68; 0.61)	0.911	0.94 (0.26; 1.62)	0.007

Table 35. Univariable analysis for velocity measures (*continued*)

	WAVZ		LAVZ	
Variable	β-estimate (95%CI)	*p* value	β-estimate (95%CI)	*p* value
Infected by any parasite	0.05 (-0.12; 0.22)	0.547	-0.08 (-0.24; 0.09)	0.378
Single parasite infection	-0.21 (-0.58; 0.16)	0.275	0.19 (-0.15; 0.53)	0.271
Giardia lamblia infection	0.11 (-0.10; 0.31)	0.307	-0.06 (-0.27; 0.16)	0.609
Cryptosporidium spp. infection	-0.43 (-0.81; -0.05)	0.025	-0.66 (-1.04; -0.27)	0.001
STH infection	0.18 (-0.04; 0.41)	0.115	0.013 (-0.22; 0.25)	0.912
Total number of infections	-0.02 (-0.07; 0.01)	0.186	-0.03 (-0.07; 0.01)	0.174
Total number of parasites	-0.02 (-0.06; 0.00)	0.101	-0.04 (-0.07; -0.00)	0.032
Total number of *Giardia lamblia* infections	-0.03 (-0.09; 0.02)	0.265	-0.05(-0.10; 0.01)	0.109
Total number of *Cryptosporidium* spp. infections	-0.09 (-0.20; 0.02)	0.114	-0.13 (-0.24; -0.01)	0.032
Total number of STH infections	-0.02 (-0.07; 0.04)	0.532	-0.02 (-0.08; 0.04)	0.495

ARI Acute respiratory infection, CF complementary feeding, LAVZ length velocity z-score, MPI Multidimensional Poverty Index, STH soil transmitted helminths, WAVZ weight velocity z-score.

Table 36. Univariable analysis for wasting and stunting (N=475)

	Wasting		Stunting	
Variable	Odds Ratio (95%CI)	*p* value	Odds Ratio (95%CI)	*p* value
Age (months)	1.05 (1.03; 1.06)	<0.001	0.99 (0.98; 1.00)	0.528
Sex (male)	1.36 (0.77; 2.39)	0.285	0.81 (0.39; 1.69)	0.583
Caué	0.51 (0.16; 1.66)	0.269	2.99 (0.74; 12.03)	0.123
Lembá	2.22 (1.01; 4.86)	0.045	3.38 (1.19; 9.59)	0.022
Improved sanitation	0.56 (0.32; 1.00)	0.052	0.22 (0.10; 0.47)	0.000
Improved water	1.00 (0.09; 10.7)	0.998	1.88 (0.10; 32.29)	0.662
Electricity	0.62 (0.24; 1.57)	0.313	0.09 (0.02; 0.29)	<0.001
Cooking with solid fuel	1.12 (0.60; 2.10)	0.709	4.10 (1.85; 9.07)	<0.001
MPI	1.23 (1.04; 1.46)	0.015	1.70 (1.35; 2.15)	<0.001
Poor household	2.15 (0.98; 4.74)	0.055	5.31 (1.83; 15.38)	0.002
Mother's height	0.97 (0.93; 1.02)	0.399	0.81 (0.76; 0.86)	0.000
Exclusive breastfeeding	0.34 (0.15; 0.78)	0.011	0.32 (0.11; 0.96)	0.043
Duration of breastfeeding	1.01 (0.90; 1.12)	0.854	1.14 (1.00; 1.32)	0.047
Age of introduction Complemtary feeding	0.64 (0.46; 0.89)	0.009	0.76 (0.49; 1.17)	0.220
Acute diarrhea	2.83 (1.91; 4.18)	<0.001	1.36 (0.93; 1.99)	0.114
ARI	1.38 (1.08; 1.75)	0.008	0.99 (0.80; 1.23)	0.988
Malaria	7.16 (2.08; 24.65)	0.002	1.21 (0.27; 5.33)	0.800

Table 36. Univariable analysis for wasting and stunting (N= 475) (*continued*)

	Wasting		Stunting	
Variable	Odds Ratio (95%CI)	*p* value	Odds Ratio (95%CI)	*p* value
Infected by any parasite	1.16 (0.64; 2.14)	0.613	1.20 (0.74; 1.93)	0.459
Single parasite infection	0.54 (0.09; 3.12)	0.498	0.85 (0.11; 6.23)	0.873
Giardia lamblia infection	1.72 (0.83; 3.54)	0.140	1.43 (0.77; 2.65)	0.246
Cryptosporidium spp. infection	0.63 (0.14; 2.80)	0.553	0.80 (0.30; 2.14)	0.669
STH infection	0.64 (0.26; 1.59)	0.342	1.81 (0.89; 3.67)	0.096
Total number of infections	1.10 (0.83; 1.46)	0.470	0.90 (0.61; 1.31)	0.586
Total number of parasites	1.07 (0.86; 1.33)	0.501	0.92 (0.68; 1.23)	0.571
Total number of *Giardia lamblia* infections	1.32 (0.93; 1.88)	0.120	1.00 (0.61; 1.65)	0.970
Total number of *Cryptosporidium* spp. infections	0.63 (0.29; 1.33)	0.228	2.21 (0.85; 5.71)	0.099
Total number of STH infections	0.99 (0.69; 1.44)	0.998	0.65 (0.40; 1.07)	0.095

ARI Acute respiratory infection, CF complementary feeding, MPI Multidimensional Poverty Index, STH soil transmitted helminths

Multivariate analysis

- Attained growth

WLZ

Significant associations of exclusive breastfeeding, acute diarrhea, and MPI with WLZ were identified (Table 37):

Infants with exclusive breastfeeding had higher mean WLZ (β-estimate = 0.48; 95% CI 0.23, 0.73; p<0.001);

Infants with acute diarrhea had lower mean WLZ (β-estimate = -0.19; 95% CI -0.27, -0.12; p<0.001);

A mean decrease of 0.07 in WLZ for each unit increase of MPI (β-estimate= -0.07; 95% CI -0.12, -0.02; p=0.005).

LAZ

Significant associations of exclusive breastfeeding, mother's height, *Giardia lamblia* infection, STH infection, and MPI with LAZ were identified (Table 37):

Infants with exclusive breastfeeding had higher mean LAZ (β-estimate = 0.39; 95% CI 0.14, 0.65; p=0.003);

For each cm increase in mother height there was a mean increase of 0.05 in LAZ (β-estimate = 0.05; 95% CI 0.04, 0.07; p<0.001);

Infants with *Giardia lamblia* infection had lower mean LAZ (β-estimate = -0.10; 95% CI -0.18, -0.02; p=0.018);

Infants with STH infection had lower mean LAZ (β-estimate = -0.16; 95% CI -0.25, -0.07; p<0.001);

A mean decrease of 0.11 in LAZ for each unit increase of MPI (β-estimate= -0.11; 95% CI -0.16, -0.06; p<0.001).

LAD

Significant associations of exclusive breastfeeding, mother's height, *Giardia lamblia* infection, STH infection, and MPI with LAD were identified (Table 37):

Infants with exclusive breastfeeding had higher mean LAD (β-estimate = 0.95; 95% CI 0.28, 1.62; p=0.006);

For each cm increase in mother's height there was a mean increase of 0.14 in LAD values (β-estimate = 0.14; 95% CI 0.10, 0.18; p<0.001);
Infants with *Giardia lamblia* infection had lower mean LAD (β-estimate= -0.32; 95% CI -0.57, -0.07; p=0.013);
Infants with STH infection had lower mean LAD (β-estimate= -0.48; 95% CI -0.76, -0.20; p<0.001);
A mean decrease of 0.26 in LAD for each unit increase of MPI (β-estimate= -0.26; 95% CI -0.41, -0.12; p<0.001).

- Growth velocity

WAVZ

Significant associations of *Cryptosporidium* spp. infection and acute diarrhea with WAVZ were identified (Table 38):
Infants with *Cryptosporidium* spp. infection had lower mean WAVZ (β-estimate= -0.43; 95% CI -0.80, -0.06; p=0.023);
Infants with acute diarrhea had lower mean WAVZ (β-estimate= -0.56; 95% CI -0.82, -0.29; p<0.001).

LAVZ

Significant associations of *Cryptosporidium* spp. infection and mother's height with LAVZ were identified (Table 38):
Infants with *Cryptosporidium* spp. infection had lower mean LAVZ (β-estimate= -0.55; 95% CI -0.94, -0.17; p=0.005);
For each cm increase in mother's height there was a mean increase of 0.02 in LAVZ (β-estimate= 0.02; 95% CI 0.01, 0.03; p=0.002).

- Wasting and Stunting

Only mild wasting and stunting degrees were analyzed, since prevalence of moderate and severe degrees was very low.
Wasting
Significant associations of exclusive breastfeeding, acute diarrhea, and MPI with wasting were identified (Table 39):

The odds of wasting was 80.3% lower in infants with exclusive breastfeeding (OR= 0.20; 95% CI 0.08, 0.49; p<0.001);
The odds of wasting was twice higher in infants with acute diarrhea (OR= 2.30; 95% CI 1.49, 3.54; p<0.001);
For each unit increase in MPI there was a 32% increase in the odds of wasting (OR= 1.32; 95% CI 1.11, 1.58; p=0.002).

Stunting

Significant associations of exclusive breastfeeding, mother's height, age of introduction complementary food, and MPI with stunting were identified (Table 39):
The odds of stunting was 83% lower in infants with exclusive breastfeeding (OR= 0.17; 95% CI 0.05, 0.53; p=0.002);
For each cm increase in mother's height there was a 19% decrease in the odds of stunting (OR= 0.81; 95% CI 0.75, 0.87; p<0.001);
For each month increase in age of introduction of complementary feeding there was a 44% decrease in the odds of stunting (OR= 0.56; 95% CI 0.35, 0.91; p=0.019);
For each unit increase in MPI total there was a 71% increase in the odds of stunting (OR= 1.71; 95% CI 1.35, 2.18; p<0.001).

Table 37. Nutritional status/ anthropometry: multivariable models for attained growth (WLZ, LAZ, and LAD).

	β-estimate (95%CI)	*p* value
WLZ		
Exclusive breastfeeding	0.48 (0.23; 0.73)	<0.001
MPI	-0.07 (-0.12; -0.02)	0.005
Acute diarrhea	-0.19 (-0.27; -0.12)	<0.001
LAZ		
Exclusive breastfeeding	0.39 (0.14; 0.65)	0.003
Mother height	0.05 (0.04; 0.07)	<0.001
Giardia lamblia	-0.10 (-0.18; -0.02)	0.018
STH	-0.16 (-0.25; -0.07)	<0.001
MPI	-0.11(-0.16; -0.06)	<0.001
LAD		
Exclusive breastfeeding	0.95 (0.28,1.62)	0.006
Mother height	0.14 (0.10; 0.18)	<0.001
Giardia lamblia	-0.32(-0.57; -0.07)	0.013
STH	-0.48 (-0.76; -0.20)	<0.001
MPI	-0.26 (-0.41; -0.12)	<0.001

ARI acute respiratory infection, LAD length-for-age difference, LAZ length-for-age z-score, MPI multidimensional poverty index, STH soil transmitted helminths, WAD weight-for-age difference, WLZ weight-for-length z-score

Table 38. Nutritional status/ anthropometry: multivariable models for growth velocity (WAVZ and LAVZ).

	β-estimate (95%CI)	*p* value
WAVZ		
Cryptosporidium spp.	-0.43(-0.80; -0.06)	0.023
Acute diarrhea	-0.56 (-0.82; -0.29)	<0.001
LAVZ		
Mother height	0.02 (0.01; 0.03)	0.002
Cryptosporidium spp.	-0.55 (-0.94; -0.17)	0.005

LAVZ length velocity z-score, WAVZ weight velocity z-score

Table 39. Nutritional status/ anthropometry: multivariable models for wasting and stunting

	Odds ratio (95% CI)	*p* value
Wasting		
Exclusive breastfeeding	0.19 (0.08; 0.48)	<0.001
Acute diarrhea	2.29 (1.48; 3.54)	<0.001
MPI	1.32 (1.10; 1.57)	0.002
Stunting		
Exclusive breastfeeding	0.17(0.05; 0.53)	0.002
Mother height	0.81 (0.75; 0.87)	<0.001
MPI	1.71(1.35; 2.18)	<0.001

MPI multidimensional poverty index.

4.2.3. *Enteric parasitic infection and neurodevelopment*

A descriptive statistics of BINS scores by categories of nutritional status and enteric parasitic infection is shown in Table 40. BINS scores were significantly higher in infants not stunted in comparison with those stunted, at 3, 6, 12 and 24 month of age.

Univariable analysis

Results of univariable analysis for high risk of poor development are presented in Table 41. Age, male sex, wasting, stunting, infection by intestinal parasites and infection by *Giardia lamblia* were significant associated with increased risk of poor development. Variables with *p* value < 0.05 were included into the multivariable model.

Multivariable analysis

Results of multivariable analysis of high risk of poor development are presented in Table 42.

- *Nutritional status*

Age, LAZ and stunting were independent predictors of high risk of poor development:
For each month increase in age there was a 7% increase in odds of poor development (OR= 1.07; 95% CI 1.05, 1.10; p <0.001);
Infants with stunting had approximately twice odds of poor development (OR= 2.22; 95% CI 1.54, 3.20; p<0.001);
For each LAZ there was a 39% decrease in odds of poor development (OR= 0.61; 95% CI 0.51, 0.74; p<0.001).

- *Enteric parasitic infection*

Age, sex (males) and *Giardia lamblia* were independent predictors of high risk of poor development:
For each month increase in age there was a 2% increase in odds of poor development (OR= 1.02; 95% CI 1.00, 1.05; p=0.033);
Males had 55% increases in odds of poor development (OR= 1.55; 95% CI 0.99, 2.32; p=0.052);

Infants infected with *Giardia lamblia* had 87% increases in odds of poor development (OR= 1.87; 95% CI 1.09, 3.19; p=0.022).

- *Nutritional status and enteric parasitic infection*

Age, sex (males), stunting, and *Giardia lamblia* infection were independent predictors of high risk of poor development:

For each month increase in age there was a 2% increase in odds of poor development (OR= 1.02; 95% CI 0.99, 1.05; p=0.055);

Males had 51% increases in odds of poor development (OR= 1.51; 95% CI 0.99, 2.32; p=0.066);

Infants with stunting had approximately twice odds of poor development (OR= 2.37; 95% CI 1.52, 3.71; p<0.001);

Infants infected with *Giardia lamblia* had 69% increases in odds of poor development (OR= 1.69; 95% CI 0.95, 3.01; p=0.071).

Table 40. Descriptive analysis of BINS scores by categories of nutritional status and enteric parasitic infection

	BINS 3 (N= 301)			BINS 6 (N=250)			BINS 12 (N= 246)			BINS 18 (N= 235)			BINS 24 (N= 270)		
	N	Median (min.max.)	*p* value	N	Median (min.max.)	*p* value	N	Median (min.max.)	*p* value	N	Median (min.max.)	*p* value	N	Median (min.max.)	*p* value
Wasting															
Not	277	9 (7-11)	0.883	226	12 (8-13)	0.007	200	9 (6-11)	0.059	204	9 (5-11)	0.067	229	10 (5-13)	0.815
Yes	24	9 (7-10)		24	11.5 (7-13)		45	9 (6-11)		28	8 (7-10)		41	10 (5-11)	
Stunting															
Not	214	9 (7-11)	0.025	176	12 (9-13)	0.010	174	10 (6-11)	0.000	151	9 (5-11)	0.168	195	10 (5-13)	0.018
Yes	87	9 (7-11)		74	12 (7-13)		71	9 (6-11)		81	8 (6-10)		76	10 (5-11)	
Infected by enteric parasite															
Not				211	12 (8-13)	0.931	174	9 (6-11)	0.368	83	9 (6-11)	0.281	155	10 (5-13)	0.584
Yes				9	12 (11-13)		57	9 (6-11)		47	9 (6-10)		85	10 (7-11)	
Giardia lamblia															
Not				215	12 (9-13)	0.824	203	9 (6-11)	0.277	102	9 (6-11)	0.301	193	10 (5-13)	0.045
Yes				5	11 (11-13)		28	9 (6-11)		28	8 (6-10)		47	10 (7-11)	
Cryptosporidium spp.															
Not				196	12 (8-13)	0.982	205	9 (6-11)	0.100	106	9 (6-11)	0.419	221	10 (5-13)	0.361
Yes				4	12.0 (12)		12	8.5 (7-10)		7	8 (6-9)		8	10.5 (8-11)	
STH															
Not				220	12 (8-13)		213	9 (6-11)	0.958	111	9 (6-11)	0.850	198	10 (5-13)	0.727
Yes				0	--------		18	9 (6-10)		19	9 (6-10)		42	10 (7-11)	

p value obtained by Mann –Whitney test

Table 41. Univariable analysis of high risk of poor development

	Odds Ratio (95%CI)	*p* value
Age	1.08 (1.06; 1.10)	<0.001
Sex (males)	1.32 (0.95; 1.85)	0.093
Nutritional status		
WLZ	0.73 (0.61; 0.88)	0.001
LAZ	0.61(0.51; 0.74)	<0.001
Wasting	1.74 (1.10: 2.77)	0.018
Stunting	2.12 (1.50; 2.99)	<0.001
Enteric parasitic infection		
Infected by any parasite	1.32 (0.85; 2.05)	0.202
Giardia lamblia	2.11 (1.24; 3.59)	0.005
Cryptosporidium spp.	1.49 (0.59; 3.76)	0.391
STH	0.84 (0.43; 1.66)	0.633

Table 42. Multivariable analysis of high risk for poor development

	Odds Ratio (95%CI)	*p* value
Nutritional status		
Age	1.07 (1.05; 1.10)	<0.001
LAZ	0.61 (0.51; 0.74)	<0.001
Stunting	2.22 (1.54; 3.20)	<0.001
Enteric parasitic infection		
Age	1.02 (1.00; 1.05)	0.033
Sex (males)	1.55 (0.99; 2.32)	0.052
Giardia lamblia	1.87 (1.09; 3.19)	0.022
Nutritional and enteric parasitic infection		
Age	1.02 (0.99; 1.05)	0.055
Sex (males)	1.51 (0.97; 2.35)	0.066
Stunting	2.37 (1.52; 3.71)	<0.001
Giardia lamblia	1.69 (0.95; 3.01)	0.071

5. DISCUSSION

This is the first birth cohort study conducted in São Tomé, represented 8.6% of live-births in STP, proportionally distributed in the 3 main São Tomé districts.

This study found association between subclinical infections by enteric parasites and growth faltering and risk of poor neurodevelopment in infants from São Tomé. For a better understanding it will firstly analyzed the specific context of São Tomé, a LMIC.

5.1. São Tomé and Principe: a low-and middle income country

Birth cohort studies conducted in LMIC have specificities such as exposures that are unique with influences on health and illness, socio-economic inequalities, food insecurity, and high prevalence of undernutrition, increasing the vulnerability of their pediatric populations (Batty 2009). Africa as a whole remains the furthest behind the world's regions in terms of health improvements and longevity, and remains the world's poorest and most under developed continent (Defo 2014). São Tome belongs to SSA region. This region is characterized by having the highest concentration under-five deaths of any region (UNICEF 2016a); the predominant causes of under-5 mortality are still due to infectious diseases and nutritional deficiencies (Global Burden Diseases GBD 2015); and around half of SSA's population lives below the international poverty line of US$1.25 *per* day (UNICEF 2014a). Beyond the monetary poverty, other dimensions of well being such access to health, food security, and education are also deprived in SSA (Watkins 2016). Malnutrition is endemic, 40% of children are stunted (UNICEF 2016a); SSA growing economies are centered on extractive production of natural resources, leaving outside the agricultural sector, on which paradoxically most of the population live and derive their livelihoods (FAO 2015a); and around one-in-five of African children of primary school age are out of school (UNICEF 2016a).

STP is one of the least populated countries with 190.344 inhabitants and similarly to other small island developing states has limited land mass and population; the economic development is constrained by its insularity, fragility, limited resources, low capacity as a small archipelago state, excessive dependence on international trade and

with a high poverty rate of 66% (IMF STP 2016). The Caué, Lembá districts and the Autonomous regional of Principe are the poorest regions (IMF STP 2016).

São Tomé, like other African countries, shows some signals of demographic and epidemiological transition despite the poor economic growth (Defo 2013). In particular, STP has been progress in child survival in which mortality in children under-5 has decreased almost half between 1990 (103/1000 live-births) and 2015 (45 /1000 live-births) (UNICEF 2015, MICS 2016). The administration of minimum health package for child survival that includes vaccines, vitamin A, and essential medicines can explain this improvement (UNICEF STP 2015). Life expectancy also increased from 55.6 years in 1970 to 66 years in 2012 (UNICEF 2015) although the fertility rate is still high (4.1)(UNICEF 2015). A health transition is also happening in STP in which the patterns of disease and cause of death are changing (Defo 2013). For instance, in STP prevalence of some infectious diseases such as HIV and malaria are diminished to less than 1% (UNICEF 2015), and new set of health problems including heart diseases, stroke, cancer and metabolic disorders are rising. Nevertheless, at the same time, communicable diseases such as acute respiratory infections and diarrhea, perinatal and maternal morbidity and mortality, and malnutrition continue to be prevalent (MICS 2016). Despite São Tomé will continue to face significant challenges, there are favorable conditions such as a high percentage (74%) of exclusive breastfeeding (MICS 2016), low prevalence (8%) of low birth weight in comparison with 16 % described for SSA (UNCIEF 2015); high proportion (91%) of woman received antenatal care (MICS 2016); 90 % of young women are literate (MICS 2016); and 94 % uses an improved source of drinking water, although nearly half of the population lives in households using improved sanitation facilities (MICS 2016). All these factors could explain a less severe pattern in our study in which a combination of subclinical enteric infection with marginally malnourished infants was observed.

5.2. Enteric parasitic infections in infants from São Tomé

To the best of our knowledge, this is the first longitudinal study providing prevalence data of enteric parasitic infections in infants in São Tomé.

We found that enteric parasites were common among infants, 58.5% of them had at least one enteric parasitic infection within the first 24 months of age, with the highest prevalence in the second year. The three most frequent pathogenic parasites, either as single or multiple infections, were by frequency, *Giardia lamblia*, STH, and *Cryptosporidium* spp. Notably, the majority of infants had not associated diarrhea at moment of stool collection, in other words the majority of parasitic infections were subclinical.

Giardia lamblia is among the most common enteropathogens detected in children in low-resource settings (Rogawski 2017). In our study, *Giardia lamblia* was the commonest parasite, with an overall prevalence of 35%, being more frequent in the second year of age. Our prevalence are similar to desceibed in MAL-ED study, in which prevalence of *Giardia* ranged from 2.1% to 28.4% in infants aged 0-11 months, and from 12.9% to 54.3% in infants aged 12-24 months in non-diarrheal stools (Platts-Mills 2015). Limited numbers of studies have addressed prevalence of enteric protozoa in STP (Ferreira 2014, Lobo 2014, Liao 2016). Ferreira *et al*. (2015) reported *Giardia lamblia* as the most common protozoa, infecting 41.7% of preschool children (Ferreira 2015), while Lobo *et al*. (2014), found a much lower prevalence of 7.5% for *Giardia* in young children from a central village. Repeated *Giardia* detection occurred in a few proportion of infants in our study, less than reported in other countries (Hollm-Delgado 2008, Donowitz 2016). In Peruvian infants, reinfection rate was 87% (Hollm-Delgado 2008); and 64% in Bangladeshi infants (Donowitz 2016). The low proportion of repeated *Giardia* detection in our study may be explained by the low frequency (every three months) of stool collection and by the fact that our infants received specific treatment for *Giardia* (Reed Book 2015) and deworming therapy (WHO 2006c). These therapies could have reduced *Giardia* detection in stools. The frequency of *Giardia lamblia* and STH co-infection was also low (1.5%-5.6%) in our infants, in spite of relatively high individual prevalence of *Giardia* and STH infections. Frequent co-infections of protozoan and STH are described in children living in the tropics and subtropics (Harnay 2010). These parasites have potential for interactions due to their occupation of similar intestinal niches, with host sharing immune defenses against both parasites (Blackwell 2013). Nevertheless, authors

suggest that *Giardia* and STH co-infections occur less frequently than expected due to an antagonism (Chunge 1991, Blackwell 2013). Given that *Giardia* and STH individually infect around 15% of the world's population, understanding the interaction between these two pathogens deserve more research (Blackwell 2013).

Molecular characterization of *Giardia* was beyond the scope of our study. It was performed in a subset of stool samples at 24 months of age, revealing a predominance (80%) of assemblage B. This is in contrast with the predominance of assemblage A found in another study in children in STP (assemblage A 55.5%, assemblage B: 45.5%) (Lobo 2014), but in accordance with the most of SSA countries, in which assemblage B prevailed in children (Squire 2017): Ethiopia (assemblage A 22.9%, assemblage B 77.1.1%) (Wegayehu 2016), Ghana (assemblage B 100%) (Anim-Baidoo 2016), Guinea-Bissau (assemblage A 11.5%, assemblage B 84.6%) (Ferreira 2012), Kenya (assemblage A 1.4%, assemblage B 88.9%, assemblage A+B 9.7%) (Mbae 2016), Rwanda (assemblage A 13.5%, assemblage B 85.9%) (Ignatius 2012), Tanzania (assemblage A 50%, assemblage B 50%) (Tellevik 2015), and Uganda (assemblage A 14.7%, assemblage B 73.5%) (Ankarklev 2012). The reason for geographic variation of *Giardia* assemblages is still unclear. It may be explained by different dynamics of transmission, the assemblage B is most likely transmitted from human to human, and assemblage A is most often responsible for zoonotic transmission (Mbae 2016).

STH are common among the youngest children living in endemic areas (Gyorkos 2001, LeBeaud 2015). In our study, STH were the second most frequent parasites with an overall prevalence of 30.4%, being more frequent in the second year of age. Among STH, *Ascaris lumbricoides* was the most frequent helminth, reaching prevalence of 20.3%, and *Trichuris trichiura* was less frequent, with prevalence less than 3.0%; no hookworms were detected. In comparison with the multinacional study MAL-ED, we found much higher prevalence of STH. In fact, MAL-ED study only reported *Ascaris* prevalence ranged from 4.7% to 6.6% in infants aged 12-24 month in non-diarrheal stools (Platts-Hills 2015). Independent cohort studies reported higher STH prevalence in infants from Peru (Gyorkos 2011) and Kenya (Lebaud 2015). For instance, in Peru, prevalence of helminth infection increased to 37.0% by 14 months of age (Gyorkos

2011) and in Kenya, STH infections have a prevalence of 19% (Lebaud 2015). In STP, studies addressing the prevalence of STH have being conducted in preschool and school children (Belo 2005, Lobo 2014, Ferreira 2015, Liao 2016). More specifically, these studies reported prevalence of *Ascaris lumbricoides* 70.8%, *Trichuris trichiura* 68.5%, and Ancylostomidae 4.6% in school children (Belo 2005) and prevalence of *Ascaris lumbricoides* 56.3% and *Trichuris trichiura* 52.5% in preschool children (Ferreira 2015). All data on STH prevalence are biased since infants received treatment with mebendazole or albendazole (WHO 2006c).

Cryptosporidium spp. was the third most frequent parasites, with an overall prevalence of 14.7%. Prevalence of *Cryptosporidium* described for infants from LMIC (Kotloff 2013, Platts-Hills 2015) are consistent with our findings. In MAL-ED study, *Cryptosporidium* prevalence ranged from 1.5% to 6.7% in infants aged 0-11months, and from 0% to 11.2% in those aged 12 -24 months in non-diarrheal stools (Platts-Hills 2015). Nevertheless, higher burden of cryptosporidiosis in infants have been reported in some national birth cohorts (Checkley 1998, Sarkar 2013, Korpe 2016, Kattula 2016) reporting prevalence of 80% in infants in Peru *(*Checkley 1998), 77% in Bangladesh (Korpe 2016), and 67% to 97% in India (Sarkar 2013, Kattula 2016). These high rates could be explained by intensive surveillance using molecular methods improving detection (Sarkar 2013). In STP, one study conducted at hospital setting found *Cryptosporidium* spp. in 8.9% of children (Lobo 2014). Repeated *Cryptosporidium* detection occurred in a few proportion of infants in or study, much lower than that described in infants from other LMIC, in which the rate of reinfections ranged from 33.8% in Bangladesh (Korpe 2016) to 49.3% in India (Kattula 2016). In our study, co-infection of *Cryptosporidium* with STH ranged from 0.4% to 2.2%, and with *Giardia* from 0.4% 1.9%. In infants, few studies have examined *Cryptosporidium* co-infections. In India, co-infection with *Giardia* and *Shigella* was reported in infants (Kattula 2016). The low prevalence of *Cryptosporidium* observed in our study may be explained by protective effect of specific antibodies in breast milk (Korpe 2013) or by less severe undernourished infants.

In our study, no *Entamoeba histolytica/dispar* complex was detected. Similarly, no cases were diagnosed in infants in Tanzania (Tellevik 2015) and in the multisite

GEMS and MAL-ED studies (Kotloff 2013, Platts-Hills 2015). This confirms the modest role of this protozoan as an enteric pathogen in infants.

Our study confirmed that this country is highly endemic for enteric parasites, infecting or colonizing infants from early ages, mainly with *Giardia lamblia* and STH. The main factor associated with this high prevalence is probably related to limited access to improved sanitation in the households (Jarquin 2016). Maternal parasitic infection described as a risk factor for infant parasitic infection, deserves further research (Menzies 2014).

Subclinical enteric parasitic infections

In this study, enteric parasites were detected mostly in infants without diarrhea. In LMIC, there is growing evidence that *Giardia lamblia* (Rogawski 2017, Donowitz 2016) and *Cryptosporidium spp*. (Korpe 2016, Kattula 2017) are etiologic agents of enteric infections in infants without overt diarrhea. Several factors may explain the excretion of enteric pathogens in the absence of diarrhea, related either to the pathogen (strain virulence, prolonged excretion of cysts), to the host (immune response, nutritional status, microbiota) and/or to the environment (Levine 2012). In relation to the pathogen, in Bangladeshi children higher odds ratios for diarrhea were observed for *Giardia* assemblage A and A2 infections, whereas higher parasite loads were observed for assemblage B infections (Haque 2005). In India, 50% of infants were shedding oocysts before or after episodes of cryptosporidial diarrhea, with important implications for transmission of disease (Ajjampur 2010). In relation to the host, barriers such as the intestinal microbiota, the mucus layer, the epithelial cell layer, and innate immune responses make explain why some pathogen may end up colonizing the human intestine for a variable (short or long) period of time without causing overt diarrhea (Levine 2012). Recent studies showed a modification in the diversity and composition of the gut microbiota and metabolism according to the parasite, either in *Giardia lamblia* (Bartelt 2017), *Cryptosporidum spp.* (Bolick 2017) and *helminth* infections (Kreisinger 2015). Immune defenses such as intestinal secretory immunoglobulin A (sIgA) antibodies, breast milk sIgA or other non-specific properties present in breast milk can prevent adherence of enteropathogens to

enterocytes or mucosal invasion without killing the pathogen (Levine 2012). In addition, in highly endemic settings, repeated infections by *Giardia* and STH seem to result in an asymptomatic clinical status, possibly reflecting acquisition of an immunoregulatory host environment (Solaymani-Mohammadi 2013, McSorley 2012). In our study, the predominance of subclinical parasitic infections in infants living in São Tomé may be explaining by the continuous exposure to enteric parasites, which induce an immune response capable of preventing clinical illness but not intestinal colonization (Levine 2012, Solaymani-Mohammadi 2013, McSorley 2012). In addition, the high proportion of exclusively breastfeed infants in our cohort may have played a role in protecting against more severe forms of diarrhea or even diarrhea at all (Kutty 2014).

5.3. Intestinal barrier function in infants from São Tomé

In our study, the intestinal barrier function analysis was based on a single measurement of fecal biomarkers at 24 months of age in a subset of 82 infants (Garzon 2017). The measurement of intestinal barrier biomarkers at 24 months, and not before, allowed exploring the cumulative effect of repeated parasitic infections on the intestinal barrier as well as could better reflect the impact of these infections on intestinal barrier, not biased by a potential effect of an incompletely development (Brandtzaeg 2006). The ideal biomarker in infants would be minimally dependent on age and breastfeeding. In addition, it should be noninvasive allowing frequent sampling and highly reproducible. Taking into account these requirements, two biomarkers were selected to assess intestinal function in our field study-addressing infants from a LMIC (Garzon 2017): fecal S100A12 for intestinal inflammation and fecal A1AT for intestinal permeability.

5.3.1. *Intestinal inflammatory response*

To the best of our knowledge, this is the first time that S100A12 is used to assess intestinal inflammatory response in in infants in a LMIC (Garzon 2017). Fecal S100A12 is a calcium-binding pro-inflammatory predominantly secreted by granulocytes (Meijer 2012). It acts as a pro-inflammatory molecule, by binding the receptor for advanced glycation end products (RAGE) on cells, with upregulation of

pro-inflammatory cytokines (Dabritz 2014). In our cohort, the fecal S100A12 median (interquartile range) concentration was 2.87 (2.41- 3.92) μg/g, five times higher than reported in healthy infants (0.5 mg/kg)(Day 2013, Ehn 2011) but below the threshold 10 mg/kg used for inflammatory bowel disease (Day 2013, Ehn 2011). This comparison should be interpreted with caution, since those reference values were obtained from a limited number of healthy children from a developed country (Day 2013, Ehn 2011). High levels of inflammatory biomarkers have been described in infants from developing countries, associated either to enteric parasite infections (Kirkpatrick 2002, 2006, Campbell 2004, Kohli 2008) or to environmental enteric dysfunction (McCormick 2016, Kosek 2012, 2017). In MAL-ED study, infants were found to have higher concentrations of inflammatory biomarkers (myeloperoxidase, neopterine), than those from developed countries (McCormick 2016, Kosek 2017). Those inflammatory biomarkers were higher in breastfed children, in infants with higher rates of pathogen detection, and in infants living in households of lower socio-economic status (McCormick 2016). In our study (Garzon 2017), the tendency to elevated fecal S100A12 concentration suggest the existence of intestinal inflammation in line with that reported in infants from LMIC (McCormick 2016, Kosek 2017), but further studies are needed to determine reference values of S100A12 in infants from LMIC.

Fecal S100A12 has advantages in field studies involving children: it is restrictively secreted by activated neutrophils (Meijer 2012), and is strongly correlated with histologically intestinal inflammation and neutrophil infiltration (Foell 2003); references values have been described for healthy infants (Day 2013, Enh 2011); its fecal levels are not increased with breastfeeding (Day 2013); it 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. (Sidler 2008, Foell 2003); it is evenly distributed throughout feces and is stable at a wide range of temperatures (4ºC to 20ºC) for several days (de Jong 2006). These characteristics make fecal S100A12 a convenient biomarker, facilitating samples collection and transportation to a laboratory for measurement, avoiding resource-consuming storage needs (de Jong 2006). Compared with other biomarkers of intestinal inflammation, S100A12 appears

to be a convenient and accurate tool for field studies in infants. Calprotectin, another calcium-binding protein, correlates well with inflammatory bowel disease activity but a meta-analysis showed a relatively specificity (0.76) in children (van Rheenen 2010). In healthy infants, fecal calprotectin levels are higher than in older children (Li 2015) and in those breastfed (Li 2013). Lactoferrin is likewise not specific as it is produced by neutrophils and epithelial cells (Perrin 2017); and breast milk may contribute to increase its fecal levels (Perrin 2017). Additionally, fecal lactoferrin may be less sensitive in malnourished children (Opitan 2010). Myeloperoxidase is produced by neutrophils, but it is also found at lower concentrations in monocytes and macrophages (Saiki 1998). Higher levels of this biomarker were associated to recent breastfeeding (McCormick 2016). Neopterin, an indicator of T-helper cell 1 activity, is used as a biomarker of intestinal inflammation (McCormick 2016, Kosek 2017). Its fecal levels were found to be much higher (26 times) in infants from developing countries than from non-tropical countries without clear explanation (Kosek 2013).
Since fecal S100A12 was herein firstly used as a biomarker of intestinal inflammatory response in enteric parasitic infection, further studies are needed to establish their reference values in children living in LMIC settings.

5.3.2. *Intestinal permeability*

A1AT is a 52-kDa glycoprotein synthesized mostly in the liver and to a lesser extent by macrophages and neutrophils (Lisowska-Myjak 2005). Since fecal A1AT has a molecular weight similar to that of albumin, its excretion is parallel to the enteric loss of albumin (Thomas 1981). In our cohort, the fecal A1AT median (interquartile range) concentration was 165.1 (66.0 - 275.6) μg/g, below the median values (299 μg/g) described in MAL-ED study (McCormick 2016, Kosek 2017). Nevertheless, it was described that A1AT changes over the first 24 months of age, with higher values in the first 12 months, declining thereafter (McCormick 2016). In fact, the median value of 165.1 μg/g we found at 24 months of age is not very different from the mean of 199 μg/g described at 13-24 months of age in infants from MAL-ED study (McCormick 2016, Kosek 2017).

Remarkably, in a subsample of infants, we found significant associations between fecal A1AT levels and risk of wasting and stunting at 24 months of age (Garzon 2017). Fecal A1AT concentrations were around 100% higher in wasted infants and 50% higher in stunted infants (Garzon 2017). The association between increased intestinal permeability and undernutrition has been described in infants from developing countries (Campbell 2004, Goto 2009, Mondal 2012, Guerrant 2016, Kosek 2013). It was suggested that increased intestinal permeability explains at least 40% of growth faltering (Lunn 2000). Fecal A1AT has been combined with two inflammatory biomarkers (myeloperoxidase and neopterin) in a score to predict growth deficit in infants (Kosek 2013); specifically, fecal A1AT levels at or above 75th centile were reported to predict a loss of 0.152 LAZ in the subsequent six months (Kosek 2013). The increased permeability in marginally nourished infants can be explaining by the inadequate or rate-limiting stores of key nutrients to repair the mucosal damage (Guerrant 2008). The increased permeability and undernutrition are part of the spectrum of environment enteropathy, characterized by small intestinal inflammation, reduced absorptive capacity, and increased intestinal permeability, that commonly affect children in developing countries (Prendergast 2012). The environmental enteropathy has been proposed as a key determinant of growth failure in children in LMIC (Kosek 2017). The assessment of environmental enteric dysfunction was beyond the scope of our study.

Fecal A1AT has several advantages: it has good correlation with Cr-labellled albumin, considered the gold standard for assessment of protein-loosing enteropathy (Crossley 1977, Thomas 1981); it a notably stable compound in stool samples, as it is neither degraded by intestinal proteases nor reabsorbed (Magazzu 1985); it was shown to be stable in stool for a minimum of 2 days, 7 days, and 3 months at room temperature, 4–8 °C and -20 °C, respectively (Erikson 2016); and it is measurable by ELISA method with acceptable performance for quantifying A1AT in stool (Erikson 2016). Several other biomarkers have been used for assess intestinal permeability, such as urinary lactulose/mannitol absorption test, serum endotoxin core antibody, and zonuline test (Zhang 2000, Goto 2002, Mondal 2012, McCormick 2016). The most commonly used lactulose:mannitol has inconveniences that limit its use in infants 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 (Erickson 2016). Furthermore, large size molecules such as A1AT (50,000 Daltons), transported through the paracellular route, may better reflect structural damage of tight junctions than small size molecules such as lactulose (342 Daltons) and mannitol (182 Daltons) (Vojdani 2013).

5.4. Infant growth in São Tomé

The refined anthropometric approach we used for growth monitoring allowed the identification of subtle deficits that would have been missed if relying solely on conventional metrics and thresholds. We employed metrics complementing the conventional z-scores for growth, including : *growth velocity*, since it accurately reflects the effect of current events (WHO 2009a), while the attained growth at a single point in time is a cumulative measure of growth rate, velocity measures display greater sensitivity in capturing influencing factors and have greater potential in predicting short-term changes in growth (Schwinger 2017); *lenght-for-age difference* (LAD) was used as a complementary more adequate measurement to assess changes in height over time (Leroy 2014), while LAZ seems to be appropriate to assess individual linear growth at determined cross-sectional point, LAD is more appropriate to assess linear growth at population-level over time (as age increases) (Leroy 2014); and the *cut-off* $\leq$ *-1 SD* to define wasting and stunting in order to include infants at risk of undernutrition (mild), and moderate-to-severe degrees (Pelletier 2003).

5.4.1. *Attained growth*

Z-scores for attained growth are the most accepted indicators to assess whether a child's growth pattern deviates from normality at particular time (WHO 2006a). To the best of our knowledge, this is the first longitudinal study to assess growth in infants from São Tomé. In our study, WLZ and LAZ were used, since WLZ reflects acute or transitory response to environment, and LAZ reflects a more permanent, longer-term response to a sustained environment effect (Richard 2012). In our infants, at the neonatal period the mean values of WLZ 0.05 were close to the WHO standards (WHO 2006a), and LAZ was more apart, with a mean of -0.71. After the neonatal

period, the mean values of these indicators remained above -1 SD with few oscillations up to 24 months of age. At this age, the mean values of WLZ, and LAZ were close to WHO standards, respectively -0.19, and -0.52 (WHO 2006a).

However, when length was expressed as LAD (Leroy 2015), the negative differences became evident from the neonatal period (around -1.5 cm) and these negative values remained practically unchanged up to 24 months of age. Studies using HAD vs. HAZ in populations of children between 2 and 5 in LMIC (Leroy 2015) found continued deterioration reflected in a decrease in mean HAD between 2 and 5 years by contrast, HAZ shows either no change or an improvement in mean HAZ (Leroy 2015). Simmilarly, in our study growth faltering was evident by using LAD but not LAZ, which confirm the usefulness of this anthropometric measurement as a complementary tool for assesses linear growth.

Several longitudinal studies provide child growth trajectories in LMIC (Shrimpton 2001, Victora 2010, Mamidi 2011, Christian 2013). An important finding in these studies addressing different LMIC was a pattern of very early linear growth faltering, noticed at birth, and continuing until 24 months of age. It differs of our results, in which a length restriction was observed at the neonatal period, probably reflecting intrauterine growth restriction, but no further deterioration was noticed after birth.

5.4.2. *Growth velocity*

Growth velocity is considered a superior quantitative measure of growth compared with attained growth (WHO 2009a). Linear growth during infancy is a dynamic process that follows a nonlinear pattern of saltation and stasis (Lampl 1992). Whereas pathogenic factors affect growth velocity directly, their impact on attained size becomes evident only after the altered rate of growth is sustained for long periods (Tanner 1952). In other words, examining velocity allow earlier identification of growth problems compared with isolate examination of attained growth (WHO 2009a). In spite of this hypothesized advantage, there are fewer references for velocity than references for attained growth, in part due to scarcity of appropriate longitudinal datasets (WHO 2009a). In our study, length velocity (LAVZ) in the first interval showed negative values (-1.34), with subsequently catch-up to values close to WHO

standards. Weight velocity z-scores (WAVZ) were within normal range during the 24 months of age. Few studies have assessed growth velocity in infants using WHO standards (Ramokolo 2015, Schwinger 2014, 2017). Growth velocity at early ages is important since it can predict growth and mortality (Iannotti 2015, Onyango 2015, Schwinger 2017). In Peruvian infants, early growth velocities positively predicted attained length and weigh by 12 months of age (Iannotti 2015); similarly, in Bangladeshi infants, two consecutive intervals of weight velocity below the threshold predicted a higher risk of stunting (Onyango 2015). In children from Congo, length and weight velocity <-3 SD were respectively associated with an 11.8 and a 7.9-fold increase in the relative risk of death in the subsequent 3-months period (Schwinger 2017). Growth velocity seems to be an accurate and precise tool in LMIC in which undernutrition is prevalent; it can detect earlier growth faltering and predict the risk of stunting and death (Onyango 2015, Schwinger 2017).

Regarding both, attained and growth velocity measures, we can conclude that linear growth (LAZ, LAD, LAVZ) was more affected than weight (WLZ, WAVZ) in infants in our study. This mild deficit in linear growth matters, since linear growth faltering in the first 2 years of age may lead to irreversible damage, including shorter adult height, lower attained schooling, reduced adult income, and decreased offspring birthweight, affecting future generations (Victora 2008). Noteworthy, a deficit in length was only observed at the neonatal period, probably reflecting intrauterine growth restriction. It is assumed that growth faltering at birth has *in utero* origins, resulting from compromised maternal nutrition, placental function and/or ability of the fetus to use the nutrients and oxygen (WHO 2002b, Christian 2013). Based on evidence from WHO multicenter studies, it appears that the major maternal anthropometric predictors of offspring' size are the maternal height and pre-pregnancy BMI, which are the result of genetic and environmental influences before pregnancy (Kramer 1987, Ramakistan 1999, WHO 2002b). Our findings should alert to the need of implement programs such as multiple micronutrient supplementation during pregnancy in order to improve growth faltering at birth.

5.4.3. *Wasting and stunting*

Wasting is a short-term response to food shortage or infectious disease, and stunting is a response to a number of different acute and chronic factors, including inadequate feeding practices and repeated infections (Richard 2012). In other words, wasting is infrequent and temporary state, whereas stunting is more likely to be chronic and irreversible (Richard 2012). In our study, at the neonatal period, 13.3% of infants were at risk of wasting (WLZ ≤-1 SD >-2SD), and 3.3% were moderately-to-severely (WLZ ≤-2 SD) wasted. Longitudinally, two peaks of prevalence of wasting was observed, respectively from 7 to 12 months of age which coincided with introduction of complementary feeding, and from 18 to 22 months of age coinciding with breastfeeding weaning. At 24 months of age, 14.36% of infants were at risk of wasting and 0.72% moderately-to-severely wasted. In relation to stunting, 29.7% of infants were at risk of stunting (LAZ ≤-1 SD >-2SD), and 7.8% moderately-to-severely (LAZ ≤-2 SD) stunted at neonatal period. Thereafter, stunting prevalence remained relatively high throughout the 24 months of age. At 24 months, 19.9% of infants were at risk of stunting and 8.9% moderately-to-severely stunted. In our study, we found a much lower overall prevalence of moderate-to-severe stunting (7.8%) in comparison to the 17.2% value described in STP for children under-5 (MICS STP 2016).

The use of the cut-off ≤-1SD in our study allowed recognition of an important proportion of infants at risk of undernutrition (mild degree), which was far higher than those with moderate-to-severe degrees, as reported (Stevens 2012). Globally, in 2011 it was estimated that 148 million of children under-5 were at risk of wasting and 110 were moderately-to-severly wasted; and 144 million were at risk of stunting and 170 million were moderately-to-severly stunted (Stevens 2012). More recently it was reported that in eight LMIC 7% of infants had WLZ <-1 SD and 31% LAZ <-1SD (Richard 2012). There are several reasons to give especial attention to mild undernutrition in infants. Firstly, although the risk of mortality in mild undernourished children is low compared with those severely undernourished, that risk is not negligible (HR 1.6 for wasting and HR 1.5 for stunting) (Pelletier 1994, Olofin 2013, Black 2013). In fact, data from 130 demographic health surveys in young children showed that variance in mild underweight had larger and more robust correlations with child mortality, across and within the countries, than variance in severe

underweight (Bhagowalia 2011). Secondly, the number of undernourished children in mild and moderately categories are far more than in the severe category (Stevens 2012). This is important from a public health perspective, since the inclusion of mild undernourished infants provides information about otherwise unobserved changes in disease exposure (Bhagowalia 2011, de Onis 2016). Determinants of mild undernutrition have been described (Oliveira 2008, Bhagowalia 2011). Analysis of demographic surveys in LMIC, found that mild underweight was more correlated with local agricultural output than severe underweight (Bhagowalia 2011). In Brazil, low family monthly income and family headed by a woman, were the main determinants of mild-to-moderate undernutrition in children under-5 (Oliveira 2008).

In our study, the higher proportion of infants at risk of undernutrition over those moderately-to-severely undernourished can be explained by particular protective factors including: a very high proportion of breastfed infants; complementary feeding commonly introduced at recommended age; and relatively literate mothers. In addition, STP is basically an agricultural country, with more than a third of the population working in agriculture, livestock, and fisheries (IMF STP 2016), which in comparison with others SSA countries, has relatively good food availability. It can also be speculated that some health benefit might resulted from the "Hawthorne Effect" with modification of mothers' behavior in response to their awareness of being observed (McCambridge 2014).

To recapitulate, this is the first longitudinal assessment of infant growth in STP using a refined anthropometric approach. In our cohort the main findings were a linear growth deficit present at the neonatal period, and high prevalence of infants at risk of undernutrition, being stunting twice more frequent than wasting.

5.5. Neurodevelopment screening in infants from São Tomé

The assessment of the neurodevelopment in the first 24 months of age is of paramount important specifically in LMIC. Within the first two years of age, the brain develops rapidly through several processes of neurogenesis, axonal and dendritic growth, synaptogenesis, cell death, synaptic pruning, myelination, and gliogenesis, that are

interconnected and happened at different times (Grantham –McGregor 2007). Any perturbation in these processes can have long-term effects on the brain's structural and functional capacity (Grantham-McGregor 2007). Particularly, children living in developing countries are exposed to poverty, undernutrition, poor health, and unstimulating home environments (Grantham –McGregor 2007). In our cohort, data on neurodevelopment are based on 1440 BINS applied at different ages along the study period. This study was the first attempt for screening neurodevelopment in children aged 0 to 24 months in São Tomé. This was also the first study using the BINS in an African country. The main finding was that the proportion of infants at high risk of poor development increased with age, from none at 3 months to 25.8% at 24 months. Concerning the developmental areas, infants showed a tendency to decrease the performance in expressive and cognitive areas, as the complexity of tasks increased. More specifically, tasks in neurologic domain were normally performed by almost all infants across ages; tasks in receptive domain were performed by more than 70% of infants; but tasks in expressive and cognitive domains were suboptimally performed.

The BINS was developed to identify infants who are developmentally delayed or at risk (Aylward 1995). In BINS four conceptual areas of basic neurological, expressive, receptive functions and cognitive processes (Aylward 1995). Very few studies have used specifically BINS in infants from LMIC (Guedes 2011, McCarthy 2012). In seven South American countries, this screening showed that than 5% of healthy infants were at high risk of poor development at 24 months of age (McCarthy 2012). This is much less than 25.8% of infants at high risk we found at the same age. This result should be interpreted with caution, since reliability of a screening tool depends on the validation and previously determined cut-offs for the especific context (World Bank 2009a). Few studies have addressed child development in younger children in LIMIC. Studies based on the Early Child Development Index data in 35 LMIC estimated that 36.8% of children aged 36 to 59 months of age did not attain basic cognitive and socio-emotional skills, with the largest number living in SSA (McCoy 2016). In the last survey in STP (MICS 2016), the Early Child Development Index showed that 45% of preschool children had not the expected development; more

specifically, 94% of children were on track in the physical function, 79 % in the learning functions, 62% in the social-emotional domains, but only 16 % were on track in the literacy-numeracy domain (cognitive tasks) (MICS 2016). Although the Early Child Development Index was applied in older children and assessed different domains (UNICEF 2009a), our results are in line with these findings.

More interestedly, infants of our study displayed an increase risk of poor development with age. For children in LMIC, it has been described that developmental scores are in normal range during the first year, but then decline during the preschool years (World Bank 2006a, 2009a). It is suggested that a less stimulating environment could be not relevant in the first months of age, but it becomes important as infant continues to develop, facing more complex processes and suffering the cumulative effect of exposure to multiple risk factors in an impoverished environment (Wagstaff 2004a, World Bank 2009a).

The poorest performance in the expressive and cognitive domains in our study deserves attention. In the first five years, the cognitive development is strongly affected by genetics and quality of the environment (Shonkoff 2000, World Bank 2009). Recent evidence links early adversity and nurturing care with brain development and function throughout the life course (Black 2017). Although genetics play a role in a child's developing abilities, the genetic-environment interactions are more important, modulating the expression of those genes (World Bank 2009). The importance of environmental influences predominates in conditions of poverty, where infants are not in stimulating and responsive environments, compromising their competencies (World Bank 2009).

The BINS has several advantages. It can be administered in 10 to 15 minutes (Aylward 1995) and can be applied by health professionals with limited training (McCarthy 2012). BINS was previously validated in Brazilian preterm infants showing high sensitivity and moderate correlation with Denver development screening test (DDST)-II and Bayley Scales of Infant Development (BSID)-II tests (Guedes 2011). In a multisite study in South American infants, BINS was feasible and appropriate for neurodevelopmental screening, regardless of their cultural, socioeconomic and languages background (McCarthy 2012). Other tests have been

developed for infants in LICM. In Kenya, the Developmental Milestones Checklist, consists of 66 items covering three broad domains: motor, language and social–emotional development (Abubakar 2009); and the Rapid Neurodevelopment Assessment Instrument consisting of 27 items and assesses several domains: primitive reflexes, gross motor, fine motor, vision, hearing, speech, cognition, behavior, and seizures (Khan 2010, 2013). Although the mentioned tests seem to be appropriate to assess neurodevelopment in infants in LMIC, they contain large number of items and are time consuming. Further studies are needed to validate BINS in African LMIC countries.

5.6. Association between enteric parasitic infection and intestinal barrier function

Enteric infections are defined as pathogen-associated disrupted intestinal absorptive and/or barrier function, with or without overt diarrhea (Petri 2008). Enteric protozoa and helminth can damage the epithelium through apoptosis, disruption of epithelial brush border, alteration of cytoskeleton, disassembly of tight junctions, and inflammation (Berkes 2013, De Genova 2016, Cliffe 2007, McKay 2017). Particularly, the increased intestinal permeability and local inflammatory response are considered main mechanisms by which enteric infections can cause intestinal damage (Berkes 2013, De Genova 2016). The assessment of these phenomena is important for the understanding of gut-infection interaction.

5.6.1. *Association between enteric parasitic infection and intestinal inflammation*

The intestinal inflammatory response to enteric parasite infections is variable and depends on the immune status of the host, initial interaction with the epithelium, parasite invasive potential and ecological niche (Farthing 2003). At intestinal level, parasite may induce a robust innate mucosal immune response that includes inflammatory infiltrate with activation of neutrophils and other cells that participate in the intestinal lesion (Kasper 2001).

Giardia lamblia and intestinal inflammation

In our cohort, the multivariable analysis showed that infants with *Giardia lamblia* infection had a tendency toward an increase of 23.6 % in fecal S100A12 concentration (Garzon 2017). Classically, *Giardia* infection is characterized by little or no inflammatory intestinal response (Roxström-Lindquist 2006). In young children from developing countries, *Giardia* infection is usually asymptomatic, probably as a result of modulation of the innate immune system or a decrease of inflammatory response in subsequent infections (Muhsen 2012, Hanevik 2007, Kohli 2008). In our study, the tendency toward increased fecal S100A12 levels may suggest local inflammatory response with participation of neutrophils. Some evidence supports the idea that *Giardia* may induce inflammatory response (Cotton 2015). In a mouse model of *Giardia lamblia* infection, mucosal influx of bacteria coincided with increases in neutrophil infiltration even after parasite clearance (Chen 2013). In rats, significant increase of inflammatory cells including neutrophils, were observed in small intestine several days after *Giardia* infection (Zahara 2012). In humans, microscopic duodenal inflammation has been reported in some adults with either acute (Hanevik 2013) or chronic (Troeguer 2007) *Giardia* infection. However, conflicting results have been reported regarding the association between *Giardia lamblia* infection and intestinal inflammation. Some studies described this association (Campbell 2004, Kohli 2008) while others did not (McCormick 2016). In Gambia, fecal neopterin was higher in infants with *Giardia* infection compared with those never infected (Campbell 2004). In Brazilian infants, lactoferrin was positive with different assemblages of *Giardia lamblia* (Kolhi 2008). Furthermore, fecal lactoferrin was associated with more prolonged illness and more episodes of diarrhea (Kohli 2008). In contrast, in MAL-ED study, the presence of *Giardia* was not associated with any marker of inflammation; paradoxically *Giardia* was associated with a decrease in neopterin concentration, suggesting that *Giardia* is not associated with intestinal inflammation (Rogawski 2016). Our results are in contradiction with MAL-ED findings. One plausible explanation may be the predominant existence of assemblage B (80.0%) in our sample (Garzon 2017), as described in other African countries (Ignatius 2012, Mbae 2016). *Giardia* Assemblage B infection elicited more extensive damage to mucosal

architecture, with infiltration of inflammatory cells in gerbil model (Bénéré 2011). Another reason could be the use of S100A12, a more sensitive and specific biomarker of neutrophil activity (Sidler 2008). Further studies are needed to clarify this discrepancy of pro-inflammatory and immunomodulatory responses in *Giardia* infection (Cotton 2015, Barlet 2016, Babaei 2016).

Soil transmitted helminths and intestinal inflammation

The multivariable analysis also showed that infants with STH infection had a tendency toward an increase of 24.1 % in fecal S100A12 concentration (Garzon 2017). Classically, helminth infections are characterized by an immunoregulatory environment compromising including regulatory set of cells and cytokines, as IL-10 and TGF-β (McSorley 2012). This depressed immune reactivity compromise the immunity response against the infecting parasite, but also protecting the host from further damage (McSorley 2012). Very few studies have explored the effects of STH infections on mucosal inflammatory response in infants (Cooper 2009). In early STH infections, Zanzibari infants showed a regulatory Th2 pattern of peripheral cytokine responses to *Ascaris* and hookworm antigens (Wright 2009), without association with acute phase proteins (Wright 2009). In our study, the tendency toward increased fecal S100A12 concentrations in concurrent 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 in regions immediately adjacent to the parasite has been described (Marimoto 2004). *Ascaris suum*-derived products can induce human neutrophil activation via a G protein-coupled receptor that interacts with the interleukin-8 receptor pathway (Falcone 2001). Furthermore, primary infections with STH, as occurring in younger children, may stimulate a strong inflammatory response in the mucosa (Cooper 2009). This association should be interpreted with caution in our study (Garzon 2017), since infants received deworming therapy. Further studies addressing specifically the effects of helminths on intestinal barrier in infants are needed.

Crytopsoridium spp. and intestinal inflammation

In our cohort, no association was found between *Cryptosporidium* spp. infection and fecal S100A12 concentrations (Garzon 2017). In LMIC, infants frequently respond to *Cryptosporidium* infection with intestinal inflammation, as demonstrated by systemic and intestinal proinflammatory cytokines in Haitian infants (Kirkpatrick 2006), or with higher fecal lactoferrin in Brazilian infants (Alcantara 2003). In spite of cryptosporidiosis is reported to be associated to intestinal inflammation in infants from similar settings, the lack of association observed could be explained by the low frequency, and less severe infections in our asymptomatic infants. In fact *Cryptosporidium* infection can trigger the expression of anti-inflammatory cytokines (IL 10) (Kirkpatrick 2002). The production of IL-10 can be considered an attempt to restrain the immune system against over exuberant inflammatory responses, minimizing damage from inflammatory response (Kirkpatrick 2002). Further studies, including the molecular characterization, are needed to clarify the association between *Cryptosporidium* infection and growth restriction observed in our study in absence of intestinal inflammatory response.

5.6.2. *Association between enteric parasitic infection and intestinal permeability*

In our cohort, the multivariable analysis showed that infants infected by any enteric parasite had a tendency toward an increase of 33.6% higher fecal A1AT concentration (Garzon 2017). This association may have limited clinical relevance, since the increased fecal A1AT concentration was associated with unspecific etiology. In infants from LMIC, several studies (most using the L:M test) described the association of enteric parasitic infection with increased intestinal permeability (Lunn 1999, Zhang 2000, Goto 2002) while others did not (Goto 2009, Campbell 2004).

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 (DiGenova 2013) as described in *Giardia duodenalis* (Teoh 2000, Troeguer 2007), *Cryptosporidium* spp. (Buret 2003, de Sablet 2016) and STH infections (McDermott 2003, Su 2011, Northrop 1987). Elevated intestinal L:M ratio was associated with *Giardia* infection in infants from Gambia (Lunn 1999) and Nepal

(Goto 2002). Similarly, transitory increase in intestinal permeability was described in *Cryptosporidium* infections in Peruvian infants (Zhang 2000). In MAL-ED study, A1AT was better associated with cumulative pathogen burden than with single contemporary pathogens (McCormik 2016). This may explain the lack of association in our study and it can be speculated that the effect of a single episode of infection on intestinal barrier is not sufficient to cause enough structural damage of tight junctions allowing the passage of large molecules such as A1AT (Vojdani 2013).

To conclude, the hypothesized association between previous exposure to 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.

5.7. Association between enteric parasitic infection and infant growth

Based on evidence, the classically linear framework of undernutrition (UNICEF 1990a) has been rearranged into a cycle model, in which enteric pathogens has a central role (Guerrant 2008). Repeated enteric infection are associated with impaired gut-function, that can exacerbate the effects of undernutrition by restricting appropriate processing of nutrients necessary for physical and cognitive development, as well as by impairing the child' s ability to resist to recurrent infections (Guerrant 2008). The quantification of the individual impact of subclinical infections by enteric parasites on growth has been less explored than in infected children with diarrhea (McCormick 2016).

In our study, the multivariable analysis showed significant associations between *Giardia lamblia a*nd STH infections and deficit in linear attained growth (LAZ and LAD) and between *Cryptosporidium* spp. infection and deficit in growth velocity (WAVZ and LAVZ). Several clinical and epidemiological studies corroborate our findings.

In infants from LMIC, conflicting results have been reported on the association between *Giardia lamblia* infection and growth restriction. Several studies described such association (Mata 1978, Farting 1986, Newman 2001, Goto 2009, Donowitz 2016, Rogawski 2017) while others did not (Lunn 1999, Campbell 2005, Hollm-Delgado 2008). Studies addressing the natural history of *Giardia* infection suggested that the duration of *Giardia* episodes (Mata 1978, Farting 1986) and the time of first infection (Donowitz 2016, Rogawski 2017) were the most important factors associated with growth restriction. In the multinational MAL-ED study, infants with high exposure to *Giardia* had some negative impact in length and weight at 2 years of age, reflected by -0.12 LAZ and -0.11 WAZ (Rogawski 2017). In Bangladesh, the presence of a least one *Giardia* detection in the first 6 months of age decreased by -0.4 LAZ at 2 years of age but not WAZ (Donowitz 2016). This early impact might be the result of a critical period of susceptibility in which the infant gut is still developing (Rogawski 2017). Our findings are in line with these last results.

Few studies have evaluated the impact of STH infection on infant growth (Moore 2001, Gyorkos 2011, LeBeaud 2015). In Peru, reduced LAZ was observed in children with moderate to heavy helminth infections than those non-infected or with light infections (Gyorkos 2011). In a Kenyan birth cohort, *Ascaris* infection at 24 months of age was significantly associated with decrease in LAZ (LeBeaud 2015). In Brazil, STH infection was associated with a linear growth faltering of 4.6 cm at 7 years of age (Moore 2001). In our study, the negative associations of STH with linear growth (LAZ and LAD) corroborate these observations in infants from similar settings (Gyorkos 2011, LeBeaud 2015).

Association between cryptosporidiosis and growth restriction have been confirmed by several longitudinal studies (Checkley 1998, Bushen 2007, Ajjampur 2010, Korpe 2016). In Peruvian infants with *Cryptosporidium parvum* infection poor weight and height gain was observed several months after the onset of infection (Checkley 1998). Despite children with symptomatic cryptosporidiosis gained less weight than did those with asymptomatic infection, asymptomatic cryptosporidiosis occurred nearly twice as often as symptomatic cryptosporidiosis, and also had an adverse effect on weight gain (Checkley 1998). In Brazilian infants, significant declines in LAZ within 3 months of

infection were described with either *C. hominis* or *C. parvum* infection; moreover, LAZ continued to decline in infants with asymptomatic infections (Bushen 2007). Bangladeshi infants with *Cryptosporidium* spp. infection had 2-fold increased risk of stunting at 2 years of age, in both non-diarrheal and symptomatic *Cryptosporidium* spp. infections (Korpe 2016). These studies confirmed that *Cryptosporidium* infection at any point in the first 2 years of age, whether diarrheal or non-diarrheal, could result in impaired growth at 2 years of age (Korpe 2016). In our study, similar negative association of subclinical *Cryptosporidium* spp. infection with growth velocity was observed.

In our study, *Giardia* and STH infections affected linear growth but not weight, which can reflect a cumulative, long-term sustained exposure to these pathogens in this endemic country (Richard 2012). In the particular case of STH, the association we found with growth deficit was mostly due to *Ascaris lumbricoides*, since *Trichuris trichiura* was detected in less than 3% of infants, and no hookworms were detected. Noteworthy, the deleterious associations between these parasites and growth were observed even though infants received regular deworming therapy, probably indicating a very high frequency of re-infection. The specific association of *Cryptosporidium* spp. infection with low growth velocity rate, but not with attained growth, may reflect acute transient rather than persistent infection in the relatively immune competent infants of our cohort, who were HIV-negative, less severely undernourished, and benefited from the protective effect of specific antibodies in breast milk (Korpe 2013).

The pathway between growth faltering and enteric infection could be related to several mechanisms, including microbial-driven nutrient deficiencies, intestinal inflammation, gut dysfunction, and increased intestinal permeability (Bartelt 2016). In experimental models, *Giardia lamblia* and *Cryptosporidium* spp. infections cause epithelial disarrangements, blunted villus architecture, and chronic inflammation, resulting in malabsorption and poor weight gain (Bartelt 2013, Costa 2011). Jejunal biopsies of Ascaris-infected children also showed histological changes in villi, crypts, and lamina propria, affecting the intestinal absorptive capacity (Tripathy 1972). Clinical studies have reported increased gut permeability in *Giardia lamblia* (Rogawski 2017),

Cryptosporidium spp. (Zhang 2000) and helminth infections (Northrop 1987). In a subsample of our cohort, we found significant association between increased intestinal permeability and undernutrition, although not between enteric parasitic infections and gut inflammation or increased permeability (Garzon 2017).

Together, experimental and clinical evidence suggest that enteric protozoa and STH may be considered as "stunting" pathogens when associated with diarrhea (Bartelt 2013) and in our opinion this concept should be extended to subclinical infections. Diarrhea-independent mechanisms are likely responsible for the growth impact, once that physiologic insult occurred without manifestation of diarrhea (Rogawski 2017). Affected infants may have a limited capacity to repair mucosal damage, which in turn could contribute to malabsorption, disturbed nutrient uptake and transport, and increased metabolic needs (Guerrant 2008). These factors, together with the yet poorly understood intestinal host-pathogen-microbiome interactions, apparently result in a negative impact on growth, thus jeopardizing the achievement of their full potential (Guerrant 2008). The combination of subclinical parasitic infections with subtle growth faltering detected in our infants is of utmost importance, since it can easily be unrecognized unless the adequate screening tools are used, resulting in a missed opportunity of targeting this at risk population in public health policies.

5.7.1. *Cofactors associated to growth faltering*

Exclusive breastfeeding

In our cohort, multivariable analysis showed that breastfed infants had a positive association with attained growth, as well as a protective effect on wasting and stunting. The benefits of breastfeeding in LMIC are well-recognized (WHO 2000a, WHO 2013a, Victora 2016). Pooled analyses of studies carried out in these countries showed that breastfeeding substantially lowered the risk of death from infectious diseases in the first two years of age (WHO 2000a), and avoided around half of all diarrhea episodes (WHO 2013a). Breast milk also provides the best combination of nutrients for child growth (Giugliani 2008) but the impact of breastfeeding on child growth is less clear (Giugliani 2008, 2015). Most studies showed positive, albeit not always significant associations with growth (Giugliani 2008). In the WHO Growth

Reference Study, it was observed that exclusively breastfed infants had accelerated growth during the first months and a slow down at older ages, compared to non-breastfed children (Giugliani 2008). In our study, the positive association of breastfeeding with attained growth was sustained along the 24 months of age. This may be explained by two reasons. Firstly, unexpected high proportion (88.4%) of infants was exclusively breastfed at 6 months of age, compared with 37% reported from 127 national demographic surveys in LMIC (Victora 2016). Secondly, prolonged breastfeeding was found in our cohort, with a mean age of 17.1 months that might have a dose-protective effect of breastfeeding on growth.

Complementary feeding

In our cohort, multivariable analysis showed that for each month increase in the age of introduction of complementary feeding a decrease in the odds of stunting. The assessment of the quality of complementary foods was beyond the scope of our study. Nevertheless, the median age at introduction of complementary feeding was 5.9 months, according to recommendations (WHO 2009b), and it may be speculated that suboptimal complementary feeding introduced not earlier than 6 months of age was protective for stunting.

Mother height

In our study, multivariable analysis showed that for each cm increase in mother's height mean increases in attained linear growth and length velocity were found, reflected by 0.05 LAZ, 0.14 of LAD, and 0.02 LAVZ. In addition, mother's height there was a protective factor for stunting. Once attained height reflects the health stock accumulated through social and environmental exposures during early childhood, maternal stature is considered a simple, stable, and useful marker for assessing intergenerational linkages in health (Ozaltin 2010, Martorell 2012). Few studies have explored its influences on offspring growth beyond the neonatal period (Subramanian 2009, Hambidge 2012, Addo 2013). In a pooled analysis in five LMIC addressing maternal height and offspring growth, it was found that each 1-cm increase in maternal height predicted an increase in 0.037 in LAZ at 2 years of age (Addo 2013). Our results corroborate these findings. Previous studies have reported that African

women are taller in comparison with Asian and Latin American women, in spite of African women have low income (Deaton 2007). One explanation is that in Africa is well endowed with land relative to its population; consequently, nutrition may be relatively plentiful, despite low national income (Deaton 2007). The protective influence of maternal height on offspring growth is explained by previous observations that tall mothers have increased reproductive success (fertility, child survival) in stressed environments (Pollet 2008).

Acute diarrhea

In our cohort, multivariable analysis showed that infants with acute diarrhea had a decrease in weight measurements. In addition, in infants with acute diarrhea the odd of wasting was twice higher. Diarrhea is common in LMIC where water and sanitation facilities are inadequate and the short-term association between diarrhea and weight is well known (Gurrant 1992, Lima 2000, Richard 2013). It has been reported that heavier and persistent diarrheal episodes in the first years of age are associated with a progressive impairment of catch-up of weight gain (Guerrant 1992). Data from 7 cohort studies in LMIC found that children who had experienced diarrhea in the past 30 days weighed less than those who had not diarrhea (Richard 2013). Although the association of acute diarrhea with infant growth was beyond the scope of this study, the association of acute diarrhea with decrease in WLZ and rate of weight gain in our cohort were in accordance with findings in LMIC (Richard 2003). Nevertheless, the associations we found should be interpreted with caution, because the proportion of infants with acute diarrhea was low.

Multidimensional poverty index

In our cohort, multivariable analysis showed that increase in MPI score, infants had a decrease in attained growth and increase in the odds of wasting and increase in the odds of stunting. We found 28% of households were classified as deprived in comparison with 47.5 % in STP in 2008/2009 (UNDP STP 2016). Socioeconomic status has been related to a wide range of health and health-related outcomes across diverse populations and at different points in the lifespan (Pollack 2007). In LMIC, collecting income data is a challenge due to fluctuations, informal work, and reporting

biases (Psaky 2014); thus, the use of non-monetary wealth measures, such as MPI, offer a more reliable measure (Chova 2010). Studies in LMIC showed that lower wealth status was associated with stunting (Chova 2010). In Bangladesh, children in the poorest 20% of households were more than 3 times likely to suffer stunting (Hong 2006); similar results were described in Cambodia (Hong 2006) and Brazil (Muniz 2007). Research in developing countries has demonstrated that assets owned by families tend to be the gateway for accessing essential services, lowering child mortality and improving development outcomes (Chova 2010).

5.8. Association between enteric parasitic infection and neurodevelopment

Cognitive impairment is the main detrimental outcome of the cycle undernutrition-enteric infections (Guerrant 2008). In children, intestinal infections may have harmful effects not only on stunting, but also on cognitive development; this seems to be independent from the effect of diarrhea on undernutrition (Guerrant 2011). The brain is the most complex and costly-energy organ in the human body (Eppig 2010). When a child cannot meet the adequate energetic demands during the rapid brain growth and development, the brain's growth and developmental will be compromised (Eppig 2010). The exposure to infectious agents may deviates energy, investing more into immune function at the expense of brain growth (Eppig 2010).

Giardia lamblia and risk of poor neurodevelopment

In our cohort, multivariable analysis showed that infants with *Giardia lamblia* infection had 69% increases in odds of poor development. Previous studies in developing countries showed robust associations between enteric infections and neurodevelopment (Guerrant 1999, Niehaus 2002, Guerrant 2011), particularly between *Giardia* spp. infections and cognitive impairment in early life (Berkman 2002, Ajjampur 2011, Yentur 2015). In Peruvian infants with more than one episode of *Giardia lamblia* infection *per* year scored 4.1 points lower in Wechsler Intelligence Scale for cognitive function at 9 years of age, than those with none episodes (Berkman 2002). Indian infants with a past history of *Giardia* associated-diarrhea showed significantly lower social quotients and lower intelligence quotients (Ajjampur 2010). Similarly, Turkish children infected with *Giardia lamblia* and other intestinal parasites

had around twice-fold of language–cognitive and fine motor development delays assessed with the Ankara Child Development Screening Inventory (Yentur 2015). In our cohort, the significant association of *Giardia* infection with higher risk of poor development is in accordance to aforementioned studies, despite these assessed children at different ages, using different tools.

There is no evidence of a specific etiopathogenic factor to explain the effect of *Giardia infection* on cognitive development in infants. Giardiasis can lead to zinc and other micronutrient deficiencies that are implicated in cognitive development (Berkman 2002). A genetic component also seems to be implicated in the effect of *Giardia* infections on brain development (Guerrant 2011). It was suggested that early cognitive development under the stress of early childhood diarrhea and undernutrition is modulated by apolipoprotein E (APOE) genotype (Oriá 2007). In Brazilian children, a significant association between *Giardia* infections and cognitive impairment was found in APOE4-negative children, but not in APOE4 carriers, suggesting a protective role of APOE genotype (Oriá 2005). APOE regulates cholesterol and fatty acid metabolism, and may mediate synaptogenesis during neurodevelopment (Oriá 2005). This supports the hypothesis that cholesterol bioavailability may be shifted from the parasite (it cannot itself synthesize cholesterol) to the developing brain (Oriá 2005). Further studies are needed to confirm these etiopathogenic hypotheses in endemic settings.

Stunting and risk of poor neurodevelopment

In our cohort, multivariable analysis showed that in stunted infants there was a twice odds of poor development, and males in particular had 51% odds of poor development. Stunting, poverty and psychosocial deprivation are the key risk factors for poor child development (Black 2017). Severe irreversible physical and neurocognitive damage associated to stunted growth is considered a major barrier to human development (de Onis 2016). The first 1.000 days represent the greatest opportunity to provide optimal nutrition to ensure normal development but also represents the time of greatest brain vulnerability to any nutritional deficit (Cusick 2016). Studies supports the biological mechanisms whereby early exposure to

undernutrition leads simultaneously to restricted linear growth and deficits in brain development (Prado 2014). Animal models showed that prenatal and early life undernutrition negatively affects axon and dendrite growth, synapse formation and connectivity within regions of the brain (Prado 2014). Neuroanatomical studies in rats found that undernutrition after birth alters cortical development, with reduction of cortical thickness, and impairment of dendritic growth (Cordero 2001). In humans, brain of undernourished infants is characterized by shortening of apical dendrite, decrement of the number of spines, and presence of dysplastic spines (Cordero 1993). It is plausible that altered higher brain functions in infants suffering early postnatal undernutrition may be a consequence of deficient development of the dendritic spine apparatus (de Onis 2016). Prospective cohort studies consistently showed associations between stunting at 24 months of age and later cognitive and educational deficits, with size of deficits varying between studies (Walker 2007, Sudfield 2015). Stunted children often exhibit delayed development of motor skills such as crawling and walking as well as diminished exploratory behavior (Kuklina 2004). Peruvian children with severe stunting in the second of age scored 10 points lower in the Wechsler Intelligence Scale test than those non-stunted (Berkman 2002). In Jamaica, stunting before 24 months of age was related to poor cognition and school achievement at 17 to 18 years (Walker 2005). In young children, underweight and stunting were also associated with apathy, less positive affect, lower levels of play, and more insecure attachment than in well-nourished children (Walker 2007). A meta-analysis including studies in 29 LMIC, LAZ was positively associated with earlier walking age and better motor scores: each unit increase in LAZ during the first 2 years of age was independently associated with shifts of +0.24 SD in concurrent cognitive ability (Sudfield 2015). Our findings are in accordance to the evidence of association between linear growth and cognitive development in infants in LMIC. In our cohort, the high prevalence of stunting already present at neonatal period could explain the relatively high proportion of infants at risk of poor development at 24 months of age, once nutrient deprivation in intrauterine life may result in mid- to long-term negative effects on brain function (Prado 2014).

To recapitulate, infants in São Tomé were at risk of poor development, with risk increasing with age. Both, exposure to *Giardia* infection and stunting were independently associated with risk of poor development. This is an important finding since São Tomé is an endemic for *Giardia*.

6. STRENGTHS AND LIMITATIONS

6.1. Strengths

This is the first study exploring several clinical aspects ever performed in infants in São Tomé.

Study design and representativeness

- This is the first birth cohort study conducted in São Tomé, representing 8.6% of live-births in São Tomé, proportionally distributed in the 3 main São Tomé districts. Our birth cohort sample size of 475 neonates seems to be representative for study purpose. In fact, the most comprehensive multinational study with similar objectives of exploring the associations of enteric pathogens with malnutrition, gut physiology, physical growth, and cognitive development in infants in LMIC included eight birth cohorts with samples varying from 233 to 314 infants *per* country (Platts-Mills 2015).
- In addition, our birth cohort sample size of 475 neonates seems appropriate to study growth. In spite of being in infants from a LMIC, they complied with most of the criteria used by WHO to construct standard growth charts (WHO 2006a). In fact, the WHO Multicentre Growth Reference Study had included six-birth cohort with similar samples with sizes varying from 208 to 328 infants *per* country (WHO 2006a).
- A birth cohort study we used has the worth of being a life course epidemiology approach, giving the opportunity to explore basic concepts of causal pathway in relation to time (accumulation of risk), timing of causal actions (critical periods), and context (LMIC) (Kuh 2003, Batty 2009, Horta 2017). Specifically, the associations of the cumulative exposure to enteric parasitic infections on intestinal barrier function, nutritional status, and neurodevelopment were examined within the critical period up to 24 months of age, in São Tomé. The understanding of the nature of these associations may be useful for planning context-specific interventions.

- The generalized additive mixed regression models we used to assess the associations of enteric parasitic infections with nutritional status and neurodevelopment is a method suitable to evaluate complex correlations of short-term effects of time-varying exposures in environmental epidemiology (Jbilou 2012).

Nutritional status/anthropometry data

- This is the first study providing longitudinal anthropometric data in São Tomé from birth to 24 months of age. Noteworthy, the enrolled infants were born at term and appropriate for-gestational-age, mostly were exclusively breastfed up to 6 months of age, around two thirds still were breastfed at 12 months of age, complementary feeding was mostly introduced at recommended age, no relevant morbidity was present, and severe undernutrition was quite infrequent. Hence, the anthropometric data obtained may be closer to standard values (WHO 2006a) rather than to reference values, and appropriate for use in this low-and middle income setting.
- A complete anthropometric approach has included, not only the conventional attained growth assessment, but also the growth velocity, LAD and a lower threshold for risk of wasting and stunting to early detect unapparent growth fathering and mild undernutrition.
- Anthropometric assessment was performed by the same trained observer, minimizing the inter-observer measurement error variability. This observer also collected and entered all data in the WHO Anthro software.

Enteric parasite data

- This longitudinal study was the first to provide prevalence data on protozoan and STH infections in São Tomé infants. This analysis allowed determining the timing of first infections as well as the associations of cumulative parasitic infections in critical periods of life with the studied outcomes. Particularly, it contributed to study the less explored subclinical enteric parasitic infections in early ages.

- The accuracy of parasitic diagnoses was guaranteed firstly by stool microscopic and staining procedures made locally by the same trained observer and secondly double-checked by an independent skilled observer at a specialized center (IHMT).

Intestinal barrier data

- This is the first study providing in São Tomé infants data on intestinal barrier function associated with enteric parasitic infections. Herein, the fecal S100A12 was firstly used as a convenient biomarker of local inflammatory response to parasitic infection.
- The longitudinal analysis of enteric parasites, preceding the assessment of intestinal inflammation and permeability at 24 months of age, was a suitable way to assess the cumulative effect of previous parasitic infections on intestinal barrier function. In addition, the assessment of intestinal barrier function at this age, and not before, reflected more accurately the impact of parasite infection, not biased by a potential effect of an incomplete development of mucosal barrier function.

Neurodevelopment data

- This is the first study screening the neurodevelopment in São Tomé infants. Herein, BINS, a convenient tool for field studies in LMIC, was firstly used in an African country.

6.2. Limitations

Representativeness

- The main limitation of this study was the high attrition rate of 40.25%. Infants who completed the study had better anthropometry indicators at birth and better socioeconomic status than those lost to follow-up. This may have biased our results in a more favorable way. The attrition rate effect did not depend on the research team, that is, it was a random lost to follow-up. No important bias is described with levels of loss up to 60% when the missing is related to random mechanism (Kristman 2004). Furthermore, in our study the loss to follow-up was a

sort of wave attrition, i.e., many infants not present at one follow-up visit returned for the next, allowing recapture for analysis (Campbell 2011).

- *Nutritional status/anthropometry data*

  Wasting and stunting based on measurements in neonatal period instead of measurements at birth were used as an indicator of intrauterine growth. This may be inaccurate because it does take into account the physiological weight loss in the first days after birth and environmental factors occurring in the first postnatal days in a LMIC may influence growth. In consequence, measured body weight might be an unreliable surrogate of birth weight, but less likely in relation to measured length.

Enteric parasite data

Two factors may have contributed to underestimate the prevalence of parasitic infections in our study.

- A single microscopic examination of stool was performed, which is reported to have a low sensitivity (Cartwright 1999, Rogawski 2017). Nevertheless, it is described that the examination of a single stool specimen may be sufficient when the prevalence of infection in the tested population is more than 20% (Branda 2006), as it was reported in São Tomé (Ferreira 2015) and observed in our study.
- The prevalence of STH and *Giardia* could be biased by the administration of anti-helminthic medications as part of mass treatment campaigns and by specific anti-*Giardia* treatment when it was indicated.

Intestinal barrier data

- In our study the assessment of intestinal barrier function was performed in a subset of infants. This subanalysis may be underpowered once the sample size was not calculated. Moreover, 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 used in the study (S100A12 and A1AT), specifically for enteric

parasitic infection in infants. In spite of the convenience size of the sample, most of the associations obtained had relatively narrow confidence intervals.

- 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 (Zhan 2000) and to a lesser degree in bacterial infections (Kukurozovic 2002). Intestinal inflammatory response is common in invasive bacterial enteric infections (McCormick 2016). Nevertheless, only asymptomatic infants with non-diarrheic stools were selected to minimize the probability of coexistence of viral and bacterial enteric infections.

Neurodevelopment data

In our study concerns on validity of the neurodevelopment screening should be acknowledged.

- Screening tests are not appropriate in samples where cutoffs have not been determined (World Bank 2009). The BINS was validated in South American countries (Guedes 2011, McCarthy 2012), but not in Africa. Thus, consistency of our results depends on further studies testing validity and reliability of BINS in this context (Sabanathan 2015).
- In spite of neurodevelopment was assessed by an experienced pediatrician, she is not specialized in neurodevelopment. Nevertheless, screening tools as used in our study are inexpensive, quick and relatively easy to administer, nd require minimal time for training (World Bank 2009, Sabanathan 2015).
- In spite of the aforemention advantages of BINS (Aylward 1995), its application was difficult because optimal conditions were not available. Frequently the health settings in São Tomé had not adequate locations for neurodevelopment screening, and the locations available were noisy and crowded. In addition, infants were unfamiliar with the material used, limiting their engagement with the test items (World Bank 2009a).

7. CONCLUSIONS

This first birth cohort ever performed study in São Tomé is innovative in exploring associations between enteric parasitic infections and the intestinal barrier function, nutritional status and neurodevelopment in infants.

This study found that:

- São Tomé is endemic for *Giardia lamblia* and STH, infecting/or colonizing children since early ages. This study also highlitinged the underestimated role of these enteric parasites as etiologic agents of subclinical enteric infections.
- The use of a refined anthropometric approach growth allowed a more reliable detection of growth faltering and risk of undernutrition, affecting around one third of infants in this cohort.
- Neurodevelopment screening, showed that around one-quarter of infants are at high risk of poor neurodevelopment at 24 months of age.

About associations, this study confirmed that:

- The hypothesized association between 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. The study confirmed a strong evidence of association between increased intestinal permeability and undernutrrtion in infants from STP.
- Strong evidence of associations between *Giardia lamblia* and STH infections and linear growth faltering, and between *Cryptosporidium* spp. infection and deficit in growth velocity.
- *Giardia lamblia* infection and stunting were independently associated with increased risk of poor neurodevelopment.

We were able to confirm the deleterious effect of subclinical enteric parasitic infections on infant growth, and neurodevelopment. Our findings are consistent with those described in the literature for LMIC. These associations are problematic in a

highly endemic setting for *Giardia lamblia* and helminthic infections and may represent an obstacle to the potential full achievement of a considerable number of infants in São Tomé. This should therefore be adequately acknowledged and incorporated in public health policies, so that not only the problem of enteric neglected parasitic diseases but also that of neglected patients is addressed.

8. GAPS AND FUTURE PERSPECTIVES

Enteric parasites can impair the growth and learning capabilities in our younger children as was confirmed in our study, nevertheless there are still several gaps that deserve future research.

First, the mechanism by which parasites affect growth and development is still unknown. Recent studies support the important role of microbiome as possible pathway in the pathogenesis of parasitic infection. Intestinal parasites, both protozoans and helminths, modify the balance between host and commensal microbiota and by the other hand, gut microbiota may strongly interfere with the pathophysiology of the infections (Berrilli 20012). It is described that normal intestinal microbiota can decrease susceptibility to infection by *Cryptosporidium parvum*, but stimulate the pathogenic expression of *Giardia lamblia* (Berrilli 20012). Helminth infection alters the composition of the bacterial intestinal microbiota and, conversely, microbiota affect helminth colonization and persistence within the hosts (Reynolds 2015).

Neverthless, the interactions between the intestinal microbiota and enteric parasites are still poorly understood. Several questions about how intestinal parasitic colonization or infection interacts with the microbiota, and how is the crosstalk between microbiome, host immune response, health, and disease are still unanswered (Berrilli 2012). Hosts and parasites do not exist in isolation, and the inclusion of microbiome in the equation will provide understanding in the complexity of host–parasite–microbiome relationships (Chabe 2017).

More importantly, the evidence on gut microbiome remain mostly focused on populations living in developed countries were typically have a low parasite burden (Chabe 2017), but little is know how the environment, diet and the high burden of enteric parasites in developing countries can shape human microbiome. In addition, it also remains unclear how the specific changes in the gut microbiota in malnourished children from low resource settings could contribute to growth faltering.

Second, intestinal helminthic and protozoan diseases impose a great burden on poor populations in the developing world, however the prevalence of enteric parasites are

underestimated due to lack of adequate detection and surveillance systems in developing countries, particularly enteric protozoa are often ignored by surveillance systems. Molecular techniques with high sensitivity and specificity as well as the ability to detect mixed infections are needed to clarify the neglected role of asymptomatic enteric parasite infections on growth faltering. A major limitation of these technologies, however, particularly for developing countries, is the costs involved.

Point-of-care diagnostic tests can enable health-care workers to provide more rapid and effective care to people in low-resource settings (Ryan 2017). These diagnostic test need to be low cost, require minimal or no external power, be able to be run on portable and easy-to-maintain equipment, be usable without extensive training, not require refrigerated reagent storage, and deliver accurate results rapidly. Several Point-of-care diagnostic tests such as lateral flow assay LFIA strip, recombinase polymerase amplification, and microfluidics based-platforms have been developed for the diagnosis of enteric parasites (Ryan 2017). Nevertheless, there is still lack of these tools in LMIC, where they are really needed.

Third, in spite of the importance to control the high burden of enteric parasite, particularly enteric protozoa in pediatric population, there is still a lack of treatment options. Currently no effective vaccine exists for *Cryptosporidium* and only one drug, nitazoxanide is available for its treatment (Squire 2017). As with *Cryptosporidium*, a human vaccine for giardiasis is not available. The most commonly antimicrobial drugs against *Giardia* are members of the 5- nitroimidazole family such as metronidazole and tinidazole. However, resistance to all major antigiardial drugs has been reported (Squire 2017). Similarly, development of variable degrees of resistance among different species of gastrointestinal nematodes has been reported for all the major groups of anthelmintic drugs (Shalaby 2012). Thereby, efforts to develop effective vaccines and effective therapies for the control of neglected intestinal protozoan/helminthic parasites are still needed.

In addition, the controversy if enteric protozoa should be included in the mass drug administration strategy remains unanswered. Chemotherapy as a rapid-impact intervention is a good strategy for immediately improving the lives of poor

populations in developing countries. Effective chemotherapeutic drugs have been developed and extensively tested against most of these parasitic diseases. However, a few months after chemotherapeutic intervention, high re-infection rates for these parasites have been reported and the administration of these drugs in endemic areas can lead to the emergence of drug resistance (Alum 2010). Thus, clinical and epidemiological studies are needed to evaluate which could be the better chemotherapy strategy for the control of enteric protozoa, namely *Giardia lamblia* in endemic settings.

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APPENDIX 1. Informed consent

Consentimento Informado

Estudo de coorte sobre as associações de infeções por protozoários intestinais com a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento em lactentes de São Tomé

Promotores: Instituto de Higiene e Medicina Tropical e Instituto Marquês de Valle Flôr
Investigador principal: Marisol Garzon Lozano. Pediatra

1. NATUREZA E FINALIDADE DO ESTUDO

O Instituto de Hugiene e Medicina Tropical e o Instituto Marquês de Vale Flor, convida-vos a participar como voluntário em un estudo para determina se as infeções por parasitas intestinais tem consecuencias negativas no estado nutricional e no neurodesenvolvimento da sua criança.

2. PARTICIPANTES E DURAÇÃO DO ESTUDO

Este estudo inclui tudas as crianças nos primer mes de idade que sejam atendidos no centros de Promoção Materno infantil em São Tomé e nos centros de saúde de Angolares, Portoalegre e Neves. O estudo terá duração de 24 meses.

3. PROCEDIMENTOS DO ESTUDO

Pare este estudo será preenchido um inquérito que comtem dados da sua criança, dados da mãe e do pai,condições da habitação, água e saneamento.

Cada criança sera medida, pesada cada mês e avalidada para o desenvolvimento neurológico cada três meses pelo pediatra do estudo. Adicionalmente, sera solicitado aos pais fazer recolla cada três meses das fezes da criança num recipiente plástico que sera fornecido pelo estudo.

4.RISCO E BENEFICIOS

Os riscos para sua criança são mínimos. A participaçãoo da sua criança terá um beneficio directo por ser avaliada directamente pelo pediatra do estudo durante os primeiros dois anos de idade. Adicionalmente, vai ter informaçãoo sobre as parasitas intestinais e receber o tratamento quando fosse indicado.

A participaçãoo da sua criança é voluntária e pode recusar a participação em qualquer momento, sem que por isso, os cuidades de saúde da sua crianças sejam afetados.

5. CONFIDENCIALIDADE

A informação e os resultados das amostras serão codificados com números, sem identificar o nome da criança e serão confidenciais.

6. ASSINATURAS

Eu, _____ responsável legal da criança _____ estou de acordo que:

Li a folha de informação dada

Comprendi o objetivo do estudo

Recibi as informações sobre o estudo

Converse com a Dra._____

Entendi que a participaçãoo de mue filho/filha é voluntária

Livremente aceito a participação voluntária de meu filho/a no estudo.

Assinatura da mãe _____

Assinatura da testemunha _____

Assinatura Investigador _____

Data_____

Se ainda tiver perguntas sobre este estudo, pode fazê-las a investigadora responsável **Dra. Marisol Garzon contacto 9841963**

APPENDIX 2. Questionnaire

Estudo de coorte sobre as associações de infeções por protozoários intestinais com a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento em lactentes de São Tomé

N. _____

Dados demográficos

Nome da criança:_____

Sexo: F_____ M_____

Data de nascimento :____/_____/_____/

Distrito:_____

Contacto:_____

Nome de mãe:_____

Idade:_____

Ocupação:_____

Educação (anos de estudo):_____

Estatura:_____ Peso: _____ IMC :_____

Nome do pai:_____

Idade:_____

Ocupação:_____

Educação (anos de estudo):_____

N. Pessoas na família:_____

N. filhos:_____

Patologias durante a gravidez: _____

Idade gestacional (semanas):_____

Via do parto: vaginal_____ cesariana_____

Sitio de atenção do parto: hospital_____ centro de saúd_____

casa_____

Pessoal do atendimento do parto: médico_____ enfermeira_____

parteira_____ outros_____

Peso ao nascimento_____ Comprimento _____ Perímetro cefálico:_____

Patologia perinatal _____

Indice de pobreza multidimensional

Educação

Crianças entre 7-13 anos que não esta na escola:_____

Crianças maiores de 13 anos que não tem mais de 6 anos de escolar:_____

Saúde

Número de mortes de crianças os últimos 5 anos: _____

Número de crianças menores de 5 anos com estatura baixa:_____

Número de adultos magros com IMC < 18.5:_____

Casa

Energia: sim_____não_____

Tem aceso a água potável: não _____ sim_____ qual _____

Tem casa de banho em casa conectada a esgoto o latrina: não _____ sim _____ qual _____

Combustível para cozinhar : _____

Material do chão :_____

Televisão/rádio: não_____ sim _____

Carro/mota (transporte): não _____ sim _____

Frigorifico: não_____ sim _____

Casa o quintal: alugado _____ propio _____

Habitação-água e saneamento

3. INQUERITO AGUA SANEAMENTO HABITAÇÃO

AGUA POTÁVEL

1	Agua canalizada
2	Fonte /furo/fossa
3	Rio
4	Nascente de agua
5	Chuva
6	Tanques comunitarios
7	Outros

TRATAMENTO DE AGUA

1	Sem tratamento
2	Filtrado
3	Fervida (min)
4	Lixivia (g/l)
5	Outros

SANEAMENTO

1	Nenhum
2	Latrina
3	Retrete sem ligação ao esgoto ou fossa septica
4	Retrete com ligação ao esgoto ou fossa septica

AGUAS RESIDUAIES

1	Na rua
2	Esgoto
3	Fossa
4	Outros

TRATAMENTO DE LIXO

1	Recolha
2	Contentor comunitario
3	Na rua
4	Quelmado
5	Enterrado
6	Outros

Data :_____ N._____

Assinatura:_____

HABITAÇÃO

N. agregado familiar	
N. pessoas/ quarto	

COZINHA

1	Lenha
2	Gas
3	Electricidade
4	Petróleo

CARACTERISTICAS DA HABITAÇÃO

Tipo de construção

1	Cimento/Tijolo
2	Madeira
3	Outros

Pavimento	1	Cimento
	2	Madeira
	3	Terra
	4	Outros

ANIMAIS DENTRO DA CASA OU QUINTAL

1	Não
2	Cão
3	Gato
4	Porco
5	Aves
6	Outros

LOCALIZAÇÃO DA HABITAÇÃO

1	Perto do mar
2	Perto do rio
3	Interior
4	Outros

Estudo de coorte sobre as associações de infeções por protozoários intestinais com a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento em lactentes de São Tomé

Dados clínicos

Data: _____ /_____/_____/

Idade:_____

Aleitamento exclusivo: sim _____ não _____

Aleitamento ótimo_____ subotimo_____

Outras leites: sim _____ não _____ qual _____

Data de inicio alimento complementar:_____

Frutas (n. Dia):_____ legumes (n. Dia)_____

proteínas (n.dia)_____carbohidratos (n. Dia) _____

Qualidade alimento complementar: ótimo _____ subotimo _____

Sintomas

Diarreia nas últimas duas semanas: sim_____ não _____

Tipo de diarreia: com sangue_____ sem sangue_____

Internamento: sim _____ não _____

Tosse ou sintomas respiratórios nas últimas duas semanas: sim_____ não _____

Internamento: sim _____ não _____

Febre: sim _____não _____ dias _____

Teste de malária :positivo _____ negativo _____ no sabe_____

Outros sintomas:_____

_____

Antropometria

Peso:_____ Comprimento:_____ Perímetro cefálico:_____

Perímetro braquial:_____

Edemas: sim _____ não _____

WHO Anthro: WAZ _____ WLZ_____ LAZ_____ BMIZ _____

MUACZ_____

Diagnostico:

_____

_____

Tratamento:

_____

_____

Observações:_____

_____

Estudo de coorte sobre as associações de infeções por protozoários intestinais com a função da barreira intestinal, o estado nutricional e o neurodesenvolvimento em lactentes de São Tomé

BAYLEY INFANT NEURODEVELOPMENTAL SCREENER

3-4 months	N	R	E	C	Total
1. Eyes follow ring					
2. Reaches for suspended ring					
3.Holds head erect and steady for 15 seconds					
4. Adjust head to ventral suspension					
5. Demonstrates optimal muscle tone in upper extremities					
6. Demonstrates optimal muscle tone in lower extremities					
7. Sits with slight support for 10 seconds					
8. Regards pellet –conjugate gaze					
9. Vocalize two different sounds					
10. Fingers hands in play					
11. Demonstrates coordinate movement of extremities					
Score					
5-6 months					
1. Regards pellet-conjugate gaze					
2. Uses partial thumb apposition to grasp cube					
3. Bangs in play					
4. Transfer object form hand to hand					
5. Looks for fallen spoon					
6. Sits with slight support for 10 seconds					
7. Moves forward using pre-walking methods					
8. Demonstrates optimal muscle tone while pulling to sitting position					
9. Demonstrates optimal muscle tone in upper extremities					
10. Demonstrates optimal muscle tone in lower extremities					
11. Vocalizes one vowel sound					
12. Demonstrates coordinate movement of extremities					
14. Imitated others					
Score					
11-15 months					
1. Removes pellet form the bottle					
2. Puts three cubes in the cup					
3. Imitates crayon stroke					

4. Demonstrates optimal muscle tone in upper extremities					
5. Demonstrates optimal muscle tone in lower extremities					
6. Walks alone					
7.Immitates words					
8. Respond to spoken request					
9. Listen selectively to two familiar words					
10. Use gesture to makes wants known					
11. Demonstrates coordinate movement of extremities					
Score					
16-20 months					
1. Imitates crayon stroke					
2. Removes pellet form the bottle					
3. Places three pieces in puzzle board					
4. Names three objects					
5. Build tower of six cube					
6. Points to three of doll's body parts					
7. Walks quickly with coordination					
8. Walks up stairs with help					
9. Imitates two-word sentence					
10.Combines word and gesture					
11. Absence of drooling and motor overflow					
Score					
21-24 months					
1. Places three pieces in puzzle board					
2. Build tower of six cube					
3. Names four pictures					
4. Identifies four picture					
5. Points to three of doll's body parts					
6. Names three objects					
7.Runs with coordination					
8. Kicks ball					
9. Jumps off floor					
10. Jumps from bottom step					
11.Used two-word sentence					
12. Speaks intelligibly					
13.Absecne of drooling and motor overflow					
Score					

APPENDIX 3. Description of the variables

1. Socio demographic variables

Variable	Description	Type
Sex	Girls, boys.	Categorical
Clusters	Agua Grande, Caué, Lembá	Categorical
Mother's age	Years	Quantitative
Mother's school	Years of literacy	Quantitative
Mother's employment	Type of employment	Qualitative

2. Multidimensional Poverty Index (adapted) variables

Dimension	Deprivation	Weight
Education	Household is deprived if mother has completed less than five years of schooling	33.4%
Health	Household is deprived if any child has died in the family.	33.4%
Standard of living	Household is deprived if has not electricity.	5.6%
	Household is deprived if sanitation facility is not improved.	5.6%
	Household is deprived if does not have access to improved water source	5.6%
	Household is deprived if has dirt, sand or dung floor.	5.6%
	Household is deprived if cooks with dung, wood or charcoal.	5.6%
	Household is deprived if does not own more than one of assets: radio, TV, telephone, bike, motorbike or refrigerator, and does not own a car or truck.	5.6%
Total score	Poor household (categorical variable)	≥ 33.4%
	Not poor household (categorical variable)	< 33.4%

3. Delivery and feeding practices variables

Variable	Description	Type
Mother age	Years	Quantitative
Mother's height	Height in centimeters	Quantitative
Mother's body mass index	Kg / m^2	Quantitative
Delivery	At health care setting (yes/not)	Categorical
Single delivery	Yes/not	Categorical

4. **Feeding practices variables**

Variable	Description	Type
Ever breastfeeding	Breastfeeding at any time during the 2 y of life (yes/not)	Categorical
Exclusive breastfeeding	Exclusively breastfed under 6 months (yes/not)	Categorical
Breastfeeding at 1 year	Still received breast milk between 12-15 months (yes/not)	Categorical
Breastfeeding at 2 year	Still received breast milk between 20-23months (yes/not)	Categorical
Duration of breastfeeding	Total of months that received breastfeeding	Quantitative
Complementary feeding	Age (months) of introduction of solid, semi-solid or soft foods.	Quantitative

5. Clinical data variables

Variable	Description	Type
Diarrhea	Acute diarrhea (lasting <14 days) or persistent diarrhea (lasting > 14 days)(yes/not)	Categorical
Respiratory symptoms	Respiratory symptoms in the last two weeks (yes/not)	Categorical
Malaria	Confirmed by Rapid Diagnostic Test and/or blood smear microscopic identification) in the last two weeks (yes/not)	Categorical

6. Anthropometric variables

Variable	Description	Type
Weight	Weight in Kilograms	Quantitative
Length	Length in centimeters	Quantitative
Head circumference (HC)	Head circumference in centimeters	Quantitative
Middle upper arm circumference (MUAC)	Middle upper arm circumference in centimeters	Quantitative
LAZ	Length-for-age *z-score*	Quantitative
WAZ	Weight-for-age *z-score*	Quantitative
WLZ	Weight-for-length *z-score*	Quantitative
HCZ	Head circumference-for-age *z-score*	Quantitative
MUACZ	Middle upper arm circumference-for-age *z*-score	Quantitative
Wasting	WLZ ≤ -1SD (yes/not)	Categorical
Stunting	LAZ ≤ -1SD (yes/not)	Categorical
Underweight	WAZ ≤ -1SD (yes/not)	Categorical
Length-for-age difference	Length-for-age difference in centimeters	Quantitative
Two months weight velocity	*z*-score for weight-velocity for two months interval	Quantitative
Two months length velocity	*z*-score for length- velocity for two months interval	Quantitative

7. Neurodevelopment variables

Variable	Description	Type
Neurologic	Muscle tone, head control, asymmetries in movement, and absence of abnormal indicators (score)	Quantitative
Receptive	Visual, auditory, and tactile input (score)	Quantitative
Expressive	Fine motor (prehension, manipulation objects with fingers, eye-hand coordination), oral motor (vocalizations, verbalizations) and gross motor (sitting, crawling, ambulating (score)	Quantitative
Cognitive	Memory/learning, thinking, reasoning, object permanence, goal directedness, attention and problem solving (score)	Quantitative
BINS score	Bayley Infant neurodevelopment screener total score	Quantitative

Risk of poor development	High risk (yes/not). Moderate risk (yes/not) Low risk (yes/not)	Categorical

8. Intestinal parasites variables

Variable	Description	Type
Infected	Infected by any parasite (yes/not)	Categorical
Single infection	Infected by one parasite (yes/not)	Categorical
Multiple infection	Infected by two o more parasites (yes/not)	Categorical
Giardia lamblia infection	Infected by *Giardia lamblia* either single or multiple (yes/not)	Categorical
Cryptosporidium infection	Infected by *Cryptosporidium* spp. either single or multiple (yes/not)	Categorical
*Entamoeba histolytica i*nfection	Infected by *Entamoeba histolytica* either single or multiple (yes/not)	Categorical
Ascaris lumbricoides infection	Infected by *Ascaris* either single or mixed (yes/not)	Categorical
Trichuris trichiura infection	Infected by *Trichuris trichiura* either single or mixed (yes/not)	Categorical
Strongyloides stercoralis infection	Infected by *Strongyloides stercoralis* either single or mixed (yes/not)	Categorical
Giardia lamblia plus STH	Infected by *Giardia* and by any helminth (yes/not)	Categorical
Cryptosporidium spp. *plus* STH	Infected by *Cryptosporidium* spp. and by any helminth (yes/not)	Categorical
Two protozoa	Infected by *Giardia lamblia* and *Cryptosporidium* spp. (yes/not)	Categorical
Two STH	Infected by *Ascaris* and *Trichuris* or *Strongyloides* (yes/not)	Categorical
Two protozoa *plus* one STH	Infected by *Giardia lamblia* and *Cryptosporidium* spp. and one helminth (yes/not)	Categorical
Two STH *plus* one protozoa	Infected by two helminths and one protozoa (yes/not)	Categorical
Number of total infections	Total number of positive stools for enteric parasites during the follow-up period (cumulative variable)	Quantitative
Number of pathogens	Total number of parasites isolated, either single or multiple, during the follow-up (cumulative variable)	Quantitative
Number of *Giardia lamblia*	Total of *Giardia lamblia* infections, either	Quantitative

infections	single or multiple, during the follow-up (cumulative variable)	
Number of *Cryptosporidium* spp. infections	Total of *Cryptosporidium* spp. infections, either single or multiple, during the follow-up (cumulative variable).	Quantitative
Number of *Entamoeba histolytica* infections.	Total of *Entamoeba histolytica* infections, either single or multiple, during the follow-up (cumulative variable).	Quantitative
Number of STH infections	Total of STH infections, either single or multiple, during the follow-up (cumulative variable).	Quantitative

9. Intestinal function variables

Variable	Description	Type
Fecal Alpha-1 antitrypsine (A1AT)	Intestinal permeability marker expressed in µg/g	Quantitative
Fecal S100A12	Intestinal inflammatory marker expressed in µg/g	Quantitative